



European Commission

# **Collection of information on enzymes**



A great deal of additional information on the European Union is available on the Internet.  
It can be accessed through the Europa server (<http://europa.eu.int>).

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## EXECUTIVE SUMMARY

### Technical Aspects of Enzymes (Chapter 3)

#### Application of enzymes (Section 3.2)

Enzymes are applied in various areas of application, the most important ones are technical use, manufacturing of food and feedstuff, cosmetics, medicinal products and as tools for research and development. Enzymatic processes - usually carried out under mild conditions - are often replacing steps in traditional chemical processes which were carried out under harsh industrial environments (temperature, pressures, pH, chemicals).

Technical enzymes are applied in detergents, for pulp and paper applications, in textile manufacturing, leather industry, for fuel production and for the production of pharmaceuticals and chiral substances in the chemical industry. Typically technical enzymes are manufactured and used as bulk enzymes in high volumes compared to other areas of enzyme application.

Food enzymes are mainly used in baking industry, for manufacturing fruit juices, in wine making and brewing as well as in cheese manufacturing. An important field of applications in terms of volumes is starch conversion to yield ingredients for foodstuff.

The use of enzymes in animal nutrition is an important and growing area of enzyme application, especially for pig and poultry nutrition. Feed enzymes offer the benefit of degrading specific feed components otherwise harmful or of no value to the livestock.

Presently, a number of enzymes are or are intended to be applied in cosmetic products. A well documented use of application is skin peeling, future applications may be skin protection and enzyme systems preserving the cosmetic product.

Notable medications of enzymes are as digestive aids, for wound cleaning, lysis of vein thromboses, acute therapy of myocardial infarction and as support in the therapy of certain types of leukaemia.

Enzymes can be used in chemical analysis and as a research tool in the life sciences. However, the volumes used in this area of applications are regarded as negligible and this type of enzymes is not considered in the course of this study.

#### Manufacturing of enzymes (Section 3.3)

The majority of enzymes currently available is manufactured from microorganism. Manufacturing process comprise large-scale fermentation to yield high volumes of microbes. Enzymes are either accumulating inside the cells or are secreted into the media of the fermentation tanks. In subsequent steps the disrupted cells (or the media including the enzymes) are subjected to further purification processes using variety of chemical, mechanical and thermal techniques (concentration, precipitation, extraction, centrifugation, filtration, chromatography). The resulting enzyme concentrate is then formulated to the final ready-to-sell product by adding stabilisers, standardizing agents, preservatives and salts. The final enzyme preparations are usually commercially marketed in granular or liquid forms.

#### The nature of enzyme products (Section 3.4)

Within the scope of this study, the terminology for enzymes used by AMFEP was applied.

Commercially available enzymes are produced as *enzyme concentrates* which result from fermentation and subsequent purification steps. The enzyme concentrate contains the active enzyme(s) and various by-products from the fermentation process. The composition and amount of by-products in the enzyme concentrate is extremely variable and depending on the organisms, the media and the conditions applied during fermentation and subsequent

downstream processing (30 - 98% by-products in the enzyme concentrate). Thus, in addition to identification and characterisation of the enzyme as the active substance, parameters applicable for characterisation of enzyme concentrate have to be implemented.

Additives are added in a subsequent step depending on the particular application and on customers demands (final ready-for-use *enzyme preparation*). Enzyme preparations should be regarded as preparations in the sense of Directive 1999/45/EC.

### **Recent developments in enzyme manufacturing (Section 3.5)**

The application of genetic engineering techniques in enzyme manufacturing is dramatically sparking the exploitation of new enzymes and the development of new enzyme properties.

Due to new technologies, new enzymes not accessible before can be cloned into and produced from a well-known host organism. Thereby, enzymes from almost any source in nature become accessible, including enzymes exhibiting unusual properties, such as extreme thermostability. Applying new technologies, the enzyme properties may be efficiently altered which will lead to an increase in the variability of enzymes available and might lead to enzymes not present in nature so far. This mainly concerns enzyme stability, catalytic mechanism, substrate specificity and range, surface activity, folding mechanisms, cofactor dependency, pH- and temperature optima, kinetic parameters. Furthermore, enzymes could be chemically modified.

With applying new technologies, the variability in enzyme structure is dramatically increased and enzyme properties are significantly enhanced. Thus, these methods are mainly contributing to technical and economic goals. However, the safety of enzyme manufacturing might also be improved by restricting to few well-known and safe-to-use production strains which are used as hosts for genes from various sources.

### **Industrial Enzymes Presently Marketed in the EU (Chapter 4)**

A list of all enzymes that are presently marketed in the EU was compiled based on information given by enzyme-producing industry (AMFEP).

This list includes 186 enzymes from 47 different catalytic types and gives information on the catalytic activity, the host and donor organisms and the various fields of application.

### **Regulation of Enzymes in Legislation Depending on their Use (Chapter 5)**

Enzymes are regulated in different legislation depending on their use. Within the scope of this study, the regulation of enzymes was investigated and analysed regarding legislation for chemicals, food additives and processing aids, animal feed additives, cosmetic products and medicinal products.

#### **Chemicals (Section 5.2)**

The analysis of the national notification systems in the EU, USA, Canada and Australia revealed that in these countries industrial enzymes are defined as chemical substances and are consequently subjected to chemicals legislation; information requirements for notification/reporting are the same as those for chemicals. In all countries, enzymes are described via their catalytic activity. Only in Canada additional information requirements for substances derived from biotechnology (including enzymes) are defined, focussing on the production organism and the enzymatic properties. In the USA, differentiation of enzymes is done on a case-by-case basis depending on the available information, but limited experience has been gained so far. In Australia, assessments on two enzymes were performed and published so far.



In the EU, difficulties in enzyme notification were identified along with the differentiation of enzymes, the interpretation of EINECS entries, the decision on notification requirement and the need for the complete testing requirements according to Directive 67/548/EEC.

### **Food additives and processing aids (Section 5.3)**

In the EU an harmonised authorisation system is only in place for food enzymes used as additives. Directive 95/2/EEC covers food additives, however, most food enzymes are applied as processing aids, which do not have a technological function in the final foodstuff. Most Member States do not have a national legislation covering these enzymes. International and national committees (SCF, JEFCA, COT), the US FDA and AMFEP issued guidelines on conditions of use, information requirements and safety evaluation of enzymes. These guidelines pertain both types of enzymes.

Information and testing requirements include basic technical data on the enzyme itself, information on the source material, additives and possible contaminants (MO, heavy metals, toxins). Long-term experience in production of a particular enzyme and use in food application is explicitly taken into account.

Safety concerns are mainly focussing on toxic properties of by-products and contaminants. Changes in the production process may lead to a re-evaluation on a case-by-case basis. Enzymes regarded as new are often compared to already approved enzymes to check if they are substantially different.

Additional information is usually required in the case of GMM on host organism. The production of toxins resulting from unintended secondary effect is regarded as the main concern. Enzymes from GMM are often evaluated on a case-by-case basis. FDA is applying the concept of substantial equivalence to enzymes from GMM. Thus, the enzyme from GMM is compared to the conventional counterpart to evaluate if relevant properties have been affected.

### **Additives in animal nutrition (Section 5.4)**

Enzymes used as feed-additives have to be authorised according to EU Directives 70/524/EEC and 87/153/EEC. According to Directive 87/153/EEC among other things the dossier has to include a characterisation of the „active enzyme” (e. g. identity, biological origin, genetic modification, possible toxins) as well as a description of physico-chemical, technical and biological properties of the enzyme preparation (i.e. additive). Further a safety assessment basing on relevant toxicity studies has to be made. This has to be done in order to prove the safe use of the enzyme preparation against target species, consumers, workers and the environment. The additives are defined by their IUB number together with their main activity and the production organism. Directive 87/153/EEC explicitly demands that the production organisms have to be non-pathogenic and non-toxicogenic. An adaptation of Directive 87/153/EEC can be expected in the near future. So far, 61 time-limited and one unlimited authorisations on enzymes containing additives are given within the EU.

It is recommended by the Scientific Committee for Animal Nutrition (SCAN) to generally regard enzymes as respiratory sensitisers (R42) unless convincing evidence to the contrary is provided. The SCAN further recognises the exposure of workers. In case of a genetic modification, both the production- and the donor-organism (source of the transferred DNA) should be described.

### **Cosmetics products (Section 5.5)**

For substances used in cosmetic products, no general authorisation procedure is foreseen, but the Annexes of the Cosmetic Directive 76/768/EEC list substances that must not form part of a cosmetic product, that are restricted or designated for specified applications (e. g. preservatives or UV filters). According to these Annexes only the enzyme „catalase” is forbidden to be used in cosmetic products, a reason therefore is not given. Prior to their listing

in one of the Annex substances have to pass a safety assessment performed by the Scientific Committee for Cosmetic and Non Food Products (SCCNFP). This is also done if concerns with regard to the safe use of cosmetic ingredients arise. No safety evaluations of the SCCNFP concerning enzymes could be revealed.

According to an answer upon an request given by DG SANCO, the question of use of enzymes in cosmetic products is a rather new issue which has not been discussed and analysed in depth by the SCCNFP so far. According to AMFEP, the potential for respiratory sensitisation has to be considered as a serious concern when applying enzymes in personal care and cosmetics products.

### **Medicinal products (Section 5.6)**

Medicinal products are subjected to Directive 65/65/EEC and Regulation (EEC) 2309/93. If medicinal products derive from a biotechnological process, they have to be authorised and the European Agency for Evaluation of Medicinal Products (EMA) is therefore performing a scientific evaluation of the product. The biotechnological production process, the genetic modification of the microorganisms and the specification of the product are taken into account. Concerning toxicological properties, at present no set of safety tests can be described which are applicable to all different product groups.

### **Parameters Applicable for the Description of Enzymes (Chapter 9)**

A clear distinction between the terms *enzyme as active compound*, *enzyme concentrate* and *enzyme preparations* is essential for the discussion of parameters applicable for a description. Within the scope of this study, the terminology used by AMFEP was applied.

Most parameters used in scientific practice for the description of enzymes focus on the *enzyme as the active compound*. Enzymes as active compounds could be characterised by their function as well as by their molecular structure.

For clearly distinguishing enzymes by function, the information on the catalytic type has to be supplemented by additional functional parameters. To unambiguously identify/distinguish enzymes, the primary structure plus information on posttranslational modification has to be specified. Functional properties of enzymes cannot reliably be deduced from enzyme structure and vice versa. Thus, functional parameters are also relevant for the description of enzymes.

In industrial and regulatory contexts, the *enzyme concentrate* is relevant, i. e. the active component (i. e. the enzyme) plus any impurities resulting from fermentation and subsequent purification steps.

A characterisation of the enzyme concentrate has to be extended to by-products or impurities resulting from fermentation and purification that may comprise 30 to 98% of a final enzyme concentrate. The enzyme concentrate is usually characterised by describing the production process and the production organism. Furthermore, the absence or level of total viable count, known pathogenic microorganisms, known toxins, as well as heavy metals are routinely estimated or verified if required by (some) legislation.

*Enzyme preparations*, the ready-to-sell products, are described by specifying the intentionally added substances, i.e. protein, carbohydrates, fat, ash, water and diluents as well as stabilisers, standardizers, preservatives, and formulating agents.

Regulatory practice for describing technical, feed, food and other enzymes largely makes use of the parameters described above, thereby focussing on parameters for enzyme identification via its catalytic activity, information required on the production organism, requirements for the production process, and additives and other ingredients used.

Additional information often required for enzymes from GMM applied in food and feedstuff often includes a description of the genetic modification and information on functional and structural aspects of the enzyme.

In the US food additive regulation, for instance, a comparative analysis of the enzyme from GMM and from wildtype is requested. For the feed sector, industry has proposed a decision tree in order to decide which data are required in case of GMM.

Data requirements specified in legislation, guidelines published by industry and parameter used in scientific practice were investigated and compared and some fundamentals of the selection of parameters for a future enzyme notification system are discussed.

## **Toxicological and ecotoxicological properties of enzymes and enzyme concentrates**

### **Irritation and Sensitisation (Chapter 6)**

The scientific literature investigated indicate that enzymes have the potential for sensitisation of the respiratory tract. At present, no validated test method exists to determine and to predict sensitisation via the inhalative route. No recommendations can be given regarding test methods that could be routinely used for the evaluation of sensitisation to the respiratory tract. It is proposed to apply the precautionary principle and generally label enzymes with R42 „May cause sensitisation via inhalation”.

Based on literature review, there are no indications that enzymes are skin sensitizers. Therefore, testing the skin sensitizing properties of enzymes is not considered to be relevant.

All enzymes may be potential skin irritants. Regarding irritation of the skin and the eye, test methods according to the OECD guideline 404 and 405 are suggested.

### **Other Toxic Properties of Enzyme Concentrates (Chapter 7)**

The discussion of toxicological properties of enzymes and the conclusions drawn are largely based on scientific literature of enzymes used in the food and feed sector (since test data could not been obtained from the industry). A position paper from the detergent enzyme industry, testing recommendations (food sector) and testing requirements (chemical and feed regulation) are also taken into consideration.

Regarding sub-chronic toxicity and mutagenicity, pure enzymes are not considered to be of concern, but this is not the case for enzyme concentrate, since a certain risk remains that toxic or mutagenic contaminants or by-products are generated in the fermentation process. Although there is no clear consensus about toxicity testing of enzyme concentrates regarding legislation, scientific literature and (detergent) enzyme industry, but a commonly applied test set exists, which comprises

- One repeated dose oral toxicity test on rodents.
- Two mutagenicity tests (one bacterial and one non-bacterial in vitro assay)

In certain well defined cases (e. g. if there is a risk for generation of certain toxins, if a critical exposure is assumed) the following toxicological endpoints may become relevant for enzymes:

- Acute oral or dermal toxicity
- Immunotoxicity and toxicokinetics

The following toxicological endpoints seem to be of no or little relevance for enzymes:

- Acute inhalative toxicity
- Reproduction toxicity, chronic toxicity and carcinogenicity

It is assumed, that enzymes produced by GMM demand no substantially „new“ toxicity testing provided that the consequences of these modifications are considered prior to the testing and are used to guide the toxicity testing. No evidence could be found which indicates that the present and foreseeable improvement of certain enzyme properties (e. g. changing pH optimum or increasing thermal stability by „protein engineering“) will demand new or altered toxicity testing .

Therefore the following recommendations are given by the authors of the study:

- Prior to toxicity testing, information should be compiled on the enzyme concentrate, the production strain, the fermentation and downstream processing. Genetic modification (donor organism, vector, transferred DNA) should be taken into account.
- A reduction of testing requirements may be considered on a case-by-case basis, if an evaluation reveals no significant product change compared to a product already described and confirmed to be safe (this is also valid for the accompanying production process). The reduction may affect the need for acute, subchronic and mutagenicity testing. Testing requirements should, however, be extended depending on the expected exposure and application. This is also true for new types of enzymes or new production organisms.
- Data already available within industry should be investigated by independent institutions.

Testing should generally be performed using the enzyme concentrate.

### **Environmental Considerations (Chapter 8)**

The studies investigated so far revealed that enzymes seem unlikely to be dangerous to the aquatic environment due to their ready biodegradability and the low effects on aquatic organisms observed.

However, enzymes derived from new technologies might have increased stability (e. g. with higher stability to temperature or pH), therefore, the ready biodegradability of such enzymes should be proved. It is suggested, only to perform a biodegradation test in the case that the enzyme has „unusual stability“. Decision criteria for „unusual stability“ have to be set. Only in the case, the enzyme is not ready biodegradable, the performance of acute toxicity testing on aquatic organisms should be discussed.

### **Regulation of Enzymes – Possible Impacts of Different Approaches in Enzyme Identification (Chapter 10)**

Within the scope of this study, two fictitious notification/registration systems were discussed, distinguishing enzymes either by their catalytic type or by their chemical structure, and the resulting impacts in terms of regulatory and safety issues were illustrated.

#### **Enzyme identification via catalytic activity (Scenario 1)**

Enzymes are presently identified by their catalytic activity. Applying this system leads, especially in the context of EINECS, to several problems.

If the identification of enzymes is exclusively basing on catalytic activity, no distinction is made between enzymes (e. g. between wildtype organism and GMM, between natural enzymes, extremozymes and structurally altered enzymes) and consequently no data on the toxicological and ecotoxicological properties of the enzymes would be reported.

### **Enzyme identification via structural characteristics (Scenario 2)**

Distinguishing enzymes exclusively by structure (primary structure, posttranslational modification) in chemical legislation would provide criteria for clearly and almost unambiguously distinctions between enzyme molecules. However, this would put a considerable burden on both the manufacturer and the regulator.

As neither advantages for the safety of enzymes become evident applying this approach, nor enzymes are regarded as highly dangerous substances of high priority at all (on the basis of present knowledge), these efforts do not seem to be justifiable. Furthermore, toxicological, and probably also exo-toxicological considerations are focusing on impurities in the enzyme concentrate. As the composition of by-products in enzyme concentrates in terms of quality and quantity is extremely variable only indirect evidence could be given by describing the production process and identifying and characterising the production organism. Thus, additional data would be required in any case.

Consequently, the structure based concept (scenario 2) might end up in similar difficulties as the activity based concept (scenario 1). Therefore, additional criteria have to be taken into account to distinguish between enzyme concentrates.

### **Regulation of Enzymes – Outlook and recommendations (Chapter 11)**

In the future, enzymes could be registered under REACH but modified data requirements are needed for enzymes, given their specific properties.

A framework of a registration system for technical enzyme is outlined based on possible impacts of the developments in enzyme manufacturing.

EINECS is not regarded as sensible for deciding on enzyme notification. In a first step, enzyme entries in EINECS should be replaced by a database, which could be generated by means of an obligation to report all enzymes presently on the EU market. The reporting into the database should include data for the characterisation of the enzyme, the enzyme concentrate and all testing data presently available.

Enzymes should be reported/registered by IUB type (catalytic activity) but equivalence of enzymes has to be justified using core and additional parameters/data. Core data/parameter are regarded as essential and have to be specified in any case. Additional data/parameter have to be specified if they are available by the applicant. The tonnage limits should be applicable for enzymes and calculated in a standardised way.

Testing requirements: With the exception of the sensitising and irritating properties, enzymes used so far do not exhibit toxic or ecotoxic properties that raises serious concern. For enzyme registration, testing of the skin irritation potential should be requested. There is no validated test method to determine sensitisation of the respiratory tract, therefore it is proposed to generally label enzymes with R42 „May cause sensitisation via inhalation”.

However, as novel enzymes become available and novel production organisms may be used, the performance of certain toxicity and ecotoxicity tests should be decided on a case-by-case basis. This testing focuses on by-products and contaminants of the enzyme concentrate (acute toxicity (oral), repeated dose toxicity (oral), mutagenicity) or on enzymes with increased stability (ready biodegradability, acute toxicity on aquatic organisms).

In case of defined changes in the enzyme manufacturing process, the manufacturer should be obliged to monitor toxicity and ecotoxicity.



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## LIST OF ABBREVIATIONS

Å	Angstrom
ACD	Allergic Contact Dermatitis
ACGIH	American Conference of Governmental Industrial Hygienists
ACNFP	Advisory Committee on Novel Foods and Processes
ACU	Allergic Contact Urticaria
ADI	Acceptable Daily Intake
AFSSA	Agence Française de Sécurité Sanitaire des Aliments
AICS	Australian Inventory of Chemical Substances
AIS	Association Internationale de la Savonnerie et de la Détergence
AMAFE	Association of Manufacturers of Animal-derived Food Enzymes
AMFEP	Association of Manufacturers of Fermentation Enzyme Products
ANZFA	Australia New Zealand Food Authority
CA	Competent Authority
CAD	Chemical Assessment Division
CAS	Chemical Abstract Service
CEC	Commercial Evaluation Chemical
CEPA	Canadian Environmental Protection Act
CEPA	Canadian Environmental Protection Agency
CFR	Code of Federal Regulations
CIAA	Confederation of the Food and Drink Industries of the EU
CNS	Central Nervous System
COLIPA	European Cosmetic Toiletry and Perfumery Association
COT	Committee on the Toxicity of Chemicals in Foods, Consumer Products and the Environment
CPMP	Committee for Proprietary Medicinal Products
CVMP	Committee for Veterinary Medicinal Products
DG ENV	Directorate General Environment of the European Commission
DNA	Deoxyribonucleic Acid
DOC	Dissolved Organic Carbon
DSL	Domestic Substance List
E.C.	Enzyme Class
EC	European Community (in most cases); Effect Concentration (rarely, depending on the context)
EDI	Estimated Daily Intake
EEA	European Economic Area

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EINECS	European Inventory of Existing Commercial Chemical Substances
ELINCS	European List of Notified Chemical Substances
ELISA	Enzyme-Linked Immunosorbent Assay
EMA	European Agency for Evaluation of Medicinal Products
ENCS	Japanese Existing and New Chemical Substances
EPA	Environmental Protection Agency
EPAR	European Public Assessment Report
ESMS	Electrospray ionisation mass spectrometry
EU	European Union
FAC	Food Safety Act
FAO	Food and Agriculture Organization
FCC	Food Chemical Codex
FDA	Food and Drug Administration
FDC	Food, Drug and Cosmetic Act IUPAC
FEFANA	European Federation of the Animal Feed Additive Manufacturers
FFDCA	Federal Food Drug and Cosmetic Act
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
FR	Federal Regulation
GILSP	Good Industrial Large Scale Practice
GLP	Good Laboratory Practice
GM	Genetically Modified
GMM	Genetically Modified Microorganism
GMO	Genetically Modified Organism
GMP	Good Manufacturing Practice
GPIT	Guinea-Pig Intratracheal Test
GRAS	Generally Recognised As Safe
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HPVC	High Production Volume Chemical
IFF/IFZ	Inter-University Research Center for Technology, Work and Culture
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IKW	Industrieverband Körperpflege- und Waschmittel
INN	international non-proprietary name
IOGTR	Interim Office of the Gene Technology Regulator
IUB	International Union of Biochemistry
IUCLID	International Uniform Chemical Information Database



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IUPAC	International Union of Pure and Applied Chemistry
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JRC	Joint Research Centres of the European Commission
Kb	Kilobase
KDa	Kilodalton
K <sub>M</sub>	Michaelis Menten Constant
LC	Lethal Concentration
LD	Lethal Dosis
LoREX	Low Releases and Low Exposures
LPVC	Low Production Volume Chemicals
Ltd	Limited Notification
LVC	Low Volume Chemical
LVE	Low Volume Exemption
M	Mol
MALDI-TOF	Matrix Assisted Laser Desorption/Ionisation-Time of Flight Mass Spectrometry
MCAN	Microbial Commercial Activity Notice
MO	Microorganism
MoD	Manual of Decisions
MoS	Margin of Safety
MSDS	Material Safety Data Sheet
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NATA	National Association of Testing Authorities
NCEL	New Chemical Exposure Limit
NDSL	Non-Domestic Substance List (Canada)
NEPA	National Environmental Policy Act
NICNAS	National Industrial Chemicals Notification and Assessment Scheme
NMR	Nuclear Magnetic Resonance
NOAEL	No Observable Adverse Effect Level
NOC	Notice of Commencement of Manufacture or Import
NOEC	No Observed Effect Concentration
NOHSC	National Occupational Health and Safety Commission
NSN	New Substances Notification
NSNR	New Substances Notification Regulation (Canada)?
OECD	Organisation for Economic Co-operation and Development
OR	Odds Ratio
PBD	Protein Data Bank

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PCR	Polymerase Chain Reaction
PEC	Priority Existing Chemicals
PE-variant	Protein Engineered Variant
PICCS	Philippines Inventory of Chemicals and Chemical Substances
PLC	Synthetic Polymers of Low Concern
PMN	Premanufacture Notice
PNP	Para Nitro Phenyl
PNPG	Para Nitro Phenyl linked to Galactose
QSAR	Quantitative Structure-Activity Relationship
R&D	Research and Development
RAST	Radio Allergo Absorbent Test
REACH	Registration, Evaluation and Authorisation of Chemicals
RNA	Ribonucleic Acid
SAR	Structure-Activity Relationship
SCAN	Scientific Committee for Animal Nutrition
SCCNFP	Scientific Committee for Cosmetic and Non Food Products SCF
SCOOP	European Scientific Cooperation Programme
SDS-PAGE	SDS Polyacrylamide Gel Electrophoresis
SNUN	Significant New Use Notification
SNUR	Significant New Use Rule
SOD	Superoxide Dismutase
SPT	Skin Prick Test
Std	Standard Notification
TLV	Threshold Limit Value
TOS	Total Organic Substance
TSCA	Toxic Substances Control Act
TSM	Technical and Scientific Meeting on issues associated with Directive 67/548/EEC
U	Units
UBA	Federal Environmental Agency
US	United States of America
UVCB	Substances of Unknown or Variable composition, Complex Reaction Products and Biological materials
w/v	Weight/Volume
w/w	Weight/Weight
WHO	World Health Organisation

## 1 TERMINOLOGY USED IN THIS REPORT

The report in hand was prepared in the context of EU chemical legislation and addressed to the Directorate General Environment of the European Commission. According to the Service Contract „Collection of Information on Enzymes”, this report includes investigations in and discussions on chemical legislation but also on sectoral legislation covering enzymes used in food, feed, cosmetic and medicinal products. The terminology used in this report is closely following those used in chemical legislation. However, as sectoral legislation is also dealt with, using other and often differing terminology, some terminological ambiguities may arise.

In the context of notification of new substances according to EU chemical legislation, the terms „exposure, hazard, risk assessment” and „risk management” are well defined and used accordingly (the principles of risk assessment and clear definition are given in the „Technical Guidance Document on Risk assessment for New and Existing Substances”. In contrast, in sectoral legislation these terms are either not used or they are used in a less defined sense.

In the context of sectoral legislation, more often the term „safety evaluation” is used to describe the procedure of e. g. assessing the risks associated with particular (properties of) enzymes on the basis of information provided by the manufacturer and performed by scientific committees. This term is neither precisely defined nor uniformly used in different sectoral contexts but usually includes certain steps of risk assessment. Therefore, depending on the particular regulatory context, the importance of particular steps of e. g. safety testing or exposure analysis in the context of those safety evaluations might be different.

Furthermore, terms as „classified” and „classes” are well defined in chemical legislation, but have a different meaning in sectoral legislation or in scientific areas dealing with enzymes.

The term „technical enzymes” is used in the context of this study to designate those types of enzymes used in industrial contexts (excluding the food, feed, and personal care segment, and diagnostic and therapeutic enzymes as well). Thus, technical enzymes do largely correspond to those enzymes which are covered by chemical legislation. For practical reasons, the term „technical enzymes”, as used in this report, is pertaining those enzymes covered by chemical legislation.

Thus, readers should be aware of these possible ambiguities while reading this report.



## 2 INTRODUCTION

### 2.1 Addressing the problems

Enzymes which are not listed in EINECS have to be notified as "New Substances" according to Directive 67/548/EEC. Consequently, the notifier has to provide the base set information according to Annex VII of 67/548/EEC, including data on physico-chemical properties, health and environmental properties and information on the identity, production and use of a substance.

EINECS contains 368 enzyme entries, both listed as generic entries without further specification and specific entries indicating the substrate or the source organism. EINECS provides information on the name, EINECS and CAS number of the enzyme.

In recent years, difficulties in the identification of the substance, the interpretation of the EINECS entries and consequently in deciding the notification requirements arose. Additionally, questions regarding toxicity and ecotoxicity testing as well as appropriate standard test methods were raised.

A further problem, that has to be addressed with enzyme products, is that enzyme products do not only contain very small amounts of the active enzyme, but a considerable proportion of by-products. The composition and proportion of these by-products is extremely variable and depends on the production process and the production organism.

The application of new technologies in modern biotechnology enables the production of new enzymes, that were not accessible before, and the development of new enzyme properties.

Difficulties in the notification of enzymes were discussed on the Technical and Scientific Meetings on issues associated with Directive 67/548/EEC (TSM) and at the meetings of the competent authorities (CA) for the implementation of Directive 67/548/EEC. A working group<sup>1</sup> concluded that EINECS was inadequate as a reference in determining notification duty and recommended to develop specific legislation for enzymes. Subsequently, the 55<sup>th</sup> CA meeting issued a mandate for a study on enzymes.

### 2.2 Terms of reference

The Directorate General Environment of the European Commission commissioned the Austrian Federal Environment Agency (UBA) to carry out a „Collection of Information on Enzymes“. An interdisciplinary project team was established comprising experts from the Inter-University Research Center for Technology, Work and Culture (IFF/IFZ) and the Austrian Federal Environment Agency.

Within the scope of this project, an investigation on the regulation, production, and testing of enzymes on the EU market should be performed. This task includes also enzymes marketed for use in foodstuff, cosmetic and medical products. In the course of this project, detailed information should be collected on individual enzymes starting off from a list of all enzymes presently marketed in the EU. Parameters and methods suitable to characterise enzymes including by-products, potential hazards of enzymes and applicable testing methods to access these hazards should be identified. Notification approaches of other countries and relevant guidelines pertaining enzymes should be investigated. Finally, recommendations to the principal on a possible future notification/registration approach suitable for enzymes should be given by the study authors.

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<sup>1</sup> Meeting of the EU-Working Group „EINECS and Enzymes“ held in Dortmund on 26 February 1998.

The results of this project should serve as a well founded basis for further discussions on a notification/registration approach suitable for enzymes within the European Union.

## 2.3 Project approach

### Characteristics of the project

According to previous experiences in investigating and assessing enzymes, one has to face the following difficulties in carrying out this task:<sup>2</sup>

Most of the relevant data on commercial enzymes are not published. The most important and sometimes exclusive source of data are the relevant enzyme manufacturers and these data are therefore not easily accessible. Thus, according to the authors' experience, one cannot rely solely on published data on enzymes.

In order to receive the relevant data from industry one has to establish contacts and build up confidence. This has already been done in the course of a technology assessment project on detergent enzymes.<sup>3</sup> Even so, the setting up of a co-operation with individual companies or more likely with relevant industry associations will need intense negotiations between the researcher and the association and also between the member companies which is of course time consuming and also difficult to handle. However, it has already been proven that this approach could result in more reliable, more comprehensive and most recent data.

Further difficulties were posed by the fact, that enzymes are applied in various fields: detergents, food processing, feeding stuff, conversions of chemicals, medicine, cosmetics etc. Depending on the final use of a particular enzyme, different technical, safety and legal requirements are applied.

However, the project team tried to overcome these difficulties and made strong efforts in order to give a representative overview of commercial enzymes.

### Overall approach

In order to overcome these difficulties, the project design included plans to establish co-operation with the industry, contacts to national competent authorities, committees and agencies as well as extensive searches for scientific literature, regulatory documents, position papers etc.

An interdisciplinary project team was set up comprising experts from the areas of chemistry, molecular biology, toxicology, allergology as well as regulators (see previous section 2.2). These experts came from different institutional background – university science department, technology assessment department, and from the Austrian competent authority.

A considerable period of the project was dedicated to intense search for literature, relevant documents, guidelines and legislation concerning enzymes. Thereby, three different sources of information were used: First, a search for published literature was carried out using international databases such as Biological Abstracts, Medline, Chemical Abstracts. This literature search focused on technical, scientific and safety aspects of industrial enzymes. Further, numerous representatives of organisations, committees and competent authorities were contacted directly in order to obtain the relevant provisions applicable to enzymes as well as position papers and guidance documents. The contacted bodies are OECD, WHO/FAO, EMEA, AMFEP/AMAFE etc. and the competent authorities in Australia, Canada, France,

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<sup>2</sup> From 1995 to 1998 a technology assessment on „Enzymes in Detergents and Cleansers“ was carried out. This study focused on biological, technical, environmental, health and regulatory aspects. The work was carried out in co-operation with industry. The results are summarised in SPÖK et al. 1998.

<sup>3</sup> Ibid.

Czech Republic, Hungary, Japan, Korea, Slovakia, Switzerland, UK, and the US. Additionally, an internet based search was carried out for position papers and guidance documents of international organisations and committees e. g. OECD, WHO/FAO, EMEA, AMFEP, scientific committees of the European Commission, the European Scientific Co-operation Programme (SCOOP). These searches resulted in more than 500 relevant articles and documents which were subsequently structured, screened and investigated.

On the basis of this information, the design of the project was adjusted and first drafts were issued outlining the major issues dealt with in the course of the project. These drafts were subsequently subjected to extensive internal discussion in the project team and in certain cases also external advice was sought. Investigations for literature and documents as well as contacts to competent authorities, organisations and industry were still going on during this period to clarify particular problems. Further information obtained was introduced in the draft documents and the discussion process. In a final step, the draft documents were elaborated to form chapters of the final report.

Communication within the project team, between the project team and additional experts as well as between the project team and industry was facilitated by six meetings and workshops.

### **Contact to industry**

Contact with representatives of the Association of Manufacturers of Fermentation Enzyme Products (AMFEP)<sup>4</sup> was established in December 2000/January 2001. AMFEP was subsequently informed on the overall project goal and approach and asked to provide data referring to requirements specified in the Technical Annex of the Service Contract and referring to questions raised by the project team. Initially, AMFEP agreed to co-operate in the course of the task. The Association of Manufacturers of Animal-derived Food Enzymes (AMAFE), representing a much smaller organisation, was integrated by the help of AMFEP.

In order to facilitate and structure the flow of information, three „Rosters of Questions” were compiled by the project team and sent to AMFEP. Roster 1 comprised detailed questions related to individual enzymes presently manufactured/marketed in the EU (see Annex, section 13.2.1) and asking for submission of data on enzyme activity, enzyme structure, enzyme formulation, safety properties, tonnage as well as manufacturing and purification process. Some questions of this Roster aimed at widening the basis of knowledge of specific and non-specific effects of genetic engineering/protein engineering on the enzyme properties. The second Roster focused on enzymes in general, especially on by-products resulting from the fermentation process and safety testing carried out by industry (see Annex, section 13.2.2). Finally, the third Roster was compiled in order to clarify regulatory questions (see Annex, section 13.2.3). Furthermore, individual companies, such as Novozymes, DSM, Henkel Biotechnology and Boehringer Mannheim, were contacted directly.

Initially, co-operation with industry was very promising. Draft answers to Roster 2 and the questions related to regulatory issues as well as a list of enzymes available from AMFEP

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<sup>4</sup> The Association of Manufacturers of Fermentation Enzyme Products (AMFEP) is an European industry association founded in 1977. The member companies of AMFEP produce and sell enzymes for use in food, feed, detergents and other non-food industries, excluding enzymes for pharmaceutical and diagnostic use. Member companies cover about 70% of the world market (and about 90% of the European market) for industrial enzymes and comprise the worlds largest enzyme manufacturers e. g. Novozymes, DSM, Genencor, Quest, Henkel Biotechnology/Biozym. The main objectives of AMFEP are in their own words to „provide a common basis for representing the interests of its members both toward the institutions of the European Union as well as national authorities; to represent the interests of its members in international organisations; to assure a free flow of information between its members on developments related to the regulatory status of enzymes in the EU; to inform its customers and other interested parties on the efficacy and safety aspects of its enzyme products; to defend and promote the products of the enzyme industry. AMFEP monitors EU activities regarding enzyme preparations, and has developed a positive relationship with the European Commission and participates in the work of Codex as an observer.

member companies and further information were provided for the project team. Contact points were established in member companies to facilitate individual contacts. A meeting of the project team and industry representatives was organised in order to clarify and discuss questions of Roster 1.

Following this meeting, discussions among AMFEP member companies came up and AMFEP contacted DG Environment directly. The questions focussed on the narrow time schedule of the project and the feasibility of the industry's submission of the detailed data requested. According to information from AMFEP, many data are difficult to provide and it would be resource and time demanding to compile them. These objections were mainly referring to Roster 1.

Consequently, to give industry more time for the collection of the requested data and in order to extend the period for further negotiations and coordination, the project team applied for an extension of the project duration at DG Environment. Hence, the project duration was prolonged until May 2002.

Finally, AMFEP came to the conclusion that it will not be possible to answer to Roster 1. However, an AMFEP position paper referring to the Roster of Questions was announced in October 2001 and finally provided in March 2002 along with some additional and updated information (see Annex sections 13.3, 13.4). This document reflects industry's position to Roster 1 and 2 also including general answers and statements. However, no data were provided on individual enzymes (Roster 1) and no data on annual production volumes either.

### **Position of the project team**

The project team had been aware that it would have been resource demanding to compile the requested data. Nevertheless, all information requested from AMFEP would have been needed to comply with the objective of the Service Contract with DG Environment.

Foremost, these data would have been essential to enable the project team to draw their conclusions and suggestions on *real data and not on opinions and/or aggregated data* from industry. Furthermore, this approach would have led to a more concrete picture what kind of data on enzymes are usually collected by / available for industry within the limits of the production process. Thus, some questions in Roster 1 did not ask to provide detailed data, rather these questions were asking if certain data were/were not available. For the suggestion of testing requirements for enzymes, information on already used test methods and availability of data would have been taken into consideration.

As these data were not provided by industry, the project team had to base their conclusions mainly on the data available from other sources. This basis is quite large in certain questions (e. g. allergic properties of enzymes) but is, in contrast, very narrow e. g. in case of otherwise toxic and ecotoxic properties, in case of impacts of genetic engineering and protein engineering on properties of enzymes and is not existing in case of annual production volumes. Thus, some information requested in the Technical Annex of the Service Contract could not be provided at all (e. g. tonnage), other information could not be provided for individual enzymes presently on the market in the EU. Nevertheless, some issues are discussed on a general basis (e. g. information and impacts on functional and safety properties of possibly „new“ enzymes especially those manufactured by genetic engineering techniques; data on toxicological properties) in the report.

As mentioned above, these data are regarded as necessary to draw more valid conclusions. As a consequence, some conclusions drawn and recommendations given in this report will have to be re-evaluated on the basis of data available within industry in the context of further negotiations.

The project team explicitly acknowledges the efforts made by some individual representatives of industry to provide information, however, also regrets not having received all data requested from industry.



## 3 TECHNICAL ASPECTS OF ENZYMES

### 3.1 Introduction

This chapter focuses on technical aspects of enzymes. First, the major fields of enzyme applications are briefly described (section 3.2). Then, the process of enzyme manufacturing from fermentation to the ready-to-sell product is described (section 3.3) and the special nature of enzyme products, containing considerable proportion of by-products and additives is discussed (section 3.4). Finally, recent developments in the manufacturing of enzymes are described and discussed in greater detail as they will be of considerable importance for any future notification system suitable for enzymes (section 3.5).

### 3.2 Application of enzymes

Enzymatic processes – usually carried out at mild conditions – are often replacing certain steps in traditional chemical processes which were carried out under harsh industrial environments (temperature, pressures, pH, chemicals) so far. Furthermore, enzymatic processes have led to a range of new products and processes.

#### 3.2.1 Enzymes in technical applications

The most important application area for enzymes in terms of volume are detergents. Enzymes are applied to remove difficult stains and soil at low washing temperatures. Commercially available enzymes for detergents are mainly proteases, lipases, amylases, and cellulases.

Pulp and paper applications use cellulases and xylanases for improving the properties of high yield pulp by means of ligninase action and for slime prevention.

Enzymes currently used in textile applications are  $\alpha$ -amylases, cellulases, catalases, and proteases. Amylases are used for the removal of starch films after the weaving process. Cellulases are now commonly used to enhance the appearance and feel of garments made with a variety of cellulose such as cotton, linen, lyocell, viscose and polynosic rayon. Catalase is used to eliminate residual hydrogen peroxide following bleaching or in dye house water recycling. Proteases are used for softening and preventing wool from pilling as well as for sandwashing of silk.

In the leather industry, enzymes (predominately proteases) are used in several steps in the processing of skins and hides for the production of leather.

In the fuel ethanol application, enzymes are increasingly used in the conversion of various agricultural feedstocks; e.g. corn in the USA, cassava and sugar in Brazil. Typically amylases and amyloglucosidases are used to convert the feedstock to a substrate that is subsequently fermented by yeast which produces the ethanol.

Enzymes are also used in the production of fine chemicals and pharmaceuticals. For instance, immobilized enzymes are used in the manufacturing of semi-synthetic penicillins and cephalosporins. In chemical industry enzymes are of particular importance for producing chiral compounds.

### 3.2.2 Enzymes in food industry

**Baking industry:** Presently, enzymes are used to make up for deficiencies in some flours, to provide for specific properties in the flour and dough on a predictable basis or to lower the protein level of flour for biscuits and crackers. Enzymes used are e.g. amylases, hemicellulases, amyloglucosidase, protease. Amylases and hemicellulases are applied to improve and standardise the quality of the bread (e.g. softness, volume, crumb quality). Amyloglucosidase is used to ensure an even browning of the crust. Proteases are applied to decrease the level of protein in flours for biscuits/crackers.

**Beverage industry:** In fruit juice manufacturing mostly pectinases are used to increase the juice yield (decrease the amount of waste) and to improve the quality (clarification, removal of pectins). Pectinases and other enzymes are also involved in the wine-making process to help maintain colour, clarity and organoleptic properties. The brewing process involves enzymes in order to break down the polysaccharides in the starting cereals to fermentable sugars. Microbial enzymes can be used to control the brewing process more precisely by compensating for differences in the quality (enzyme content) of the malt. Other enzymes (e.g.  $\beta$ -glucanase) are used to prevent chill-haze (the beer becoming cloudy upon cooling).

**Dairy Products:** For the making of cheese the enzyme chymosin is used. Other areas where enzymes find application are in the improvement of organoleptic qualities of some cheese and to break down lactose (milk sugar) in order to make dairy products digestible for people with lactose intolerance.

**Starch and Sugar:** Amyloglucosidase and glucose isomerase are widely used in the hydrolysis of starch to glucose and in the conversion of glucose to fructose. Fructose the naturally occurring sweetener in honey and fruits, is 40% sweeter than sugar but has about the same calorific value and is thus widely used as a sweetener.

### 3.2.3 Enzymes in feed industry

For the animal feed industry, adding feed enzymes to feed stuff represent a means of improving feed utilisation and reducing pollution from excretory products. The use of enzymes in animal diets started in the 1980s and exploded in the 1990s. Figures from the United Kingdom show that the percentage of broiler feed receiving enzyme treatment rose from zero in 1988 to 95% in 1993 (POWER & WALSH, 1994). Similarly, the use of enzymes by the industry in pig and poultry feed stuff increased. As a consequence, world-wide efforts by the relevant regulatory authorities were intensified to control and to regulate the use of these additives in terms of quality, safety and efficacy. Feed enzymes are degrading specific feed components which are otherwise harmful or of little or no value. In doing so, a wider range of ingredients may be used in diet formulation. Feed enzymes may be used to increase the amount of nutrients available from vegetable proteins or to substitute a previously unacceptable energy source for another (e. g. barley for wheat in broilers). A further benefit lies in the reduction on faecal nutrient level, which may be important where faeces are applied to land with restrictions. Feed enzymes in combination with endogenous enzymes degrade compounds so that can be utilised by animal. Consequently, enzymes often are added in order to complement the endogenous enzyme activities. Multi-enzyme products have the potential to release more nutrients than single enzyme products. Research interest into the potential value of feed enzymes has occurred in the field of ruminant nutrition and aqua-culture and on the development of rapid, cheap and reliable assays for measuring enzyme activity (D'MELLO, 2000: 405 - 421).

### 3.2.4 Enzymes in cosmetics

Past and present areas of enzyme application in cosmetics seem to be few. This is partly due to unsolved technical problems associated with the product itself (e. g. achieving or maintaining enzyme stability and activity). An area of application is the use of enzymes, particularly proteases, to clean and smoothen the skin. Therefore, papain and bromelain contained in plant extracts from pineapple and papaya are used in „fruit enzyme peeling“. The products are applied as skin creams with the intention to peel off dead or damaged skin. It is yet mentioned in scientific literature, that problems are associated with this type of application, since the reaction is difficult to stop and the „enzyme keep eating its way down into the skin, causing irritation“ (BROOKS et al., 1997; LODS et al., 2000).

Due to the „positive image“ of enzymes, efforts are made to find new areas of application in cosmetic products (BROOKS et al., 1997). This becomes a realistic goal since stable enzyme systems are created. Some examples according to scientific literature are given below.

Great expectations are attributed to the application of enzymes in skin protection: Enzymes with the ability to capture free radicals and thereby preventing damage to the skin caused by environmental pollution, bacteria, smoke, sunlight or other harmful factors. In this case, the most protective enzyme type is superoxide dismutase (SOD). It is proposed to use a combination of SOD and peroxidase as free radical scavengers in cosmetic products because of their ability to reduce UV-induced erythema when topically applied. Superoxide dismutase could be extracted from yeast, the peroxidase is found in aqueous extracts of fennel (LODS et al., 2000).

Another targeted application is the use of a peroxidase (lacto peroxidase) to prevent cosmetic formulations from bacterial attack. This system is based on enzymes that consume the oxygen present in a formulation (BUSSMANN, 1996).

A patent search reveals patents concerning skin protective compositions containing catalase (PUGLIESE, 2000), skin treatment compositions comprising protein and enzyme extracts (YOUSSEFYEH, 2000), hair dye composition including laccase (L'OREAL, 1999); a composition containing a lipase, vitamin precursor and a fatty alcohol (L'OREAL, 2000), cosmetics containing enzymes (GOLZ-BERNER, 2000); and enzymes coupled with polymeric molecules for skin care (NOVO NORDISK, 1998).

Furthermore enzymes are widely used in contact lens cleaners. For that purpose proteolytic enzymes such as subtilisin are applied to remove protein films and other deposits from contact lens. Although this application is associated with personal care contact lens cleaners are not regarded as cosmetic products according to EU harmonised legislation and will therefore not be dealt with in this report.

### 3.2.5 Enzymes in medicinal products

Important routes of enzyme administrations are orally as digestive aids, topically for wound cleaning, or parenterally. The use of enzymes as digestive aids is a wide spread application. For example, pancreatin (containing trypsin, chymotrypsin, amylase and lipase) or substitutes such as bromelain or papain (containing cellulases, proteases and amylases) are used for curing pancreatic insufficiency. Proteases are recommended for removal of fibrin layers from wound to improve healing. Also, heterologous proteins are applied such as bovine plasmin, trypsin or collagenase. Proteases from snake venoms are improving blood fluidity and therefore able to cure peripheral arterial diseases. Plasminogen activators (serine proteases) are responsible for the therapeutic lysis of blood clots (thrombolysis), which lead to thromboses of veins, lung embolism and myocardial infarction. The therapy is performed either with streptokinase or urokinase. A further use is supporting the therapy of malignancies (certain types of leukaemia) using asparaginase.

Factors complicating the therapeutic use of enzyme are their large molecular weight which makes distribution to body cells uneasy. Another important factor is, that they are foreign proteins to the body (antigenic) and can elicit an immune response (allergic reaction). Efforts are made to overcome these problems partly by (genetically) modification of the enzyme structure. A further important question is the purity (absence of toxic by-products) of the enzyme preparation (e. g. more stringent if administered intravenously).

### 3.2.6 Enzymes for scientific and analytical use

Enzymes can be used as chemicals to determine the concentration of substrates, measure the catalytic activity of enzymes present in biological samples and serve as labels in immunoassays to determine the concentrations of enzymatically inert substances.

For instance, enzymes are routinely used in determination of glucose (glucose oxidase, horse-radish peroxidase), urea (urease, glutamate dehydrogenase), triglycerides (lipase, carboxylesterase, glycerol kinase etc.) in clinical diagnosis. Carbohydrates, organics acids, alcohols and other food ingredients are routinely determined in food analysis using enzymes.

Research and development in life science is often using genetic engineering techniques which in turn are largely depending on various types of DNA-modifying enzymes: restriction endonucleases, ligases, polymerases etc. The variety of such enzymes is huge: For instance, about 3000 restriction enzymes have been identified so far; several hundreds of which are commercially available at present.

The enzyme market for scientific and analytical uses is also large, probably several hundred million dollars, based on retail prices. The market is characterised by several thousand different products. However, these type of enzymes are manufactured in very small quantities and might be often bellow 10 kg/year worldwide (KOPETZKY et al., 1994) and in case of restriction endonucleases, presumably even far bellow 1 kg/year worldwide.<sup>5</sup>

## 3.3 Manufacturing of enzymes

In this section relevant aspects in the manufacturing process of enzymes are briefly described. Section 3.3.1 deals with the fermentation process. Some basic information on the fermentation process of enzymes is given and relevant aspects in enzyme fermentation are concisely described. Section 3.3.2 describes the downstream processing, i.e. the purification of the fermentation broth containing the enzyme and the subsequent formulation of the enzyme preparation ready to be sold. This section also touches upon purification procedures from plant and animal tissue which are however rarely practiced in enzyme manufacturing. The description largely follows those in SPÖK et al. (1998), ULLMANN'S Encyclopaedia of Industrial Chemistry (2000), and CHAPLIN & BUCKE (1990).<sup>6</sup>

### 3.3.1 Large-scale fermentation of enzymes

Use of an aerobic submerged culture in a stirred tank reactor is the typical industrial process for enzyme production involving a microorganism that produces an industrial enzyme. Figure 1 shows a flowchart of a typical production process. In the following, relevant aspects of the

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<sup>5</sup> Therefore, and because these enzymes are neither covered by EU chemical legislation nor by sectoral regulation these type of enzymes are regarded as being out of the scope of this report.

<sup>6</sup> More basic and detailed information can be found in handbooks on bioprocessing microbiological principles and methods.

fermentation process as displayed in Figure 1 are briefly described: the role of the organism in enzyme fermentation, the media as the „raw material”, the requirement of sterile environments for enzyme production and the fermentation process itself.

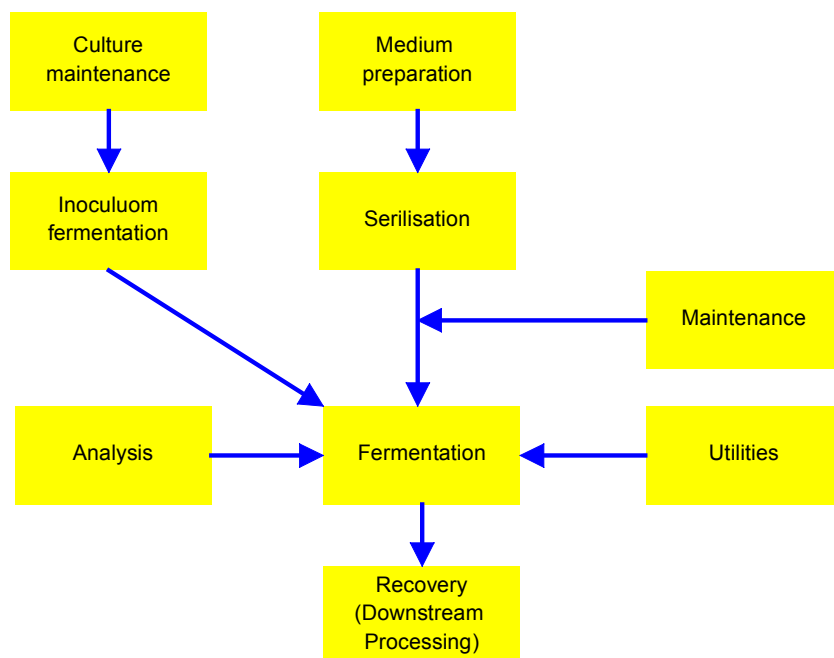


Figure 1: Flowchart of a fermentation process

### 3.3.1.1 Organism and enzyme production

The basic mechanisms of enzyme synthesis, including transcription, translation, and post-translational processing, seem to be highly conservative. However, several differences exist between various classes of organisms, as well as some fundamental differences between prokaryotic and eukaryotic organisms. The enzymes themselves differ enormously in molecular mass, number of polypeptide chains, isoelectric point, and degree of glycosylation. In addition, a variety of enzyme-producing species exist. Although all the differences may influence the characteristics of synthetic patterns, the basic mechanisms underlying enzyme synthesis are similar enough to allow a general treatment of the microbiological production process. However, the differences in production kinetics among various species are large enough to make individual optimisation programs necessary.

Different organisms may also differ in their suitability for fermentation; such process characteristics as viscosity or recoverability, legal clearance of the organism, and available knowledge about the selected organism, must be considered.

As cellular enzyme expression is heavily influenced by regulatory mechanism, enzyme synthesis rates range from no synthesis to maximum synthesis allowed by the synthetic apparatus, as in a normal control loop. The complexity of mechanisms ranges from relatively simple and well-understood induction and repression systems, to very complex global regulatory networks.

Process development must deal with the complexity of the enzyme synthesis system either by changing the structural characteristics, including structural elements of the regulatory systems (strain improvement; see also section 3.4), or by selecting optimal environmental conditions.

### 3.3.1.2 Media

Substrates for industrial applications are usually included in complex media, which must be supplemented with special compounds, such as a nitrogen source, various nutrient salts, or certain trace elements.

The main sources for microbial processes consist of sugars such as sucrose or hexoses. They represent the source for both carbon and energy. Typical feedstock materials are molasses, unrefined sugar, sulfite liquor from cellulose production plants, hydrolysates of wood and starch, or fruit juices, such as the grape juice used in wine making. In many cases starches from various cereals (barley, corn, rice, rye, and wheat) or tubers (potatoes) are used as inexpensive raw materials. Starch-containing substrates from wheat are mostly used in raw form, i.e. in the form of grist, and therefore contain a number of additional substances.

Nitrogen, phosphorus and potassium are important nutrients to maintain growth. They are added in the form of inorganic compounds such as phosphates, ammonium compounds, or potassium chloride. Soy meal, fish meal, cotton seed, low-quality protein materials such as casein or its hydrolysates, millet, stillage, and especially corn steep liquor are also used as low-price nutrients. In addition, these chemically complicated mixtures contain trace elements and growth promoters. A number of trace elements are provided in sufficient quantity by the tap water that is used for the preparation of the medium.

In general, the raw materials are dissolved or suspended in water and the resulting medium is heated, filtered and sterilized. The complex composition of the media used in industry causes considerable problems. For downstream processing (harvest, concentration, and purification of product) or for analytical assays during the process, additional pre-treatment of the raw material is needed to avoid unfavourable side effects.

### 3.3.1.3 Sterilization

Most bioprocesses for the production of enzymes are based on pure cultures. Because contamination would prevent proper functioning of the process, bioprocessing must be carried out under aseptic conditions.

Solid substrates, such as grist used for amylase production or treated soil used in mushroom cultivation, are kept in rooms at elevated temperatures for an appropriate amount of time. Liquid media are sterilized in situ, e. g. in the reaction vessel or in separate containers, usually by external heating. In some cases, media are prepared in a concentrated form and steam is injected directly into the solution, with the resulting condensate making up the final volume.

The temperature for sterilization is normally above 100 °C for an appropriate period. Flow sterilization through heat exchangers also applies temperatures far above 100 °C, but residence times are shorter than in batch sterilization. In general, the duration of the heat treatment does not only depend on the material to be processed but also on the pH of the medium and the initial number of viable germs or spores.

In aerobic processes the culture must be supplied with sterile air. Air is usually filtered through glass wool filters, sintered materials, or membranes of appropriate design.

### 3.3.1.4 Inoculation

After inoculation with the microorganisms, the process should start immediately and the reaction should proceed fast. The amount of active cell culture added is therefore critical and

depends on the size of the batch. Scaling-up from the original starter culture to the inoculation broth is done in several steps in large-scale industrial processes. The starter culture is kept deep frozen (-70 to -90 °C) for preservation.

In fungal inoculates proper wetting of the spores is achieved by adding small amounts of surfactants to the broth. If inoculation by spore suspensions is not optimal, mycelial pellets can be used for start-up. Bacterial spores must be activated by thermal treatment before they can be used for inoculation. During the exponential growth phase of the bioprocess cells can be harvested for following inoculations.

### 3.3.1.5 Fermentation

For enzyme production, economy of scale leads to the use of fermenters with a volume of 20-200 m<sup>3</sup>. The higher energy yield from aerobic combustion results in the use of aerobic processes which require continuous transfer of poorly soluble oxygen into the culture broth.

Process design is the complicated task of choosing the optimal conditions for maximal process outcome. The number of interdependent factors is high and the available physiological knowledge is seldom complete. The relationship between synthesis rate and growth rate could be very complex and depends on the presence of inductors or the absence of repressors. The use of genetically developed strains may considerably ease process design. The designed process is usually first tested on a pilot-plant scale and optimised in a number of fermentation runs; it is then scaled up to production size. The total synthesis rate depends not only on growth rate but also on biomass concentration.

Two types of cultivation can be distinguished:

In batch culture, the growth rate cannot be controlled by dosed feeding, because all substrates are added at the beginning. Batch processes are rarely used today.

In fed-batch processes, a low initial biomass concentration should be chosen to maintain the desired growth rate for a certain period, without exceeding the transport capacity of the equipment. In addition, fed-batch processes can be designed in a way that the enzyme concentration at harvesting is higher than in batch or continuous cultures, and the productivity of fed-batch processes can be increased several-fold compared to batch processes. Fed-batch processes probably constitute the most frequently used process type.

Continuous cultures are ideally suited for high productivity because the excess of biomass is continuously withdrawn, and both synthesis rate and biomass concentration will be optimal. Therefore, in principle, continuous culture is preferred for biotechnological production. However, enzyme concentrations are lower than those reached in fed-batch cultures. In addition, the use of continuous cultures is limited by technical reasons such as the higher contamination risk and the problem of strain degeneration.

### 3.3.2 Downstream processing

Downstream processing is a very important step in biotechnology because costs for collection, concentration and purification of the final product are substantial. High product concentrations in the supernatant or inside the cells and efficient purification are therefore important aspects in the overall economy of enzyme manufacture.

The degree of purity of commercial enzymes ranges from raw enzymes to highly purified forms and depends on the application. Raw materials for the isolation of enzymes are animal organs, plant material and microorganisms.

Often enzymes may be purified several hundred-fold but the yield of the enzyme may be very poor, frequently below 10% of the activity of the original material. In contrast, industrial en-

zymes will be purified as little as possible, only other enzymes and material likely to interfere with the process which the enzyme is to catalyse, will be removed. Unnecessary purification will be avoided as each additional stage is costly in terms of equipment, manpower and loss of enzyme activity. As a result, some commercial enzyme preparations consist essentially of concentrated fermentation broth, plus additives to stabilise the enzyme's activity.

However, the content of the required enzyme should be as high as possible (e. g. 10% w/w of the protein) in order to ease the downstream processing task. This may be achieved by developing the fermentation conditions or, often more dramatically, by genetic engineering. It may well be economically viable, e. g. to spend some time cloning extra copies of the required gene together with a powerful promoter back into the producing organism in order to get „over-producers”.

Downstream processing involves isolation and purification steps and ends up in the formulation of the enzyme preparation.

Enzymes are universally present in living organisms; each cell synthesizes a large number of different enzymes to maintain its metabolic reactions. The choice of procedures for enzyme purification depends on their location. On the one hand, isolation of intracellular enzymes often involves the separation of complex biological mixtures. On the other hand, extracellular enzymes are generally released into the medium with only a few other components. Enzymes are very complex proteins and their high degree of specificity as catalysts is manifest only in their native state. The native conformation is attained under specific conditions of pH, temperature, and ionic strength. Hence, only mild and specific methods can be used for enzyme isolation. Figure 2 shows the sequence of steps involved in the recovery of enzymes.

### **3.3.2.1 Preparation of biological starting materials**

**Animal Organs:** Animal organs must be transported and stored at low temperature to retain enzymatic activity. Frozen organs can be minced with machines generally used in the meat industry, and the enzymes can be extracted with a buffer solution. Besides mechanical grinding, enzymatic digestion can also be employed.

**Plant Material:** Plant material can be ground with various crushers or grinders, and the desired enzymes can be extracted with buffer solutions. The cells can also be disrupted by previous treatment with lytic enzymes.

**Microorganisms:** Enzymes might accumulate inside the cells or be released into the medium. Most enzymes used commercially are extracellular enzymes, and the first step in their isolation is the separation of the cells from the solution. For intracellular enzymes, which are being isolated today in increasing amounts, the first step involves grinding to rupture the cells. A number of methods for the disruption of cells are known, corresponding to the different types of cells and the problems involved in isolating intracellular enzymes. However, only a few of these methods are used on an industrial scale.

#### **Cell disruption by mechanical methods**

High-pressure homogenisation is the most common method of cell disruption. The cell suspension is pressed through a valve and hits an impact ring. The cells are ruptured by shearing forces and simultaneous decompression.

The wet grinding of cells in a high-speed bead mill is another effective method of cell disruption. Glass balls with a diameter of 0.2-1 mm are used to break the cells. The efficiency of this method depends on the geometry of the stirrer system.



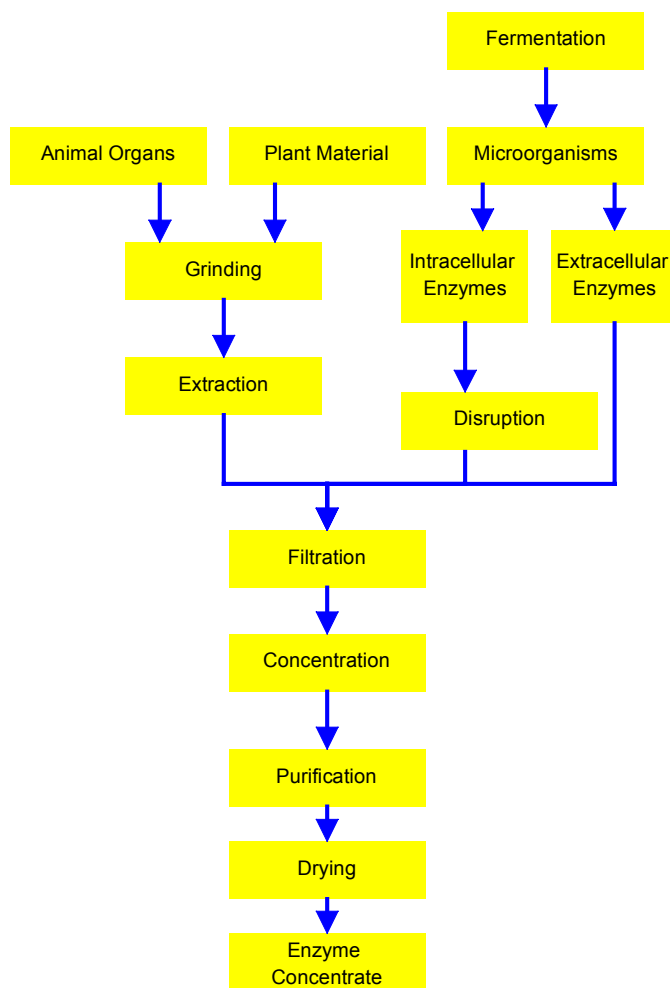


Figure 2: Flowchart of the downstream processing of enzymes

### Cell disruption by non-mechanical methods

Cells may frequently be disrupted by chemical, thermal, or enzymatic lysis. The drying of microorganisms and the preparation of acetone powders are standard procedures in which the structure of the cell wall is altered to permit subsequent extraction of the cell contents. Methods based on enzymes or autolysis have been described in the literature.

#### 3.3.2.2 Separation of solid matter

After cell disruption, the next step is separation of extracellular or intracellular enzymes from cells or cellular fragments, respectively. This operation is rather difficult because of the small size of bacterial cells and the slight difference between the density of the cells and that of the fermentation medium. Continuous filtration is used in industry. Large cells, e. g. yeast cells, can be removed by decantation. Today, efficient centrifuges have been developed to separate cells and cellular fragments in a continuous process. Residual plant and organ matter can be separated with simpler centrifuges or filters.

#### Filtration

**Pressure Filters:** A filter press (plate filter, chamber filter) is used to filtrate small volumes or to remove precipitates formed during purification. The capacity to retain solid matter is lim-

ited, and the method is rather work-intensive. However, these filters are highly suitable for the fine filtration of enzyme solutions.

**Vacuum Filters:** Vacuum filtration is generally the method of choice because biological materials are easily compressible. A rotary vacuum filter is used in the continuous filtration of large volumes. The suspension is usually mixed with a filter aid (e. g. kieselguhr) before being applied to the filter.

**Cross-flow Filtration:** In recent years, a new method of filtration, cross-flow filtration, has been devised. In conventional methods, the suspension flows perpendicular to the filtering material. In cross-flow filtration, the input stream flows parallel to the filter area, thus preventing the accumulation of filter cake and an increased resistance to filtration. Cross-flow filtration can be conveniently used in recombinant DNA techniques to separate organisms in a closed system.

### **Centrifugation**

The sedimentation rate of a bacterial cell with a diameter of 0.5  $\mu\text{m}$  is less than 1  $\mu\text{m}/\text{h}$ . An economical separation can be achieved only by sedimentation in a centrifugal field. The range of applications of centrifuges depends on the particle size and the content of the solids.

Decanters (scroll-type centrifuges) work with low centrifugal forces and are used in the separation of large cells or protein precipitates. Solid matter is discharged continuously by a screw conveyor moving at a differential rotational speed.

Tubular bowl centrifuges are built for very high centrifugal forces and can be used to sediment very small particles. However, these centrifuges cannot be operated in a continuous process. Moreover, solid matter must be removed by hand after the centrifuge has come to a stop. A further disadvantage is the appearance of aerosols.

Separators (disk stack centrifuges) can be used in the continuous removal of solid matter from suspensions. Solids are discharged by a hydraulically operated discharge port (intermittent discharge) or by an arrangement of nozzles (continuous discharge). Bacteria and cellular fragments can be separated by a combination of high centrifugal forces, up to 15,000  $\times$  gravity, presently attainable, and short sedimentation distances. Disk stack centrifuges that can be sterilized with steam are used for recombinant DNA techniques in a closed system.

### **Extraction**

An elegant method used to isolate intracellular enzymes is liquid-liquid extraction in an aqueous two-phase system. This method is based on the incomplete mixing of different polymers, e. g. dextran and poly(ethylene glycol), or a polymer and a salt in an aqueous solution. The first extraction step separates cellular fragments. Subsequent purification can be accomplished by extraction or, if high purity is required, by other methods. The extractability can be improved by using affinity ligands or modified chromatography gels, e. g., phenyl-Sepharose.

### **Flocculation and flotation**

The flocculation of bacterial cells to form larger particles can be achieved by the addition of mineral colloids, salts, or organic polymers. The neutralization of charges on the cell surface and the formation of bridges between individual cells lead to agglomeration. Agglomerates can then be removed by filtration or centrifugation.

#### **3.3.2.3 Concentration**

The enzyme concentration in starting material is often very low. The volume of material to be processed is generally very large, and substantial amounts of waste material must be removed. Thus, if economic purification is to be achieved, the volume of starting material must

be decreased by concentration. Only mild concentration procedures which do not inactivate enzymes can be employed. These procedures include thermal methods, precipitation, and to an increasing extent, membrane filtration.

### **Thermal methods**

Only brief heat treatment can be used for concentration because enzymes are thermolabile. Evaporators with rotating components which achieve a thin liquid film (thin-layer evaporator, centrifugal thin-layer evaporator) or circulation evaporators (long-tube evaporator) can be employed.

### **Precipitation**

Enzymes are very complex protein molecules possessing both ionisable and hydrophobic groups which interact with the solvent. Indeed, proteins can be made to agglomerate and, finally, precipitate by changing their environment. Precipitation is actually a simple procedure for concentrating enzymes.

**Precipitation with Salts:** High salt concentrations act on the water molecules surrounding the protein and change the electrostatic forces responsible for solubility. Ammonium sulfate is commonly used for precipitation; hence, it is an effective agent for concentrating enzymes. Enzymes can also be fractionated, to a limited extent, by using different concentrations of ammonium sulfate. Sodium sulfate is another precipitating agent used.

**Precipitation with Organic Solvents:** Organic solvents influence the solubility of enzymes by reducing the dielectric constant of the medium. The solvation effect of water molecules surrounding the enzyme is changed; the interaction of protein molecules is increased; and therefore, agglomeration and precipitation occur. Commonly used solvents are ethanol and acetone.

**Precipitation with Polymers:** The polymers generally used are polyethylenimines and polyethylene glycols of different molecular masses. The mechanism of this precipitation is similar to that of organic solvents and results from a change in the solvation effect of the water molecules surrounding the enzyme. Most enzymes precipitate at polymer concentrations ranging from 15 to 20 %.

**Precipitation at the Isoelectric Point:** Proteins are ampholytes and carry both acidic and basic groups. The solubility of proteins is markedly influenced by pH and is minimal at the isoelectric point at which the net charge is zero. Because most proteins have isoelectric points in the acidic range, this process is also called acid precipitation.

### **Ultrafiltration**

A semipermeable membrane permits the separation of solvent molecules from larger enzyme molecules, because only the smaller molecules can penetrate the membrane when the osmotic pressure is exceeded. This is the principle of all membrane separation processes including ultrafiltration. In reverse osmosis, used to separate materials with low molecular mass, solubility and diffusion phenomena influence the process, whereas ultrafiltration and cross-flow filtration are based solely on the sieve effect. In processing enzymes, cross-flow filtration is used to harvest cells, whereas ultrafiltration is employed for concentrating and desalting.

**Desalting:** The desalting of enzyme solutions can be carried out conveniently by diafiltration. The small salt molecules are driven through a membrane with the water molecules. The permeate is continuously replaced by fresh water.

### 3.3.2.4 Purification

For many industrial applications, partially purified enzyme preparations will suffice; however, enzymes for analytical purposes and for medical use must be highly purified. Special procedures employed for enzyme purification are crystallization, electrophoresis, and chromatography. However crystallization and electrophoresis are not relevant for large scale purifications. Chromatography, in contrast, is of fundamental importance to enzyme purification. Molecules are separated according to their physical properties (size, shape, charge, hydrophobic interactions), chemical properties (covalent binding), or biological properties (bio-specific affinity).

In gel chromatography (also called gel filtration), hydrophilic, cross-linked gels with pores of finite size are used in columns to separate biomolecules. In gel filtration, molecules are separated according to size and shape. Molecules larger than the largest pores in the gel beads, i.e. above the exclusion limit, cannot enter the gel and are eluted first. Smaller molecules, which enter the gel beads to varying extent depending on their size and shape, are retarded in their passage through the column and eluted in order of decreasing molecular mass. Gel filtration is used commercially for both separation and desalting of enzyme solutions.

Ion-exchange chromatography is a separation technique based on the charge of protein molecules. Enzyme molecules possess positive and negative charges. The net charge is influenced by pH, and this property is used to separate proteins by chromatography on anion exchangers (positively charged) or cation exchangers (negatively charged). The ability to process large volumes and the elution of dilute sample components in concentrated form make ion exchange very useful.

For hydrophobic chromatography, media derived from the reaction of CNBr-activated Sepharose with aminoalkanes of varying chain length are suitable. This method is based on the interaction of hydrophobic areas of protein molecules with hydrophobic groups on the matrix. Adsorption occurs at high salt concentrations, and fractionation of bound substances is achieved by eluting with a negative salt gradient. This method is ideally suited for further purification of enzymes after concentration by precipitation with such salts as ammonium sulfate.

In affinity chromatography the enzyme to be purified is specifically and reversibly adsorbed on an effector attached to an insoluble support matrix. Suitable effectors are substrate analogues, enzyme inhibitors, dyes, metal chelates, or antibodies. The insoluble matrix is contained in a column. The biospecific effector, e. g. an enzyme inhibitor, is attached to the matrix. A mixture of different enzymes is applied to the column. The immobilized effector specifically binds the complementary enzyme. Unbound substances are washed out and the enzyme of interest is recovered by changing the experimental conditions, for example by altering pH or ionic strength.

Immunoaffinity chromatography occupies a unique place in purification technology. In this procedure, monoclonal antibodies are used as effectors. Hence, the isolation of a specific substance from a complex biological mixture in one step is possible. In this procedure, enzymes can be purified by immobilizing antibodies specific for the desired enzyme. A more general method offers the synthesis of a fusion protein with protein A by „protein engineering.“ Protein A is a Staphylococcus protein with a high affinity for many immunoglobulins, especially of the IgG class of antibodies. In this way, enzymes that usually do not bind to an antibody can be purified by immunoaffinity chromatography.

Covalent chromatography differs from other types of chromatography by forming a covalent bond between the required protein and the stationary phases.

### 3.3.3 Formulation of the final enzyme product

Once the enzyme has been purified to the desired extent and concentrated, the manufacturer's main objective is to retain the activity. Enzymes for industrial use are sold on the basis of overall activity. Often a freshly supplied enzyme sample will have a higher activity than that stated by the manufacturer. This is done to ensure that the enzyme preparation has the guaranteed storage life. The manufacturer will usually recommend storage conditions and quote the expected rate of loss of activity under those conditions. It is of primary importance to the enzyme producer and customer that the enzymes retain their activity during storage and use. Some enzymes retain their activity under operational conditions for weeks or even months. However, most enzymes do not.

Most industrial enzyme preparations contain a relatively little amount of active enzyme (see section 3.4), the rest being due to inactive protein, stabilisers, preservatives, salts and the diluent which allows standardisation between production batches of different specific activities.

The key to maintaining enzyme activity is maintenance of conformation, so preventing unfolding aggregation of the enzyme molecules and changes in the covalent structure. Three approaches are possible:

- use of additives
- the controlled use of covalent modification
- enzyme immobilisation.

In general, proteins are stabilised by increasing their concentration and the ionic strength of their environment. Neutral salts compete with proteins for water and bind to charged groups or dipoles. This may result in the interactions between an enzyme's hydrophobic areas being strengthened causing the enzyme molecules to compress and making them more resistant to thermal unfolding reactions. Not all salts are equally effective in stabilising hydrophobic interactions, some are much more effective at their destabilisation by binding to them and disrupting the localised structure of water (the chaotropic effect). From this it can be seen why ammonium sulphate and potassium hydrogen phosphate are powerful enzyme stabilisers whereas sodium thiosulphate and calcium chloride destabilise enzymes. Many enzymes are specifically stabilised by low concentrations of cations which may or may not form part of the active site, for example  $\text{Ca}^{2+}$  stabilises  $\alpha$ -amylases and  $\text{Co}^{2+}$  stabilises glucose isomerases.

At high concentrations (e. g. 20% NaCl) salt discourages microbial growth due to its osmotic effect. In addition, ions can offer some protection against oxidation to groups such as thiols by salting-out the dissolved oxygen from solution.

Low molecular weight polyols (e. g. glycerol, sorbitol and mannitol) are also useful for stabilising enzymes, by repressing microbial growth due to the reduction in the water activity, and by the formation of protective shells which prevent unfolding processes. Glycerol may be used to protect enzymes against denaturation due to ice-crystal formation at sub-zero temperatures. Some hydrophilic polymers (e. g. polyvinyl alcohol, polyvinylpyrrolidone and hydroxypropylcelluloses) stabilise enzymes by a process of compartmentalisation, whereby the enzyme-enzyme and the enzyme-water interactions are somewhat replaced by less potentially denaturing enzyme-polymer interactions. They may also act by stabilising the hydrophobic effect within the enzymes.

Many specific chemical modifications of amino acid side chains are possible which may (or, more commonly, may not) result in stabilisation. A useful example of this is the derivatization of lysine side chains in proteases with N-carboxyamino acid anhydrides. These derivatization result in polyaminoacylated enzymes with various degrees of substitution and length

of amide-linked side chains. This derivatization is sufficient to disguise the proteinaceous nature of the protease and prevent autolysis.

Enzymes are much more stable in the dry state than in solution. Solid enzyme preparations sometimes consist of freeze-dried protein. More usually they are bulked out with inert materials such as starch, lactose, carboxymethylcellulose and other poly-electrolytes which protect the enzyme during a cheaper spray-drying stage.

Other materials which are added to enzymes before sale may consist of substrates, thiols to create a reducing environment, antibiotics, benzoic acid esters as preservatives for liquid enzyme preparations, inhibitors of contaminating enzyme activities and chelating agents. Additives of these types must, of course, be compatible with the final use of the enzyme's product.

In order to ensure safe handling, stability, suitable mixing, functionality etc. in the various applications, most enzyme preparations are formulated in a variety of liquid and granular forms. Some enzyme preparations are immobilised. Often the precise details of the methods used to stabilise enzyme preparations are kept secret or revealed to customers as a confidential information only.

### 3.4 The nature of enzyme products

The nature of enzyme products is influenced by (i) the enzyme itself and its properties „as active compound“, (ii) the enzyme source, the fermentation media and conditions and the purification steps (resulting in the „enzyme concentrate“), (iii) the additives used to formulate the final „enzyme preparation“. Thus, one has to differentiate between „enzyme“, „enzyme concentrate“, and „enzyme preparation“. Table 1 describes the terminology that AMFEP (AMFEP's Position on Roster of Questions, 2002-02-27/PE) is using for enzymes.

*Table 1: Terminology used in enzyme manufacturing*

Nomenclature	Description
Enzyme	A pure protein with a specified activity, on basis of which the enzyme is identified. The activity has an IUB and a CAS number. „Enzyme“ may also be referred to as „active enzyme“ or „active enzyme protein“.
Enzyme concentrate	An enzyme-containing mixture in its most concentrated form as it occurs during commercial production, thus before formulation. Enzyme concentrates are not commercially available as such, but some may be used directly by the producer as a processing aid for food or to synthesize certain chemicals (i. e. „captive use“). „Enzyme concentrate“ may also be referred to as „enzyme isolate“.  The enzyme concentrate is measured as Total Organic Substance (TOS; $TOS = 100\% - (Water + Ash + Diluents)$ ).
Enzyme formulation	An enzyme concentrate, diluted to a standardised activity, stabilized and commercially available on the market.
Enzyme blend	A formulation of an intended mixture of enzyme concentrates originating from different sources.
Enzyme preparation	Enzyme formulation or enzyme blend.
Declared enzyme	The enzyme component(s) in the preparation of significance in terms of application and regulation.

Enzyme preparations vary considerably in terms of purity and formulation (additives) among companies and depending on the particular application. Most enzyme applications do not re-

quire high enzyme concentration of the isolate or the preparation. A look on the range of main components of industrial enzyme preparations (Table 2) reveals, that the contents of active enzyme protein in the final enzyme preparation is usually very low, typically in the order of 1 - 5%. Sugars and inorganic salts are used for stabilising the finished product for storage and distribution. Salts and sometimes carbohydrates such as starch, maltodextrins and sugar alcohols are used to dilute extracted enzymes (the enzyme concentrate) to a standard activity. Preservatives are generally restricted to use in liquid preparations. Thus, by-products from the fermentation process (other proteins, carbohydrates, sugars, salts), additives and preservatives comprise up to 99% of the enzyme preparation. Manufacturing costs are not the only issue, in some cases limited purity and concentration are desirable in terms of formulation of the preparation, improving stability/performance, facilitating homogeneous blending etc. The increasing use of GMM and new recovery techniques have generally increased purity and amount of enzyme present in the enzyme concentrate.

*Table 2: Typical range of main components of industrial enzymes in enzyme preparations. Source: GODFREY & WEST (1996)*

<b>Component</b>	<b>Range of content (% dry solids)</b>
Proteins and amino acids	10-15
Active enzyme protein	1-5
Complex carbohydrates	5-12
Sugars (and sugar alcohols)	2-20
Inorganic salts	3-40
Preservatives	0-0.3

Table 3 gives an overview on the typical ranges of enzyme concentrations in enzyme concentrate and enzyme preparation for different applications. The ranges for technical, food and feed enzymes largely correspond with the typical ratios published in a study on detergent enzymes (2 - 10%; SPÖK et al., 1998) and the ratio given in Table 2 (1 - 5%).

*Table 3: Typical range of active enzyme contents in enzyme isolate and preparation*

<b>Application</b>	<b>aep/TOS</b>	<b>TOS/enzyme preparation</b>	<b>aep/enzyme preparation</b>
Technical, Food, and Feed enzymes	25-75%	2-20%	0.5-15%
Personal Care, Therapeutic and Analytical/Diagnostic application <sup>a</sup>	50-100%	10-50%	5.0-50%

*Source: AMFEP's answers to Roster of Questions not related to individual enzymes (2); DRAFT/2001-06-06/PE; Comments to 'Governing Remarks'. <sup>a</sup>...approximate range, limited range within AMFEP; aep... active enzyme protein; TOS...total organic solids*

Only in case that the active enzyme present in a concentrate reaches a purity of 80% or higher (w/w on basis of dry matter), it becomes possible to determine the purity fairly accurate even if the specific activity is unknown. This can be done by measuring the protein content in the concentrate in combination with the relative amount of the enzyme protein in a SDS-PAGE profile. For less pure products, this method does not result in accurate figures.

Based on the limited data presently available, it can be stated that the percentage of active enzyme in enzyme concentrates may range between 2% and 70% (w/w on basis of dry matter) (AMFEP's Position on Roster of Questions, 2002-02-27/PE).

### 3.4.1 The enzyme concentrate

Impurities in the enzyme isolate/concentrate result from the fermentation broth and subsequent purification and consist of proteins, peptides and amino acids, carbohydrates, minerals and other minor components. The relative amounts of these components vary considerably within and between categories of enzyme concentrates. Enzyme content and purity is similar for technical, food and feed enzymes. Enzymes used in personal care products, for therapeutic and analytical or diagnostic application may generally be of higher purity and concentration. Ash constituents comprise small amounts of minerals and diluents are additives for granulation, liquid formulation, stabilisation, preservation etc. Table 4 and Table 5 show the approximate ranges of impurities as stated by AMFEP.

*Table 4: Approximate ranges of impurities for technical, food, and feed enzymes*

Fraction	Range
Proteins, peptides and amino acids	15-50%
Carbohydrates (excluding enzyme glycosylation)	10-20%
Minerals and other minor components	0-15%

*Source: AMFEP's answers to Roster of Questions not related to individual enzymes (2); DRAFT/2001-06-06/PE; Comments to 'Governing Remarks'.*

*Table 5: Approximate ranges of impurities of enzymes for personal care, therapeutic and analytical/diagnostic application*

Fraction	Range
Proteins, peptides and amino acids:	0-40%
Carbohydrates, minerals, other:	0-10%

*Source: AMFEP's answers to Roster of Questions not related to individual enzymes (2); DRAFT/2001-06-06/PE; Comments to 'Governing Remarks'.*

Enzymes used for food and feed have to comply with purity specifications comprising limits for heavy metals and contaminating microorganisms and absence of mycotoxins, antibiotics, and the production strain.

The type and range of impurities, which are present in the enzyme concentrate, is highly varying and depend on the production strain, the media used, the fermentation conditions and the subsequent purification steps as outlined in section 3.3.2. Some of the impurities may also fulfil a technical function during enzyme application. This is particularly true for side activities.

An enzyme concentrate will typically contain other enzymes usually referred to as „side activities„ (not to be confused with „side activities“ of a particular enzyme protein). The type and range of these side activities are largely depending on the enzyme manufacturing conditions and the production strain.

These side activities may be more or less important in any given application. An example of this is the use of amylases in baking. Fungal alpha-amylase is added to dough as part of a „flour improver“. The objective is to hydrolyse starch to provide more fermentable sugars for the yeast. Different enzymes, however, result in different effects on the rheology of the dough and the final product quality. The reason for this is that the enzyme preparation contains a side activity of endo-beta-xylanase which cleaves the hemicellulose in the flour. This enzyme is now manufactured solely for this application.



Side activities can partly be inactivated during the enzyme manufacturing process. Nevertheless, it is quite normal to find a variety of different activities in an industrial enzyme preparation. If a preparation is marketed as a protease, e. g. glycosidases and lipases might be present as well.

#### **3.4.1.1 Factors affecting the purity of enzyme concentrates**

The purity of the enzyme concentrate is largely influenced by (i) the fermentation and purification processing applied, (ii) the production organism used, and (iii) the media used for fermentation. Whereas fermentation and purification processing is described elsewhere in this report (section 3.3) the importance of the production strain chosen and the media will be considered in the following.

##### **Production organisms**

The particular production strain is obviously affecting the nature as well as the range of by-products present in the enzyme concentrate. As microorganisms are known to produce toxins, the production organisms may themselves be sources of hazardous materials and have therefore been a chief focus of attention by the regulatory authorities. Some of these toxins are quite well known. However, there is always the possibility of introducing new toxins. Therefore, production strains which are investigated not to produce toxins and which do have a long history of safe use are preferred.

##### **Media for enzyme production**

Media used have a bearing on the cost of the enzyme and media components often find their way into commercial enzyme preparations. Details of components used in industrial scale fermentation broths for enzyme production are not readily obtained. Not surprisingly, as manufacturers do not wish to reveal information that may be of technical or commercial value to their competitors. Also some components of media may be changed from batch to batch as availability and cost of e. g. carbohydrate feedstock change. Such changes reveal themselves in often quite profound differences in appearance from batch to batch of a single enzyme from a single producer. The effects of changing feedstock must be considered in relation to downstream processing. If such variability is likely to reduce the efficiency of the standard methodology significantly, it might be economical to use a more expensive defined medium of easily reproducible composition.

Clearly defined media are usually out of question for large scale use on cost grounds but may be perfectly acceptable when enzymes are to be produced for high value uses, such as analysis or medical therapy where very pure preparations are essential. Less-defined complex media are composed of ingredients selected on the basis of cost and availability as well as composition. Waste materials and by-products from the food and agricultural industries are often major ingredients. Thus, molasses, corn steep liquor, distillers solubles, and wheat bran are important components of fermentation media providing carbohydrate, minerals, nitrogen and some vitamins. Extra carbohydrate is usually supplied as starch, sometimes refined but often simply as ground cereal grains. Soybean meal and ammonium salts are frequently used sources of additional nitrogen. Most of these materials will vary in quality and composition from batch to batch causing changes in enzyme productivity.

#### **3.4.2 Enzyme preparations**

Enzyme preparations contain a varying amount of additives for the purpose of stabilising the enzyme activity, preservation, granulation, coating or as (de)colouring aids.

The choice of formulation ingredients (additives) is not specific for a given application category, but certain substances used for e. g. technical enzymes may not comply with food, feed and cosmetics regulations, specifications etc. Some applications may require special substances and technologies, e. g. in the case of immobilised enzymes or enteric coating of digestive aid preparations.

AMFEP (AMFEP's Position on Roster of Questions, 2002-02-27/PE) only outlined typical kind of additives and their functionalities (Table 6) as product formulation often comprises company specific and proprietary information.

*Table 6: Formulation additives used in enzyme preparations*

<b>Type of formulation</b>	<b>Type of additive (typical examples)</b>	<b>Purpose</b>	<b>Range (%)</b>
Liquid/slurry	Sucrose, sorbitol, glycerol, propylene glycol	Stabiliser	20-50%
Liquid/slurry	Ethoxylated fatty alcohols (technical products only)	Stabiliser	20-40%
Liquid/slurry	Sodium chloride	Stabiliser	10-20%
Liquid/slurry	Sodium benzoate, potassium benzoate, BIT (isothiazoline derivative, technical products only)	Preservative	0.1-0.5%
Granular	Inorganic salts, e. g. sodium chloride, sulphate, calcium carbonate	Stabilising, granulation	50-75%
Granular	Cellulose, dextrin, sucrose	Stabilising, granulation, coating	10-75%
Granular	Kaolin, titanium dioxide (Technical products only)	(De)colouring	0-20%
Granular	Polyethylene glycol (all PEG variants, Technical products only)	Stabilising, granulation, coating	0-10%
Granular	Vegetable oil (e. g. hydrogenated palm oil)	Stabilising, coating	5-15%

*Source: AMFEP's Position on Roster of Questions, 2002-02-27/PE.*

Enzymes going to be introduced to the market should conform to a number of quality procedures including regulatory requirements. This is provided by the quality assurance within the company. Enzyme products must be consistent as appropriate to their intended use. This may be ensured by good manufacturing practice (GMP) and further checked by quality control.

Additives used in the manufacturing of food enzymes must e. g. be of food grade. In the context of EU chemical legislation, additives have to be listed on EINECS or already notified.

Parameters applicable for the description of (i) the active enzyme protein, (ii) by-products and impurities accompanying the enzyme concentrate, and (iii) additives used to formulate the final enzyme preparation are outlined in chapter 9, followed by a comparison of the regulatory practice.

## 3.5 Recent developments in enzyme manufacturing

### 3.5.1 Introduction

The exploitation of new types of enzymes, improvements of enzyme properties and of the production process<sup>7</sup> are overall goals of innovation in the enzyme manufacturing industry.

Although considerable improvements had been made in process engineering, large scale fermentation and downstream purification of enzymes remained to be a significant bottleneck in innovation. Until the mid 1980s, screening for interesting enzymes was mainly confined to groups of organisms which promised a realistic chance of developing a cost efficient production process. Microorganisms which are adapted to extreme environments and therefore, are difficult to be accessed and cultured, but which are producing enzymes with promising properties, could not be exploited.

Moreover, strain improvement in general as well as of enzymes properties in particular were largely restricted to statistical methods such as induced mutagenesis. These methods are both time-consuming and undirected.

Only when genetic engineering techniques was introduced into routine research and development of enzyme manufacturing companies a promising tool became available to circumvent these problems. The introduction of genetic engineering is most probably the most important breakthrough in enzyme manufacturing during the last 30 years. Much progress have been made since the first enzyme from genetically modified microorganisms (GMM) was introduced onto the market in 1987 (SPÖK et al, 1998).

This section will therefore focus on the state of the art in genetic engineering techniques applied in the improvement of both production strains and enzyme properties. Furthermore, some related technologies such as chemical modification of enzymes will be briefly described. Finally, potential impacts for enzyme regulation of the widespread application of this technology in enzyme manufacturing process will be discussed.

### 3.5.2 Genetic engineering

From the perspective of an enzyme-producing company, genetic engineering serves as a core technology which is offering the following advantages (MENRAD et al., 1999: 289):

- The exploitation of new types of enzymes and new source organisms, even enzymes from organisms which are difficult to handle or non-culturable (e. g. extremophiles).
- Drastic shortening of development times from screening to marketing.
- Significant cost reductions in the development and production process of enzymes .
- Genetic engineering is a prerequisite for the optimisation of enzyme molecular properties by protein engineering.
- Improved product safety and fewer production risks, due to the production of enzymes from a wide variety of different source organisms in a small number of well-characterised enzyme production organisms.

Table 7 gives an overview how these advantages are achieved by genetic engineering techniques. Important techniques and most recent trends are briefly described in the following section.

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<sup>7</sup> The rapid growth of biocatalysis is also sparked by efforts to engineer the (micro)environments of enzymes (e. g. non-aqueous environments). However, this is not being dealt with in the course of this study.

Table 7: Contribution of genetic engineering techniques to the goals of innovation in enzyme manufacturing

Goals of innovation	Technical approaches due to genetic engineering
Reduction of manufacturing costs	Increase of enzyme yield by <i>increasing enzyme expression</i> in the production organism. No need for a de-novo design of a production process for a new production organisms. Instead, the enzyme gene of interest is cloned into a well known production strain.
New enzymes	Increase of accessibility of new enzymes especially from extremophiles. Isolation of the respective genes and expression in known production strains.
Improved enzyme properties	Rational protein engineering / directed molecular evolution.
Improved product safety	Use of well-characterised production strains instead of new less characterised strains and sometimes strains that might be less safe.

### 3.5.2.1 Increasing enzyme expression

Before the era of genetic engineering, an increase in enzyme yield could only be attempted by traditional strain selection from natural sources or by random mutagenesis induced by mutagenic agents.

Using genetic engineering techniques enzyme yield could be further increased dramatically. This increase is made technically feasible either by *multiplying the enzyme gene* or by constructing an *artificial expression system*. Both approaches aim at increasing the transcription/translation of the enzyme gene(s) into proteins on the cellular level.

Multiplying enzymes genes could be done just by amplifying the number of copies of the enzyme gene in the source organisms thereby not necessarily ending up in a GMM as defined in by the regulator.<sup>8</sup> Alternatively, the gene could be isolated from the source organism, cloned onto a plasmid which is then introduced in a production strain. Whereas, normally only one set of genomic genes (two sets in the case of diploid organisms) is present in a microorganism, up to 1000 and more copies of plasmids and consequently of plasmid related enzyme genes could be present in one cell.

The continuous improvement of artificial expression systems is sparked by the fast growing knowledge on regulatory DNA sequences. Strong promoters, enhancing sequences and efficient terminators often derived from different organisms are used in order to enhance the transcription rate of the enzyme gene. Frequently, ribosome binding sites are also optimised in order to increase the translation rate. However, this approach could lead to changes in the amino acid sequence of the enzyme protein.

Applying these techniques enzyme yield could be enormously increased. E. g. heterologous expression of a xylanase in *E. coli* lead to an accumulation of the enzymes up to 70% of total

<sup>8</sup> „Self-cloning consisting in the removal of nucleic acid sequences from a cell of an organism which may or may not be followed by reinsertion of all or part of that nucleic acid (or a synthetic equivalent) with or without prior enzymic or mechanical steps, into cells of the same species or into cells of phylogenetically closely related species which can exchange genetic material by natural physiological processes where the resulting micro-organism is unlikely to cause disease to humans, animals or plants.” (Council Directive 98/81/EC of 26 October 1998 amending Directive 90/219/EEC on the contained use of genetically modified micro-organisms. Official Journal of the European Communities - 5.12.1998 - No L 330 P. 0013 - 0031, Annex II, Part A: Techniques or methods of genetic modification yielding micro-organisms to be excluded from the Directive)

cell protein (KULKARNI & SHENDYE, 1999). An increase in enzyme yield of 3 to 50 was reported in several studies. In case of a yeast  $\beta$ -glucosidase a factor of 300 was achieved (RUTTLOFF, 1994).

### 3.5.2.2 New enzymes

Until the mid 1980 the access to new microbial enzymes was largely depended on to the availability of new source organisms which could be used in a production process. Of course the variety of existing microorganisms is huge and it was estimated that probably much less than 1% of all microorganisms are cultured until present (AMANN et al., 1996; PACE, 1997 according to ADAMS & KELLY, 1998). However, this approach is not only time consuming but often turns out not to be feasible in practice because of economic reasons or technical problems that cannot be hurdled. Consequently, the range of enzymes which are accessible for large scale processing was considerably narrow.

Again it was genetic engineering which enabled the manufacturer to tackle this problem. Using these techniques the gene for the enzyme of interest can be isolated and introduced into a well known production organism. Consequently, this has led to an enormous increase in the availability of interesting enzymes: every organism which could be cultivated in the lab was accessible at this point. In combination with polymerase chain reaction techniques (PCR), almost every enzyme in nature – and recently also those from non-cultivable organisms – are accessible and thereby exploitable (DALBØGE & LANGE, 1998). Screening and characterisation of new and potentially interesting enzymes is a big issue within industry: According to DALBØGE & LANGE (1998) between 1993 and 1997 more than 130 fungal enzyme genes have been cloned at Novozymes alone.

Lipolase, a lipase from Novozymes is a well known example. This enzyme was originally produced by *Humicola lanuginosa*. Though, a large scale process using this strain as production organism was not economically feasible. Consequently, the lipase gene was isolated and expressed in *Aspergillus oryzae*. Lipolase is presently being manufactured as a bulk enzyme mainly for use in detergents.

The availability of this technique and the increasing knowledge on microorganisms living in extreme habitats (extremophiles) also sparked the interest on enzymes from these organisms. Adapted to such extreme conditions as Arctic sea, glaciers, hot springs or smokers in the deep sea, these organisms must have enzymes that work under these extreme conditions (extremozymes). As the properties of these enzymes are considerably different to those marketed so far, extremozymes are of considerable importance for the industry.

Table 8 shows an overview on enzymes from extremophiles and their industrial importance. However, as thermophiles, extreme thermophiles and psychrophiles are the far most important organisms these organisms will be dealt with in more detail.

**Table 8: Industrial applications of enzymes isolated from extremophiles. Source: DEMIRJIAN et al. (2001)**

Extremophile	Habitat	Enzymes	Representative applications
Thermophile	High temperature	Amylases	Glucose, fructose for sweeteners
	Moderate thermophiles (45–65°C)	Xylanases	Paper bleaching
	Thermophiles (65–85°C)	Proteases	Baking, brewing, detergents
	Hyperthermophiles (<85°C)	DNA polymerases	Genetic engineering
Psychrophile	Low temperature	Proteases	Cheese maturation, dairy production
		Dehydrogenases	Biosensors
		Amylases	Polymer degradation in detergents
Acidophile	Low pH	Sulfur oxidation	Desulfurization of coal
		Chalcopyrite concentrate	Valuable metals recovery
Alkalophile	High pH	Cellulases	Polymer degradation in detergents
Halophile	High salt concentration		Ion exchange resin regenerant disposal, producing poly( $\gamma$ -glutamic acid) (PGA) and poly( $\beta$ -hydroxy butyric acid) (PHB)
Piezophile	High pressure	Whole microorganism	Formation of gels and starch granules
Metalophile	High metal concentration	Whole microorganism	Ore-bioleaching, bioremediation, biomineralization
Radiophile	High radiation levels	Whole microorganism	Bioremediation of radionuclide contaminated sites
Microaerophile	Growth in <21% O <sub>2</sub>		

### Thermophilic enzymes

These enzymes are well adjusted to temperature above 50°C. Table 9 lists some thermophilic enzymes. As perhaps an outstanding example amylase from *Pyrococcus furiosus* is one of the most stable enzymes presently known with significant half-life at 130°C (DEMIRJIAN et al., 2001). Whether this already represents the upper limits of thermal stability, remains however a question to be solved. Physical limits seem to be reached around 250°C. At this temperature, peptide bond hydrolysis reactions rapidly occur in (denatured) proteins (DANIEL, 1996). For comparison, the temperature optima of „normal“ mesophilic enzymes are in the range of 30° to 40°C with denaturation starting around 50° to 60°C. Interestingly, it could be shown that resistance to high temperature is often accompanied with resistance to proteases, chaotropic agents, low pH, oxidation and high salt concentration.

The high stability of enzymes from extreme thermophiles generally resides in the amino acid sequence and three-dimensional structure. This is shown by stability which is retained upon purification and when genes for stable enzymes are expressed in mesophiles (TANAKA et al., 1981; PATCHETT et al., 1989). A few proteins might also require posttranslational modification to become fully thermostable. Up to present – this means during the last 5 years – more than 100 genes from hyperthermophiles have been cloned and expressed in mesophiles (VIEILLE & ZEIKUS, 2001).

As a consequence, these enzymes are highly attractive for biotechnological applications, such as starch processing, leaching of low grade ores, sugar conversion, detergents, or as a tool for molecular biology (PCR) – as these processes are carried out at elevated temperatures.

### Psychrophilic bacteria

These group of organisms optimally grow at or below 15°C, having an upper limit of growth of about 20°C, and a lower limit of growth of 0°C or below. Psychrophilic enzymes have a high specific activity at low and moderate temperatures, and are inactivated easily by a moderate increase in temperature. Typically, the specific activity of these cold adapted enzymes is higher than that of their mesophilic counterparts at temperatures of approximately 0 - 30°C. This increased activity is accompanied by lower thermal stability (GERDAY et al., 2000).

These properties could be of interest for various applications. According to (GERDAY et al., 2000) cold-adapted cellulase could be used in biopolishing and stone-washing of cotton

garments. In fabric production, tissues often have cotton fibre ends protruding from the main fibres which reduce smoothness and alter the appearance of the garment. Treatment with cellulases could excise protruding ends. The current treatment, however, is accompanied by a loss of mechanical resistance. A cold-adapted enzyme would enable the decrease of processing temperature and rapid inactivation as a result of thermal liability would be possible. The mechanical resistance of the final product would also be improved as a result of rapid inactivation of the enzyme.

In the beverage industry, pectinases are added in the juice extraction process in order to reduce viscosity and to clarify the final product. In the meat industry, proteases are applied to tenderise meat, and in baking processes amylases, proteases and xylanases are used to reduce the dough fermentation time. The use of psychrophilic enzymes in all these processes could be advantageous not only because of their high specific activity, thereby reducing the amount of enzyme needed, but also for their easy inactivation<sup>9</sup>. Psychrophilic enzymes could therefore become interesting alternatives to mesophilic enzymes in brewing and wine industries, cheese manufacturing, animal feed and other applications.

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<sup>9</sup> An easy inactivation method of enzymes used in food processing is important because the time period of catalytic activity could be easily controlled. Psychrophilic enzymes could be easily terminated for example by moderate heat treatment thereby keeping heat sensitive ingredients in the food stuff. This may also be important if enzymes should not remain functional in the final product because of security reasons.

Table 9: Biocatalytically relevant extremozymes. Source: DEMIRJIAN et al. (2001)

Enzyme	Organism	Host/induction	Stability/activity
Hyperthermophilic esterase	<i>Pyrococcus furiosus</i>	<i>E. coli</i> /heterologous (own promoter)	T opt = 100°C t <sub>1/2</sub> = 50 min at 126°C
Thermophilic esterase	<i>Bacillus licheniformis</i>	<i>E. coli</i>	T opt = 45°C t <sub>1/2</sub> = 1 hr at 64°C
Thermophilic esterase	<i>Bacillus acidocaldarius</i>	<i>E. coli</i>	Active at 70°C
Thermophilic esterase	<i>Archaeoglobus fulgidus</i>	<i>E. coli</i>	Active at 70°C
Thermophilic lipase	<i>Bacillus Stearothermophilus</i>	<i>E. coli</i> /IPTG	T opt = 68°C stable 30 min at 55°C
Thermophilic lipase	<i>Bacillus thermocatenulatus</i>	<i>E. coli</i> DH5α/pUC18	T opt = 60–70°C
Psychrophilic lipase	<i>Moraxella</i> TA144	<i>E. coli</i> /pULG	T opt = 35°C*, 45°C† stable only as whole cell
Psychrophilic lipase	<i>Pseudomonas</i> sp. B11-1	<i>E. coli</i> /pUC118, IPTG	T opt = 45°C, activated by MeOH, EtOH, DMSO, DMF
Hyperthermophilic pullulanase	<i>Thermococcus aggregans</i>	<i>E. coli</i>	T opt = 95°C t <sub>1/2</sub> = 2.5 h at 100°C
Thermophilic pullulanase	<i>Bacillus acidopullulyticus</i>	<i>Bacillus acidopullulyticus</i>	55% active after 30 min at 60°C/pH 5.5
Thermophilic and acidophilic α-amylase	<i>Alicyclobacillus acidocaldarius</i>	<i>E. coli</i>	Optimum activity at 75°C and pH = 3
Halophilic β-galactosidase	<i>Haloferax alicantei</i>	<i>Haloferax alicantei</i>	Active only at 4 M NaCl
Halophilic class I fructose aldolase	<i>Haloarcula vallismortis</i>	<i>Haloarcula vallismortis</i>	Optimal activity at 2.5 M KCl
Hyperthermophilic fructose aldolase (Type II)	<i>Thermus aquaticus</i>	<i>Thermus aquaticus</i> YT-1	Active and stable at 90°C for >2 hr
Hyperthermophilic Fructose aldolase (Type I)	<i>Staphylococcus aureus</i>	<i>Staphylococcus aureus</i>	Stable at 97°C for 1.6 hr T opt = 37°C
Thermophilic 2-keto-3-deoxygluconate aldolase	<i>Sulfolobus solfataricus</i> nalidixic acid	<i>E. coli</i> JM109/pREC7	t <sub>1/2</sub> = 2.5 hr at 100°C
Psychrophilic protease	<i>Bacillus</i> TA39	<i>Bacillus</i> TA39	Low temperature optimum
Halophilic protease	<i>Halobacterium halobium</i>	<i>Halobacterium halobium</i>	Max activity at 4 M NaCl
Thermophilic nitrile hydratase-amidase (whole cell)	<i>Bacillus</i> spp.	<i>Bacillus</i> spp.	Optimal growth at 65°C
Thermophilic nitrile hydratase	<i>Bacillus pallidus</i>	<i>Bacillus pallidus</i>	Thermostable up to 55°C
Hyperthermophilic alcohol dehydrogenase	<i>Pyrococcus furiosus</i>	<i>Pyrococcus furiosus</i>	t <sub>1/2</sub> = 160 hr at 85°C; 7 hr at 95°C
Barophilic glutamate dehydrogenase	<i>Pyrococcus furiosus</i>		36 times more stable at 105° and 750 atm
Psychrophilic phosphatase	<i>Shewanella</i> sp.	<i>E. coli</i>	Low temperature optimum
Psychrophilic alanine racemase	<i>Bacillus psychrosaccharolyticus</i>	<i>E. coli</i> /pYOK3	Low temperature optimum (0°C)

\*Recombinant enzyme. †Wild-type enzyme.

### 3.5.2.3 Improving enzyme properties – protein engineering

As described above industry is interested in new enzymes exhibiting properties some of which are not easily found/accessible in nature in order to meet the demands of the enzyme applying industry and to open up new areas of application. Another approach towards these goals is to modify the properties of enzymes which are already in use and for which a production process is already established.

Before the introduction of genetic engineering, this approach was limited to induced random mutagenesis, natural recombination, or fusion of protoplasts. These methods, however, are randomly affecting the whole genome. Consequently, only a small number of genotype variants created by this method will result in mutations in the gene of interest. Subsequent high



throughput screening is necessary to select mutants with interesting properties. Another disadvantage is that this method does not provide a tool to specifically change the properties that are supposed to be changed.

Thus, protein engineering is – no doubt – a more promising approach. Protein engineering is based on three areas of intellectual endeavour: bioinformatics,<sup>10</sup> including functional genomics and proteomics,<sup>11</sup> protein chemistry, and genetic engineering. Protein engineering takes advantage of the fact that all functional properties of enzymes reside in the structure of the protein. In turn the 3-D structure of proteins is largely determined by the sequence of amino acid residues it comprises (see section 9.1.3). It is the amino acid sequence that can be easily altered by the means of genetic engineering techniques. Protein engineering comprises approaches and methods to alter protein structure, usually by exchanging or deleting amino acids inside or inserting amino acids into one given amino acid sequence. At present, this is done on a routine basis by altering the corresponding nucleotide sequence in the enzyme gene.

Basically three approaches exist for generating enzymes with improved properties (RYU & NAM, 2000):

- First, the enzyme properties can be modulated by an exchange of a single or a few amino acid residues. This is mainly achieved by *oligonucleotide-directed mutagenesis*.
- Second, by exchanging functional domains between related enzymes, which lead to hybrid enzymes. This could technically be achieved by *DNA shuffling*.
- Third, the active site of an enzyme can be introduced into a small protein fragment scaffold of the enzyme that is devoid of its original active site.

These modifications could be made on a „rational” basis directed to particular changes with supposed impacts on the enzyme properties (*rational protein design*). Alternatively, modifications are made randomly, however, restricted to the gene of interest and not affecting the whole genome as described above (*directed evolution; molecular evolution of enzymes*).

As a prerequisite of rational protein design some hypothesis on the functional effects of well defined alterations is needed – usually affecting one or a few amino acids. The latter approach takes into account the limited knowledge of structure – function relations in enzymes. Virtually, no information on how enzyme structure relates to function is needed. This strategy is even more useful as the problems and demand from enzyme application are usually complex and multifactorial. According to industry, the directed evolution approach might therefore be the more promising one.<sup>12</sup>

Major goals for improving enzyme properties are:

- (i) increase in catalytic activity (under conditions applied in practice),<sup>13</sup>
- (ii) enzyme stability, and

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<sup>10</sup> Bioinformatics is a rapidly developing discipline that makes use of biological data stored in computer databases, complemented by computational methods and data analysis, to retrieve and/or derive new biological information. At the beginning bioinformatics was used in comparative studies and has now developed further to predict structures and functions out of sequence information at a high percentage of security.

<sup>11</sup> Proteomics: The scientific study of an organism's proteins and their role in an organism's structure, growth, health, disease (and/or the organism's resistance to disease, etc.). Those roles are predominantly due to each protein molecule's tertiary structure/conformation.

<sup>12</sup> Whereas academics might be more interested in explaining the structure-function relationship and to find a causative explanation, industry is more focusing on the effects of alterations without the need to fully understand the underlying mechanism; DSM pers. comm.

<sup>13</sup> Catalytic activity as described by distinct assay conditions – usually optimal for the enzyme – is sometimes different compared to conditions applied to enzymes in practice. For example the „specific washing performance” of detergent enzymes is not only dependent on catalytic activity but also on stability against temperature, detergents, bleaching agents, increased pH values, availability of co-factors, presence of inhibitors.

(iii) improved substrate range.

Stability to pH and temperature are important factors e. g. for detergent enzymes. Starch conversion into sugars needs low pH and high temperature, and so the demand for thermostable  $\alpha$ -amylase and amyloglucosidase from thermophile organisms is a logical consequence. Enzymes which are able „to do their job” at lower temperatures are also of interest. For example, in dairy industry,  $\beta$ -galactosidase is used at low temperature to reduce the amount of lactose.

Subtilisin, a bacterial serine protease often used in detergents, might illustrate the manifold goals of protein engineering. Up to present, mutations in well over 50% of the 275 amino acids have been reported in the scientific literature. Although enhanced stability has been the predominant target, these alterations also resulted in changes in catalytic mechanism, substrate specificity, surface activity, folding mechanisms and also in new activities (reviewed in (BRYAN, 2000)).

In the following section the different approaches and methods in use to modify protein structure will be briefly described.

### Oligonucleotide-directed mutagenesis

This method is based on computer modelling of individual amino acid changes, followed by site-directed mutagenesis of the corresponding DNA with pre-designed oligonucleotide primer, and expression of the recombinant protein in mutants for testing and evaluation. This method makes it possible to modify one or a few amino acids in the protein.

To enhance the exploitation of biomolecular diversity, „error prone PCR” (CALDWELL 1994; KE et al., 1997) has been used. Error prone PCR employs a low-fidelity replication step to introduce random point mutations at each cycle of amplification. This method is limited by the size of the gene and brings a lot screening work with it.

Applying this technique the stability of protease BPN from *Bacillus amyloliquefaciens* in the chelating environment of detergents was improved by deleting the strong calcium binding site (residues 75-83) and restabilising it through interactions which do not involve metal-ion binding (STRAUSBERG, 1995).

Replacing seven residues of a  $\beta$ -turn in *T. thermophilus* isopropylmalate dehydrogenase by a 13-residue sequence modelled on an  $\alpha$ -helix and loop in highly divergent *E. coli* isocitrate dehydrogenase, together with four additional substitutions, cause a dramatic shift in preference from NAD to NADP by a factor 100,000 without significantly affecting performance (CHEN et al., 1996).

### DNA (gene) shuffling

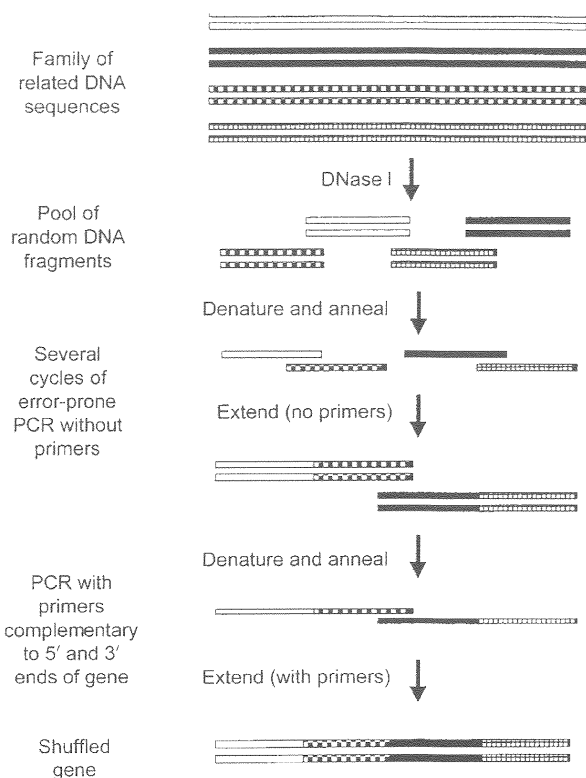
The technique of DNA shuffling has been derived from imitating natural recombination by allowing in vitro homologous recombination of DNA (STEMMER, 1994). With this method, a population of related genes<sup>14</sup> is randomly fragmented and subjected to denaturation and hybridisation, followed by extension with PCR reaction. As a result of repeated PCR cycles, the length of fragments is expanded. DNA recombination occurs when a fragment derived from one template primes a template with a different sequence (Figure 3). Combined with well focused selection procedures, this technique makes it possible to rapidly develop a huge variety of enzyme variants.

DNA shuffling has already been introduced in industrial R & D on a routine basis. Several enzymes resulting from DNA shuffling are already on the market including improved  $\alpha$ -amylases, lipases and subtilisins for laundry applications. Improved thermostable esterases

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<sup>14</sup> These genes could be either mutated or naturally homologous (family shuffling).

and dehydrogenases are also being commercialised (SCHMIDT-DANNERT & ARNOLD, 1999); TOBIN et al., 2000; DSM pers. comm.).



**Figure 3: DNA shuffling methodology.** Source: REID (2000). Genes possessing various useful mutations are mixed and randomly fragmented by DNase I. This produces fragments of various lengths that after denaturation, hybridise to form an equal mixture of 5' and 3' overhangs. The fragmented DNAs are subsequently reassembled by error-prone PCR without using primers. Recombination occurs when a fragment derived from one template primes a template with a different sequence. Finally PCR with two primers complementary to the 5' and 3' ends of the gene is carried out to obtain full-length DNA segments.

The enormous technical potential of directed evolution sometimes complemented with rational protein design could be demonstrated by the following examples:

A 14°C increase in the thermostability of an evolved esterase and 1000-fold change in substrate specificity for a fucosidase evolved from a glucosidase were reported (IVERSON & BREAKER, 1998; see also ZHANG et al., 1997); SCHMIDT-DANNERT & ARNOLD (1999) presented results on a hybrid enzyme with increased catalytic activities at all temperatures resulting from a mesophilic esterase and a thermophilic subtilisin.

- In the case of trypsin, the exchange of four residues in the active site and two non-structural surface loops gave the substrate specificity of chymotrypsin (HEDSTROM et al., 1992 according to RYU & NAM, 2000). Using a combination of rational design and directed evolution methods, the catalytic activity of an indole-3-glycerol-phosphate synthase (EC 4.1.1.48) was switched to that of a phosphoribosylanthranilate isomerase (EC 5.3.1.24; ALTAMIRANO et al., 2000 according to PETROUNIA & ARNOLD, 2000).
- A novel specificity was created by the fusion of a specific DNA-binding motif from Ubx homeodomain of *Drosophila* sequences with the non specific cleavage site of the restriction enzyme FokI. The resulting hybrid was able to recognise and cleave specific DNA targets (KIM et al., 1995, according to RYU & NAM, 2000).
- Design of hybrid enzymes using highly homologous enzymes has been demonstrated by exchanging residues or structures of their homologous regions. For instance, a hybrid of an *Agrobacterium tumefaciens*  $\beta$ -glucosidase (optimum at pH 7.2-7.4 and 60°C) and a *Cellvibrio gilvus*  $\beta$ -glucosidase (optimum at pH 6.2-6.4 and 35°C) was developed by exchanging the homologous regions. The resulting hybrids showed the optimal activity at pH 6.6-7.0 and 45-50°C (SINGH & HAYASHI, 1995, according to RYU & NAM, 2000).
- Further examples of DNA shuffling are given in Table 52 and Table 53 (Annex).

Up to present a variety of different protocols for DNA shuffling has been developed in order to increase the frequency of chimeria formation between homologous genes. Applying techniques like „Random CHimeragenesis on Transient Templates” (RACHITT) chimeric libraries averaging 14 crossovers per gene can be observed (COCO et al., 2001). But while these techniques have been quite successful applied in improving enzyme activity, substrate specificity, and physical properties, directed evolution of enzymes that catalyse a fundamentally different reaction has not yet been demonstrated. Drastic changes in protein function will require considerable conformational rearrangements, and these are not likely to result from shuffling of highly homologous genes.

In order to overcome these restrictions, new approaches have been described. Exon shuffling (KOLKMAN & STEMMER, 2001) for eucaryotic proteins e. g. the „Incremental Truncation for the Creation of HYbrid enzymes” (ITCHY) are being developed in combination with DNA shuffling for carrying out non-homologous recombination (for review see PETROUNIA & ARNOLD, 2000).

### 3.5.2.4 New enzyme production systems

In the last few years, several attempts have been made to express enzymes from microbial and animal sources in plants. In fact, the first two proteins produced from transgenic plants to be sold are enzymes; avidin from a chicken gene, and  $\beta$ -glucuronidase from *E. coli*, both of which produced in maize (HOOD & JILKA, 1999). However, both enzymes are produced at low volumes and used in laboratory research and diagnostics.

Until present, several enzymes of industrial interest have been reported to be expressed in plants: thermophilic cellulases in *Arabidopsis thaliana* (ZIEGLER et al., 2000); a fungal xylanase in canola (LIU et al., 1997); and bacterial thermophilic xylanase from *Clostridium thermocellum* in tobacco (KULKARNI & SHENDYE, 1999).

ProdiGene and Genencor has established an alliance to develop and produce industrial enzymes from plants. This alliance has already made significant progress and a first product is expected to be sold soon. ProdiGene already hold patents on the commercial production of enzymes in plants, e. g. proteases (HOWARD & HOOD, 2000).

According to JILKA et al. the manufacturing of enzymes in plants has several advantages compared to manufacturing in microbes. Despite some process related advantages, espe-

cially the posttranslational processing of eucaryotic enzymes (glycosylation, terminal proteolytic processing) may be more accurate compared to bacteria (no glycosylation) or yeast (sometimes hyperglycosylation).

### 3.5.2.5 Improved product safety

In the course of screening for new enzyme producing microorganisms, it sometimes turns out that growing of technically interesting microorganisms under industrial conditions will not be possible. Either the microorganism is pathogenic or toxic or not safe to handle. Enzyme purification could be prohibitively expensive, e. g. because the enzyme was cell associated or contaminated with undesirable compounds. Using production strains without long-term experience will anyhow be accompanied by a higher risk of harmful by-products in the final enzyme isolate (NIELSEN et al., 1994).

The cloning of enzymes following heterologous expression makes it possible to confine oneself to a small number of production strains which already have been used for enzyme production for decades and even might be considered as GRAS.<sup>15</sup>

### 3.5.3 Chemical modification of enzymes

Chemical modification of enzymes was in fact the first method available to alter enzyme properties in the sixties. Since the mid eighties, interests on chemical modification have been rekindled. One major goal for chemical modification is to increase enzyme stability and activity, particularly in non-aqueous applications for biocatalysis in organic synthesis (DESANTIS & JONES, 1999).

Examples: Various approaches were reported on covalent modification of enzymes: incorporation of vanadium into phytase for example, which has the in vivo role of catalysing phosphate ester hydrolysis is sufficient to convert phytase to a vanadium-dependent peroxidase that could catalyse enantio-selective sulfoxidation (GEORGIU & DEWITT, 1999). VIJAYALAKSHMI described the use of chemical glycosylation as means for producing thermostable soluble RNaseA (PLOU & BALLESTEROS, 1999).

Table 10 gives an overview of approaches of covalent chemical modification described in literature.

Table 10: Covalent chemical modification of enzymes in vitro (according to DESANTIS & JONES, 1999, modified)

Approach	Description	Goals	Examples
Protein crosslinking	Intra- and intermolecular crosslinking of enzymes with bi- and poly-functional reagents	Increase stability	Subtilisin, penicillin G acylase, lipase
Modification of mono-functional polymers	Binding of specific monomeric or polymeric functionalities e. g. with polyethylene glycol	Increase stability	Lipase, horseradish peroxidase, catalase
Introduction of small moieties and atom replacement	e. g. glycosylation	Increase stability	RNase A, trypsin, subtilisin

<sup>15</sup> GRAS: generally recognised as safe; see section 5.3.6.

Approach	Description	Goals	Examples
Cofactor introduction	Covalent attachment of cofactors onto protein templates	Induce new enzymatic activities, increase activity	Phytase, RNase S
Combined site-directed mutagenesis and chemical modification approach			Subtilisin

According to very recent reports the production of enzymes containing synthetic amino acids *in vivo* (!) might also be technically feasible in the near future, thereby resulting in proteins with „properties that natural proteins do not possess“ (MEJHEDE et al., 2001). These approaches will even more extend the variability of enzymes available for industrial exploitation.

### 3.6 Summary

#### Enzyme application

Enzymes are applied in various areas of application, the most important ones are technical use, manufacturing of food and feedstuff, cosmetics, medicinal products and as tools for research and development. Enzymatic processes – usually carried out under mild conditions – are often replacing steps in traditional chemical processes which were carried out under harsh industrial environments (temperature, pressures, pH, chemicals).

Technical enzymes are applied in detergents, for pulp and paper applications, in textile manufacturing, leather industry, for fuel production and for the production of pharmaceuticals and chiral substances in the chemical industry. Typically technical enzymes are manufactured and used as bulk enzymes in high volumes compared to other areas of enzyme application.

Food enzymes are mainly used in baking industry, for manufacturing fruit juices, in wine making and brewing as well as in cheese manufacturing. An important field of applications in terms of volumes is starch conversion to yield ingredients for foodstuff.

The use of enzymes in animal nutrition is an important and growing area of enzyme application, especially for pig and poultry nutrition. Feed enzymes offer the benefit of degrading specific feed components otherwise harmful or of no value to the livestock. Thereby, a wider range of ingredients may be used in diet formulation. Furthermore, a wider range of nutrients from vegetable proteins becomes available. An extension of application can be expected on the field of ruminant nutrition and aqua-culture.

Scientific literature as well as the „Inventory of Ingredients employed in Cosmetic Products“ indicate that amylase, nuclease, peroxidase, proteinase, glucose oxidase, superoxide dismutase and urease are applied or intended to be applied in cosmetic products. Currently no detailed information on the present status of application was provided to the project team by industry. A well documented area of application is skin peeling (fruit enzyme peeling), future applications may be skin protection and enzyme systems preserving the cosmetic product. Furthermore, microbial, animal and plant cell extracts containing various enzymes are also applied.

Notable medications of enzymes are as digestive aids, for wound cleaning, lysis of vein thromboses, acute therapy of myocardial infarction and as support in the therapy of certain types of leukaemia.

Enzymes can be used to determine the concentration of substrates, measure the catalytic activity of enzymes present in biological samples, serve as labels in immunoassays and as tools in research and development especially in the life sciences. However, the volumes used in this area of applications are regarded as negligible and these enzymes are not considered in the course of this study.

### **Enzyme manufacturing**

The majority of enzymes currently available is manufactured from microorganism. Manufacturing process comprise large-scale fermentation to yield high volumes of microbes. Enzymes are either accumulating inside the cells or are secreted into the media of the fermentation tanks. In subsequent steps the disrupted cells are (or the media including the enzymes) are subjected to further purification processes using variety of chemical, mechanical and thermal techniques (concentration, precipitation, extraction, centrifugation, filtration, chromatography). The resulting enzyme concentrate is then formulated to the final ready-to-sell product by adding stabilisers, standardizing agents, preservatives and salts. The final enzymes preparations are usually commercially marketed in granular or liquid forms.

### **The nature of enzyme products**

Within the scope of this study, the terminology for enzymes used by AMFEP was applied.

Commercially available enzymes are produced as *enzyme concentrates* which result from fermentation and subsequent purification steps. The enzyme concentrate contains the active enzyme(s) and various by-products from the fermentation process. The composition and amount of by-products in the enzyme concentrate is extremely variable and depending on the organisms, the media and the conditions applied during fermentation and subsequent downstream processing (30 - 98% by-products in the enzyme concentrate). Thus, in addition to identification and characterisation of the enzyme as the active substance, parameters applicable for characterisation of enzyme concentrate have to be implemented.

Additives are added in a subsequent step depending on the particular application and on customers demands (final ready-for-use *enzyme preparation*). Enzyme preparations should be regarded as preparations.

### **Recent developments in enzyme manufacturing**

The application of genetic engineering techniques in enzyme manufacturing is dramatically sparking the exploitation of new enzymes and the development of new enzyme properties.

Enzyme expression is dramatically increased by the use of strong expression or multi-copy systems. New enzymes not accessible before can be cloned into and produced from an well-known host organism. Thereby, enzymes from almost any source in nature become accessible, including exotic sources such as extremozymes, exhibiting unusual properties such as extreme thermostability.

Combinatorial approaches of rational protein design and directed evolution methods turns out to efficiently alter the properties of enzymes: enzyme stability, catalytic mechanism, substrate specificity and range, surface activity, folding mechanisms, cofactor dependency, pH- and temperature optima, kinetic parameters have been successfully modified. Recently, also the enzyme activities were switched. Protein shuffling and related techniques dramatically increase the variability of enzymes and might lead to enzymes not present in nature so far. Apart from manufacturing enzymes from MO also plants are investigated for the production of enzymes. Furthermore, enzymes could be chemically modified, e. g. by incorporation of cofactors, chemical glycosylation.

Applying these methods, the variability in enzyme structure is dramatically increased and enzyme properties are significantly enhanced. This in turn will keep up with the demands of the enzyme applying industry and open up new areas of application.

Thus, these methods are mainly contributing to technical and economic goals. However, the safety of enzyme manufacturing might also be improved by restricting to few well-known and safe-to-use production strains which are used as hosts for genes from various sources.



## 4 INDUSTRIAL ENZYMES PRESENTLY MARKETED IN THE EU

This section is largely based upon the information given by an updated overview on enzymes presently produced/used of AMFEP in the EU (status: October 2001). The data given in this list are of considerable relevance as the portion of the world market of industrial enzymes covered by AMFEP member companies is estimated to be about 75% (SPÖK et al., 1998).<sup>16</sup>

### List of enzymes produced/used in the EU

According to this list, 186 enzymes are presently marketed in the EU (Table 54, Annex). This overview comprises 47 different catalytic types as differentiated by E.C. numbers. As depicted in Table 54 (Annex), enzymes identical in functional type (E.C.) are produced from different sources. Therefore, one could conclude that these enzymes exhibit also different properties. Moreover, enzymes identical in catalytic type and source organism are produced by different production organisms. However, it cannot be concluded that these enzymes might be identical in structure as it is not shown in the overview, if these enzymes are encoded by the same genes (one organisms might produce more than one isoform of a particular enzyme type; alternatively the enzyme structure could e. g. be modified in different ways). If these aspects would be considered, the number of enzymes might presumably be twice as long. Furthermore, this list does not consider the different formulations used. Many enzyme concentrates are formulated to various enzyme preparations usually depending on the field of application. Some are even mixed to form so called „blends“. The total number of all different formulations of enzymes is unknown but might be in the magnitude of thousand.

Almost all enzymes are produced from microbes as production organisms (prokaryotic and eucaryotic sources), only three enzymes are produced from plant or animal origin. These enzymes are produced by means of 66 different production organisms (Table 55, Annex).

### Use of enzymes

The greatest variety of enzymes is used in food industry (158 enzymes), followed by technical application (64 enzymes) and use in (processing of) feedstuff (57). More than the half of the enzymes are used in just one field of industry (either technical, food or feed), 90 enzyme are used exclusively in food industry. About 24 enzymes are used in three industrial sectors.

### Tonnage of enzymes produced/used in the EU

However, the AMFEP list does not reflect the amount of enzymes produced or used. In case of technical enzymes, several dozens or even hundreds of tons might be produced from one particular enzyme (e. g. for use in detergents) per year<sup>17</sup> whereas the tonnage of enzymes e. g. used in personal care products might be of magnitude lower. Generally, the tonnage of technical enzymes used (e. g. in detergents) is rather high in comparison to other industrial sectors. However, no information was provided by the industry on the overall tonnage of particular enzymes or groups of enzymes. As the only evidence, the tonnage of 5 to 10 enzyme types comprising many enzymes of the AMFEP list might exceed 10 tons/year. According to AMFEP, information on the tonnages of some enzymes were reported to the European Chemicals Bureau (Joint Research Center, Ispra) in accordance with the Existing Sub-

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<sup>16</sup> This overview does not include enzymes used in very small quantities in R & D, for analytical or for therapeutic purposes as AMFEP member companies (with very few possible exceptions) do not produce such enzymes. The variety of these enzymes is much higher than those of industrial enzymes. Especially for analytical and R & D purposes hundreds of different enzymes are used. No information could be obtained from the industry on the tonnages of these categories of enzymes. However, the annual tonnage might be often below 10 kg/year worldwide (KOPETZKY et al., 1994) and in case of enzymes for R & D, such as restriction endonucleases, presumably even far below 1 k/year worldwide. As these enzymes are not covered by EU chemical regulation they will not be dealt with in this chapter.

<sup>17</sup> The actual numbers are depending on the calculation basis. See also section 11.3.4 for discussion.

stances Regulation (AMFEP 2002).<sup>18</sup> Data sets on 1 HPVC and 8 LPVC were submitted (SCHEER, pers. comm.).

According to the AMFEP list, 15 enzymes (six of which are manufactured from GMM) are not covered by EINECS entries. According to Chemical Abstracts, only six of these enzymes are registered in other inventories (TSCA, ENCS, DSL/NDL, PICCS) and might therefore pertain exported products. At least, four enzymes seem not to be registered in any of these inventories. It should be clarified, if these 15 enzymes are covered by one of the EINECS entries, if they fall under any exception of Directive 67/548/EEC or if they possibly should be notified as new substance. This task cannot be done within the scope of this study as essential information is not available, but could be performed if requested by the Commission.

Laccase was notified as a new substance according to Directive 67/548/EEC.

Two laccase are listed on the AMFEP-list produced by the same production organism but with differing donor organism (the source of the DNA encoding the enzyme of interest), *Myceliophthora* sp. and *Polyporus* sp.. As these enzymes are of different origin they will most probably be structurally different. However, the notification as a new substance according to Directive 67/548/EEC of laccase was specified for the donor organism *Polyporus* sp. It has to be clarified, if the two laccase are covered by the ELINCS entry.<sup>19</sup>

## Enzymes from GMM

The first industrial enzyme from GMM, a lipase from Novo Nordisk was introduced in the EU market in 1987. 65 of 186 enzymes marketed by AMFEP member companies in the EU are presently manufactured from GMM<sup>20</sup> (Table 56, Annex), this comprises 30 out of 42 different catalytic types (according to IUB). Approximately two thirds are used in the food industry, the half of which is dedicated to technical application (e. g. detergent enzymes) and to application feed industry (Table 56, Annex). In 1995, about 40 enzymes (out of 116) were manufactured by means of genetic engineering (SPÖK et al., 1998). This makes clear that the va-

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<sup>18</sup> The Existing Substances Regulation (EEC 793/93) concerns existing substances, i. e. those that are listed in EINECS. In a first step, information on high production volume chemicals (> 1000 t/yr; HPVC) had to be submitted. In a second step, information on low production volume chemicals (> 10 - 1000 t/yr; LPVC) had to be submitted. The reporting period is 1990 - 1994.

<sup>19</sup> The following procedure is proposed: First, AMFEP should be asked to communicate the identity of the manufacturer/importer of Laccase (donor organism: *Myceliophthora* sp.). In case, the Laccase are manufactured by a different company, the responsible national authority should request additional information from the company. On the basis of the additional information, the (two) responsible national authorities should decide if the substance is identical with the one notified and whether, consequently, a re-notification or a new substance notification has to be submitted.

Within the scope of this study, the study authors identified a need to characterise the enzyme concentrate that is the result from the fermentation and subsequent purification processes, containing the enzyme as active substance as well as by-products. The composition and the amount of by-products is extremely variable and depends on the production process and production organisms. Therefore, additional parameters to those currently applied are needed to characterise the enzyme product and should be requested.

The formation of harmful metabolites in the enzyme concentrate could be influenced by changes in either the production process or the production organisms. In order to get (indirect) evidences on toxicity of an enzyme concentrate, the following information is essential: Specifications on the production organism, genetic modification (donor organism, inserted DNA, vector), and the manufacturing process (e. g. fermentation conditions, downstream processing). (see sections 7.5 and 7.6).

<sup>20</sup> AMFEP did not specify if an enzyme is produced by means of genetic engineering. However if a donor organism is specified it can be concluded that this enzyme is manufactured from GMM. If no donor is specified this does not exclude that genetic engineering is involved because donor and production organism could be identical. Consequently, the numbers stated for enzymes from GMM can be considered as minimum numbers and these enzymes are not included in Table 56.

riety of enzymes available in general and of enzymes produced from GMM in particular is rapidly increasing.

It has to be pointed out that several techniques are not resulting in a GMM according to the definition of Directive 90/219/EEC.<sup>21</sup> Even it is most probably, it still remains unclear if these techniques are applied for commercial production. Consequently, these enzymes could not be included in Table 56 (Annex).

Table 11, Table 12, and Table 13 list Enzymes from GMM presently manufactured/used in the EU and specify the corresponding host and source organisms used (derived from AMFEP Oct. 2001).

*Table 11: Types of enzymes manufactured from genetically modified microorganisms. Source: AMFEP Oct. 2001*

Types of enzymes from GMM	
Acetolactate decarboxylase (alpha)	Lipase, triacylglycerol
Aminopeptidase	Maltogenic amylase
Amylase (alpha)	Mannanase (endo-1,4-beta)
Arabinofuranosidase	Pectate lyase
Catalase	Pectin lyase
Cellulase	Pectin methylesterase or Pectinesterase
Chymosin	Penicillin amidase
Cyclodextrin glucanotransferase	Phospholipase A
Galactosidase (alpha)	Phospholipase B
Glucanase (beta)	Phytase
Glucoamylase or Amyloglucosidase	Polygalacturonase or Pectinase
Glucose isomerase	Protease (incl. milkclotting enzymes)
Glucose oxidase	Pullulanase
Hemicellulase	Xylanase
Laccase	
Lactase or Galactosidase (beta)	

<sup>21</sup> „Self-cloning consisting in the removal of nucleic acid sequences from a cell of an organism which may or may not be followed by reinsertion of all or part of that nucleic acid (or a synthetic equivalent) with or without prior enzymic or mechanical steps, into cells of the same species or into cells of phylogenetically closely related species which can exchange genetic material by natural physiological processes where the resulting micro-organism is unlikely to cause disease to humans, animals or plants.

Self-cloning may include the use of recombinant vectors with an extended history of safe use in the particular micro-organisms.” (COUNCIL OF THE EUROPEAN UNION: COUNCIL DIRECTIVE 98/81/EC of 26 October 1998 amending Directive 90/219/EEC on the contained use of genetically modified micro-organisms, Annex II (4).

Table 12: Host organisms used in enzyme manufacturing using genetic engineering techniques. Source: AMFEP Oct. 2001

Host organisms	
Alcaligenes faecalis	Cryphonectria or Endothia parasitica
Aspergillus niger	Klebsiella planticola
Aspergillus niger var. awamori	Kluyveromyces lactis
Aspergillus oryzae	Pseudomonas alcaligenes
Bacillus alcalophilus	Saccharomyces cerevisiae
Bacillus amyloliquefaciens or subtilis	Streptomyces lividans
Bacillus halodurans or lentus	Streptomyces rubiginosus
Bacillus licheniformis	Trichoderma reesei or longibrachiatum
Bacillus subtilis	

Table 13: Donor organisms for enzyme genes. The name of the species is usually not specified by the industry due to confidentiality reasons. Source: AMFEP Oct. 2001

Donor organisms	
Actinomadura sp	Klyveromyces sp.
Alcaligenes sp.	Myceliophthora sp.
Aspergillus sp.	Myceliophthora sp.
Bacillus sp.	Peniophora sp.
Calf stomach	Polyporus sp.
Candida sp.	Pseudomonas sp.
Cryphonectria sp.	Rhizomucor sp.
Fusarium sp.	Streptomyces sp.
Guar plant	Thermoanaerobacter sp.
Hormoconis sp.	Thermomyces sp.
Humicola sp.	Thielavia sp.
Klebsiella sp.	Trichoderma sp.

It could not be derived from the AMFEP list which enzymes are modified using protein engineering. SPÖK et al. (1998) revealed that for use in detergents at least seven enzymes (status: 1995) out of 16 are structurally modified.

As mentioned above, the numbers of enzyme stated are not reflecting the quantitative ratios. As shown in SPÖK et al. (1998) genetic engineering and protein engineering techniques are applied in an extremely high degree in bulk enzymes such as detergent enzymes. In Austria about 82% (tonnage pure enzyme) of all enzymes used in detergents came from GMM (SPÖK et al., 1998). As detergent enzymes comprise a large portion of the overall tonnage of enzymes this lead to the conclusion that the mere number (66 out of 186 enzymes from GMM) might distort the picture. Presumably more than 60% of all industrial enzymes are produced by GMM (also supported by COWAN, 1996; according to MENRAD et al., 1999).

## 5 REGULATION OF ENZYMES – REVIEW

### 5.1 Introduction

Enzymes and enzyme notification in the European Union and in non-EEA countries are regulated in different legal provisions depending on their use e. g. as technical enzymes, food enzymes, feed enzymes, cosmetic enzymes or medicinal products.

A particular aim of this study is to investigate the regulation of enzymes on the EU market as a whole. Therefore, this chapter gives an overview on the regulation of enzymes that is broader than the scope of Directive 67/548/EEC.

Concerning enzymes used for technical purposes, the project team investigated the chemical regulation and chemicals notification systems in the EU, USA, Canada and Australia (section 5.2.1 to 5.2.9). Section 5.2.10 gives a compilation and analysis of these investigated notification systems.

In case of food enzymes EU harmonised legislation, national legislation in EU Member States and the US as well as guidelines from international and national committees are investigated and compared (section 5.3).

For feed enzymes, cosmetic enzymes and enzymes used in medicinal products the EU harmonised legislation and relevant guidelines were investigated (sections 5.3, 5.5, and 5.6).

Special attention is paid to the treatment of enzymes produced by means of GMOs and structurally modified enzymes. Current praxis and gained experiences regarding the regulation of enzymes in some EU member states are summarised in section 5.3.

### 5.2 Enzymes in the context of the regulation of chemicals

#### 5.2.1 EU – present state and practice

Technical enzymes are considered to be chemicals and are amongst others subject to the notification requirements of Directive 67/548/EEC.

Difficulties arising from the notification requirements for enzymes were discussed at the Technical and Scientific Meetings on issues associated with Directive 67/548/EEC (TSM) and at the meetings of the Competent Authorities (CA) for the implementation of Directive 67/548/EEC: The major problem are the criteria to show that two enzymes are identical (EINECS-listed and not listed enzymes. CAs agreed that this includes the chemical structure and the catalytic activity. However, the question was raised, if this includes the three-dimensional structure of the enzyme, as this was not reported on EINECS and it is difficult to analyse. While enzymes extracted from natural occurring organisms may be covered by the EINECS entry „natural occurring substances”, CAs agreed that in principle enzymes produced by GMOs are new substances<sup>22</sup>). EINECS is considered inadequate as a reference for enzymes<sup>23</sup>.

A provisional guidance document on enzyme notification was agreed at the 8<sup>th</sup> TSM (NOTIF/4/99 rev. 2; dated: 15/11/99). This document should ensure that Member States apply a consistent approach to potential notifiers of new enzymes in order to decide what is or is not notifiable and the data to be submitted with the notification. It was discussed at the 57<sup>th</sup> CA meeting (November 1999) and the adoption postponed until a satisfying proposal would

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<sup>22</sup> 3<sup>rd</sup> TSM.

<sup>23</sup> Conclusion at 55<sup>th</sup> CA Meeting.

be presented. The guidance document is was considered confidential by CAs and can therefore not be quoted in this report.

### 5.2.2 Enzymes in EINECS

EINECS is the European inventory of those chemical substances which were on the European Community market between 1. January 1971 and 18. September 1981. The basis for the introduction of EINECS was Article 13 of Council Directive 79/831/EEC. Substances listed in the inventory are considered as Existing Substances and do not need to be notified.

The list includes well defined substances as well as UVCB substances which cannot be represented by a complete chemical structure diagram nor by specific molecular formula, such as reaction products, plant products, post-reacted naturally occurring substances or naturally occurring substances.

When setting up EINECS, enzymes were regarded as biological material, i. e. UVCB substances, which had to be reported if synthesised [sic!] or if obtained after processing of a natural product.<sup>24</sup> An article describing enzymes under EINECS was planned but not published so far.

In EINECS, enzymes are both listed as generic entries without further specification (like the source organism) and specific entries indicating the substrate or the source organism. 368 entries in EINECS are actually covering enzymes including those with generic entries. The enzymes are primarily characterised on the basis of their catalytic activity. The following information is in general indicated for a substance in EINECS: chemical name (IUPAC), EINECS No, CAS No, molecular formula and substance definition description. Concerning enzymes, the chemical name, the EINECS No and the CAS No are listed in EINECS. A list of all enzymes in EINECS is given in Annex (Table 57).

The document „Reporting for the EINECS Inventory“ (EUROPEAN COMMISSION 1992) set criteria for the definition of naturally occurring substances which are included in EINECS as one generic entry and those substances which were not regarded as naturally occurring substances. Criteria for reporting substances for EINECS are also set in the Manual of Decisions (non-confidential version):<sup>25</sup>

- *“Substances occurring in nature as such, unprocessed, or processed only by manual, mechanical or gravitational means; by dissolution in water, by flotation, or by heating solely to remove water, or which are extracted from air by any means, will be listed in EINECS under the collective name „naturally occurring substances“ and should not be reported individually. However, substances as such or as part of mixtures which are produced by chemical modification of naturally occurring products or are separated from them by physical processing can be reported.*
- *Enzymes can be reported if synthesised [sic!] or if obtained after processing of a natural product.*
- *Substances which are produced by chemical modification of bacteria, fungi, yeasts and their metabolic products (or are separated from the living materials by physical processing) can be reported. This will apply whether the substance is isolated or is a component of a mixture.”*

Concluding, these definitions do not clearly define the status of enzymes / enzymes derived from GMOs. For example, from the statement *„Enzymes can be reported if obtained after*

<sup>24</sup> Compilation of EINECS: Descriptions and definitions used for UVCB substances.

<sup>25</sup> Citation from the original document in italic letters.

*processing of a natural product*” it could be concluded that effectively all enzymes are covered, depending on the definition of natural product. “*Substances which are produced by chemical modification of bacteria, fungi, yeasts and their metabolic products*” could lead to the interpretation that chemical modification refers to genetic engineering.

Discussions on EINECS as a reference for determining liability of new enzymes to notification were raised on TSM and CA level. The working group „EINECS and Enzymes“ discussed the fundamental problems treating enzymes under the Directive 92/32/EEC using EINECS as decision basis. According to this group, EINECS in its present status seems not to be suitable for a decision about the duty to notify enzymes. Consequently, the problems and open questions when using EINECS as a decision basis for enzyme notification were described. The working group recommends „*to develop and implement a new legislation specific to enzymes, also covering substances produced from genetically modified organisms (GMOs) and including a tailored testing programme*“.<sup>26</sup> Furthermore, it was proposed to compile a list containing all enzymes reported by Industry on the market until a fixed date. All enzymes marketed after this date and not on this list would have to be notified.

### 5.2.3 Enzymes notified according to Directive 67/548/EEC

One enzyme, Laccase, has been notified in the European Union so far. Due to data security reasons no further information can be given in this report.

### 5.2.4 Enzymes in Annex I of Directive 67/548/EEC

Annex I of Directive 67/548/EEC contains 16 types of enzymes that were added within the 22<sup>nd</sup> Adaptation to Technical Progress (96/54/EC). All 16 types of enzymes are within three classification categories: subtilisins, non-subtilisin proteases and non-proteases. A list of these enzymes is given in Annex (Table 58).

In general, all enzymes listed in Annex I of Directive 67/548/EEC are classified with the risk-phrase R42 (May cause sensitisation by inhalation). 9 of these enzymes are additionally classified with the risk-phrases R 36/37/38 (Irritating to the eyes, respiratory system and skin.). One enzyme (i. e. subtilisin) was additionally classified with the risk-phrases R 37/38-41 (Irritating to the respiratory system and skin, Risk of serious damage to eyes).

The classification of these enzymes for environmental effects was discussed in the relevant working group. There was a considerable lack of environmental data for these substances, especially for alpha-amylases and proteases. The working group on classification and labelling agreed not to classify the listed enzymes for environmental effects due to lack of data.

### 5.2.5 Possibly enzyme-relevant issues in the „Manual of Decisions“ – non-confidential version (MoD)

The Manual of Decisions (update 2002) includes some issues that seemed relevant in the context of enzyme notification. Citations of the manual are printed in italic letters followed mostly by comments and open questions made by the authors of this study.

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<sup>26</sup> Meeting „EINECS and Enzymes“, Dortmund, 26 February 1998.

### 5.2.5.1 Definition of substance

The MoD entry point 5.12. Applicability of the Directive to microorganisms (not genetically modified) is as follows:

*... Taking into account that substances obtained by any production process have to be notified according to Directive 92/32/EEC, substances produced by micro-organisms (e. g. enzymes produced by biotechnology) have to be notified, if no exemptions are to be applied. ...*

*... „Substances” produced from killed microorganisms have to be notified. However, for instructions on how to describe these substances the guidance „Reporting for the EINECS Inventory” should be consulted. ...*

*... Conclusions: Since there is no exemption to the general rule that substances obtained by any production process have to be notified, those killed microorganisms which can be defined as substances (e. g. protein or amino acid hydrolysates) have to be notified. Living organisms, in contrast, do not have to be notified. ...*

#### Discussion:

According to this MoD entry it is clearly stated that enzymes are considered as chemical substances and have to be notified accordingly. However, the following item is not applicable to enzymes.

### 5.2.5.2 Identification of mixtures

The MoD defines ELINCS rules for the identification of mixtures (point 3.3.):

*... Intentional mixtures made by mixing or blending with other substances were neither reportable for EINECS nor are covered by Directive 92/32/EEC. Mixtures listed in EINECS or notified as new substances are reaction products not being separated into individual components, including additives to preserve the stability of the product and any impurity deriving from the production process used. These mixtures are placed on the market as such, e. g. mixed isomers and plant extracts. ...*

*... The 53<sup>rd</sup> CA meeting agreed that ELINCS rules should follow EINECS, as follows: A notified substance listed as a (mono-) substance must contain at least 80% of this component. If not, it is a mixture and several components have to be listed in ELINCS. ...*

#### Discussion:

According to Directive 92/32/EEC, intentional mixtures are considered to be „preparations“. Intentional mixtures are termed as enzyme formulations, blends and preparations according to AMFEP (definition see section 3.4).

An „enzyme concentrate” is considered to be an enzyme-containing mixture in its most concentrated form as it occurs during commercial production, thus before formulation. Besides the active enzyme protein, the enzyme concentrate will also contain substances derived from the microorganism and the fermentation medium (e. g. proteins, peptides, amino acids, carbohydrates, minerals and other minor components). The relative amount of these by-products can vary considerably.<sup>27</sup> The percentage of active enzyme in enzyme concentrates may range between 2% and 70% (w/w on basis of dry matter).

Taking into account the high degree of by-products within the „enzyme concentrate”, the following question raises: What is considered to be the substance to notify? Are by-products

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<sup>27</sup> Definitions according to AMFEP (see: AMFEP Position on Roster of Questions, section 13.3, Annex).



considered to be impurities resulting from the production process? The present approach seems not applicable to enzyme concentrates that contain the active protein in a varying range of 2-70 %.<sup>28</sup>

### 5.2.5.3 Extracts from genetically-modified plants

A CA decision concerning the notification of extracts from genetically-modified plants is included in the MoD under 2.14.:

*... The CA had received a query from a company asking whether the oil extracted from a genetically modified form of Brassica napus was covered by the EINECS entry for the extract from the „natural“ B. napus? ... It was decided at the 53<sup>rd</sup> meeting of the Competent Authorities that the oil has to be notified. The description in ELINCS will include the plant name and the „extra“ gene. Competent Authorities agreed that it was important to distinguish between „new“ plant extracts obtained from hybrids by normal agriculture techniques and those from plants in which another gene is introduced deliberately. ...*

#### Discussion:

According to expertise it is common practice to provoke mutations in natural plants by means of irradiation. The question is raised, if this is considered as normal agriculture technique<sup>29</sup>. For further discussion see the following issue.

### 5.2.5.4 Substances produced by means of biotechnology

The MoD includes under 5.8. a decision concerning complex polysaccharide gums, etc., produced by biotechnology:

*The 42<sup>nd</sup> CA meeting discussed the question of complex polysaccharide gums produced by biotechnology.*

*EINECS lists various products, such as certain gums (complex polysaccharides) derived by implication from natural sources. It is now possible to make what appears to be the same, products by fermentation (i. e. biotechnology). However, it is difficult to prove that natural and biotechnology-derived gums are identical. The question is: can it be assumed that an EINECS entry covers both types?*

*It was agreed that prospective notifiers should provide analytical proof that their biotechnology product is identical to the natural product. ... In principle, substances used producing the new technologies should be notified if they are not on EINECS. If they are on EINECS, the notifier should prove that it is the same substance as the naturally occurring one in order to be exempted from notification<sup>30</sup>.*

#### Discussion:

The question, if a substance produces by means of biotechnology is covered by the EINECS entry for the „natural“ substance, was raised under both points 5.2.5.3 and 5.2.5.4.

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<sup>28</sup> Enzymes are also manufactured as „intentional mixtures“, namely as „enzymes blends“ of separately manufactured. However, enzyme blends are considered to be covered by Directive 88/379/EEC. In contrast „multi-enzyme-preparations“ are the result of a single manufacturing process intentionally resulting in an enzyme concentrate containing more than one enzyme of technical importance.

<sup>29</sup> If the oil extracted from Brassica napus is intended to be used in food, this product would be subjected to the Regulation (EC) No 258/97 of the European Parliament and of the Council of 27 January 1997 concerning novel foods and novel food ingredients.

<sup>30</sup> Decision at 43<sup>rd</sup> CA meeting.

This is also an open question with enzymes: Are enzymes produced by means of new technologies considered to be identical to those from natural sources? How to provide analytical proof of identity of enzymes is in question.

The question is raised, what criteria are used for the differentiation of substances – the production process, the chemical identity, etc. In case of the polysaccharide gums, the identity of the resulting product is important. While in case of oil extract, the genetically modification of the medium from which the oil is extracted is of relevance. From our point of view, this is an inconsistent situation. If the chemical identity of a substance is not available, it cannot unambiguously be proven whether this substance is identical to another or not.

We conclude, that substance identity is an extensive problem with all substances, for which the chemical structure is not sufficient for unambiguous identification, or where the chemical structure is not available. This especially concerns products from biotechnology.

The Canadian new substances notification regulation defines information requirements for biotechnology products (see section 5.2.8). Recommendations, on how to deal with this problem, are given in chapter 11.

#### **5.2.5.5 Notification of substances (enzymes) produced and processed in different Member States and substance to test**

At the 14<sup>th</sup> TSM a text proposal was agreed which will be added in the MoD as new paragraph 12.4 Notification of substances (enzymes) produced and processed in different member states:

*... An enzyme is generated in one member state in a fermentation process using a GMO. The resulting culture „broth” is purified in another member state for use as a disposable catalyst. The final concentrate comprises only 4 % enzyme (dry weight) prepared for use as a dilute solution, 10 % total dry matter in water.*

*Notification in either member state, respectively as a UVCB or pure substance, might be warranted. Moreover, to notify either the culture broth or the solution may trigger unrealistic tonnage thresholds, where essentially water would be the assayed material.*

*It was agreed that the country of notification should be the one where production (broth fermentation) originates, and where testing of the dry matter (i. e. increasing dose level by 10-fold, to correct the 90 % dilution) would be appropriate.*

#### **Discussion:**

In this case, Total Organic Substance (TOS), that is the pure enzyme protein plus defined impurities, was considered as the substance to notify.

### **5.2.6 Experience of EU Member States in enzyme notification**

#### **5.2.6.1 Austria<sup>31</sup>**

As a result of the Austrian national chemicals regulation up to 1995, laboratory chemicals had to be notified. Within the period of 1989 - 1995, 162 enzymes used in laboratories were notified in Austria. As all EINECS-listed substances are excluded from the notification procedure, the Austrian CA had to decide on the differentiation between new (notifiable) and existing (non-notifiable) substances. This posed problems in the interpretation of generic

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<sup>31</sup> Umweltbundesamt, Austrian comments on the notification of new enzymes, Wien (1996)

EINECS-entries and in cases where the substance was produced by means of biotechnology.

The Austrian CA came to the conclusion, that a substance is generally characterised by its chemical identity, and not by its catalytic activity. They concluded to exclude enzymes only from notification if there was a proof for the chemical identity to an enzyme already listed in EINECS (e. g. amino acid sequence, prove of identical tertiary structure, etc.). In many cases, data on the chemical composition (amino acid sequence) of the enzyme could not been made available.

Thus, if there was no proof that enzymes were chemically identical, solely those enzymes with the same catalytic activity (= name) and extracted from the same organism were regarded as chemically identical, and therefore were excluded from notification. The following criteria formed the basis for differentiation between new and existing substances:

- Proteins having the same names (and EC No) but being extracted from different natural sources (organisms) were considered as individual substances. Consequently, proteins extracted from a specific organism having the same name as a substance in EINECS without a specification from which organism it had been extracted, had to be notified.
- Proteins being modified by means of biotechnology, that still had the same name and activity as a naturally occurring protein, were considered as individual substances.
- Proteins from GMO's (being expressed in a host organism, using recombinant DNA) that had the same name and activity as the naturally occurring protein extracted from an organism, were considered as individual substances.

These criteria were applied due to the following reasoning:

- According to chemicals regulation, a substance is defined via its chemical identity. The chemical properties of enzymes are characterised by their primary, secondary, tertiary and quaternary structure (i. e. unique alignment of amino acids, methylation, glycosylation etc. and its specific folding).
- The production of an enzyme in different organisms may even result in different folding but almost certainly in different glycosylation and methylation even though the amino acid sequences are identical.
- Some enzymes are formed in various species but it cannot automatically be concluded, that they are identical in a „chemical“ sense.
- The amount and kind of impurities contained in the product depends on the (host) organism from which the protein is extracted.

#### 5.2.6.2 Germany<sup>32</sup>

The German CA had to decide on the testing program for an enzyme following an inquiry from a company. The following decision concerning the test program has been taken:

Substance to test: The enzyme is not isolated but marketed as 5 % solution. The substance should be tested as aqueous solution. The tests should be conducted using a (most possible) concentrated enzyme solution.

Tests on physico-chemical properties: are not requested because of the specific properties of the polypeptide chain and the enzyme is in aqueous solution.

Concerning toxicological properties the following tests were requested: acute toxicity (oral, dermal - limit test), irritation (skin, eye), skin sensitisation, 28 days toxicity (96/54/EEC - limit test), Ames test (Salmonella; test design for proteinaceous substances) and mutagenicity

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<sup>32</sup> Information made available after the 13<sup>th</sup> TSM.

(chromosomal aberration in-vitro). It is supposed that the enzyme solution has no cell toxic effects.

The following ecotoxicological tests were requested: acute toxicity for fish and daphnia (limit test), growth inhibition on algae, respiration inhibition test and biodegradation.

#### 5.2.6.3 Denmark<sup>33</sup>

Denmark had to decide on the testing of a new enzyme. The Danish CA departed from the usual demands concerning certain tests because they were judged meaningless or inapplicable to an enzyme.

Denmark supports the use of the „dry matter“ (TOS) as the basis for calculation of tonnage and testing. The notifier stated that from a toxicological point of view TOS is generally recognised as the most relevant measure of the dry matter.

Physical chemical testing: As the normally required identification and physical / chemical characterisation are not relevant for enzymes, some alternative identification methods and activity were accepted including: determination of enzyme activity (LACU-assay), purification and characterisation data, occurrence of recombinant DNA in TOS, characterisation of the fermentation and purification processes used.

Sensitisation: The notifier claimed that enzymes were known respiratory sensitisers, but not skin sensitisers. Classification as a skin sensitiser without substance-specific data was therefore inappropriate. The notifier claimed that it would be incorrect to test the enzyme in a GMP test. Nor would a Buehler test be appropriate according to the notifier. No alternative test for skin sensitisation was suggested by the notifier. The Danish CA agreed that a GMP test would not be appropriate for a protein but could not accept not testing and no classification based on anecdotal evidence for lack of skin sensitising potential of enzymes in general. Therefore, the Danish CA requested a Buehler test. The test turned out to be negative.

#### 5.2.7 Premanufacturing notification of chemical substances in the U.S.A.

The Environmental Protection Agency's (EPA's) New Chemicals Program is mandated by Section 5 of the Toxic Substances Control Act (TSCA) that was established in 1976 to help manage the potential risk from chemicals new to the marketplace.

The final regulations for microbial products of biotechnology under section 5 of TSCA (Code of Federal Regulations (CFR) Part 725) establish reporting requirements and notification procedures for review of certain new microorganisms before they are introduced into commerce. The regulation focuses on (the living) microorganisms that are manufactured for commercial purposes including research & development for commercial purposes, e. g. microorganisms used for the production of industrial enzymes. The review process is designed to prevent unreasonable risk of injury to human health and the environment without imposing unnecessary regulatory burdens on the biotechnology industry. However, new microorganisms that meet criteria defining eligible microorganisms and specified use conditions are exempted.

The product manufactured by the use of new microorganisms is considered as a substance and therefore is subject to the notification procedures and reporting requirements defined under section 5 of TSCA.

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<sup>33</sup> Information made available after the 13<sup>th</sup> TSM.

Chemical substances and mixtures whose manufacture, processing, distribution in commerce, use, or disposal may present an unreasonable risk of injury to health or the environment are identified and regulated under TSCA. Adequate data should be developed with respect to the effect of chemical substances and mixtures on health and the environment. The development of such data are the responsibility of the manufacturer of the chemical substance.

Under TSCA, EPA has authority to issue regulations designed to gather health/safety and exposure information on, require testing of, and control exposure to chemical substances and mixtures and to take action with respect to chemical substances and mixtures which are imminent hazards.

EPA shall carry out its task in a reasonable and prudent manner, and shall consider the environmental, economic, and social impact of any action EPA takes or proposes to take under TSCA.

### 5.2.7.1 Scope of TSCA

(1) Substances excluded from notification:

The following new substances fall under the jurisdiction of other federal laws and are reviewed by other federal programs: tobacco and certain tobacco products, nuclear materials, munitions, foods, food additives (this matter is dealt with in section 5.3.6), drugs, cosmetics, and substances used solely as pesticides (statutory excluded categories).

The following are excluded from premanufacturing notification (PMN) reporting under certain conditions: naturally-occurring materials, products of incidental reactions, products of end-use reactions, mixtures (but not mixture components), impurities, by-products, substances manufactured solely for export, non-isolated intermediates, and substances formed during the manufacture of an article.

*Research and Development (R&D) – the substance is manufactured in small quantities for research and development purposes (synthesis of new chemical substances for analysis, experimentation, research on new or existing chemicals, including product development activities). The quantity may vary depending on the nature of the R&D activities. The company has to apply for a R&D exemption.*

Test Marketing Exemption (TME): A company may apply for an exemption from PMN if the intended activity is not commercial production and is not appropriately considered to be research and development. Test marketing involves the distribution of a predetermined limited amount of a chemical, or of a mixture or article containing the chemical, to specified number of customers to explore market acceptability before general distribution.

(2) Exemptions from PMN reporting:

- Low Volume Exemption (LVE) – For substances that will be manufactured or imported 10,000 kilograms or less each year; recordkeeping requirements are defined.
- Low releases and low exposures (LoREX) – Eligibility for this exemption category is independent of production volume level. Performance standards for this include both absolute criteria (e. g. an upper limit on surface water releases) and goals (e. g. no worker exposure).
- Polymer – This exemption is available for certain classes of polymers which are not chemically active or bioavailable. Recordkeeping requirements are defined.

To summarise, industrial enzymes are considered as chemical substances and are subjected to the reporting requirements of TSCA section 5. Enzymes used in any aspect of food production/processing and as feed additives are excluded from reporting under TSCA and fall

under the Federal Food Drug and Cosmetic Act (FFDCA, see section 5.3.6). This includes also enzymes used as processing aids, food and feed additives.

For inventory reporting purposes, complex biologicals, including enzymes, organisms and products of the biotechnology industry are considered to be UVCB substances. Many naturally occurring enzymes were placed on the original TSCA Inventory in 1979. EPA has not yet developed guidance for their Inventory representation.

According to EPA, chemically modified enzymes for industrial uses have been assessed. However, EPA's experience is limited and the number of reviewed enzymes could not be communicated.

#### **Microbial Products of Biotechnology: Final Regulations Under the Toxic Substances Control Act:**

These rules published by EPA implement a screening program for „new“ microorganisms under Section 5 of TSCA. „New“ microorganisms are those microorganisms formed by combining genetic material from organisms in different genera (intergeneric). Intergeneric microorganisms are considered new chemicals under TSCA section 5. This includes new microorganisms used commercially for such purposes as production of industrial enzymes and other speciality chemicals.

Persons intending to use intergeneric microorganisms for commercial purposes in the US have to submit an MCAN (Microbial Commercial Activity Notice) to EPA at least 90 days before such use. Certain intergeneric microorganisms are exempt from the requirement to submit a MCAN if the manufacturer meets criteria defining eligible microorganisms and specified use conditions. This exemption is most applicable to the manufacture of speciality and commodity chemicals, particularly industrial enzymes.

#### **5.2.7.2 Definitions in TSCA**

##### *Chemical substance*

means any organic or inorganic substance of a particular molecular identity, including –

- (a) any combination of such substances occurring in whole or in part as a result of a chemical reaction or occurring in nature, and
- (b) any element or uncombined radical.

Such term does not include

- (a) any mixture,
- (b) any pesticide (as defined in the FIFRA) when manufactured, processed, or distributed in commerce for use as a pesticide,
- (c) tobacco or any tobacco product,
- (d) any source material, special nuclear material, or by-product material (as such terms are defined in the Atomic Energy Act 1954 and regulations issued under such Act),
- (e) any article the sale of which is subject to the tax imposed by section 4181 of the Internal Revenue Code of 1986 (26 U.S.C. 4181), and
- (f) any food, food additive, drug, cosmetic, or device (as defined in the Federal Food, Drug, and Cosmetic Act) when manufactured, processed, or distributed in commerce for use as a food, food additive, drug, cosmetic, or device.

*Distribute in commerce; distribution in commerce*

means to sell, or the sale of, the substance, mixture, or article in commerce; to introduce or deliver for introduction into commerce, or the introduction or delivery for introduction into commerce of, the substance, mixture, or article; or to hold, or the holding of, the substance, mixture, or article after its introduction into commerce.

#### *Manufacture*

means to import into the customs territory of the United States, produce, or manufacture.

#### *Mixture*

means any combination of two or more chemical substances if the combination does not occur in nature and is not, in whole or in part, the result of a chemical reaction; except that such term does include any combination which occurs, in whole or in part, as a result of a chemical reaction if none of the chemical substances comprising the combination is a new chemical substance and if the combination could have been manufactured for commercial purposes without a chemical reaction at the time the chemical substances comprising the combination were combined.

#### *New chemical substance*

means any chemical substance which is not included in the chemical substance list compiled and published in the TSCA Inventory.

#### *Article*

is defined as a manufactured item which:

- (1) is formed to a specific shape or design during manufacture;
- (2) has an end use function(s) dependent in whole or in part upon its shape or design during end use; and
- (3) either has no change of chemical composition during its end-use or only those changes in composition which have no commercial purpose separate from the article of which it is a part and that may occur as described in 40 CFR §710.4(d)(5) and 40 CFR §720.30(h)(5). Articles are excluded from PMN requirements.

#### *Unreasonable risk*

is not defined in the TSCA. The legislative history, however, indicates that unreasonable risk involves the balancing of the probability that harm will occur and the magnitude and severity of that harm against the effect of a proposed regulatory action on the availability to society of the expected benefits of the chemical substance.

### **5.2.7.3 The distinction between existing and new substances**

EPA classifies chemical substances as either „existing“ chemicals or „new“ chemicals. The only way to determine if the substance is a new chemical is by consulting TSCA Inventory.

Any substance that is not on the Inventory is classified as a new chemical. If a substance is new, it can be manufactured for a commercial purpose only if it is subject to an exemption from PMN reporting or a TSCA reporting exclusion. A new chemical is eligible for addition to the TSCA Inventory after the PMN review has been completed.

Once a substance is listed on the TSCA Inventory, it is considered an existing chemical. For existing substances the Inventory can be used to determine if there are restrictions on manufacture or use under the TSCA. The Existing Chemicals and Chemical Testing Program focus on 3,000–4,000 HPV (High Production Volume) chemicals.

Most enzymes were placed on the original TSCA Inventory in 1979 which includes a subset for enzymes. For inventory reporting purposes, enzymes are considered to be UVCB sub-

stances (of Unknown or Variable composition, Complex reaction products, Biological materials). These substances lack an accepted molecular formula representation and cannot be represented by unique structure and molecular formulas. In general, chemical substances which fall within the UVCB listing do not need to be individually reported under the Inventory.

EPA makes its decision on equivalence of enzymes based on the substrate specificity and any other distinguishing characteristics the substance may exhibit. If an enzyme product retains the same substrate specificity as other enzymes included under the Inventory UVCB listing, the substance is considered to fall within the existing Inventory listing and is thus not subject to the notification requirements of Section 5 of TSCA. There is no guidance document available for this policy.

#### **5.2.7.4 The premanufacturing-notification procedure**

##### **A description of administration, duties of the notifier and the Authority including**

The term chemical substance also covers industrial enzymes. Therefore, the notification procedure is described for chemical substances in general. In case, there are special requirements concerning industrial enzymes, are they described in the relevant part of the text.

Anyone who plans to manufacture or import a new chemical substance for a non-exempt commercial purpose is required by Section 5 of TSCA to provide EPA with a premanufacture notice (PMN) at least 90 days prior to the manufacture or import of the chemical. After receiving a PMN form, EPA sends an acknowledgement letter that includes the PMN number assigned to the submission and the date the PMN review began. EPA controls if the PMN is complete and valid. If the notice is declared incomplete due to chemical identity or nomenclature errors, it is returned to the submitter for resubmittal.

If the identity of some reactants for substance synthesis, or of the substance itself is unknown to the manufacturer, a letter of support can be used to enable EPA to have full identity information.

EPA evaluates chemistry, hazard and exposure of the new chemical substance and then performs a risk assessment<sup>34</sup> on the new chemical substance in order to determine if an unreasonable risk may or will be presented by any aspect of the new substance. If, based on an assessment of the potential exposures and releases associated with the new chemical, EPA concludes that the new substance may pose an unreasonable risk to human health or the environment, testing and restrictions may be required. EPA develops risk management options and, if necessary, negotiates consent agreements aimed at controlling the potential risks. The current status of the PMN is published on the EPA web page.

If the Agency has any concerns about the substances, the notifier will be informed before the end of the review period. Otherwise, the notifier will receive no subsequent notification and is free to manufacture or import the substance the day after the review period ends.

The review period of a new chemical is structured in four distinct, successive technical phases dropping substances of low-risk in an early stage and focusing on those substances of greater risk when the review progresses:

- (1) the chemistry review phase
- (2) the hazard (toxicity) evaluation phase
- (3) the exposure evaluation phase and
- (4) the risk assessment / risk management phase.

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<sup>34</sup> Risk assessment is the characterization of the potential for adverse health or ecological effects resulting from exposure to a chemical substance.



EPA groups PMN chemicals into categories depending on the chemical and toxicological properties (45 categories in total) allowing the review to be facilitated. The inclusion in a category is determined by molecular structure, boundary conditions such as molecular weight, equivalent weight, the log of the octanol/water partition coefficient (log P), or water solubility, and standard hazard and fate tests. When a new substance is identified as being a member of a category, the chemical is evaluated in the context of the potential health or environmental concerns associated with that category.

Concerning enzymes, EPA's experience is still limited as most enzymes were placed on the TSCA Inventory in 1979. The decision on equivalence of enzymes is mainly based on the substrate specificity but any other distinguishing characteristics, the substance may exhibit, will be used if available. The submitter is asked to supply any information and characteristics that would help to identify the enzyme. Specific characteristics used to describe enzymes are the same whether produced by GMO's or naturally occurring microorganisms.

According to EPA, enzymes that are produced by genetically modified organisms or extremophilic microorganisms are reviewed the same way. If the substrate specificity is affected but not completely changed, EPA will evaluate that along with any other factors to determine equivalency. In summary, EPA reviews each enzyme on a case-by-case basis and determines its equivalency to another enzyme based on the specific characteristics it exhibits. However, if an enzyme is substantially equivalent to another, even modulation of its existing biological activity may not make it a „new” enzyme (pers. comm.)

For the vast majority of PMN substances, EPA is unable to reach a decision based on the submitted data alone. Therefore, EPA utilizes a number of technical approaches to overcome the lack of data during risk assessment, e. g. chemistry review, analysis of structure-activity relationships (SARs), analysis of quantitative structure-activity relationships (QSARs), etc.

Currently, the EPA receives approx. 2,500 PMNs annually. Based on current information, the Agency takes action to control potential risks to health or the environment on approximately 10% of the PMNs submitted. Only 2-3% of the total number of PMNs submitted (20-30% of the above 10%) now undergo a standard review, while the remaining 7-8% are identified as members of the New Chemicals Program chemical categories.

### **5.2.7.5 Content of the premanufacturing-notification dossier**

The PMN form indicates the type of notice (exemptions etc.) and includes a certification statement that the information provided is correct and to the best knowledge of the submitter, and the test data submitted are in compliance with 40 CFR 720.50. The submitter of a PMN may claim any information in the notice as confidential.

The PMN dossier consists of 3 parts and has to contain the following information: submitter identification, chemical identity, description of uses, production / importation volume, description of by-products, description on human exposure, description of disposal practices, any available health & environmental effects test data. Unless a substance is imported this requires a description of the production process. A description of its content in more detail is given below.

There are no information requirements addressing the biological activity of an enzyme or modified enzyme except to the extent it that it describes the required information in the notice: biological activity is especially pertinent to chemical identity and use. The PMN submitter is asked to supply any information / characteristics that would help to identify the enzyme, e. g. the substrate specificity, the actual chemical formula/structure and any other distinguishing characteristics the substance may exhibit.

The submitter is required to provide health & environmental effects test data, including physicochemical properties data that are in his possession or control and a description of any

other effect data known to or reasonably ascertainable to the submitter. Specific testing prior to the premanufacturing-notification is not required. EPA may require testing if they are unable to find that the chemical will not present an unreasonable risk.

### **Detailed content of the PMN dossier**

The PMN dossier (notice) consists of three forms (part I-III). Any information in the PMN notice may be claimed as confidential by the submitter.

#### ***Part I – General Information***

##### Section A – Submitter identification

- name and address of submitter / agent / joint submitter / technical contact
- type of notice (manufacture, import).

##### Section B – Chemical identity information

- class of substance (Class1 or Class2<sup>35</sup>)
- chemical name (CA nomenclature)
- molecular formula and CAS number
- chemical structure diagram for Class1 substances; for Class2 substances the precursor substances (incl. CAS No.), description of nature of reaction or process, composition, chemical structure diagram have to be reported
- polymers
- impurities
- synonyms
- trade identification (trade names)
- generic chemical name
- by-products.

##### Section C – Production, import, use information

- production volume
- use information: category of use, percentage of production volume, formulation of the new substance)
- hazard information (incl. hazard warning statement, label, MSDS, etc.).

#### ***Part II – Human Exposure and Environmental Release***

##### Section A – Industrial sites controlled by the submitter

- operation description: site, amount and duration, process description, manufacture/processing operations diagram

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<sup>35</sup> Class1: a single molecular entity that can be represented by a single, definite structural diagram. Class2: a substance whose composition cannot be represented by a single definite chemical structural diagram. Class2 substances include UVCB's or well-defined substances without specific structures.

- occupational exposure: worker activity and maximum duration of activity, protective equipment/engineering controls, physical form and % new substance, number of workers exposed
- environmental release and disposal: release point identified in the process description, amount of new substance released, media of release, description of control technology and efficiency, destination of releases to water.

#### Section B – Industrial sites controlled by others

- operation description
- worker exposure / environmental release.

#### Optional pollution prevention and recycling information:

The PMN submitter may provide information not reported elsewhere regarding his efforts to reduce or minimize potential risks associated with activities surrounding manufacturing, processing, use and disposal of the PMN substance (e. g. source reduction, recycling activities and safer processes or products available due to the new chemical substance).

#### ***Part III – List of attachments***

The list of attachments includes the MSDS and all attachments to the PMN, e. g. individual toxicity studies, GPC data, spectra, exposure monitoring evaluations, physical and chemical properties data, SAR modelling, test sample characterization data, explanatory comments, etc. The PMN submitter is required to submit all test data in his possession or control and provide a description of all other data known to or reasonably ascertainable by him, if those data are related to the health and environmental effects of the manufacture, processing, distribution, use or disposal of the new chemical substance.

#### Physical and chemical properties worksheet:

EPA recommends to submit this worksheet as it will simplify the review process. When these data required for chemical evaluation are not reported in a PMN submission, EPA finds or estimates values for the missing data using literature and database sources, estimation of physicochemical properties from structural analogs or using computer estimation programs. This measured or estimated properties should be for the neat chemical substance.

The submission of the physical and chemical properties worksheet is optional and may include information on: the physical state of the neat substance, vapour pressure, solubility in a solvent and water, melting temperature, boiling temperature, spectral data (to verify the identity as well as to identify the presence of unreacted functional groups and unknown, possibly toxic by-products of the substance), dissociation constant, particle size distribution, octanol/water partition coefficient, Henry's Law constant, volatilisation from water and soil, pH, flammability, explosability, adsorption coefficient, etc.

#### **5.2.7.6 Testing of the substances properties**

TSCA does not require toxicity testing of a new chemical substance prior to submission of the PMN. EPA receives the data that are available and then determines whether there may be an unreasonable risk associated with the chemical.

The New Chemicals Program can require submission of any additional data, including the development of data through testing, when the information included in the PMN as well as the data available to the risk reviewer from internal archives is not adequate to clarify whether the likely risk is unreasonable.

If reasonably ascertainable test data or other information, which adds to or makes more complete the determination of the potential unreasonableness of risk becomes available within the PMN review period, the submitter is obliged to send this information to EPA. This obligation includes additional toxicological information, details on manufacture, processing, use, and disposal, likely worker exposures and environmental releases, and facts on innovations and improvements in product chemistry and safety practices.

#### 5.2.7.7 Risk assessment

*Risk* is defined as the probability that a substance will produce harm under specified conditions, and is a function of the intrinsic toxicity of a substance and the expected or known exposure to the substance. In practical situations, the critical factor is not the intrinsic toxicity of a substance, but the risk is associated with its use.

The *risk assessment* is the characterization of the potential for adverse health or ecological effects resulting from exposure to a chemical substance. The New Chemicals Program focuses on substances of greater risk. Therefore, a risk assessment that is the final step of the review process will be performed by EPA experts only on substance of substantial risk.

The potential risk of the chemicals substances is estimated by toxicologists, chemists, biochemists, engineers and by experts of other disciplines. As many PMN dossiers do not include toxicological data structure-activity relationships (SAR) are used to predict toxicity. An exposure and release modelling is done using information and data submitted with the PMN form and other information available. The risk is predicted as a function of hazardousness of the substance and expected exposure to it ( $\text{hazard} \times \text{exposure} = \text{risk}$ ).

*Risk management* is the weighing of policy alternatives and selecting the most appropriate regulatory or non-regulatory action after integration of risk assessment with social and economic considerations.

#### 5.2.7.8 Further actions after premanufacturing-notification

##### Publication of test data

The *TSCA Inventory* is an inventory that includes chemical substances that were manufactured, imported, processed or used as an intermediate between 1 January 1975 and 30 June 1979, plus substances added after completion of the 90-day PMN review and submission of a Notice of Commencement of Manufacture or Import (NOC). Once a substance is listed on the TSCA Inventory, it is considered an existing chemical. The Inventory currently contains over 70,000 existing chemicals.

A new chemical is eligible for addition to the TSCA Inventory after the PMN review has been completed. The company that submitted the PMN must provide a Notice of Commencement of Manufacture or Import (NOC) to EPA within 30 days of the date the substance is first manufactured or imported for non-exempt commercial purposes. After a NOC is reviewed, the chemical will be listed.

Substances on the Inventory are divided into two classes for ease of identification.

*Class 1* substances have discrete molecular formulas and fully-defined structural diagrams. *Class 2* substances cannot be represented by a single definite chemical structural diagram and can further be divided into three subgroups. Class 2 substances includes substances of unknown, variable, or uncertain composition, complex mixtures or reaction products, or well-defined substances without specific structures (UVCB substances). Each name for a UVCB substance includes more than one molecular entity. Each UVCB can be considered to be a category of molecules, often closely related.

Enzymes are considered to be UVCB substances (mentioned in section D<sup>36</sup>: substances that are biological materials or made from biological materials). Although some of these more complex biological substances were reported and included on the original TSCA Inventory in 1979, EPA has not yet developed guidance for their Inventory representation.

The following information is included in TSCA Inventory:

<u>non-confidential:</u>	<u>confidential:</u>
CAS No.	chemical identity
chemical name	statement of intent to manufacture or import for commercial purposes
molecular formula	
UVCB	description of R&D activities
some trade names	description of intended application or use
	infrared (or alternate) spectra
	estimated data of PMN submittal
	address of manufacturing or processing facility
	description of manufacturing process

### **Actions taken: risk reduction, prohibition and restrictions measures**

Depending on the risk management phase within the review process the possible outcomes of a PMN review may be the following: prohibition or limiting activities (Consent Orders), Significant New Use Rules (SNURs) or Actions which are described in the following. Concerning enzymes, there have been no actions under TSCA section 5 so far.

### **Consent Orders**

Almost 90 percent of PMNs submitted to EPA complete the review process without being restricted or regulated in any way. However, EPA may issue a TSCA Section 5(e) Order to prohibit or limit activities associated with the substance.

In practice, these Section 5(e) Orders are almost always issued as „Consent Orders“ that are signed by both EPA and the chemical manufacturer.

Consent orders are set when there is insufficient information to evaluate the human health and environmental effects of the substance, and the substance may present an unreasonable risk of injury to human health or the environment (the „risk-based“ finding), or when the substance will be produced in substantial quantities and may be anticipated to enter the environment in substantial quantities or there may be significant or substantial human exposure (the „exposure-based“ finding).

Risk-based Consent Orders typically contain requirements regarding toxicity or environmental fate testing by a certain production volume, worker personal protective equipment, New Chemical Exposure Limits (NCEL's), distribution/use/disposal restrictions, hazard communication (e. g. MSDS, labels), restrictions on release to water and/or air, recordkeeping requirements, production/importation volume testing trigger. Exposure-based Consent Orders consist primarily of a requirement to conduct triggered testing plus recordkeeping and risk notification in case the test data indicates a risk.

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<sup>36</sup> Toxic Substances Control Act Inventory Representation for Chemical Substances of Unknown or Variable composition, Complex reaction products and Biological materials: UVCB Substances.

### Significant New Use Rules (SNURs)

Together with the development of Consent Orders EPA promulgates TSCA Section 5(a)(2) Significant New Use Rules (SNURs) for a certain new chemical substance. The SNUR requires that manufacturers, importers and processors of certain substances notify EPA at least 90 days before beginning any activity that EPA has designated as a „significant new use“ and the submission of a Significant New Use Notification (SNUN). These new use designations are typically those activities prohibited by the Section 5(e) Consent Order. The notification required by SNURs allows EPA to prevent or limit potentially adverse exposure to or effects from the new use of the substance.

SNURs can include for example environmental release activities and certain industrial, commercial, or consumer activities, as well as protection in the workplace and hazard communications.

It is always the obligation of the manufacturer or processor selling a chemical substance to notify the user of the SNUR status of that substance.

### Limitation, restriction, prohibition

If EPA determines that a new chemical will present unreasonable risk, EPA may limit the amount or impose other restrictions on the substance or completely prohibit the substance by issuing a proposed order or applying to a US District Court for an injunction (TSCA Section 5(f) Actions).

## 5.2.8 Notification of technical enzymes in Canada

The Canadian Environmental Protection Act (CEPA) was promulgated on 30 June 1988. The provisions for the control of substances new to Canada in CEPA are intended to ensure that no new substance is introduced into the Canadian marketplace before an assessment of its risks to human health or the environment has been completed. Features of the new substances program include criteria for identifying new substances, a mechanism for assessing new substances and, when necessary, the enabling powers to implement specific controls.

The CEPA approach to controlling new substances is preventative, employing a pre-import or pre-manufacture assessment process. Substances suspected of being toxic may be controlled as necessary including a prohibition of their import or manufacture.

The definition of „substance“ in CEPA includes both inanimate and animate matter. As a result, the Act provides the ability to address substances ranging from chemicals to organisms. The manufacture or import of biotechnology substances which are either organisms, biochemicals or biopolymers (the products of microorganisms such as enzymes) are regulated under the Biotechnology Regulations and Guidelines accompanying the New Substances Notification Regulation (NSNR).

The Information Note *Reporting Substances that are Products of Microorganisms under the New Substances Notification Regulation* gives guidance on how to deal with these substances.

### 5.2.8.1 Scope of the New Substances Notification Regulation (NSNR)

#### Substances subjected to Notification

Notification is required if the material proposed for import or manufacture is a substance as defined in the Act, „new“ in the context of CEPA and neither excluded nor exempted from notification.

#### Substances not subjected to the New Substances Provisions of the CEPA

New substances do not require notification when the following criteria are met:

- Substances covered under the Feeds Act, Fertilizers Act, Health of Animals Act<sup>37</sup>, Pest Control Products Act and Food and Drugs Act are subject to notification under CEPA if used in applications other than those regulated by these Acts. In these instances, notification under the NSNR of CEPA is required before manufacture or import commences for the new use.
- Transient reaction intermediates that are not isolated and are not likely to be released into the environment.
- Impurities, contaminants and partially unreacted materials the formation of which is related to the preparation of a substance.
- Substances produced when a substance undergoes a chemical reaction that is incidental to the use to which the substance is put or that results from storage or from environmental factors.
- A substance is exempted from notification when it is manufactured or imported in a quantity that does not exceed the maximum quantity prescribed.

The NSNRs do not apply in respect of a substance that is loaded on a carrier outside Canada and moved through Canada to a location outside Canada, whether or not there is a change of carrier during transit.

Substances described by the following definitions are not subject to the New Substances Provisions of CEPA and therefore excluded from notification.

- Mixtures are not considered substances and, consequently, not candidates for notification.
- A manufactured item that possess a definite shape or design necessary to its final function.
- Material contained in effluents, emissions, and wastes is excluded from the statutory definition of a new substance. However, if a material in this category is subsequently used as a commercial product, it will be considered a notifiable substance and, if not listed on the Domestic Substances List (DSL), will be subject to the notification regulations.

#### **Proteins subject to the „two percent rule“<sup>38</sup>**

A protein that is not on the DSL can be considered to be substantially equivalent to a protein that is listed on the DSL. Proteins that are substantially equivalent are not subjected to the NSNR. A protein is considered substantially equivalent if:

- (i) the function of the protein has not been changed from the protein listed on the DSL; and
- (ii) (a) the protein has 98 % amino acid sequence homology with the listed protein, based on amino acid or DNA sequence; or  
(b) the protein is 98 % identical to the listed protein based on all of the following items: molecular weight, isoelectric point (pI), amino acid composition, peptide map, and N-terminal sequence.

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<sup>37</sup> This Act defines a „veterinary biologic“ as „a helminth, protozoa or microorganism, a substance or mixture of substances derived from animals, helminths, protozoa or microorganisms, or a substance of synthetic origin that is manufactured, sold or represented for use in restoring, correcting or modifying organic functions in animals or for use in diagnosis, treatment, mitigation or prevention of a disease, disorder or abnormal physical state, or the symptoms thereof, in animals“. Therefore, all veterinary biologics that are biochemicals or biopolymers are subject to the Health of Animals Act and its Regulations and exempt from the NSNR, provided that its use as a veterinary biologic is the only use for that substance in Canada.

<sup>38</sup> Reporting Substances that are Products of microorganisms under the NSNR.

In certain circumstances, substantial equivalence could be applicable beyond the established 2% limit. The rationale for substantial equivalence above the 2% limit must be discussed with officials from Environment Canada to determine whether it is applicable.

### 5.2.8.2 Definitions

*Substance* (as defined in Section 3 of CEPA)

is any distinguishable kind of organic or inorganic matter, whether animate or inanimate, and includes

- any matter that is capable of being dispersed in the environment or of being transformed in the environment into matter that is capable of being so dispersed or that is capable of causing such transformations in the environment;
- any element or free radical;
- any combination of elements of a particular molecular identity that occurs in nature or as a result of a chemical reaction; and
- complex combinations of different molecules that originate in nature or are the result of chemical reactions but that could not practicably be formed by simply combining individual constituents.

In some instances, materials derived from natural sources and complex reactions cannot be characterised in terms of constituent chemical compounds because their composition is too complex or variable. These materials are commonly referred to as Unknown or Variable composition Complex reaction products and Biological materials (UVCBs) and are considered a single substance for notification purposes.

Mixtures that are deliberately prepared formulations, or reaction mixtures that are fully characterized in terms of constituent substances (except for minor impurities) are not considered substances and, consequently, not candidates for notification. However, if any constituent of a mixture is a new substance, that constituent is considered to be a notifiable substance.

*Biotechnology*

is defined as the application of science and engineering in the direct or indirect use of living organisms or parts or products of living organisms in their natural or modified forms. Items covered under the biotechnology term are all organisms, their parts and products. Although this is a very broad definition only certain products of biotechnology are regulated by Environment Canada or other federal government departments/agencies.

*Biotechnology product*

is defined as a substance that is produced by means of biotechnology. The term biotechnology product covers a wide variety of potential substances including microorganisms and organisms other than microorganisms, as well as products of these microorganisms such as biochemicals and biopolymers. The term biotechnology product does not include commercial products that are deliberate formulations of different individual substances. However, care should be taken to consider whether a deliberate formulation may also contain a new substance other than a biotechnology product such as a chemical or a polymer that may also be subject to the NSNR.

Inanimate products of biotechnology are dealt with as „substances“ under part 5 of CEPA. Part 6 of CEPA establishes an assessment process for new animate products of biotechnology (such as living organisms).

*Biochemical*



means a biotechnology product, other than an organism or biopolymer, that is produced by a microorganism, including killed microorganisms.

#### *Chemicals*

include all substances that are not polymers, biopolymers, organisms, or biochemicals.

#### *Toxic*

A substance is toxic if it is entering or may enter the environment in a quantity or concentration or under conditions having or that may have an immediate or long-term harmful effect on the environment; constituting or that may constitute a danger to the environment on which human life depends; or constituting or that may constitute a danger to human life or health.

#### *Transitional period; transitional chemical*

means the period beginning on January 1, 1987 and ending on June 30, 1994.

Chemicals, not listed on the DSL, that were manufactured or imported in a quantity greater than 20 kg in any calendar year during the transitional period are defined as transitional chemicals.

#### *Post-transitional period*

For a biochemical first manufactured or imported into Canada between the end of the transitional period (June 30, 1994) and when the amended Regulations come into force (April 1, 1997) (post-transitional substance) a notification must have been provided before April 1, 1997. This is required in order that the manufacture or importation of the biochemical continue, subject to the results of the assessment, once the Regulations come into force. If these biochemicals have not been notified and assessed by April 1, 1997, they will be considered new substances and their manufacture and importation will have to halt until a notification is provided and the assessment period has expired.

### **5.2.8.3 The distinction between existing and new substances**

The Domestic Substances List (DSL) is the sole basis for determining whether a substance is new for the purposes of CEPA. Substances on the DSL are considered to exist in Canadian commerce and do not require notification. Substances not appearing on the DSL are considered to be new to Canada and are subject to notification and their potential for adverse environmental and human health effects must be assessed before they can be manufactured in, or imported into, Canada.

### **5.2.8.4 Exemptions**

Exempted from notification requirements of NSNR are:

- product development substances,
- site-limited intermediate substances, or
- substances that are being manufactured or imported by a person for export only, if the quantity of the substance does not exceed 10,000 kg.

### **5.2.8.5 The notification procedure**

#### **A description of administration, duties of the notifier and the Authority**

The assessment process begins when Environment Canada receives a notification prepared by the individual or company that proposes to import or manufacture a new substance.

After receipt and preliminary screen of the NSN, an acknowledgement will be issued specifying the starting date of the assessment period and the NSN Reference Number. Acknowledgement indicates that the administrative information is satisfactory and that all required information has been received but not yet reviewed. A rejection or interruption notice will be issued if the NSN contains significant omissions or errors in the mandatory information requirements.

When additional time is required to complete an assessment, the notifier will be advised of an extension of the assessment period before the end of the initial assessment period.

The notifier will be advised in writing, before the end of the assessment period, whether or not the government suspects that the substance is toxic, and what action, if any, will be taken by the government. When the substance is suspected to be toxic, control measures (prohibition and restrictions) may be applied to minimise any risk to human health or the environment. If there is no suspicion that the substance is toxic, the notifier may proceed with import or manufacture after the assessment period has expired.

After the assessment period Environment Canada is obliged to place the substance on the DSL if certain criteria have been met.

### Information requirements

Appropriate information requirements for assessment of new substances are specified in the relevant part in the NSNR:

- Part I applies to non-polymeric substances referred to as chemicals and biochemicals, e. g. enzyme (except for biotechnology products derived from a whole plant or animal or from parts of a whole plant or animal. Cell cultures are not considered to be part of a whole plant or animal);
- Part II for polymers and biopolymers.

Microorganisms, parts of microorganisms and substances produced by microorganisms or cell cultures (i. e. biochemicals and biopolymers) are subject to information requirements similar to those for chemicals and polymers and are prescribed in the biotechnology portion of the Regulation.

The information requirements for biochemicals depend on the quantity manufactured/imported and are those prescribed in the relevant Schedule (I - III) – together with certain additional information that addresses the nature of the production process and the potentially unique biological activity of enzymes and nucleic acids (detailed information see section 5.2.8.7).

Information requirements in NSNR are tailored to the use and quantity of the biochemical. Decision schemes are provided to help select the appropriate Schedule of information. The schemes specific to chemicals also apply for biochemical substances. Before using the schemes, it should be determined:

- Whether the substance exists on the Non-domestic Substances List (NDSL) that specifies substances believed to be in international commerce. Substances listed on the NDSL require less detailed notification packages for the assessment.
- Whether the substance meets the criteria for a transitional or post-transitional substance (see section 5.2.8.2). The information requirements and notification time periods for transitional chemicals vary depending on when trigger quantities were exceeded.
- Whether the substance falls within any of the prescribed special categories which are exempted from notification requirements of NSNR: research & development; product development; export only; site-limited intermediates.

- An estimate of the yearly and cumulative manufacture or import quantities: The notifier must develop an accurate estimate of the annual quantities and the accumulated total amount of the new chemical imported and/or manufactured. This information is needed to ensure that additional information requirements are submitted before higher „trigger“ quantities are reached. The prescribed trigger quantities relate to the actual amount of chemical manufactured and imported, not to the quantity of formulation containing the chemical.

The notified substance may be manufactured or imported at the earliest

5 days – for Schedule I

45 days – for Schedule II

90 days – for Schedule III

#### **5.2.8.6 Post-notification responsibilities**

- Correction of Information: Information generated after a notification that reasonably supports the conclusion that the substance is toxic, or is capable of becoming toxic, must be provided to Environment Canada.
- If, subsequent to the notification of a new substance, a notifier obtains new information that reasonably supports the conclusion that the substance is toxic, or is capable of becoming toxic, the notifier is obliged to provide that information to the Environment Canada without delay.
- Notice of Excess Quantity: The notifier has to advise Environment Canada within 30 days of exceeding the quantity when the manufactured or imported quantity exceeds
  - 5,000 kg in any calendar year or an accumulated total of 25,000 kg for chemicals listed on the NDSL; or
  - 10,000 kg in any calendar year or an accumulated total of 50,000 kg for chemicals not listed on the NDSL; or
  - 10,000 kg in any calendar year or an accumulated total of 50,000 kg for polymers; or
  - 5,000 kg in any calendar year or an accumulated total of 25,000 kg for transitional chemicals provided these quantities are exceeded after the NSNR come into force.

#### **5.2.8.7 Content of the notification dossier**

The New Substances Notification (NSN) form should be sent in two copies by mail or courier to the New Substances Division – Commercial Chemicals Branch of Environment Canada. All information must be provided in one of the two official languages - English or French.

A complete notification must contain administrative and substance identity information (details see under „Administrative and substance identity information“), technical information (details see under „Technical information requirements“), all laboratory reports (including the name and address of the head of the quality assurance unit of every laboratory that developed test data), waiver justifications, and other attachments necessary to fulfil the requirements set by regulation. Substance identity and technical information requirements vary depending on the quantity manufactured/imported and are defined in detail in the relevant Schedule (I – III). This does not apply for product development substances, site-limited intermediate substances and substances manufactured or imported for export only. For products of biotechnology additional information (to those described in Schedule I – III ) is required (see Technical information requirements).

#### **Administrative and substance identity information**

must include the following information:

- (1) Certification statement including the name, address and signature of the person manufacturing or importing the substance.
- (2) Corporate headquarters (name and address).
- (3) Proposed site of manufacture or port of entry.
- (4) Canadian agent, if the corporate headquarters is located outside Canada (name and address).
- (5) Technical contact (name, position, company, address, and telephone and facsimile numbers).
- (6) Amount (Indication of the trigger quantity that the notifier expects to exceed on the date given).
- (7) Date of exceedance (on which manufacture or import is anticipated to exceed the trigger(s) quantity indicated).
- (8) Activity (substance will be manufactured and/or imported).
- (9) Schedule information (indication of the schedule provided and substance type).
- (10) Official language of correspondence.
- (11) Substance information (complete and unambiguous identification of the new substance including chemical name, trade names, synonyms, CAS No, structural formula, gram molecular weight, purity, impurities, additives and stabilizers and MSDS).
- (12) Confidentiality requests (indication if the information shall considered or non-confidential)
- (13) Information sharing agreement.

### Technical information requirements

The information requirements for biochemicals are those prescribed in Schedule I, II, III depending on the quantity manufactured/imported together with certain additional information required for biotechnology products (Schedule XIV) that are considered necessary to address the nature of the production process and the potentially unique biological activity of enzymes and nucleic acids. A detailed list of the required information depending on the Schedule is given in Table 15. In order to find out which Schedule is applicable Table 14 should be consulted.

The technical information required include: data on physico-chemical properties, mammalian toxicity and ecotoxicity, exposure information, indication if the substances has been notified in other agencies and additional information.

*Table 14: Quantity of the substance manufactured/imported Schedules I – III\**

Substance not listed on DSL and	Not specified on the NDSL	Specified on NDSL (that are believed to be in international commerce)
Schedule I	20 – 1,000 kg / yr	1,000 – 5,000 kg / yr or accumulated total of 5,000 – 25,000 kg
Schedule II	1,000 – 10,000 kg / yr or accumulated total of 5,000 – 50,000 kg (data on adsorption-desorption and hydrolysis as a function of pH are not	> 5,000 kg / yr or accumulated total of > 25,000 kg

Substance not listed on DSL and	Not specified on the NDSL	Specified on NDSL (that are believed to be in international commerce)
	required)	
Schedule III	> 10,000 kg / yr or accumulated total > 50,000 kg	-
Schedule XIV	Applies for biotechnology products that are not derived from a whole animals or whole plants. Information requirements are additional to those defined in Schedule I – III.	

\* This description does not take into account substances manufactured/imported during the transitional period

Table 15: Tonnage-depending technical information requirements specified in the Schedule

	Information	Schedule		
		I	II	III
Substance identification	<ul style="list-style-type: none"> <li>- chemical name of the substance</li> <li>- trade names, synonyms</li> <li>- CAS No</li> <li>- structural formula;</li> <li>- gram molecular weight;</li> <li>- purity, impurities, additives and stabilizers</li> <li>- MSDS</li> </ul>	x	x	x
Data on physico-chemical properties	<ul style="list-style-type: none"> <li>- melting point</li> <li>- boiling point</li> <li>- density</li> <li>- vapour pressure</li> <li>- water solubility or fat solubility</li> <li>- octanol-water partition coefficient</li> <li>- infra-red, ultra-violet, mass or nuclear magnetic resonance spectrum;</li> <li>- dissociation constants;</li> <li>- adsorption-desorption screening test data;</li> <li>- hydrolysis rate as a function of pH, identification of the products of the hydrolysis;</li> <li>- particle size or fibre length distribution data</li> </ul>		x	x
Mammalian toxicity and ecotoxicity	<ul style="list-style-type: none"> <li>- acute mammalian toxicity (test on the most appropriate way of uptake: oral, dermal or inhalation)</li> <li>- skin irritation, skin sensitisation</li> <li>- repeated dose mammalian toxicity</li> <li>- mutagenicity data</li> <li>- acute toxicity on fish and daphnia</li> <li>- ready biodegradability</li> <li>- all other information and test data that are relevant to identifying hazards to human health and</li> </ul>	x	x <sup>a</sup>	x <sup>b</sup>

	Information	Schedule		
		I	II	III
	the environment and that are in the person's possession			
Additional information	- use	x	x	x
	- manufacture, importation and disposal information (incl. estimate of the quantity manufactured and imported annually, intended uses, the methods for destruction or disposal, transportation modes),		x	x
	- precautions and emergency measures,		x	x
	- anticipated nature and extent of release into the environment,		x	x
	- estimated number of persons who may become exposed to the substance,		x	x
	- identification of other government agencies where the substance has been notified so far	x	x	x
	- descriptions of the analytical test methods that can be used for the detection and determination of the substance concentrations at or below the limit test results or the LC50 results of ecotoxicity tests.			x
Information required for products of biotechnology (according to Schedule XIV)	- identity of microorganism	x	x	x
	- description of adverse human health or environment effects associated with the production organism	x	x	x
	- concentration of viable microorganism	x	x	x
	- description of separation methods		x	x
	- description of enzymatic properties		x	x
	- description of nucleic acids		x	x

<sup>a</sup> one uptake route; <sup>b</sup> two uptake routes

### Identification of production microorganism

The notifier must provide an accurate identification the microorganism utilised to produce the biochemical. The identification should include a taxonomic designation to at least the species level. Taxonomic designations should follow International Codes of Nomenclature and standard taxonomic sources. Where the microorganism is genetically modified, the host microorganism and all organisms that were sources of genetic material must be identified.

The identification of the microorganism must also include any known synonyms, common or superseded names for the microorganism, and information on the source and history of the microorganism. This information should include its original source, any strain bank and accession number (e. g. ATCC) and information describing any genetic modifications.

### Concentration of viable production microorganism

Production microorganisms that are present in the commercial form of the notified substance are considered impurities. The level of these microorganisms should therefore be determined

and a description of the assay method provided<sup>39</sup>. The presence of viable microorganisms in a substance is of importance because of the potential health hazard that may result from exposure to a microorganism or its metabolic products.

### **Separation method(s)**

A description of the method or methods used to separate the production microorganism from the notified substance must be provided for biochemicals subject to Schedules II and III.

### **Substances with enzymatic properties**

If the biochemical or biopolymer possesses known catalytic activity the following information must be provided to characterise that activity:

- (a) a description of all known catalytic functions
- (b) the International Union of Biochemistry registry number (IUB), if known
- (c) known substrate specificity for each known catalytic function
- (d) optimum pH and temperature for the most appropriate substrate(s) specified in clause (b);
- (e) the catalytic constants  $K_M$  (Michealis-Menten constant) and  $K_{cat}$ , and the
- (f) conditions under which they were measured;
- (g) known cofactors necessary for enzymatic activity; and
- (h) the activity per unit weight of the final product.

This information must be provided, regardless of whether the catalytic activity is necessary for the intended use of the biochemical. This information is only required for biochemicals subject to Schedules II and III.

Requests for waiver of information must be accompanied by justifications. Information requirements for new substances or significant new activities involving a substance may be waived if the information is not needed to assess a substance, the substance can be contained in a way that protects the environment and human health or it is not practical or feasible to obtain the test data.

### **Nucleic Acids**

For biochemicals or biopolymers that are nucleic acids (repeating units of deoxyribonucleotides or ribonucleotides) the identity of any known product encoded by the nucleic acid must be provided. In addition, a description of any known biological activity (e. g. antibiotic resistance) or adverse environmental or human health effects associated with the encoded product or nucleic acid must be provided. These information requirements only apply to biochemicals subject to Schedules II and III.

## **5.2.8.8 Testing and assessment of the substance's properties**

Notification information is jointly assessed by the departments of Environment and Health to determine whether there is a potential for adverse effects of the substance on the environment and human health.

Evaluators within Environment Canada and National Health and Welfare assess the notification package to determine the acceptability of: substance identity and masked names; claims

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<sup>39</sup> This information is not required for research & development, product development substances as well as site-limited intermediates.

for confidential business information; test protocols and procedures; test data; rationales for requests for waivers of information; and exposure information.

The determination of whether a substance is or is suspected of being toxic involves assessment of the potential exposure to humans and components of the environment as well as of the adverse effects of the substance on humans or the environment. A substance may be suspected of being toxic if either the adverse effects of a substance or the potential exposure to a substance is of concern.

#### **5.2.8.9 Standards for testing and data**

The conditions and test procedures used for the development and reporting of test data must be consistent with OECD Guidelines for Testing of Chemicals that are current at the time of testing. Any deviations from the OECD Guidelines should be clearly noted and explained.

The laboratory practices used to develop test data for a new substance notification must be consistent with the Principles of Good Laboratory Practice (GLP) set out by the OECD.

The Health Protection Branch mutagenicity test guidelines should be regarded as the standard methods for developing mutagenicity test data for new substance notifications. The OECD or other mutagenicity test guidelines will be acceptable when they are equally or better suited to measure the mutagenic potential of the substance under investigation.

Information in support of a notification from alternative test protocols or from calculation or estimation methods will be acceptable when they are equally or better suited to measure the endpoint under investigation. In addition, protocols developed by individual companies or associations may also be acceptable. The method used must be clearly referenced and described in sufficient detail to permit evaluation.

In order to predict the physical, chemical, toxicological, and ecotoxicological properties of a substance structure–activity relationships (SARs) and quantitative structure–activity relationships (QSARs) can be used. Calculation or estimation methods will be acceptable if the validity of the provided data is demonstrated. Other methods used to calculate data for a notification will be accepted on a case-by-case basis.

UVCB substances are considered a single substance under the New Substances provisions of CEPA. Therefore, all tests should be performed on the entire UVCB substance. Where a prescribed test is not appropriate (e. g. melting point) the use of alternative methods should be considered (e. g. softening point). Also, the provision of information on any of the known components of the UVCB substance will assist in the interpretation of data generated on the UVCB substance.

#### **Impurities**

In the case of high levels of impurities tests should be performed on a high-purity sample of the substance. However, if a further purification of the substance is neither technically feasible nor practical, tests should be performed on the crude product. In all cases, the purity of the tested material must be stated. Furthermore, information on the physico–chemical or toxicological properties of any of the impurities will assist in the interpretation of the data generated on the impure substance.

#### **5.2.8.10 Further actions after notification**

##### **Publication of test data**

##### Domestic Substances List (DSL)



The DSL is an inventory of approximately 23,000 substances manufactured in, imported into or used in Canada on a commercial scale. It is based on substances manufactured in or imported into Canada, under certain conditions (defined as „substance”; in a quantity not less than 100 kg/yr, between 1 January 1984 and 31 December 1986 and was published on 4 May 1994 in Part II of the *Canada Gazette*. The DSL is the sole standard against which a substance is judged to be new to Canada. After the assessment of a chemical substance Environment Canada is obliged to place the substance on the DSL if certain criteria have been met (Section 30 of CEPA).

Mixtures<sup>40</sup> should not be reported. For example, a product which is a mixture of pure cultures and enzymes, should not be reported, but the individual pure cultures and enzymes should be reported. Mixtures derived from natural sources that cannot be characterized because their composition is too complex or variable are considered single substances and are reportable.

Substances occurring in nature as such, unprocessed or processed only by manual, mechanical, or gravitational means, by dissolution in water, by flotation, or by heating to remove water, should not be reported to DSL. Isolated substances, or those which are parts of mixtures, which are produced by chemical modification of naturally occurring products or are separated from them by physical processing should be reported.

Initially 45 enzymes were listed in the DSL by IUB EC number.

#### Non-Domestic Substances List (NDSL)

The NDSL specifies substances other than those on the DSL, that are in world commerce and is based on the United States Environmental Protection Agency's TSCA Chemical Substances Inventory of 1985. It contains more than 58,000 entries. The NDSL was published in Part I of the *Canada Gazette* and is maintained by Environment Canada. Substances that are not on the DSL but are listed on the NDSL are subject to lesser information requirements.

Chemicals appearing on the DSL and the NDSL are named using Chemical Abstracts Service (CAS), preferred nomenclature (English) and International Union of Pure and Applied Chemistry (IUPAC) nomenclature (French).

#### **Control measures: prohibition and restrictions**

When a substance is suspected of being toxic the Canadian government may apply the following measures (as defined in Section 29 of CEPA) in order to minimize any risk to human health or the environment:

- (a) to permit the manufacture or import of the substance subject to specified conditions;
- (b) to prohibit the manufacture or import of the substance for a period not exceeding two years (this prohibition lapses at the end of this two-year period unless a notice of proposed regulation under Section 34 of CEPA is published in the *Canada Gazette*); or
- (c) to prohibit the manufacture or import of the substance until supplementary information or test results have been submitted to the government and assessed.

Measures under Section 29 of CEPA must be taken by the government before the expiration of the assessment period. The notifier must comply with these measures or withdraw the notification.

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<sup>40</sup> Defined as a commercial product formed by the intentional mixing of two or more substances.

### 5.2.9 Notification of chemicals in Australia

Chemical or chemical product registration in Australia is conducted by a number of National Chemicals Assessment and Registration Schemes covering food, industrial chemicals, pharmaceuticals and agricultural and veterinary chemicals. The registration of enzymes would fall into one or more of these schemes depending on the use of the enzyme. The Australia New Zealand Food Authority (ANZFA) has responsibility for the registration of foods and food additives e. g. processing agents, preservatives, flavouring and colouring.

Australians National Industrial Chemicals Notification and Assessment Scheme (NICNAS) was established in 1990 with the enactment of the Industrial Chemicals (Notification and Assessment) Act 1989 (Cwlth), in short referred to as the Act. It is a Commonwealth Government scheme administered within the Chemical Assessment Division of the National Occupational Health and Safety Commission (NOHSC) and forms an integral part of the Commonwealth regulatory framework for chemicals.

The object of the Act is to provide a national system of notification and assessment of new industrial chemicals in order to find out the risks to occupational health and safety, to public health and to the environment that could be associated with the importation, manufacture or use of the chemicals and to provide information and make recommendations on these chemicals to Commonwealth, State and Territory bodies with responsibilities for the regulation of industrial chemicals.

Under this system information about the properties and effects of the chemicals are obtained from importers and manufacturers of the chemicals. The risks are minimised if potentially harmful effects are identified at the earliest possible time - prior to the chemical's importation and/or manufacture. NICNAS has established a comprehensive scientific program of international standard for assessing industrial chemicals in Australia and makes recommendations for safe use.

The Interim Office of the Gene Technology Regulator (IOGTR) is a temporary body to oversee the introduction of new legislation for the regulation of genetically modified organisms (GMOs) and GMO products not covered by the above existing regulatory schemes. The new legislation will regulate only GMOs and GMO products which do not fall within the scope of other schemes.

#### 5.2.9.1 Scope of the Industrial Chemicals (Notification and Assessment) Act

All new industrial chemicals must be notified to NICNAS and assessed prior to their import or manufacture in Australia. In general, the amount of information which is required in the notification statement increases in accordance with the proposed introduction volume of the new chemical and the associated hazards, use, handling and disposal of the notified chemical.

It is the responsibility of the importer or manufacturer of the new industrial chemical to notify NICNAS through the preparation of a notification statement.

Chemicals which are used solely for the following use categories are outside the scope of NICNAS and do not require notification:

- (a) use as an agricultural chemical or a constituent of an agricultural chemical; or
- (b) use as a veterinary chemical or a constituent of a veterinary chemical; or
- (c) therapeutic use or use as an ingredient or component in the preparation or manufacture of goods for therapeutic use; or
- (d) use as food intended for consumption by humans or animals or a constituent of such food; or

- (e) use as a food additive in food referred to in paragraph (d)

Articles, radioactive chemicals and mixtures are excluded from notification.

### **Gene Technology Act 2000 – regulation of genetically modified products**

A genetically modified (GM) product is defined in the Gene Technology Act 2000 (GT Act) as „a thing other than a GMO (genetically modified organism) derived or produced from a GMO”. GM products are derived from GMOs but are not viable, capable of reproduction or capable of transferring genetic material.

The GT Act regulates all dealings with live, viable organisms that have been modified by techniques of gene technology, regardless of whether these are also examined by other regulators. However, in the case of GM products, these are generally regulated by other regulatory agencies. The relevant product regulator in the case of GM industrial chemicals is the National Industrial Chemicals Notification and Assessment Scheme (NICNAS).

The GT Act requires that when the relevant regulatory agency receives an application for approval of a GM product, the agency must seek the advice of the Gene Technology Regulator. When the relevant regulatory agency is considering the application, the agency must take into account any advice provided by the Gene Technology Regulator. The relevant authority must notify the Gene Technology Regulator of the decision regarding the GM product, so that the Gene Technology Regulator can include the information on the Record of GMO and GM Product Dealings, the comprehensive database of GMOs and GM products approved for use in Australia.

#### **5.2.9.2 Definitions according to the Act**

*Chemical* includes:

- (a) a chemical element, including a chemical element contained in a mixture; or
- (b) a compound or complex of a chemical element, including such a compound or complex contained in a mixture; or
- (c) a UVCB substance; or
- (d) a naturally-occurring chemical (meaning unprocessed chemicals occurring in nature, or chemicals occurring in nature which have been extracted from the parent material through certain defined processes without chemical change);

but does not include an article; or a radioactive chemical; or a mixture.

*Industrial chemical*

is any chemical that has an industrial use, whether or not it also has an excluded use. This includes specialty chemicals, dyes, solvents, adhesives, plastics, laboratory chemicals, chemicals used in mineral and petroleum processing, refrigeration, printing and photocopying, paints and coatings, as well as chemicals used in the home, such as cleaning products, cosmetics and toiletries.

*Relevant industrial chemical* means an industrial chemical that is not intended for an excluded use and that is not:

- (i) a naturally-occurring chemical; or
- (ii) biological material; or
- (iii) an incidentally-produced chemical; or
- (iv) a reaction intermediate.

### *Article*

means an object that:

- (a) is manufactured for use for a particular purpose, being a purpose that requires that the object have a particular shape, surface or design; and
- (b) is formed to that shape, surface or design during manufacture; and
- (c) undergoes no change of chemical composition when used for that purpose except as an intrinsic aspect of that use;

### *Mixture*

means physical combinations of chemicals resulting from deliberate mixing or from chemical reactions, but does not include UVCB substances. Although a mixture itself is not notifiable, new industrial chemical components in the mixture are notifiable unless exempt.

*UVCB substance* means

- (a) a chemical of unknown or variable composition; or
- (b) a complex product of a chemical reaction; or
- (c) biological material, other than a whole animal or a whole plant.

## **5.2.9.3 The distinction between existing and new substances**

A new chemical is defined as one which is not listed in the Australian Inventory of Chemical Substances (AICS). Existing chemicals are also assessed by NICNAS after nomination under the Priority Existing Chemicals (PEC) program.

## **5.2.9.4 Exemptions**

Exempt from notification are substances that are

- manufactured or imported only for research, development or analytical work in a quantity of 50 kg or less per year;
- imported *solely* for an excluded use, that is use as an agricultural chemical, veterinary chemical, therapeutic good or use as a food or food additive.

Reduced information submission is required when the substance is

- manufactured in Australia in quantities larger than 50 kg per year, solely for the purpose of research, development or analytical work, provided it is site-limited and manufactured in an apparatus which cannot operate effectively to produce smaller quantities;
- introduced in quantities of less than 10 kg per year, for non-cosmetic use, and poses no unreasonable risk to occupational health, public health or the environment;
- introduced in quantities of less than 10 kg per year, for cosmetic use, poses no unreasonable risk to occupational health, public health or the environment and meets the defined criteria:

## **5.2.9.5 The notification procedure**

### **A description of administration, duties of the notifier and the Authority**

There are five main categories relating to notification for a new chemical assessment, with each category depending on the type of chemical, the amount being introduced and the period of use required (see Table 16). The notification certificate or permit is valid for a limited time.

- (1) *Commercial Evaluation Chemical (CEC)* notifications are for small volume chemicals to be introduced solely for the purpose of market evaluation where the maximum quantity to be introduced is 2 tonnes in a maximum period of two years.
- (2) *Low Volume Chemical (LVC)* notifications are for small volume chemicals to be introduced at a rate of up to 100 kg per year for a maximum of three years.
- (3) *Synthetic Polymers of Low Concern (PLC)*
- (4) *Limited Notifications (Ltd)* which are small volume chemicals (up to one tonne per 12 month period) or site-limited chemicals (manufactured at a rate of not more than 10 tonnes per 12 month period) or research, development or analytical chemicals which are manufactured or imported in a quantity of more than 50 kg but not more than one tonne per 12 month period.
- (5) *Standard Notifications (Std)* are for chemicals imported at greater than 1 tonne per year and which do not fulfil the requirements of any other category

Table 16: Summary of new chemicals notification categories

	Categories				
	CEC	LCV	PLC	Ltd	Std
<b>Outcome</b>	permit	permit	certificate	certificate	certificate
<b>Chemical amount introduced</b>	1 t/yr	100 kg/yr	unlimited	< 1 t/yr	> 1 t/yr
<b>Duration of certificate or permit</b>	1-2 years	36 months	5 years	5 years	5 years
<b>Assessment timeframe</b>	14 days	20 days	90 days	90 days	90 days

After the receipt of a notification NICNAS reviews first if the notification is in the approved format, contains all the relevant data and meets the required standards in terms of level of detail. If the submission is not complete, the applicant will be advised of the outstanding data items required for the assessment, and also that the assessment has not commenced. If the submission is grossly deficient, it will be returned to the notifier with an explanation of the deficiencies. The assessment will not commence until the notification is considered complete.

In normal circumstances, an assessment for a new industrial chemical is completed within 90 days from the date of acceptance of an application for an assessment certificate, that is, the date of receipt of the complete notification package (PLC, Ltd and Std notification). The application for a CEC and LCV is usually processed within 14 and 20 days of receipt of a valid application, respectively.

During the assessment process NICNAS can ask the notifier to provide additional information or test data. If the notifier becomes aware of new information relevant to the assessment of the chemical during the assessment, he must submit it as soon as practicable.

Following the assessment of a notified chemical under the Standard Notification and Limited Notification and PLC categories, the following reports will be written and sent to the notifier:

- assessment report (which may contain exempt information)
- full public report (the assessment report without the exempt information); and

- summary report (condensed version of the full public report for publication in the *Chemical Gazette*).

Within 14 days of receipt of an assessment report, the notifier may ask for variation to the report. If NICNAS has not received any request for changes to an assessment report from the notifier within 28 days of first forwarding the report, then it may be published in the *Chemical Gazette*.

#### Date for placing on the market:

The assessment certificate for a new chemical is given to the notifier within seven days of publishing the assessment report. Manufacturers and importers who have received an assessment certificate for a chemical must notify the Director in writing within seven days of introducing the chemical.

Under certain circumstances, a chemical may be eligible for an early introduction permit allowing it to be introduced into Australia before its assessment is complete, these are non-hazardous new industrial chemicals and chemicals where it can be shown that their immediate introduction is in the public interest. The conditions for this early introduction permit are described in section 30A and 30 of the Act.

#### Fees:

Depending on the notification category the notifier has to pay a fee when submitting a notification as well as when he applies for variation of Schedule requirements or for exempt information.

In the case the notifier provides an acceptable written draft assessment report with their notification statements he may get a rebate on the assessment fee. The notifier may also be granted a fee reduction if he submits a report of the chemical assessed under an assessment scheme from any OECD country. Therewith, NICNAS aims to avoid duplication and save time and costs.

### **5.2.9.6 Content of the notification dossier**

All notification documents should be sent to the director of Chemicals Notification and Assessment at NICNAS in Sydney, must be clearly legible and submitted in English. For each notification category a specific standard form and a notification checklist is available which should be used as far as possible in the submission of data and information required in the notification package.

A notification dossier consists of:

- the application for an assessment certificate or permit, depending on the notification category;
- the technical dossier of information about the chemical, comprising Parts A, B and C of the Schedule (Part D only for a polymer). The detailed content of this technical dossier is given below.
- any other information available to the notifier which may assist in the proper assessment of the chemical;
- any application for variation of Schedule requirements;
- any application for exempt information;
- the checklist of the relevant notification category, indicating which items in the Schedule have been submitted, items for which an application for variation has been made, and items for which an application for exempt information has been made;

- a statement that the notifier is entitled to use and to give the Director of NICNAS all the data in the notification statement;
- a declaration that all available information has been submitted;
- the appropriate fee.

### The technical dossier of information on the chemical

The technical dossier defines the data necessary to submit with the application for new chemical assessment. Data requirements depend on the notification category which are described in Table 17.

Table 17: Data requirements for different notification categories

Category	Data to be submitted		
	Non-polymer	Synthetic polymer	Biopolymer
PLC		as per Form 1-PCL	
CEC	Part B (only identity of chemical, use, exposure data on occupational and public health, environmental impact, label, MSDS)		
LVC	Part A (only summary of the chemicals occupational health, public health and environmental effects) and Part B (only identity of chemical, use, exposure data on occupational and public health, environmental impact, label, MSDS)		
Ltd	Parts AB	Parts AB or ABD	Parts ABD
Std	Parts ABC	Parts ABCD	Parts ABCD

#### Part A

- type of notification
- summary of the chemical's occupational health, public health and environmental effects (summary of the most significant results of Part C)
- summary of how the chemical meets the definition of a hazardous substance (in accordance with the NOHSC Approved Criteria for Classifying Hazardous Substances). If the chemical is a hazardous substance, the notifier should provide a health hazard classification including the appropriate risk and safety phrases and the basis of the classification
- details of any notification made in another country; Where information is available about a new chemical because of its listing on either EINECS, TSCA Chemical Substance Inventory or the two existing chemicals list in Japan, the notifier may apply for an assessment certificate under the flexibility provisions of NICNAS
- bibliography of all references used in the notification package.

#### Part B

- identity: chemical and other names, marketing name, CAS No, molecular and structural formulae, molecular weight, spectral data; confirmation of the identity of biological materials derived from animals and gene technology is required
- composition: purity, toxic or hazardous impurities, non-hazardous impurities, additives/adjuvants
- physicochemical properties of the chemical:

- melting point/boiling point (OECD<sup>41</sup> 102/103)
- specific gravity/density (OECD 109)
- vapour pressure (OECD 104)
- water solubility (OECD 105)
- hydrolysis as a function of pH (OECD 111)
- partition coefficient n-octanol/water (OECD 107)
- adsorption and desorption (OECD 106)
- dissociation constant (OECD 112)
- particle size (distribution)/fibre length (OECD 110)
- flashpoint (open or closed cup method)
- flammability limits (%) (explosive limits)
- auto ignition temperature
- explosive properties
- reactivity
- the provision of estimated values for physico-chemical parameters derived from QSARs is not encouraged and will only be accepted as a last resort after all other efforts to obtain data, including from the literature, have been exhausted
- use
- manufacture/import volume (incl. approx. percentage of total manufacture or import for each use, fields of use, methods of application, concentration in mixture)
- exposure data (occupational health, public health and environmental)
- methods of detection and determination
- proposed label(s) for the notified chemical in accordance with the NOHSC *National Code of Practice for the Labelling of Workplace Substances*
- MSDS: compiled in accordance with the NOHSC National Code of Practice for the Preparation of Material Safety Data Sheets
- (occupational and environmental) emergency procedures.

### Part C

Health and environmental effect data. The complete study reports have to be provided.

- acute toxicity data: acute oral toxicity (OECD 401<sup>1</sup>), acute dermal toxicity (OECD 402), acute inhalation toxicity (OECD 403)
- irritation/corrosion: skin irritation (OECD 404), eye irritation (OECD 405)
- sensitisation: skin sensitisation (OECD 406), respiratory sensitisation, repeated dose toxicity
- genetic toxicity. induction of point mutations (OECD 471, 472), induction of germ cell damage (OECD 478), chromosome damage (OECD 473, 474, 479)
- ecotoxicity data: fish, acute toxicity test (OECD 203), daphnia, acute immobilisation test and reproduction test (OECD 202), algal growth inhibition test (OECD 201)
- biodegradation: ready biodegradability (OECD 301 A-F), bioaccumulation.

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<sup>41</sup> refer to the OECD Guidelines for the Testing of Chemicals. An equivalent test can be used.



**Part D** (specific for polymers)

- molecular weight data
- residual monomer and impurity data; and
- stability data.

**Confidential information:**

The notifier can claim certain items of information as confidential and should give reasons to substantiate each claim. These items would not be published in the publicly available versions of the assessment report of the chemical.

**Variation of data requirements**

In the case that the provision of a data item may be scientifically inappropriate, not technically possible or not economically feasible the notifier can apply for a variation of data requirement. Data exemptions can be gained by a written application and subsequent approval from NICNAS. Sufficient information must be available in order to enable the performance of an adequate assessment of the chemical.

In some cases, NICNAS may allow or recommend the substitution of certain data items with alternatives. Where the notifier considers that it would not be technically possible to carry out a specified test, or to obtain results for the test, an application for a waiver for the test or relevant data item may be submitted.

The notifier may consider that a specified test or data item is irrelevant, unnecessary or scientifically inappropriate in the evaluation of the potential occupational health, public health and environmental hazards of the chemical. Any omission on such grounds must be justified, and included under the appropriate item heading.

The notifier may consider that the generation of a particular data item required by the Schedule is not economically feasible, and that the data item is not essential for adequate occupational health, public health and environmental assessment of the chemical. Such omissions on economic grounds must be fully justified by the notifier.

**Substance information submitted within the scope of a limited notification of a new enzyme**

So far, one public report on the assessment of a new enzyme is available (NICNAS, 1998).

The notified enzyme „Laccase“ is intended to be used as a textile bleach. The quantity imported of the enzyme is up to 1 tonne per annum for each of the first five years. The enzyme (active) and impurities are expressed together as the total organic substance (TOS).

Although, for a Limited Notification the submission of toxicological and ecotoxicological test data is not required, data on these (below listed) properties have been provided .

The assessment was performed and the full public report of the notified enzyme „Laccase“ was published in 1998, it is available in public at <http://www.nicnas.gov.au/publications/car/new/na/nasummr/na0500sr/na510.htm>.

The following substance information has been made available according to the full public report:

- (1) identity of the chemical: chemical name, CAS No, trade name, molecular weight, enzyme activity, method of detection and determination, comments on chemical identity.
- (2) physical and chemical properties: appearance, density, water solubility, comments on physico-chemical properties. Standard methods on physico-chemical characterisation

are not valid for enzymes. The enzyme is expressed as the total organic substance (TOS).

- (3) purity index, impurities, additives
- (4) use, volume and formulation
- (5) occupational, public and environmental exposure
- (6) toxicological data: The enzyme in the batch is expressed as total organic solid (TOS). All tests were performed similar to OECD guidelines.
  - acute oral toxicity (rat)
  - acute inhalation toxicity (rat)
  - skin irritation (rabbit)
  - eye irritation (rabbit)
  - skin sensitisation (guinea pig)
  - repeated dose toxicity (rat)
  - genotoxicity
    - Reverse Mutation (liquid culture) Assay (*Salmonella typhimurium*)
    - Plate Incorporation Assay (*Salmonella typhimurium*/*Escherichia coli*)
    - Chromosomal Aberrations Assay (Human Peripheral Lymphocyte Cultures)
- (7) ecotoxicological data: All test were carried out according to OECD test methods.
  - acute toxicity on fish
  - acute immobilisation of *Daphnia magna*
  - growth inhibition on algae
  - respiration inhibition (aerobic waste water bacteria)
- (8) MSDS
- (9) recommendations for the handling
- (10) publications.

### Substance information available for an existing enzyme

NICNAS has performed an assessment of the chemical „Savinase“ within the scope of the Existing Priority Chemicals program (NICNAS, 1993). Savinase is one of the commercial names for proteolytic enzymes used in the detergent industry known as proteinases. The assessment report considers also other proteinases used in the detergent industry and focuses on the use of proteinases in the manufacture of enzymatic detergents especially on use in laundry detergents.

Information was collected from a range of sources, including the information dossiers documenting toxicology, manufacturing process and data relevant to occupational exposure obtained from applicants, a literature search, site visits and information from overseas industry associations.

The following information is available in the full public report:

- (1) chemical identity of the substance: common names, CAS No, IUB No, other names, trade names, molecular weight, composition of enzyme preparations

- (2) physical and chemical properties: water solubility, vapour pressure, pH and thermal stability, enzyme activity
- (3) methods of detection and analysis (describe in general for proteinase enzymes)
- (4) use
- (5) manufacturing process of detergents containing proteinase
- (6) occupational exposure (no measured data available, exposure described in words)
- (7) animal toxicological data
  - oral acute toxicity (rat)
  - inhalation toxicity (rat)
  - skin irritation (rabbit)
  - eye irritation (rabbit)
  - skin sensitisation (guinea pig)
  - repeated dose toxicity (rat)
  - genotoxicity
    - Reverse Mutation (*Salmonella typhimurium*)
    - In vivo Chromosome Aberration Assay (Chinese hamster)
    - Dominant lethal study (mouse)
  - respiratory sensitisation (guinea pig, monkeys)
- (8) data available on human health effects: literature review on observed health effects, skin sensitisation test performed on humans
- (9) environmental data
  - acute toxicity on fish
  - biodegradation
- (10) exposure (occupational, public and environmental exposure)
- (11) publications.

#### 5.2.9.7 Prescribed standards for testing and data quality

The full test reports must be submitted. The test methods used should be described in sufficient detail to enable the assessor to determine the acceptability of the method. It should be indicated whether a standard guideline was used. NICNAS recommends that tests on physicochemical, toxicological and ecotoxicological properties should be carried out in accordance with OECD Guidelines for the testing of chemicals or equivalent guidelines. Other techniques may be accepted on a case-by-case basis if they constitute a valid method for determining the required information.

For testing carried out in Australia, notifiers must indicate whether the testing has been carried out in accordance with National Association of Testing Authorities (NATA) standards.

It is preferred that data submitted should have been obtained in accordance with currently accepted principles of good laboratory practice, for example, the *OECD Principles of Good Laboratory Practice*. A quality assurance report should also be provided with each study.

#### 5.2.9.8 Assessment of unreasonable risk

In assessing whether a risk posed is unreasonable both the potential risk (hazard and exposure level) and how that potential risk might be minimised are taken into consideration. If the potential risk is high and/or cannot be minimised, the risk posed may be considered by NICNAS to be unreasonable. If this is the case, the notifier will need to apply for an assessment permit or certificate.

The hazardous nature of the chemical is determined by using the NOHSC *Approved Criteria for Classifying Hazardous Substances*.

The processing of an assessment of new (and existing) chemicals

NICNAS officers within the Chemical Assessment Division (CAD) of NOHSC carry out the toxicological and occupational health and safety assessments. The Commonwealth Department of Health and Aged Care undertake the public health assessment for NICNAS under a service agreement. The Chemical Products Assessment Section, which is located within the Chemicals and Non-Prescription Drugs Branch of the Therapeutic Goods Administration, performs evaluations. The Risk Assessment and Policy Section of Environment Australia conduct the environmental assessment for NICNAS under a service agreement.

The type and degree of risk assessment varies depending on the type of notification. For a priority existing chemical a quantitative risk assessment may be conducted, whereas a qualitative assessment usually occurs for a new chemical. The risk assessment entails some or all of the following elements:

- hazard identification,
- hazard assessment, incorporating the dose-response relationship,
- exposure assessment, and
- risk characterisation, where the hazard and exposure assessments are integrated.

The assessment consists of chapters on occupational health and safety, public health and environment. Assessments are conducted on a case-by-case basis and will be based on a weight of evidence approach, taking into account scientific judgment, knowledge of the mechanism of action of effects, and recognition of the inherent uncertainty in extrapolating animal data to humans.

From the risk assessment, recommendations are formulated to manage the risk (regulatory and non-regulatory controls), taking into account existing risk management strategies.

The assessment reports of new and priority existing chemicals are published on the NICNAS webpage (<http://www.nicnas.gov.au/publications/CAR>). The following assessments of enzymes have been conducted so far:

New chemicals: Laccase

Priority existing chemicals: Savinase

#### 5.2.9.9 Further actions after notification

##### Classification and labelling

The hazardous nature of the chemical is determined by using the NOHSC *Approved Criteria for Classifying Hazardous Substances*. This criteria focus solely on hazards to health.

The health effects criteria as well as the relevant risk phrases are the same as those used by the European Communities in EC Council Directive 67/548/EC for classifying substances hazardous to health and its subsequent amendments.

Concentration cut-off levels for ingredients which have satisfied the health effects criteria are taken from EC Council Directive 1999/45/EC relating to the classification, packaging and labelling of dangerous preparations. For classifying mixtures which may be hazardous due to the additive effects of ingredients which have satisfied the health effects criteria formulae are used.

Physicochemical hazards and the appropriate labelling are addressed under the *National Code of Practice for the Labelling of Workplace Substances [NOHSC: 2012(1994)]* which is currently in revision.

### **Safety data sheets**

A MSDS must be submitted together with the notification dossiers.

### **Publication of data**

Five years after the assessment report has been issued NICNAS automatically lists a chemical on the non-confidential section of the AICS.

#### Australian Inventory of Chemical Substances (AICS)

is a list of approximately 40,000 chemicals and is the mechanism for distinguishing between new and existing chemicals in Australia. It consists of chemicals nominated for listing between 1 January 1977 and 28 February 1990, along with chemicals for which five years have elapsed since the issuing of a NICNAS assessment certificate. The Inventory consists of a non-confidential section and a confidential section. The following substance information is available in both the non-confidential section and confidential section of AICS:

- chemical name,
- CAS number,
- molecular formula, and
- synonyms.

AICS does not contain health hazard and/or safety information, listings of commercial products which contain the chemical, MSDS, or information linking a listed chemical with the notifier or nominator of that chemical.

Chemicals added to the AICS are periodically published in the monthly Chemical Gazette.

#### Record of GMO and GM Product Dealings

Information on GMO's and GM products are listed on this comprehensive database of GMOs and GM products approved for use in Australia by the Gene Technology Regulator.

### 5.2.10 Comparative Analysis of enzymes in chemicals regulation in the EU, USA, Canada and Australia

In the investigated countries as well as within the EU, enzymes are regulated in different legislation depending on their use, e. g. for technical purposes, in the food or feed sector, in personal care products or in medicinal products. In all countries, industrial enzymes fall under the definition of chemical substance and are consequently subjected to the notification of chemicals. Information requirements are in principle those for chemicals. Canada is the only country, where additional information addressing the special nature of enzymes are defined and requested.

This chapter gives an overview on the different approaches of industrial enzymes regulation in the EU, USA, Canada and Australia and comparatively analyses the different notification systems. A detailed description of the chemicals notification systems is given in chapter 5.2.7 (USA), 5.2.8 (Canada) and 5.2.9 (Australia). The current practice of enzyme notification within the European Union and related problems are discussed in section 5.2.1.

In order to facilitate the comparison of the investigated national systems, the most important issues of chemicals notification concerning the regulative framework, definitions, differentiation between new and existing substances, information requirements, etc. are summarised in the following table where special attention was paid to the requirements concerning enzymes.

#### 5.2.10.1 Legislative framework

European Union	USA	Canada	Australia
The legislative framework relevant for notification of industrial enzymes			
Directive 67/548/EEC and its amendments	Toxic Substances Control Act (TSCA of 1976)	Canadian Environmental Protection Act (CEPA of 1998) and New Substances Notification Regulation (NSNR): A part within NSNR addresses biotechnology products including products of microorganisms (such as enzymes).	Industrial Chemicals (Notification and Assessment) Act 1989 (Cwlth)
Authority that is responsible for the notification of chemicals and the review / assessment of the chemical			
Competent Authorities of the Member State where the substance is notified	Environmental Protection Agency (EPA)	New Substances Division – Commercial Chemicals Branch of Environment Canada	NICNAS within the Chemical Assessment Division of the National Occupational Health and Safety Commission (NOHSC)  In case of a GMO product, NICNAS seeks advice by the Gene Technology Regulator.

In the European Union, an enzyme used for industrial purposes falls under the definition of a chemical and therefore is subjected to Directive 67/548/EEC. Information required for notification is those defined for chemicals. The Member State where the enzyme is notified is responsible for notification and also takes decisions on waiving /derogation of testing.

In the USA, an enzyme manufactured by the use of new microorganisms falls under the definition of a chemical substance and is subject to the notification procedures and reporting requirements defined for chemicals. The *Final regulations for microbial products of biotechnology* focus in particular on living microorganisms used for the production of industrial enzymes.

The Canadian new substances notification system addresses biotechnology and biotechnology products. An enzyme is considered to be a biochemical that is defined as a substance produced by means of biotechnology. Biochemicals are subjected to the same notification requirements as chemicals plus some additional information requirements which address the special nature of biotechnology products.

In Australia an enzyme is covered by the definition of a chemical. Addressing the development in new technologies, Australia has established the Gene Technology Act 2000 that only regulates GMO's and GMO products which do not fall within the scope of other schemes. A GM product is defined as a „thing“ derived or produced from a genetically modified organism (GMO). A GM product used for technical purposes is assessed by the National Industrial Chemicals Notification and Assessment Scheme (NICNAS). Within the scope of an assessment NICNAS takes into account the advice given by the Gene Technology Regulator.

#### 5.2.10.2 Identity of an enzyme

As an enzyme cannot be described by means of its chemical structure, alternative approaches addressing the special nature of enzymes have been developed for notification purposes. In all investigated countries, an enzyme is described via its catalytic activity.

European Union	USA	Canada	Australia
<b>The definition of „enzyme“</b>			
Enzymes fall under the definition of „chemical substance“.	Enzymes fall under the definition of „chemical substance“.	Enzymes are considered to be biochemicals.  A biochemical is defined as a biotechnology product other than an organism or biopolymer, that is produced by a microorganism, including killed microorganisms.	Enzymes do fall under the definition of „chemical“.  A GM product is defined as a thing other than a GMO (genetically modified organism) derived or produced from a GMO; it is not viable, capable of reproduction or capable of transferring genetic material).
<b>Parameters for enzyme identification</b>			

European Union	USA	Canada	Australia
Enzymes are described via their catalytic activity.	Enzymes are described via their substrate specificity and any other available distinguishing characteristic.	Enzymes are described via their catalytic activity.	Enzymes are described via their catalytic activity.
Information requested for identification of a chemical substance			
<ul style="list-style-type: none"> <li>- name, other names (usual name, trade name, abbreviation), CAS No and name</li> <li>- molecular and structural formula</li> <li>- composition of the substance (purity, impurities, stabilizing agent, inhibitors or other additives, spectral data, HPLC, GC)</li> <li>- methods of detection and determination</li> </ul>	<ul style="list-style-type: none"> <li>- chemical name</li> <li>- molecular formula</li> <li>- CAS No</li> <li>- chemical structure diagram or precursor substances, description of nature of reaction or process, composition, chemical structure diagram</li> <li>- impurities</li> <li>- synonyms and trade names</li> <li>- generic chemical name</li> <li>- by-products</li> </ul>	<p>Chemical identity information that is usually requested for a chemical substance:</p> <ul style="list-style-type: none"> <li>- chemical name</li> <li>- trade name, synonyms</li> <li>- CAS No</li> <li>- structural formula</li> <li>- gram molecular weight</li> <li>- purity, impurities, additives and stabilizers</li> </ul> <p>Identity information required for biotechnology products (&gt; 1t/yr):</p> <ul style="list-style-type: none"> <li>- identification of the production organism (synonyms, common names, the source and history)</li> <li>- concentration of the viable production organism</li> <li>- description of the separation method</li> <li>- description of enzymatic properties (catalytic function, IUB No, substrate specificity, optimum pH and temperature, catalytic constants <math>K_m</math> and <math>K_{cat}</math>, cofactors, activity per unit weight).</li> </ul>	<ul style="list-style-type: none"> <li>- chemical and other names, marketing name,</li> <li>- CAS No,</li> <li>- molecular and structural formulae,</li> <li>- molecular weight,</li> <li>- spectral data;</li> <li>- confirmation of the identity of biological materials derived from animals and gene technology</li> <li>- composition (purity, impurity, additives)</li> <li>- methods of detection and determination</li> </ul>

Identity information usually requested for enzymes:



In the USA, the submitter of a premanufacturing notification has to supply any information and/or characteristics that would help to identify the enzyme. This includes the substrate specificity but any other distinguishing characteristics the substance may exhibit.

In Canada, certain information addressing the nature of biochemicals is requested additional to the information required for chemicals. This includes the identity of the microorganism, the concentration of viable microorganism, the description of the separation methods as well as the enzymatic properties and the description of nucleic acids.

### 5.2.10.3 Differentiation between new and existing substances

In all investigated countries, a distinction is made between substances that are present on a chemicals inventory and that may be used furthermore without need for more information, and substances new on the market which need to be notified. The inventories of chemical substances of the investigated countries are listed in the table below. For the differentiation between new and existing chemical substances usually the chemical identity is used. As enzymes are identified via their catalytic activity, this parameter is applied to differentiate between new and existing substances.

In the EU, this issue rose problems and was discussed at the Technical and Scientific Meetings on issues associated with Directive 67/548/EEC (TSM). The major problem is the criteria to show that two enzymes are identical (EINECS-listed and not listed enzymes). EINECS was considered inadequate as a reference for enzymes. A provisional guidance document on enzyme notification was agreed at the TSM which should ensure that Member States apply a consistent approach on the decision, if to notify an enzyme, and the data requirements. The guidance document was considered confidential by CAs and can therefore not be quoted in this report.

In the USA, any other information or characteristics that helps to identify the enzyme is taken into account, in case any information has been submitted. The submitter is asked to supply any information/ characteristics that would help to identify the enzyme. Enzymes that are produced by genetically modified organisms or extremophilic microorganisms are reviewed the same way. If the substrate specificity is affected but not completely changed, EPA will evaluate that along with any other factors to determine equivalency.

In Canada, proteins are subject to the „two percent rule“. A protein that is not on the DSL can be considered to be substantially equivalent to a protein that is listed on the DSL. Proteins that are substantially equivalent are not subjected to the new substances notification regulation. A protein is considered substantially equivalent if:

- (i) the function of the protein has not been changed from the protein listed on the DSL; and
- (ii) (a) the protein has 98 % amino acid sequence homology with the listed protein, based on amino acid or DNA sequence; or  
(b) the protein is 98 % identical to the listed protein based on all of the following items: molecular weight, isoelectric point (pI), amino acid composition, peptide map, and N-terminal sequence.

European Union	USA	Canada	Australia
<b>Differentiation between new and existing chemicals/enzymes; determination of the equivalence of enzymes</b>			
<p>Substances not listed on EINECS are considered to be new to the market and are subjected to notification.</p> <p>This issue poses problems as enzyme listing in EINECS is inconsistent.</p> <p>A provisional guidance document on enzyme notification was agreed according to which any enzyme not listed in EINECS has to be notified. Also, criteria on how to decide in case the enzyme is listed on EINECS were defined.</p>	<p>Substances not listed on the TSCA Inventory are considered to be new to the market and are subjected to notification unless they are exempted.</p> <p>The decision on equivalence of enzymes is based on the substrate specificity and any other available distinguishing characteristics.</p>	<p>Substances not listed on the Domestic Substances List (DSL) are considered to be new and therefore subject to notification.</p> <p>For proteins the „two-percent-rule“ is applied to determine equivalence taking into account the function of the protein, homology of amino acid sequence or certain enzyme characteristics (further clarification in the above text).</p>	<p>A chemical is considered to be new when it is not listed in the Australian Inventory of Chemical Substances (AICS).</p>
<b>Inventories of chemical substances</b>			
<p>European Inventory of Existing Commercial Chemical Substances (EINECS)</p> <p>European List of Notified Chemical Substances (ELINCS)</p>	<p>TSCA Inventory of Chemical Substances</p>	<p>Domestic Substances List (DSL): lists substances existing on Canadian market.</p> <p>Non- Domestic Substances List (NDSL): specifies substances that are believed to be in international commerce.</p>	<p>Australian Inventory of Chemical Substances (AICS)</p>

#### 5.2.10.4 Tonnage thresholds and notification requirements

European Union	USA	Canada	Australia
<b>Tonnage thresholds relevant for notification</b>			
<p>10 – 100 kg/yr (VII C notification)</p> <p>100 – 1,000 kg/yr (VII B notification)</p> <p>&gt; 1 t/yr (VII A notification)</p>	<p>&gt; 10 t/yr</p> <p>Substances that will be manufactured/imported 10 tonnes or less each year are required less information (Low Volume Exemption).</p>	<p>For substances not specified on NDSL (not believed to be in international commerce):</p> <p>20 – 1,000 kg/yr</p> <p>1 – 10 t/yr or accumulated total of</p>	<p>&lt; 100 kg/yr (low volume chemicals)</p> <p>&lt; 1 t/yr (limited notification)</p> <p>&gt; 1 t/yr (standard notification)</p>

European Union	USA	Canada	Australia
When exceeding a manufacture /import volume of 10 resp. 100 t/yr additional information may resp. must be required.		5 – 50 t > 10 t/yr or accumulated total > 50 t Substances that are specified on NDSL (believed to be in international commerce) require less information.	The period of the chemical's use is limited. There are five main categories of notification schemes depending on the type of chemical, the quantity introduced and period of use.
Information requirements for chemical notification tailored to the below stated manufacture/import volume			
> 1 t/yr (VII A notification)	> 10 t/yr	1 – 10 t/yr or accumulated total of 5 – 50 t	> 1 t/yr (standard notification)
<p>Information requirements for enzymes are those requested for new chemical substances according to Annex VII A of Directive 67/548/EEC.</p> <ul style="list-style-type: none"> <li>- chemical identity/structure,</li> <li>- information on the substance (description of the production process, proposed use including exposure estimates, tonnage, precautions, emergency measures, packaging)</li> <li>- physico-chemical properties of the substance</li> <li>- experimental data on toxicological properties</li> <li>- experimental data on ecotoxicological properties</li> <li>- possibility of rendering the substance harmless</li> </ul>	<p>Information requirements for enzymes are those requested for new chemical substances and include the following:</p> <ul style="list-style-type: none"> <li>- chemical identity,</li> <li>- production/import volume,</li> <li>- use information,</li> <li>- hazard information,</li> <li>- human exposure and environmental release (operation description, worker exposure, environmental release and disposal)</li> <li>- any available health and environment effects data,</li> <li>- data on physical and chemical properties (optional)</li> </ul>	<p>Information requirements for biochemicals are those requested for new chemical substances plus additional information that address the nature of biochemicals.</p> <p>Information requirements are tailored to the quantity manufactured/imported (prescribed in Schedules) and can include:</p> <ul style="list-style-type: none"> <li>- chemical identity,</li> <li>- MSDS,</li> <li>- experimental data on physico-chemical properties,</li> <li>- experimental data on toxicological and ecotoxicological properties,</li> <li>- information on manufacture or import incl. annual quantity, disposal,</li> <li>- use,</li> <li>- precautions and emergency measures,</li> <li>- information on environmental</li> </ul>	<p>Information requirements for enzymes are those requested for new chemical substances and include the following:</p> <ul style="list-style-type: none"> <li>- chemical identity</li> <li>- composition</li> <li>- physicochemical properties</li> <li>- use</li> <li>- manufacture/import volume</li> <li>- exposure data</li> <li>- methods of detection and determination</li> <li>- proposed label(s)</li> <li>- MSDS</li> <li>- emergency procedures</li> <li>- health and environmental effect data incl. complete study reports</li> </ul>

European Union	USA	Canada	Australia
		release and human exposure - other agencies where sub- stance is notified	

#### 5.2.10.5 Information required on the production organism

In Canada, certain information on the production organism of an enzyme as well as on the produced substances has to be submitted.

These requirements include:

- the identity of the production microorganism: taxonomic designation to at least the species level, its source and history; description of any genetic modification as well as sources of genetic material in the case the microorganism is genetically modified;
- description of adverse human health or environment effects associated with the production organism;
- concentration of viable microorganism; the production microorganism present in the commercial product is considered as impurity. This is of importance because of the potential health hazard that may result from exposure to a microorganism or its metabolic products
- description of separation methods.

In the other investigated countries, no information on the production organism is explicitly requested.

#### 5.2.10.6 Information required for substances with enzymatic properties

Canada was the only investigated country where specific information on substances with catalytic activity is explicitly requested. The following must be provided to characterise the activity, regardless of whether the catalytic activity is necessary for the intended use of the biochemical:

- a description of all known catalytic functions
- the International Union of Biochemistry registry number (IUB)
- known substrate specificity for each known catalytic function
- optimum pH and temperature for the most appropriate substrate(s)
- the catalytic constants  $K_M$  (Michealis-Menten constant) and  $K_{cat}$ , as well as the conditions under which they were measured
- known cofactors necessary for enzymatic activity
- the activity per unit weight of the final product.

### 5.2.10.7 Data available on toxicological and ecotoxicological effects

The below table lists required toxicological and ecotoxicological information for substances manufactured/imported at an amount between 1-10 t per year.

In the EU, further toxicological and ecotoxicological testing may resp. must be requested at higher tonnage triggers (10 resp. 100 t/yr).

In the USA, the notifier has to submit all health and safety data in his possession in the premanufacturing notification dossier. If all available data are not adequate to determine a likely unreasonable risk, additional data may be requested (including the development of data through testing).

In Canada, required data on toxicological and ecotoxicological properties are tailored to the quantity manufactured or imported. Chemical substances that are in international commerce (which means that they are listed on the NDSL) require less detailed information for the assessment. The following table lists required toxicological and ecotoxicological information for substances manufactured/imported at an amount between 1-10 t per year. Toxicity and ecotoxicity data requested additionally requested at the next higher tonnage threshold (> 10 t/yr) include the following: skin irritation and skin sensitisation, repeated dose mammalian toxicity, fish and daphnia acute toxicity, ready biodegradability.

In Australia, the submission of health and environmental effect data depends on the type of chemical, the tonnage and the period of use.

European Union	USA	Canada	Australia
Required information on toxicological and ecotoxicological properties of the substance at the below stated manufacture/import volume			
> 1 t/yr (specified in Annex VII A of the Substance Directive)	> 10 t/yr	1 – 10 t/yr or accumulated total of 5 – 50 t	> 1 t/yr (standard notification)
<ul style="list-style-type: none"> <li>- acute toxicity (oral, dermal, inhalative) - two routes,</li> <li>- irritation (skin, eye),</li> <li>- skin sensitisation,</li> <li>- repeated dose toxicity,</li> <li>- other effects (mutagenicity, toxicity to reproduction, assessment of toxicokinetic behaviour),</li> <li>- ecotoxicity (data on fish, Daphnia, growth inhibition on algae, bacterial inhibition),</li> </ul>	Those health and environmental effect data that is in the possession of the submitter.	<ul style="list-style-type: none"> <li>- acute mammalian toxicity (oral, dermal, inhalative) - one route,</li> <li>- mutagenicity,</li> <li>- all other information and test data that are relevant to identify hazards to human health and the environment that are in the person's possession.</li> </ul> <p>Chemical substances that are in world commerce (listed on NDSL) need less information requirements.</p> <p>Additionally, for biotechnology</p>	<ul style="list-style-type: none"> <li>- acute toxicity (oral, dermal, inhalative),</li> <li>- irritation/corrosion (skin, eye),</li> <li>- sensitisation (skin, respiratory tract, repeated dose toxicity),</li> <li>- genetic toxicity,</li> <li>- ecotoxicity (data on fish, daphnia, algae),</li> <li>- biodegradation.</li> </ul>

European Union	USA	Canada	Australia
<b>Required information on toxicological and ecotoxicological properties of the substance at the below stated manufacture/import volume</b>			
<ul style="list-style-type: none"> <li>- degradation,</li> <li>- absorption/desorption.</li> </ul> <p>When scientifically reasoned, test can be waived. The individual Member States make decisions on the modification of the testing programme on a case-by-case basis.</p>		<p>products the following has to be indicated:</p> <ul style="list-style-type: none"> <li>- the identity of the production organism (source and history),</li> <li>- a description of any known adverse human health or environmental effects of the product organism,</li> <li>- the concentration of the production organism in the final product</li> </ul>	

#### 5.2.10.8 Experience gained in the notification and assessment of enzymes

In general, the experience gained in the notification of new enzymes is limited in all investigated countries.

European Union	USA	Canada	Australia
<b>Experience gained in the notification and assessment of enzymes by the authority</b>			
<p>So far, there is one notification (&gt; 1 t/yr) of a new enzyme. A risk assessment has been performed on this new notified substance. The Member State decided on the amendment of the testing regime on a case-by-case basis.</p>	<p>Naturally occurring enzymes are listed on TSCA Inventory</p> <p>EPA has limited experience with enzyme submissions.</p>	<p>According to Environment Canada, approximately 15 biochemicals and biopolymers have been reviewed and assessed. So far, 5 have been rejected, 1 has been completed and 9 are still under review.</p> <p>No information on experience gained in notification and assessing enzymes by the authority could be communicated.</p>	<p>Two assessments of enzymes have been conducted so far: on Laccase (a new chemical) and on Savinase (an existing chemical).</p>

In the European Union, only one notification of a new enzyme was submitted so far. The responsible Competent Authority decided on the testing regime. According to an agreement with the notifier, enzymes in general must be classified as sensitising by inhalation with the symbol Xn (harmful) and with the R-phrase R42 („May cause sensitisation by inhalation“).

In the USA, many naturally occurring enzymes were placed on the original TSCA Inventory in 1979 and consequently are not subject to premanufacturing-notification reporting. EPA has assessed enzymes that have been chemically modified for industrial uses. A count on how many enzymes/modified enzymes were reviewed, could not be communicated by EPA. But, EPA has limited experience with enzyme submissions. There is no requirement for information addressing the biological activity of an enzyme/modified enzyme except to the extent it that it describes the required information in the notice (biological activity is especially pertinent to chemical identity and use). And there is no toxicity data requirement when submitting a premanufacturing-notification notice under TSCA (other than health and safety data in a submitters possession).

EPA reviews each enzyme on a case-by-case basis and determines its equivalency to another enzyme based on the specific characteristics it exhibits. Enzymes that are produced by genetically modified organisms or extremophilic microorganisms are reviewed the same way. If the substrate specificity is affected but not completely changed EPA will evaluate that along with any other factors to determine equivalency. EPA has no guidance document for their policy.

So far, Australia has performed assessments on one new enzyme within the scope of the notification of new substances and on one existing enzyme within the scope of the existing priority chemicals program. The assessment reports are accessible to the public on NICNAS homepage. The major outcomes of the assessments are summarised below.

#### Assessment of the new enzyme Laccase:

The information on the identity of the enzyme includes chemical name, CAS No, trade name, molecular weight, enzyme activity, method of detection and determination, the production strain and origin of genetic material. Furthermore, purity, hazardous and non-hazardous impurities, additives as well as the use and exposure information are indicated. The enzyme is intended for use in the textile industry. Although, for Laccase a limited notification (for small volume chemicals) was submitted not requiring the submission of toxicological and ecotoxicological test data, data on these properties have been made available, including acute toxicity (oral, inhalative), irritation (skin, eye), sensitisation (skin), repeated dose toxicity, genotoxicity. Concerning ecotoxicity, data on fish, daphnia, algae and bacteria were made available. The tests provided were pertinent to the isolated enzyme preparation to which workers are exposed.

The major outcomes of the assessment are: The enzyme preparation (sections 3.4; 3.3.3) is not likely to be harmful by acute oral exposure, nor by repeated exposure. On the basis of the investigated toxicological studies the major concern seems to lie in the skin and eye irritation potential, but not in sensitisation of the skin. There is concern over the potential for dusts and aerosols of enzymes to induce pulmonary sensitisation. It is unlikely that genotoxic effects will result from exposure to the enzyme preparation. The environmental hazard from the proposed use of the notified substance is rated as low. The assessment of Laccase resulted in recommendations on the handling of the enzyme on the working place in order to minimise occupational exposure.

#### Assessment of the existing enzyme: Savinase

Savinase is the trade name for one proteinase product. This assessment was performed on the generic category known as proteinases. The report focuses on proteinases used in laundry detergents.

Enzyme identity information includes common names, CAS No, IUB No, other names, trade names, molecular weight, composition of enzyme preparations. Furthermore, methods of detection and analysis, the use, the manufacturing process of detergents containing proteinase are described and exposure information is given. In the section physical and chemical properties, the water solubility, vapour pressure, pH and thermal stability and enzyme activity are specified.

Available animal toxicological data include data on acute toxicity (oral, inhalative), irritation (skin, eye), sensitisation (skin, inhalative), repeated dose toxicity, genotoxicity. A literature review on observed human health effects and on skin sensitisation tests performed on humans is given. Concerning ecotoxicity, data on fish and biodegradation are indicated.

The evaluation of animal toxicity revealed that all, in this report investigated proteinases have low acute oral toxicity but high acute inhalative toxicity and cause slight skin irritation and moderate eye irritation. The inhalation toxicity results present a worst case result. Evidence of skin sensitisation in animals is questionable. The 28 day repeated-dose gavage studies showed no specific target organ toxicity. The bacterial mutation assay and mammalian genotoxicity studies revealed negative results for the investigated proteinases. But respiratory sensitisation to proteinases has been documented.

Concerning effects on humans, it has been proven that proteinases, which include Savinase, are respiratory sensitisers and that exposure to proteinases can lead, to a lesser extent, to skin irritation. There are no data to suggest that proteinases are human skin sensitisers.

The enzyme preparation should be labelled with the risk phrase R42 („May cause sensitisation by inhalation“) and the safety phrase S 38 („In case of insufficient ventilation, wear suitable respiratory equipment“). As proteinases are irritants and respiratory sensitisers, inhalation and dermal exposure to these enzymes should be avoided. The assessment report gives recommendations on the basis of which proteinases can be used safely. Finally it is concluded that, if the appropriate control measures are implemented, proteinases can be used safely in Australia.



## 5.3 Enzymes in the context of food regulation

### 5.3.1 Introduction

In terms of food regulation enzymes can be distinguished into food additives and processing aids<sup>42</sup>. The main distinction between food additives<sup>43</sup> and processing aids<sup>44</sup> is that additives do have a technological function in the final food whereas processing aids do not. Processing aids are used during the manufacturing process of foodstuff. Most food enzymes are considered as processing aids only a few (e. g lysozyme and invertase) are used as additives.

From a regulatory point of view this distinction is very important because the regulation of food additives has already been harmonised in the EU while the regulation of processing aids is still governed by national legislation. National regulations on enzymes used as processing aids differ a lot among the EU Member States: in some states these enzymes are subjected to an authorisation procedure in others no regulation is in place. Furthermore, the range of enzymes permitted by national legislation varies among the Member States.

The increasing use of food enzymes and the introduction of recombinant DNA techniques in the manufacturing of these enzymes raised concerns among consumer organisations and competent authorities. Questions came up, whether the safety evaluation procedures of food enzymes from GMM are still adequate or if there is a need for additional safety requirements.

However, it seems that both the European Community and the industry are well aware about these lack of harmonised regulation. In November 1999 a task in the framework of the European Scientific Cooperation Programme (SCOOP task 7.4) was initiated and designated as „Study of the enzymes used in foodstuffs and collation of data on their safety”. This study comprises a survey on (i) enzyme preparations being used by Member States, (ii) on the extent these enzymes have been evaluated for safety, and (iii) on the current legislative situation in EU Member States. Very recently this survey was completed and the drafted final report has already been submitted to the Directorate General Health and Consumer Protection (SANCO). However, the final report has not been published so far.

Very recently, in June 2001, food enzymes were also put on the agenda of the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The JECFA is carrying out evaluations of food additives which normally lead to the calculation of an acceptable daily intake (ADI) and to the development of specifications for identity and purity.

Overall, the issue of food enzymes seems to be a very lively issue within the EU, at present.

This section briefly describes the regulatory contexts of food enzymes in the EU, in selected EU Member States and the United States. Position papers of the industry are also considered. The different requirements for safety evaluation either embodied in legislation or in guidelines are briefly described and compared.

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<sup>42</sup> Theoretically enzymes might also be considered as food ingredients. In this case enzymes from GMO would be regulated by the Regulation (EC) No 258/97 of 27 January 1997 concerning novel foods and novel food ingredients. However, in practice this is not done.

<sup>43</sup> Food additives „means any substance not normally consumed as a food in itself and not normally used as a characteristic ingredient of food whether or not it has nutritive value, the intentional addition of which to food for a technological purpose in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food results, or may be reasonably expected to result, in it or its by-products becoming directly or indirectly a component of such foods.” (Directive 89/107/EEC, Article 1.2).

<sup>44</sup> Processing aids „means any substance not consumed as a food ingredient by itself, intentionally used in the processing of raw materials, foods or their ingredients, to fulfil a certain technological purpose during treatment or processing and which may result in the unintentional but technically unavoidable presence of residues of the substance or its derivatives in the final product, provided that these residues do not present any health risk and do not have any technological effect on the finished product.” (Directive 89/107/EEC, Article 1.2).

Subsections 5.3.3.1, 5.3.3.2 and 5.3.3.4 are partly based on unpublished results of the SCOOP task mentioned above which are quoted with kind permission of Marc Chambolle<sup>45</sup> and DG SANCO.

### 5.3.2 Harmonised regulation in the EU

Enzymes in the context of EU food legislation are regulated by Directive 95/2/EEC for the regulation of food additives others than colours and sweeteners if they are considered as food additives. Furthermore, enzymes are also covered by specific legislation, e. g. Directive 93/77/EC permits the use of several enzymes in the production of fruit juices, Commission Regulation (EC) No 1622/2000 permits and sets out criteria for the use of enzymes for wine making.

Nevertheless, an harmonised authorisation system is only in place for food additives.

Food additives are authorised at EU level for all of the fifteen Member States, as well as for Norway and Iceland. The Community legislation on food additives is based on the principle that only those additives that are explicitly authorised may be used. Most food additives may only be used in limited quantities in certain foodstuffs. If no quantitative limits are foreseen, they must be used according to good manufacturing practice, i. e. only as much as necessary to achieve the desired technological effect.

Food additives may only be authorised if:

- there is a technological need for their use
- they do not mislead the consumer
- they present no hazard to the health of the consumer.

Prior to their authorisation, food additives are evaluated for their safety by the Scientific Committee on Food (SCF). Figure 10 (Annex) provides a flowchart overview of the evaluation procedure.

As far as enzymes are concerned the Community legislation on food additives consists of the following Directives:

- Council Directive 89/107/EEC, as amended by Directive 94/34/EC, that provides the framework for the authorisation of food additives.
- European Parliament and Council Directive 95/2/EC, as amended by Directives 96/85/EC, 98/72/EC and 2001/5/EC, that lay down detailed rules for authorisation of all food additives other than colours and sweeteners.

Furthermore, all authorised food additives have to fulfil purity criteria which are set out in detail in three commission directives: Directive 96/77/EC as amended by Directive 96/86/EC and Directive 2000/63/EC for additives other than colours and sweeteners. Further detailed recommendations are given by the SCF (SCF, 2001).

Enzymes covered by Directive 95/2/EC are listed in the Annexes which are regularly updated. So far, only lysozyme and invertase are authorised as food additives (Table 18).

*Table 18: Enzymes authorised as food additives under Directive 95/2/EEC.*

Enzyme	E No.	Application	References
Invertase	E1103	Not stated	Annex 1 „Food additives generally permitted

<sup>45</sup> Marc Chambolle (DERNS - Unité d'appui scientifique et technique à l'expertise Agence française de sécurité sanitaire des aliments – AFSSA, France) was the co-ordinator of the SCOOP task.

Enzyme	E No.	Application	References
			for use in foodstuff not referred to in article 2 (3)”
Lysozyme	E1105	Ripened Cheese	Annex 3 „Conditionally permitted preservatives and antioxidants”, part C „Other preservatives”

### 5.3.2.1 SCF-Guidelines

As with other food additives according to Directive 95/2/EC the evaluation of enzymes is carried out by the SCF. The SCF has also been asked for advice on enzymes before harmonised regulations on food additives came into force or on enzymes covered by other pieces of food legislation (see Table 19).

The SCF therefore issued guidelines for the presentation of data on food enzymes in 1992 (SCF, 1992). These guidelines include conditions of use, requirements for information, documentation and testing and do occasionally refer to concrete methods of testing.

Interestingly, the SCF questioned in the introductory statements that enzymes as processing aids should be subjected to a different safety evaluation than enzymes used as additives: From „a toxicological point of view it is not pertinent to distinguish between [enzymes used as processing aids or food additives] since, in both cases, the enzyme preparations may remain in the food” (p. 13). Consequently, these guidelines are applicable and obviously have been applied to both types of enzymes (see Table 19).

The guidelines focus on enzyme safety evaluation and consider themselves as minimum requirements for information to be supplied. These requirements refer to potential hazards and to the exposition of the enzyme preparation to the final consumer. With respect to hazards, the guidelines are mainly focussing on toxicological requirements of enzyme preparation, on the safety of the source organism and on unintended reaction products in the food caused by enzymatic reactions in the final foodstuff. Exposition is mainly dealing with the quantity of enzyme consumed. Allergies or irritative effects are just briefly mentioned by stating that these effects are considered primarily as occupational problems.

A complete list of information requirements is given in Table 59 (Annex). Whereas the SCF considers enzymes from (edible) plants or animal species as posing no health problems, the toxicological evaluation of enzymes from microbial sources is deemed to be far more important. These toxicological tests should investigate known toxins as well as unknown toxic compounds that might be present in the enzyme preparation.

Toxicological testing has to be done on a batch from the final purified fermentation product before the addition of carriers, diluents etc. on „each specific enzyme preparation” and should be performed according to EC/OECD guidelines. However, the SCF acknowledges the fact that contaminants and ingredients of one particular enzyme preparation could vary: „If changes occur in the manufacturing process or in the purification of the enzyme preparation it will be considered as new unless it can be demonstrated that the final product can be considered the same as that prepared by the original procedures” (p. 17).

On the other hand the SCF admits that „if [...] one enzyme preparation from a specific strain has been thoroughly tested and the manufacturing process does not differ significantly for other enzymes from the same strain, the full testing battery may be waived for these enzymes.” This will be evaluated on a case-by-case basis.

Further exemptions from full testing requirements may be justifiable if the production strain of an already tested and approved enzyme preparation is substituted by a mutant strain<sup>46</sup> or in case of highly pure and specific enzyme preparations (which will become possible due to the use of non-toxin-producing GMM as hosts).

An enzyme preparation may even be accepted without specific toxicological testing if the production organism has a long history of safety in food use, and belongs to a species where no toxins are produced, and if the particular strain is of well documented origin.

### Enzymes from GMO

The guidelines also refer to enzymes from GMO<sup>47</sup>. The main concern of the SCF regarding to GMO was the potential for unintentionally introducing toxin production into the production organism. Furthermore, the potential for secondary effects due to the genetic rearrangements is also attributed: „each recombinant product is to be evaluated on a case-by-case basis considering the host, the vector and the insert and taking into account that the potential hazard from the final product might be more than simply the sum of the single elements.” (page 16). At that time the guidelines were issued protein engineered enzymes have not been taken into account.

So far these guidelines have not been updated. However, the SCF recently issued a general guidance on submissions for additive evaluations which does not explicitly refer to enzymes but are generally applicable to substances produced by GMO. Most likely these additional requirements will be taken into account when evaluating an enzyme as food additive. According to the guidance further information has to be provided on the effect of transgenic DNA on the host organism, the genetic stability of the GMM, the probability of transfer of inserted genetic material to human gut flora and its likely consequences, and the ability of the GMM to survive in and to colonise the human gut (SCF, 2001)

Table 19: Opinions of the SCF on the use and safety of enzyme preparations. *n. r.* not revealed

Enzyme	Application	EU Regulatory Context	Opinion of the SCF
Urease from <i>Lactobacillus fermentum</i>	Wine production	Regulation 822/87/EEC amended by Regulation 536/97/EC, Art. 4 (c); presently Regulation (EC) No 1622/2000 of 24 July 2000	10 December 1998
Papain from papaya fruit (additional information)	Meat tenderising agent	N. i.	20 September 1997
Papain from papaya fruit	Meat tenderising agent	N. i.	2 June 1995
Invertase from <i>Saccharomyces cerevisiae</i>	Food additives	presently Directive 95/2/EEC.	23 September 1994
Three chymosins from GMM	Cheese	N. i.	25 June and 10 December 1993
Activated lactoperoxidase system	N. i.	N. i.	19 June 1992

*N. i.* ... not investigated.

<sup>46</sup> Whether the term „mutant strain“ also includes GMM is not specified.

<sup>47</sup> At the time the guidelines were issued (1992) GMO in the context of enzyme production were mainly understood as genetically modified microorganisms (GMM).

In general, the evaluation of enzymes as food additives should proceed as described in Figure 10. After submitting a dossier (which should have been set up on the basis of the SCF guidelines) the SCF will ascertain the safety in use of the particular enzyme preparation. Thereby either acceptable conditions for use will be described or an ADI based on the NOEL in the sub-chronic rodent study with the application of a suitable safety factor will be allocated.

The SCF evaluations are regarded to be confined to a particular enzyme preparation described in the submission and cannot „automatically be considered to cover other preparations of the same enzyme prepared from other sources or by other processes” (p. 21). Accordingly, whether an enzyme preparation is regarded as being new/different to an already approved enzyme preparation might be considered on a case-by-case basis: e. g. if changes in the manufacturing and purification process would result in an enzyme preparation that does/does not substantially differ from the original one. These changes may also include the replacement of the production strain by a mutant. However, no further details were specified.

In summary, the guidelines could be understood that any changes in the manufacturing and purification process of a particular enzyme preparation have to be examined for their relevance according to safety. This examination has to be carried out by the manufacturer. If the enzyme preparation is substantially different from the already approved one, the manufacturer has to submit a new dossier. If the enzyme preparation does not substantially differ from the already approved one, the manufacturer has to justify this fact and the SCF might consequently either acknowledge the modifications to be covered by the original approval or waive from the full set of toxicological requirements. However, as only a few enzymes have been evaluated by the SCF so far (see Table 19), it is not quite clear how this will work in practice.

### **5.3.3 National regulation in EU-Member States**

The national regulatory context of enzymes applied as processing aids in food production differs significantly among EU Member States: In France and Denmark authorisation for enzymes used as processing aids (with the possible exception of enzymes covered by other pieces of harmonised food legislation) is mandatory, in UK an approval is voluntary. In UK enzymes are evaluated for safety and for their need of use as well. In other Member States there seems to be no statutory notification or approval system for these kind of enzymes. Furthermore, the range of enzymes permitted by national legislation varies among the Member States.

#### **5.3.3.1 Denmark**

In Denmark the National Veterinary and Food Administration approves the enzyme preparations following a safety evaluation carried out by its Institute of Food Safety and Toxicology.

The information requirements laid down in Order No. 282 of 19 April 2000 covering all enzyme preparations used in food production are identical with the information mentioned in the SCF guidelines and are also including enzymes from GMM. The Danish administration is not distinguishing between enzymes used as additives and those used as processing aids.

An enzyme preparation is permitted as a specific preparation with a trade name. This permission does not cover other preparations of the same enzyme prepared from other sources or by other processes.

So far (2000) 92 enzyme preparations (based on trade names) were evaluated and approved by the Danish administration.

### 5.3.3.2 France

The French Order of 5 September 1989 covers the use of enzyme preparations in the production of certain foods and beverages for human consumption. Since September 2000 the Agence Française de Sécurité Sanitaire des Aliments (AFSSA) is responsible for evaluating the technological and toxicological aspects of enzyme preparations. Authorisation is given by Direction Générale de la Concurrence, de la Consommation et de la Répression des Fraudes. In case of enzymes from GMM the Commission du Génie Génétique and – if necessary – the Commission du Génie Biomoléculaire is asked for their advice.

Approval is given for a particular enzyme activity (no trade names) and is confined to a particular MO and for a specific use.

Authorised sources of enzymes are listed in the annex of the Order. MO must be known as non pathogenic for man, animals and plants. In case of GMM the manufacturer must have assessed the safety of the MO and of the enzyme preparation.

Information requirements are similar than those given by the SCF. However, some divergent or additional requirements are cited in Table 62.

So far more than 60 enzyme preparations were evaluated and approved by the French administration (1999).

### 5.3.3.3 United Kingdom

In UK the use of processing aids is controlled by general provisions of the Food Safety Act 1990. The Food Advisory Committee is responsible for the technical specifications and evaluations of enzyme preparations. The FAC considers the advice of the Committee on the Toxicity of Chemicals in Foods, Consumer Products and the Environment (COT), the Committee on Carcinogenicity, the Committee on Mutagenicity, and – in case of enzymes from GMO – the Advisory Committee on Novel Foods and Processes (ACNFP) (FAC 2000; ATKINS, 1992; pers. comm.).

The COT issued guidelines in 1992 (BATTERSHILL, 1993) which are fairly similar to those of the SCF. Likewise the SCF the COT considers the distinction between enzymes used as additives or as processing aids not being relevant for safety evaluation. The COT also acknowledged the difficulties in evaluating toxicological data derived from one strain only as production strains are constantly subjected to strain improvements. Consequently, the COT recommended to include a suitable non-specific biological screening test and that „this test be repeated after every strain improvement to enable comparison with the original enzyme preparation subjected to toxicological tests” (ATKINS, 1992).

Figure: 11 (Annex) shows a flowchart of the evaluation procedure of the COT.

In contrast to the SCF guidelines, the COT guidelines do not cover enzymes from plant or animal sources. Nevertheless, they touch upon some additional points:

- The control of the processes during enzyme manufacturing with the requirement to analyse 3-5 different batches.
- The evaluation of the possibility for unexpected enzyme reaction – if unintended reaction products are detected, additional toxicity studies may be required.
- The potential for allergenicity, primarily via occupational exposure.

Based on the advice of the COT, the FAC may give a clearance which could be limited in time. In case of enzymes from GMO the ACNFP is consulted.

So far (1999) 12 enzyme preparations were evaluated and approved by the British administration.

#### **5.3.3.4 Other Member States**

Finland, Germany, Netherlands<sup>48</sup>, Portugal and Spain, do not have any regulatory or voluntary approval system or a safety assessment procedure for enzymes used as processing aids. The safety of this type of enzymes is evaluated only on a voluntary basis by the manufacturers. Enzymes from GMM are dealt with in the same way as enzymes from conventional sources.

In Greece an approval system for certain foodstuff and ingredients is also applied for the approval of enzymes no matter whether they are used as additives or processing aids. However, no information is available on this approval system.

No information is available from Austria, Belgium, Italy, Ireland and Sweden as this countries were not included in the SCOOP task.

#### **5.3.4 JECFA Guidelines**

About ten years before the SCF guidelines on enzyme safety evaluation were issued the Joint FAO/WHO Expert Committee on Food Additives (JECFA) had been concerned with this matter.

The JECFA is an international expert scientific committee that is administered jointly by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO). JECFA serves as a scientific advisory body to FAO, WHO, their Member States, and the Codex Alimentarius Commission, primarily through the Codex Committee on Food Additives and Contaminants and the Codex Committee on Residues of Veterinary Drugs in Foods.

It has been meeting since 1956, initially to evaluate the safety of food additives. For food additives, contaminants and naturally occurring toxicants, the Committee, (i) elaborates principles for evaluating their safety; (ii) conducts toxicological evaluations and establishes acceptable daily intakes (ADI) or tolerable intakes; (iii) prepares specifications of purity for food additives; and (iv) assesses intake. The Committee accomplish their task by preparing reports of their meetings and publishing specifications or residue monographs and toxicological monographs.

JECFA started evaluation of enzymes as early as 1971 and issued their first guidelines „General specifications for enzyme preparations used in food processing” in 1981 (FAO/WHO, 1981). These guidelines described requirements for technical data, source material, additives and processing aids used in enzyme preparations as well as for hygiene and contaminants. Since then the guidelines have been further amended and supplemented (e. g. FAO/WHO, 1990). Right from the beginning the JECFA specification provided detailed description of methods for measuring enzyme activity, for the detection of antibiotic activity as well as for testing for contaminating heavy metals and MO.

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<sup>48</sup> In the Netherlands there is probably a specific legislation for enzymes from GMO under the Dutch Novel Food Regulation. These information from the SCOOP task has to be questioned as the enzymes are not covered by the EU Novel Food Regulation. However, this is regarded to be beyond the scope of this report.

In 1991 the „General considerations and specifications for enzymes from genetically manipulated microorganisms” were published to supplement the „General specifications” (FAO/WHO, 1990). In this supplement the Committee stated that in order to properly evaluate enzymes from GMM it is considered very important to provide adequate information on the source material, the „genetic manipulation techniques”, and the fermentation and recovery process employed.

In addition to these enzyme specific guidelines the „Principles for the safety assessment of food additives and contaminants in food” (WHO, 1987) specified especially toxicological testing requirements.

In the revision of the „General specifications” in 1989 (35<sup>th</sup> meeting) the Committee concluded that a complete definition of all of the components of an enzyme preparation can rarely, if ever, be achieved and that therefore the identity and purity of preparations can best be ensured by defining the manufacturing process and by establishing criteria limiting the presence of contaminants and possible toxic metabolites derived from the source or contaminating organisms. Consequently, the Committee considered that the source organism should be defined not only by genus and species but also by strain or variant and that the culture conditions employed in manufacturing should be the same as those used for preparing the batches subjected to toxicity testing (ATKINS, 1992).

In the view of the Committee, differences in either the strain of the source organism or the conditions under which it was cultured would imply a change in the composition of the preparation and would therefore require a re-evaluation (ATKINS, 1992).

The requirements to define the strain of the production organism were somewhat loosened in 1999 when it was concluded that citation of genus and species of host organisms is usually adequate for those that have been determined to be safe and suitable. The reason given for this amendment was, that identification at the strain level may impose unnecessary constraints on the development of production micro-organisms used to produce food-grade enzymes.

The guidelines including all amendments and supplements until 1999 were recently published in an updated version of the „Compendium of food additive specifications” (Annex 1, originally published as FAO Food and Nutrition Papers 52). The detailed requirements described in the guidelines are presented in Table 62.

Very recently, in June 2001, the Committee revised their guidelines on enzyme preparations again (JECFA 2001a, b). This revision concerning threshold limits for heavy metals and contaminating MO, testing requirements for mycotoxins, and the need for evaluation of the allergic potential are pointed out in Table 62.

Although the task of JECFA is limited to additives (according to self-portrayal), evaluation practice does not distinguish between additives and processing aids. The Committee normally evaluates dossiers submitted by the manufacturers. The toxicological monographs are based upon working papers which are often based on proprietary unpublished reports. These were voluntarily submitted to the Committee by the manufacturers and in many cases these reports represent the only safety data available on these substances. All these studies were available to the Committee when it made its evaluations.

An (temporary) approval of enzymes from the Committee results either in the allocation of an ADI or – more often – in general statements that, e. g. in the opinion of the Committee, the enzyme preparation does not represent a hazard to health (for that reason, and for the reasons stated in the individual evaluations, the establishment of an ADI expressed in numerical form is not deemed necessary).

Although the evaluation procedure by the JECFA is not legally obliged and an approval or rejection has therefore no legal consequences – at least in the EU – on the marketing of



such enzymes, the results of the evaluations will be considered note in many states all over the world.

So far (2001) about 50 enzyme preparations have been evaluated by the JECFA including at least seven enzymes from GMM (see Table 60).

### 5.3.5 Perspectives from the industry

In the view of the enzyme manufacturers the different ways of regulating enzymes (as processing aids) in the EU (see section 5.3.3) poses problems to the free movement of enzymes and foodstuff manufactured using enzymes. Therefore the enzyme manufacturing industry in the EU believes that an EU enzyme regulation would be the best solution (AMFEP, 1997; AMFEP/CIAA). In the view of the industry such a regulation should provide a uniform and centralised authorisation procedure. Furthermore, this prospective regulation should cover all enzymes used as processing aids, both from traditional MO and from GMM, provide for an authorisation of the enzymes presently used in the EU, provide for a „centralised, speedy and predictable procedure for the authorisation” of new enzymes, provide for clear criteria used in the safety evaluation (AMFEP, 1997).

In order to distinguish more precisely between additives and processing aids and as a step towards a harmonised regulation the industry also proposes to divide food enzymes into four categories (AMFEP/CIAA) – all definitions apply to the state of the enzyme in the final product:

- (1) „Active enzyme: An enzyme which is added at any stage of the production process with the purpose of still exerting its catalytic activity in the final foodstuff. Thus, the enzyme plays a specific, intentional technological function in the final foodstuff.
- (2) Non-active enzyme: An enzyme which is added with the purpose to exert its catalytic activity during food processing. In the foodstuff itself, the enzyme is still present but not able to exert its catalytic activity due to external circumstances, such as depletion of the substrate, wrong pH, wrong redox conditions, lack of cofactors, lack of water, etc. If it were technically possible to remove the non-active enzyme from the final foodstuff at the moment of purchase, this would not have any influence on the characteristics of the foodstuff itself.
- (3) Denatured or degraded enzyme: An enzyme which is added with the purpose to exert its catalytic activity during food processing. At some point during the processing, the enzyme is not removed, but irreversibly denatured or degraded. This can be brought about by heat denaturation or degradation by proteolytic enzymes naturally present in the food. If it were technically possible to remove the denatured/degraded enzyme from the final foodstuff at the moment of purchase, this would not have any influence on the characteristics of the foodstuff itself.
- (4) Removed enzyme: An enzyme which is used with the purpose to exert its catalytic activity during food processing. At some point during the processing a step to either remove or separate the enzyme from the final food is carried out. The final food therefore contains either no or only trace amounts of residual enzyme protein.”

According to AMFEP and CIAA case 1 is considered as a food additive whereas cases 3 and 4 are processing aids. Case 2 is considered to be a food processing aid, if it can be shown that the anticipated processing conditions exclude the possibility of the enzyme being or becoming functional in the final product.

As the safety evaluation of enzymes used as processing aids is not regulated in most EU Member States it is up to the industry to design an appropriate process of safety evaluation. However, industry acknowledges the guidelines for safety evaluation issued by JECFA

(AMFEP, 1992), SCF and COT (AMFEP, 1996) and came up with their own ones as well (AMFEP, 1992).

According to AMFEP it is more appropriate to approve the source rather than each particular enzyme activity as an enzyme preparation also consists of accompanying substances which highly depend on the source organism. Therefore enzymes are obtained from non-pathogenic and non-toxicogenic MO grown on materials which do not contain components which might be hazardous to health.

Information and documentation which must be available for a detailed review and assessment of the safety of use according to AMFEP can be seen in Table 62.

In order to further facilitate the evaluation of the production organism, which is crucial in the view of AMFEP as mentioned above, AMFEP established criteria to develop a list of MO that can be recognized as safe for food production (AMFEP, 1992).

AMFEP, however, generally admits the importance of evaluating the possible impact on safety of enzyme preparations in case of strain improvements or when production conditions are altered on a case-by-case basis.

### 5.3.6 Regulation of food enzymes in the US

Enzymes used in food are regulated by the United States Food and Drug Administration (FDA) under the Food, Drug and Cosmetic (FDC) Act. Since 1958 the FDA has perceived enzymes as food additives and therefore subjected to the restriction pertaining food additives. In general food enzymes may either be classified as (i) substances that are „generally recognised as safe” (GRAS), (ii) substances that are not GRAS which are defined as food additives, or (iii) substances approved for use in food prior to September 6, 1958 by the FDA or by the Department of Agriculture. The FDC Act requires approval of food additives prior to marketing. GRAS substances, in contrast, are not subjected to approval or notification to the FDA prior to marketing.

In contrast to EU food legislation the FDC act does not distinguish between additives and processing aids.<sup>49</sup> Therefore, both type of enzymes used as additives and processing aids are regulated by the FDC Act.

GRAS status may be based either on a history of safe use in food prior to 1958 or on scientific procedures which require the „same quantity and quality of evidence as would be required to obtain a food additive regulation”. GRAS status may be either affirmed by the FDA or determined independently by qualified experts.

Table 61 shows a compilation of enzymes which are either approved as food additives or affirmed as GRAS by the FDA.

The regulatory status of food additives or substances affirmed as GRAS is established through a petition process. Section 409(b)(2) of the FDC Act prescribes the statutory requirements for food additive petitions. The requirements for food additives petitions are discussed in greater detail under title 21 of the Code of Federal Regulations (CFR) (part 171.1). However, the FDC Act does not provide specific statutory requirements for GRAS affirmation petitions. The eligibility requirements for classification of a substance as GRAS are described under title 21 in CFR 170.30 and for GRAS affirmation petitions in CFR 170.35.

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<sup>49</sup> A food additives is defined as „any substance, the intended use of which results or may reasonably be expected to result, directly or indirectly, in its becoming a component or otherwise affecting the characteristics of any food (including any substance intended for use in producing, manufacturing, packing, processing, preparing, treating, packaging, transporting, or holding food”: FDC Act Section 201(s) according to FORDHAM & BLOCK (1987).

Detailed recommendations for enzymes are given in a guidance document (FDA 1993). These recommendations intend to aid petitioners in assembling the chemical and technological data currently considered appropriate for a food additive or GRAS affirmation petition for an enzyme preparation. They cover data requirements in the following areas: identity, manufacturing process, purity, use, analytical methodologies, technical effects, and probable human exposure (shown in Table 62, Annex). The recommendations do not address other data needs, such as those pertaining to microbiological, toxicological and environmental considerations.<sup>50</sup>

In order to evaluate the safety of an enzyme preparation, the petitioner and FDA are comparing the enzyme to be assessed with other enzymes which have already been approved or with those that have been „safely consumed as part of the diet throughout human history“. According to FDA „enzymes that have the same function and that are identified by the same name and EC number often differ slightly in structure and properties when they are obtained from different sources. For example, the structure of an enzyme isolated from one tissue (such as liver) of one animal species, may differ slightly from that of the same enzyme isolated from a different tissue from the same species, or from the liver of another animal species. In part because of this variability, the diet routinely contains many thousands of different protein molecules“ (FDA, 1995).

This approach is based on the concept of Substantial Equivalence (in earlier documents also referred as substantial similarity) which is also used in the safety evaluation of novel food. According to the definition if „a new food or food component is found to be substantially equivalent to an existing food or food component, it can be treated in the same manner with respect to safety. No additional safety concerns would be expected.“ Consequently, the food or food component can be concluded as safe as conventional food or food component (OECD, 1993; FAO/WHO, 1996). This concept was further recommended by international expert groups to be applied in the assessment of „substances intentionally added to food.“ For example, a carbohydrase preparation and a protease preparation from *Bacillus subtilis* and *Bacillus amyloliquefaciens* were assessed to be substantially equivalent to carbohydrase and protease enzymes from other MO that have been evaluated and found to be safe by FDA before (e. g. mixed carbohydrase protease preparation from *Bacillus licheniformis*, carbohydrases from *Rhizopus niveus*, *Rhizopus oryzae*, *Aspergillus niger*) (FDA 1999).

### 5.3.7 Comparison of information requirements

Within the EU an harmonised authorisation system is only in place for enzymes used as food additives. However, most food enzymes are applied as processing aids. This type of enzymes is only in the US, France, and Denmark covered by special legislation. International and national committees (SCF, JECFA, COT) and an industry organisations (AMFEP) issued guidelines on conditions of use, information requirements and safety evaluation of enzymes. Most committees do not consider the distinction between food additives and processing aids as being relevant for safety evaluation. Consequently their guidelines pertain both type of enzymes.

In Table 62 the guidelines from SCF, JECFA, AMFEP, as well as the requirements from the FDA and the French Order covering enzymes are given and compared.

Although the requirements differ between the guidelines, the overall structure is fairly similar and includes:

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<sup>50</sup> The extent of toxicological testing of food additives is depending on the on the assignment of a „concern level“, on structural features and on an estimation of exposure. Minimum testing requirements are recommended for each concern level as well as each structural and exposure group (FDA 1997). However, these requirements are not investigated in the course of the project are therefore not included in Table 62.

- basic technical data on the enzyme itself, as the active compound of the preparation
- information on the source material; special attention is given to microbial sources
- substances added to the enzyme isolate (or used during processing)
- possible contaminants (MO, heavy metals, toxins) present in the final enzyme preparation.

Additional information is asked for in the case of GMM on

- host organism, donor organism, vector, introduced DNA.

Enzymes are identified by their catalytic activity in all guidelines and provisions. In general guidelines and provisions do not precisely describe the requirements. Thus, much is left to interpretation. Most detailed technical information on the enzyme is required by the FDA (enzymatic function, mode of action, substrate specificity, molecular weight, isoelectric point, kinetic properties, specific activity, temperature, pH, inorganic ions). In general, more detailed information is requested in case of GMM and for potential contaminants.

FDA requires the most detailed information on enzymes from GMM, including detailed technical data on the enzyme itself, on structural modifications, on the GMM (genetic stability, growth properties), vector, introduced DNA, donor organism as well as more detailed information on the manufacturing and purification process. Also the JECFA guidelines require more detailed technical data on enzymes from GMM whereas the SCF asks in greater detail on the vector. Only JECFA and FDA point to antibiotic resistance genes which might be unintentionally present in the final enzyme preparation. FDA also specifies a list of parameters which should be used in comparative analysis to justify substantial equivalence (enzymatic activity, kinetic parameters, amino acid composition, amino sugar composition, amino acid sequence, molecular weight, isoelectric point, gel-migration, chromatographic properties).

SCF describes basic toxicological requirements, that are toxicological tests to be performed and possible exemptions from testing within the enzyme guidelines. The JECFA laid down general principles for toxicological testing in a separate publication. The concrete requirements for toxicological testing are primarily depending on the nature of the microbial source, e. g. if it is a microbe that also naturally occur in food, if it is a GMM etc.

Potential allergenic properties are mentioned by the SCF only in the introduction whereas no requirements are given in the guidelines itself. Allergenic properties are mentioned by the JECFA only in case of GMM.

Only SCF includes requirements on data for the manufacturing process, the usage and stability in food.

Detailed descriptions of methods or references for methods to be used in testing and data production are given by both SCF and JECFA.

The AMFEP guidance does in general not include detailed description of requirements. However AMFEP does acknowledge the guidelines from JECFA, SCF and COT.

Safety approvals are often confined to particular enzyme preparations (SCF). Changes in the production process or application of genetic engineering lead to a re-evaluation on a case-by-case basis. Waivers from toxicological testing are also dealt with on a case-by-case basis.

FDA is taking into account long-term experience with certain enzymes. Such enzymes may be assigned the status of GRAS.

### 5.3.8 Summary

In the EU an harmonised authorisation system is only in place for food enzymes used as additives. Directive 95/2/EEC covers food enzymes used as additives and evaluations are performed by the SCF. So far, only two enzymes are authorised as food additives. Most food enzymes are applied as processing aids, which do not have a technological function in the final foodstuff. This type of enzymes is covered by sectoral legislation in the US, France and Denmark. In the United Kingdom a voluntary approval system is in place. Many other Member States do not have a regulation in place covering these enzymes. International and national committees (SCF, JEFCA, COT), governmental bodies (FDA) and an industry organisations (AMFEP) issued guidelines on conditions of use, information requirements and safety evaluation of enzymes. These committees and the FDA do not consider the distinction between food additives and processing aids as being relevant for safety evaluation. Consequently their guidelines pertain both types of enzymes.

Although information and testing requirements differ between these guidelines, the overall structure is fairly similar and includes basic technical data on the enzyme as the active compound of the preparation and information on the source material. Special attention is given to microbial sources, substances added to the enzyme isolate (or used during processing), possible contaminants (MO, heavy metals, toxins) present in the final enzyme preparation. FDA and SCF are explicitly taking into account long-term experience in production and use of certain enzymes in food application. The status of GRAS may thereby be assigned to enzymes by the FDA.

Safety concerns are mainly focussing on toxic properties of by-products and impurities including possible contamination with antibiotic active proteins, heavy metals, pathogens, production strains, microorganisms, and also DNA encoding proteins of concern.

Safety approvals are often confined to particular enzyme preparations (SCF). Changes in the production process as well as application of genetic engineering lead to a re-evaluation on a case-by-case basis. As a means enzyme preparation regarded as new are often compared to the already approved enzymes to check if they are substantially different. Waivers from toxicological testing, e. g. in cases where a mutant strain replaces the original production strain, are also dealt with on a case-by-case basis. However, no detailed guidance is given for such cases.

Additional information is usually asked in the case of GMM on host organism, donor organism, vector and introduced DNA. According to SCF and COT the main concern are unintended secondary effects due to the genetic modification that might subsequently and potentially lead to toxin production by the production organism.

Enzymes from GMM are often evaluated on a case-by-case basis. FDA is applying the concept of substantial equivalence to enzymes. Thus, the resulting enzyme from GMM is compared to the conventional counterpart to evaluate if relevant properties have been affected.

## 5.4 Enzymes used as additives in animal nutrition

### 5.4.1 Harmonised regulation in the EU

#### Legislation

Within the European Union, legislation covers enzymes and microorganisms used in animal feeding-stuffs by the definition of feed additives stated in Directive 70/524/EEC concerning additives in feeding-stuffs. As stated in the Directive these substances or preparations are likely to „*affect the characteristics of livestock production*” and a registration procedure has to be passed as article 3 of Directive 70/524/EEC generally states: „*only those additives should*

be marketed in the member states, which are already registered". Enzymes have to be authorised by the Commission according to a procedure described in Article 4 of Directive 70/524/EEC. Directive 93/113/EC (concerning the use and marketing of enzymes, microorganisms and their preparation in feeding-stuffs) historically marks the beginning of a harmonisation process concerning the regulation, use and marketing within the European Union. A collection of information on enzyme products registered in the Member States was subsequently initiated. Dossiers about the nationally permitted products had to be sent to the Commission. These dossiers were examined and a preliminary permit was granted by 1<sup>st</sup> July 1999 to 42 enzymes products. The data requirements for the registration procedure are detailed in Directive 87/153/EEC which forms the basis for the data compilation and examination of the enzymes. The Annex of the Directive provides basic guidelines for the assessment of additives in feeding-stuffs. This Annex consists of five sections: Section I (summary of the data in the dossier); section II (identity, characterisation and conditions of use of the additive – methods of control); section III and IV (studies); section V (form of monograph). Since the original Directive does not include specific requirements for microorganisms and enzymes, appropriate criteria were supplemented by Directive 94/40/EC and Directive 95/11/EC. The data requirements are further outlined in an Opinion of the Scientific Committee for Animal Nutrition (SCAN).

### Registration procedure and authorised products

For registration, the applicant (e. g. manufacturer) submits a dossier including all necessary product information to a national competent authority that acts as a rapporteur within the assessment procedure. After examination and clearance by the national competent authority, the dossier is then handed over to the Commission and to all other Member States and submitted to various EC boards for consultation. Before the final version of the dossier is approved, the Commission consults SCAN as an advisory body on the safety of enzyme preparations and the Standing Committee for Feeding-stuffs<sup>51</sup>. The Commission (DG Health and Consumer Protection) grants a market authorisation by including the enzyme preparation into a list of approved enzymes which will be published. The authorisation may either be time-limited or can be given unlimited when sufficient data regarding the efficacy are presented. The preliminary and the final authorisations are valid in all Member States. Both, the permits and the detailed information for application have been published; the authorisation periods are limited between 2002 and 2005.

To give an example, entry Number 23 in Annex II of Regulation (EC) 2200/2001 is listed in Table 20. This entry provides information about the enzyme *Endo- 1,4-beta-xylanase* (E.C. 3.2.1.8) produced by *Trichoderma longibrachiatum* (CNCM MA 6-10 W). The activity is defined as the amount of enzyme which liberates one micromole of reducing sugars (xylose equivalents) from oat xylan per minute at pH 4.8 and 50° (1 IFP), the target species are chickens and turkeys for fattening, the enzyme preparation is either applied as solid or liquid, in each case the enzyme activity is defined. Enzyme preparation Number 23 only consists of one type of enzyme, but authorisation is also given to preparations which form mixtures of two or more different enzymes. For example entry Number 27 authorises a blend of *Endo-1,4-beta-xylanase* (E.C. 3.2.1.8) and *Endo-1,3(4)-beta-glucanase* (E.C. 3.2.16). In that case, the information about production strain or the activity in the final preparation has to be provided for each enzyme type separately.

Table 20: Entry Nr. 23\* in Annex II of Regulation (EC) 2200/2001

N o.	Additive	Chemical formula, description	Species, category of animal	Minimum content	Date of authorisation
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<sup>51</sup> From the 19 February 2002 on the Standing Committee for Feeding-stuffs is renamed into „Standing Committee on the Food Chain and Animal Health“.

N o.	Additive	Chemical formula, description	Species, category of animal	Minimum content	Date of authorisation
23	Endo-1,4-beta-xylanase E.C. 3.2.1.8.	Preparation of Endo-1,4-beta-xylanase from <i>Trichoderma longibrachiatum</i> (CNCM MA 6-10 W) with a minimum activity:  Solid preparation: 70,000 IFP/g  Liquid preparation: 7,000 IFP/ml	Chickens for fattening	1,050 IFP**	30.6.2004
			Turkeys for fattening	700 IFP**	28.2.2005

\*.... The columns „maximum content” and „other provisions” are not rendered. \*\*.... 1 IFP represents the amount of enzyme, which sets free 1 micromole of sugar (xylose) in 1 minute at a pH of 4.8 and a temperature of 50°C from oat xylane.

Table 63 (Annex) lists the commercial names and the corresponding approved enzyme preparations according an information from the Austrian Federal Ministry of Agriculture, Forestry, Environment and Water Management (Department. for Feeding-stuffs) from 21 February 2002. The first column refers to the 61 provisional time limited authorisations and one unlimited authorisation granted by the EU. The second column gives the commercial name, by which the authorised preparation is marketed. Therefore, the commercial name of product entry Number 23 (*Endo-1,4-beta-xylanase*) is „Safizym X”. Table 63 (Annex) gives detailed information on the types of enzymes used in the authorised products which are 3-phytase, 6-phytase, alpha galactosidase, glucanase, xylanase, alpha amylase, polygalacturonase, subtilisin and bacilloylisin.

### Present and future data requirements for the authorisation process

For registration and authorisation, the dossier provided by the manufacturer, has to meet the data requirements defined by Directive 87/153/EEC. Therefore, all relevant data for safety assessment are compiled in the dossiers and have to assure appropriate safety to animals, users (workers), consumers and to the environment. Data requirements for authorisation are:

- Name according to main enzyme activities, i.e. IUB/IUPAC, EINECS or CAS number.
- Name and place of culture collection, where the strain is deposited and depositing number, genetic modification and all relevant properties its identification including genetic data. The activities toward relevant chemically pure model substrates and other physico-chemical characteristics.
- Degree of purity by checking the level of contaminating microorganisms, heavy metals, absence of toxins derived from the source organism (e. g. mycotoxins). Absence of antimicrobial activity at feed concentration level has to be determined by a suitable method. Composition of the non-enzymatic components (content of Total Organic Substance – TOS).
- Relevant properties such as pH optimum or temperature optimum.
- Details on the presence of unexpected reaction products formed by either enzymatic or chemical reactions or of the enzyme preparation with feed constituents or by degradation of the enzyme preparation during storage of the feeding-stuff.
- Assessment of the resistance to degradation or loss of biological activity in the digestive tract or by in vitro simulation.
- Toxicological tests for enzymes derived from edible parts of animals or plants are not required.

- If the enzyme is produced by a microorganism the level of the respective viable organism in the enzyme preparation should be determined.
- Acute oral toxicity studies are not considered as relevant for enzyme preparations.
- Mutagenicity: In case of enzyme preparations derived from microorganisms, the subsequent tests are normally required; a test for gene mutations in bacteria and a test for chromosomal aberrations (preferably in vitro). If possible the toxicological test should be performed using a batch from the final purified fermentation product before adding carriers, diluents and other substances.
- In case of enzyme preparations derived from microorganisms, a 90-day oral toxicity test in a rodent species may be sufficient.
- Chronic toxicity/cancerogenicity studies are not considered as relevant for enzyme preparations

To give a proposal for an adaptation of Directive 87/153/EEC to the scientific and technical progress the Scientific Committee on Animal Nutrition published a document (Opinion on the Revision of the Guidelines for the Safety Assessment of Additives in Animal Nutrition, 22 October 1999). While part I of this document deals with chemically specified additives; Part II is dedicated to enzymes and microorganisms. Meanwhile part I is covered by Directive 2001/79/EC, Annex 1 (a corresponding Directive concerning enzymes can be expected to be published this year). The document has to be seen in the light of a future revision and not as a interpretation of the present status of Directive 87/153/EEC. It includes a chapter (General Aspects) which describes the position of the Scientific Committee according to risks arising from the application of enzymes in feeding-stuffs. There it is stated, that *„studies necessary for the evaluation of risks to human health or the environment will depend essentially on the nature of the additive and the circumstances of its use. In this respect, no strict rule is applicable. However, it will be assumed, that all enzymes and microorganisms are respiratory sensitisers (R42) unless convincing evidence to the contrary is provided. Consequently, attention will be paid to the physical nature of the formulation, which should minimise this risk to workers handling the product. In other respects, enzymes per se are not expected to be harmful to the target species, consumers of products from animals fed enzyme-treated feed or to the wider environment. Microorganisms should be selected from taxonomic groups not normally able to induce clinical symptoms in healthy humans or animals and should be demonstrated unable to produce any toxins or virulence factors associated with related organisms”* (SCAN, 1999: II, 2).

### 5.4.2 Summary

Within the EU a comprehensive sectoral regulation for animal feed additives exists, since enzyme preparations used as feed additives are exempted from Directive 67/548/EEC (dangerous substances) and Directive 1999/45/EC (dangerous preparations). Instead, an authorisation procedure is foreseen basing on Directive 70/524/EEC and Directive 87/153/EEC. So far 61 time-limited authorisations and one unlimited authorisation on enzyme preparations used as feeding-stuffs were given within the EU. In the respective Regulations, the authorised enzymes are defined by their IUB number together with a description of activity and the production organism. The catalytic types of the enzymes authorised so far are: 3-phytase, 6-phytase, alpha galactosidase, glucanase, xylanase, alpha amylase, polygalacturonase, subtilisin and bacillolysin. For authorisation, data have to be provided in form of a dossier by the manufacturer and thereupon a safety assessment is performed by the authority. The assessment has to prove, that the use of the preparation is save to consumers, workers, the environment and also to the target species. Data requirements are given in Directive 87/153/EEC and generally include the compulsory description of the physico-chemical, technical and biological properties of the additive. For assessing consumer safety, mutagenicity testing and at least a long term oral study is provided.



SCAN elaborated a guideline document which forms the basis for a future adaptation of Directive 87/153/EEC (a corresponding amending Directive can be expected within 2002). The guideline document states notable presumptions and recommendations on enzymes and their effects:

- Enzymes are generally assumed to be respiratory sensitisers (R42) unless convincing evidence to the contrary is provided.
- It is assumed that the impact of enzymes to the environment is negligible.
- Enzymes per se are not expected to be harmful to the target species, consumers of products from animals fed enzyme-treated feed or to the wider environment.
- Microorganisms should be selected from taxonomic groups not normally able to induce clinical symptoms in healthy humans or animals and should be demonstrated to be unable to produce any toxins or virulence factors associated with related organisms.
- In case of a genetic modification both the production- and the donor-organism have to be described and identified and evidence of compliance with the requirements of Directive 2001/18/EC or 90/219/EEC has to be made. Enzymes from a GM source meeting these requirements should be treated as any other additive.

## 5.5 Enzymes used in cosmetics products<sup>52</sup>

The following specifications about present and possible future applications of enzymes in cosmetic products are mainly based on scientific literature and on examinations of the „Inventory of Ingredients employed in Cosmetic Products”,<sup>53</sup> since no detailed information could be received from the cosmetic industry (COLIPA) upon request. Furthermore, only enzymes which remain „active” (i. e. able to act as a catalyst to a substrate) in the cosmetic product are taken into account excluding hydrolysed or inactivated proteins (e. g. gluten, hydrolysed soybean or silk proteins).

### 5.5.1 The harmonised EU cosmetic regulation

Within the EU, the control, marketing and surveillance of cosmetic ingredients as well as cosmetic products is harmonised by Directive 76/768/EEC (Cosmetic Directive). Enzymes are covered by Directive 76/768/EEC if they comply with the criteria of Article 1 and 2 of the Cosmetic Directive: *„A Cosmetic product shall mean any substance or preparation intended to be placed in contact with the various external parts of the human body or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance and/or correcting body odours and/or protecting them or keeping them in good condition...A cosmetic product must not cause damage to human health when applied under reasonably foreseeable conditions.”*

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<sup>52</sup> Enzyme manufacturers often refer to enzymes in „personal care products”. While the term „cosmetic product” is embodied in the EU cosmetic regulation, this is not the case with the term „personal care product”. If an enzyme forms part of a cosmetic product, it is covered by the cosmetic regulation. Within the EU the control, marketing and surveillance of cosmetic ingredients as well as cosmetic products is harmonised by Directive 76/768/EEC (Cosmetic Directive). „Personal Care Products” are covered by cosmetic legislation if they comply with the criteria of article 1 of the Cosmetic Directive for a cosmetic product. For instance, enzymes are widely used in contact lens cleaners. For that purpose proteolytic enzymes such as subtilisin are applied to remove protein films and other deposits from contact lens. Although this application is associated with personal care within the EU contact lens cleaners are not covered by the Cosmetic Directive but have to fulfil the requirements of the Medical Device Directive 93/42/EEC. Requirements are for example a (national) registration of the product and the implementation of a quality assurance system in production.

<sup>53</sup> The „Inventory of Ingredients employed in Cosmetic Products” is described in section 5.5.1.

To ensure consumer safety, the Cosmetic Directive includes lists of substances which must not form a part of cosmetic products (Annex II) or are subject to restrictions or specific conditions (Annex III). Substances used for well defined applications in cosmetics such as colouring agents, preservatives or UV filters have to pass a safety assessment prior to their listing in Annex IV, VI or VII of the Cosmetic Directive. This safety assessment is performed by the Scientific Committee for Cosmetic and Non Food Products (SCCNFP) and is based on data supplied by the cosmetic industry. Substances (ingredients) other than those listed in the annexes mentioned above and other than colouring agents, preservatives or UV filters may be applied in cosmetic products by the manufacturer or importer if they meet the basic criteria of Article 2 („*cause no damage to human health*”). The manufacturer has to provide a dossier for each cosmetic product he is going to place on the market. Among other things, the dossier has to include a safety evaluation of the product based on physico-chemical and toxicological data of the ingredients. This dossier has to be accessible for control purposes by national authorities and does not represent an authorisation or registration.

Enzymes are also explicitly mentioned in the annexes of Directive 76/768/EEC and in the „Inventory of Ingredients employed in Cosmetic Products”:

Annex II of Directive 76/768/EEC, which is a list of substances which must not form part of the composition of cosmetic products includes Catalase (entry 74). No further information is provided for this entry and no other entries explicitly referring to enzymes could be found in the articles and annexes of the Cosmetic Directive.

The Inventory of Ingredients (first update) was compiled by the Commission on the basis of information provided by the industry. The Inventory is however not part of the cosmetic legislation and can therefore not be considered as a complete list of cosmetic ingredients. Section 1 includes 6300 entries, section 2 refers to perfumes and aromatic raw materials and has therefore not to be considered. Within section 1, prior to the listing of the substances, nomenclature conventions are made which theoretically cover enzymes generated from (industrial) fermentation. Insofar biotechnological materials are defined as „*substances derived from the action of microorganisms, such as bacteria or yeast, by fermentation, metabolism, hydrolysis, lysis or other processes. The process may involve the use of nutrients or other materials such as enzymes. The resulting product is referred to as a “culture” or “ferment”. The ferment can be further processed by extraction, filtration, and/or other procedures to yield the final product*” (Inventory of Ingredients, p. 12).

A electronic search of section 1 using the terms „enzyme” and „-ase” as keywords revealed several enzyme entries which are shown in Table 21.

Table 21: Enzyme entries in the Inventory of Ingredients employed in cosmetic products

INCI Name	CAS No	EINECS/ ELINCS	Chem/IUPAC Name	Function
Amylase	9000-92-4	232-567-7	Amylase	Skin conditioning
Amyloglucosidase	9032-08-0	232-877-2	Amylase, gluco	Skin conditioning
Desoxyribonuclease	9003-98-9	232-667-0	Nuclease, desoxyribo-	Skin conditioning
Glucose oxidase	9001-37-0	232-601-0	Oxidase, glucose	Stabilising
Lactoperoxidase	9003-99-0	232-668-6	Peroxidase	Stabilising
Oxido reductases	-	-	Yeast, enzymes, mixture	Skin conditioning
Placental enzymes	85195-59-5		Enzymes, mammalian placenta	Skin conditioning
Protease	9001-92-7	232-642-4	Proteinase	Skin conditioning
Superoxide dismutase	9054-89-1	232-943-0	Dismutase, superoxide	Antioxidant

INCI Name	CAS No	EINECS/ ELINCS	Chem/IUPAC Name	Function
Subtilisin	9014-01-1	232-752-2	Enzymes, subtilisin	Keratolytic
Suttilains	12211-28-8	235-390-3	Proteinase, Bacillus subtilis, suttilains	Keralolytic
Urease	9002-13-5	232-656-0	Urease	Viscosity controlling

The Notes of Guidance for Testing of Cosmetic Ingredients for their safety evaluation (adopted by the SCCNFP on 24 October 2000) are dedicated to public authorities as well as to the cosmetic industry within the scope of interest in the safety evaluation of cosmetic products and their ingredients, as requested by Directive 76/768/EEC and especially by the Sixth amendment to this Directive. The document does not have a compulsory character, but is intended to be used for the safety evaluation and information provision of cosmetic products to which the manufacturer is obligated. Annex 7 provides recommendations concerning the information requirements of *complex ingredients derived from biotechnology* (p. 67). Although enzymes are not explicitly mentioned, it can be assumed that they are covered by this annex. Accordingly, in case of biotechnologically derived ingredients, and if a „modified“<sup>54</sup> microorganism or a potential toxin has not been fully removed, amongst other things specific data must be available, which can comprise:

- description of organisms involved: donor organisms, recipient organisms, modified micro-organisms
- host pathogenicity
- toxicity, and if possible, identity of metabolites, toxin produced by the organisms
- fate of the viable organisms in the environment -survival potential for the transfer of characteristics to e. g. natural bacteria
- physico-chemical specifications
- microbiological quality
- additional external contamination
- preservatives added.

The question of the use of enzymes in cosmetic products is a new issue which has not yet been discussed and analysed in depth by the SCCNFP.<sup>55</sup> It is stated, that it is too early to present any conclusions (DG SANCO, pers. comm.).

The enzyme entries found in the Inventory of Ingredients employed in Cosmetic Products (see Table 21) as well as the investigated scientific literature indicate that several types of enzymes form part of cosmetic products. Yet the Inventory neither gives information about the extent of use nor can it be considered as a complete list of enzymes, since it is no approval list. Therefore, only the cosmetic industry itself or COLIPA as its representative, could provide detailed and accurate information about the extent of enzyme application in cosmetic products. Several requests were made to COLIPA but no answer was received in the period of the project.

<sup>54</sup> No specifications are given if the term „modified“ only refers to methods of selective culture, induced mutagenesis or also includes genetic engineering methods.

<sup>55</sup> The main task of the SCCNFP is to evaluate cosmetic ingredients for their safe use on behalf of the European Commission.

In 1999 AMFEP published a position paper to inform potential producers and users of personal care products and cosmetics about the potential health hazards of enzymes and to provide a guidance for risk assessments (AMFEP's Position on Consumer Risk Assessments for Enzyme Containing Personal Care Products and Cosmetics; Internet; 9 December 1999). AMFEP encourages that an appropriate risk assessment has to be conducted prior to the introduction of enzymes in personal care and cosmetic products. It is emphasised in this paper that the main hazard of enzymes is their potential to be Type I respiratory sensitisers causing allergic reactions like asthma but do not have significant skin sensitisation potential. The primary hazard comes from inhalation, but exposures via mucous membranes and damaged skin has also to be considered. In case of proteases, it has to be considered that in some cases they are skin and eye irritants. To carry out a consumer risk assessment, a dose-response analysis is necessary. No applicable dose-response relationships are currently available and one has to rely on *benchmark values*. Benchmark values are derived from clinical studies in which a specific biological effect, e. g. sensitisation or lack thereof, can be associated with an exposure level. In relation to clinical studies for the generation of the benchmark values, three types of studies are mentioned: Retrospective („look back”), prospective (individuals that have never been previously exposed to the enzymes, typically more than a year), provocative (conducted on known sensitised populations). The document does not provide an indication, if and to what extent benchmark values are available. According to AMFEP, the general concern with the use of enzymes in cosmetics and personal care products was demonstrated in a 6-month at-home pilot use test (KELLING et al., 1998). In this study, about 7 % of the individuals became sensitised via inhalation (detection of IgE specific for protease enzyme used) after using a cleansing product for showering containing a protease.

### 5.5.2 Summary

Enzymes applied in cosmetics are exempted from the Dangerous Substance Directive 67/548/ECC and are covered by Directive 76/768/EEC. Only catalase is exempted from the application in cosmetics. Since no mandatory authorisation procedure is foreseen, the safety for human health of cosmetic ingredients is evaluated by the Scientific Committee for Cosmetic and Non Food Products (SCCNFP) either if concerns arise or if manufacturers request for incorporation in one of the annexes of the Cosmetic Directive.

The question of enzymes is a new issue to the SCCNFP which has not been discussed so far (information of DG SANCO). However, since no safety assessments of enzymes used in cosmetics could be made available the practice of safety assessments could not be evaluated within the scope of this study.

Primarily the potential for sensitisation of the respiratory tract has to be considered when applying enzymes in personal care and cosmetics products (AMFEP Position Paper, 1999).

## 5.6 Enzymes used in medicinal products

### 5.6.1 The EU system for the authorisation of medicinal products

The EU regulation of medicines/drugs aims to regulate the quality, safety, efficacy and supply of medicinal products. No medicine may be sold or supplied without prior authorisation or registration by the government. Therefore, authorisation procedures comprise thoroughly toxicological, pharmaceutical and clinical testing together with standardised data acquisition.

A medicinal product may only be placed on the market in the EU if a marketing authorisation has been issued by the competent authority of the Member State for its own territory (na-

tional authorisation) or an authorisation has been granted in accordance with Regulation (EEC) 2309/93 for the entire EU (community authorisation). The European system for the authorisation of medicinal products is based on co-operation between the national competent authorities of the Member States and the European Agency for Evaluation of Medicinal Products (EMA). The EMA acts as a focal point co-ordinating the scientific resources made available by national authorities. The principal scientific bodies of the EMA are the Committee for Proprietary Medicinal Products (CPMP) and the Committee for Veterinary Medicinal Products (CVMP). A marketing authorisation is only granted to a single authorisation holder who is responsible for placing the medicinal product on the market. The marketing authorisation includes, when available, the international non-proprietary name (INN) and when branded, a single invented name (brand name).

#### **Authorisation at national level:**

In order to obtain a national marketing authorisation, an application must be submitted to the competent authority of the Member State. For marketing authorisation in more than one Member State (mutual recognition), the company which is responsible for placing the product on market must first apply in one of the Member States. Once the marketing authorisation has been granted, an application can be made in another Member State, requesting them to mutually recognise the marketing authorisation already granted. Alternatively, parallel applications in different Member States can be made.

#### **Authorisation at EU level:**

For medicinal products which are derived from a biotechnological process the European Community is responsible for granting marketing authorisations. In order to obtain a Community authorisation, an application must be submitted to the EMA. The scientific evaluation of the application is carried out by the scientific committees of the agency. Then these committees sent a scientific opinion to the European Commission which drafts a decision. Following consultation with the relevant Standing Committee, the Commission normally adopts the decision and grants a marketing authorisation.

Directive 92/32/EEC and Regulation (EEC) 2309/93 constitute the basis of Community pharmaceutical legislation and sets out the legal requirements and the procedures for applying for a marketing authorisation. Directive 75/318/EEC harmonises the criteria for the chemical, pharmaceutical and biological testing of medicinal products within the European Union. An application dossier for marketing authorisation has to include physico-chemical, biological and microbiological tests as well as pharmacological and toxicological studies and clinical tests.

If a marketing authorisation is developed for a medicinal product by means of a biotechnological process utilising

- recombinant DNA technology
- controlled expression of genes coding for biologically active proteins in prokaryotes and eukaryotes including transformed mammalian cells, or
- hybridoma and monoclonal antibody methods.

The application must be submitted to the EMA. This procedure finally leads to an European Public Assessment Report (EPAR) issued by the CPMP and will be made available at the date of the Commission's decision to grant marketing authorisation.

### **5.6.2 EMA Guidelines for medicinal products for human use**

Tests and studies for centralised authorisations have been harmonised within the EU and beyond the requirements are described in the guidelines (Rules Governing Medicinal Products in the European Union, Vol. 1 - 9) provided by EMA. The guidelines are not legally

binding but intend to assist in preparing applications for marketing authorisation. Volume 3A and 3B of the guidelines are concerning medicinal products for human use. Both documents do not explicitly mention enzymes, but provide recommendations for biotechnological derived products.

Volume 3A includes a chapter (Biotechnology Guidelines) to support applications for marketing authorisation within the EU for polypeptide based products derived by rDNA technology and intended for medicinal use in human. It is recommended to take into account all steps of the production process:

- genetic development (expression, stability)
- control of cell banks (well defined master and working cell bank system)
- fermentation of cell culture (a clear definition of the „batch”)
- product purification
- active substance (characterisation, comparison with reference and natural material, post-translational modifications, conformational data for macromolecules, purity and contaminants)
- routine batch control
- specification of the product.

Volume 3B of the guidelines includes pharmaco-toxicological and environmental guidelines and refer to the information on medicinal products. Chapter *Pre-clinical biological safety testing on medicinal products derived from biotechnology* pertains to polypeptides and proteins as hormones, cytokines, blood products, monoclonal antibodies or vaccines. Testing should be carried out on the final product and as far as possible information should be obtained, whether the observed adverse effects are due to the intended therapeutic substance or to the contained impurities. By pharmaco-kinetic studies the pattern and time course of absorption, distribution, metabolism and elimination should be determined as far as possible. Pharmaco-dynamic studies should provide evidence on primary effects and mode of actions and should address the dose-response relationships.

For all toxicological tests, justification for the selection of species, dose, route of administration, duration of treatment/experiment and the number of animals should be given, taking into account data available on pharmacokinetic, metabolism, pharmacodynamic and immunoreactivity of the product. This is, for instance, true for single dose toxicity and repeated dose toxicity.

It is concluded, that at present no set of safety tests can be described which would be applicable to all types of product groups and all biochemical groups that have been available so far. The usefulness of performing various combinations of tests should be discussed amongst pharmacologists, toxicologists and clinicians, both from the pharmaceutical company (industry) and the competent authorities.

### **5.6.3 Discussion of selected centrally authorised medicinal products including protein with enzymatic activity**

EMA provides a register including all medicinal products with a community marketing authorisation for human use (last update: December 2001) together with the corresponding EPAR providing non-confidential and consolidated information about the authorised product. The register provides information on: brand name, non proprietary name (INN), the company, therapeutic area, presentation (form, strengths) and further information on the date of authorisation. This register also includes enzymes. In order to give an impression of the nature of the safety evaluation two EPAR reports, included in this register, are described below.

The reports are referring to dossiers but give no detailed information about (toxicity) testing, calculations or conclusions.

*Cerezyme (CPMP/76/97)*: The active substance of Cerezyme, Imiglucerase, is a recombinant macrophage targeted  $\beta$ -glucocerebrosidase. The indication is for use as long-term enzyme replacement therapy in patients with a confirmed diagnosis of Type I gaucher disease. Imiglucerase is the recombinant form of alglucerase. Acute toxicity was performed on rats, repeated dose toxicity was performed on rats and cynomolgus monkeys (13 weeks). Ames test was performed but no reproduction or carcinogenicity studies. Clinical aspects, pharmacokinetics and efficacy were described, a risk/benefit ratio was also estimated by the CPMP.

*Rapilysin (CPMP/472/96)*: The active substance of Rapilysin is a modified recombinant non-glycosylated tissue type plasminogen activator (t-PA) produced in *E. coli*. The indication is for thrombolytic therapy of acute myocardial infarction. The natural t-PA is a serine protease with 527 amino acids and a molecular mass of 65,000. The derived reteplase contains a 355 amino acid chain, since 3 major domains of the molecule have been removed. The key manufacturing steps are described as: cell line production (construction of plasmids and transfer into production strain); description of master cell bank and working cell bank (nucleotide sequences; absence of bacteria, yeast, fungi bacteriophages); fermentation and re-folding process; purification process and finished product. A batch-to batch consistency has been shown for the active ingredient and the final product. Acute toxicity testing was performed on rats and cynomolgus monkeys; a 14 days repeat toxicity was done on rats, dogs and monkeys. Reproductive toxicity was performed on rats, local tolerance and genotoxicity tests was done by in vitro (gene mutations in bacteria and mammalian cells, chromosomal aberrations in mammalian cells) and in vivo tests (micronucleus test in mice and rats). No carcinogenicity studies were performed. A phase of environmental risk assessment revealed a negligible risk. Further clinical aspects, pharmacodynamics, pharmacokinetics and dose finding studies were performed.

#### 5.6.4 Summary

Medicine products are exempted from the Dangerous Substance Directive 67/548/EEC and are subjected to Directive 65/65/EEC and Regulation (EEC) 2309/93. Medicinal products, which are derived from a biotechnological process have to be authorised by the European Agency for Evaluation of Medicinal Products (EMA). EMA guideline documents refer to hormones, cytokines, blood products, monoclonal antibodies or vaccines. Emphasis is put on the biotechnological production process from the genetic modification of the microorganisms to the specification of the product. Concerning toxicological properties at present no set of safety tests can be described which are applicable to different biotechnological produced product groups.

A review of the summaries of the application dossiers (EPAR reports) revealed that there are particularly in depth studies on primary structure (e. g. unequivocally determination of amino acid sequence) and extensive animal studies on different species (rats, dogs, rabbits, monkeys). Mutagenicity studies, which have usually be performed in food and feed regulations are sometimes not performed if literature does not provide evidences of a mutagenic potential. Emphasis is put on the description of the biotechnological production process including the description of the genetic engineering procedure.

Enzymes for therapeutic applications are beyond the scope of AMFEP. Therefore, no information was obtained from the industry. For medical applications a very high purification and safety standard is required, especially when they are administered intravenously.

## 5.7 Summary of „Regulation of Enzymes“

Enzymes are, depending on their use, regulated in different EU legislation. Table 22 gives an overview on different fields of enzyme application and the relevant harmonised EU legislation covering enzymes and bodies that carry out safety evaluations (either mandatory or voluntary).

Technical enzymes are presently defined as chemical substance and are consequently subjected to the notification requirements of the Dangerous Substances Directive. According to the preamble of Directive 92/32/EEC „... any new substances placed on the market should be notified ...” (see also Article 2 (1) e) and MoD 8). In practice, EU Member States identified difficulties in notification of enzymes. The main problems are the identification of enzymes, the interpretation of EINECS entries and consequently the decision on the duty to notify an enzyme, as well as testing requirements for these substances. The Manual of Decisions does not include clear guidance for the notification requirements of enzymes.

Within the scope of this study, the notification procedures and information requirements in three non-EEA countries have been described in detail and comparatively analysed. Regarding technical enzymes in the USA, Canada and Australia, they are also regulated under chemicals legislation, consequently, information requirements are those for chemicals. Enzymes are described via their catalytic activity. In the Canadian New Substances Notification Regulation, additional information requirements for substances derived from biotechnology are defined including information on the production organism and enzymatic properties. For a pre-manufacturing notification in the USA, there are no specific information requirements for enzymes. The authority decides on a case-by-case basis but limited experience has been gained so far. In Australia, information requirements for enzymes are not defined in legislation. However, authorities have performed assessments on two enzymes and published so far (one new substance and one existing substance).

In the EU an harmonised authorisation system is only in place for food enzymes used as additives. Directive 95/2/EEC covers food additives, however, most food enzymes are applied as processing aids, which do not have a technological function in the final foodstuff. Most Member States do not have a national legislation covering these enzymes. International and national committees (SCF, JEFCA, COT), the US FDA and AMFEP issued guidelines on conditions of use, information requirements and safety evaluation pertaining both types of enzymes.

Information and testing requirements include basic technical data on the enzyme itself, information on the source material, additives and possible contaminants (MO, heavy metals, toxins). Long-term experience in production of a particular enzyme and use in food application is explicitly taken into account.

Safety concerns are mainly focussing on toxic properties of by-products and contaminants. Changes in the production process may lead to a re-evaluation on a case-by-case basis. Enzymes regarded as new are often compared to already approved enzymes to check if they are substantially different.

Additional information is usually required in the case of GMM on host organism. The production of toxins resulting from unintended secondary effect is regarded as the main concern. Enzymes from GMM are often evaluated on a case-by-case basis. FDA is applying the concept of substantial equivalence to enzymes from GMM. Thus, the enzyme from GMM is compared to the conventional counterpart to evaluate if relevant properties have been affected.

For enzymes used as feed-additives an authorisation procedure is foreseen based on Directive 70/524/EEC and Directive 87/153/EEC. The authorisation dossier has to include a description of the physico-chemical, technical and biological properties of the enzyme. For as-



sessing consumer safety mutagenicity testing and at least a long term oral study is provided. A safety assessment is performed by the authority in order to prove that the use of the preparation is safe to consumers, workers, the environment and also to the target species.

So far, 61 time-limited and one unlimited authorisations on enzyme preparations used as feeding-stuffs were given within the EU. The enzymes are defined by their IUB number together with a description of activity and the production organism.

An adaptation of Directive 87/153/EEC is planned in the near future. The Scientific Committee for Animal Nutrition (SCAN) recommended to consider enzymes as sensitizers to the respiratory tract. Microorganisms should be selected from taxonomic groups that are considered to be „safe“. In case of a genetic modification, both the production- and the donor-organism should be described.

Concerning cosmetic products, no mandatory authorisation procedure is foreseen. The Cosmetic Directive (76/768/EEC) lists those substances that must not form part of a cosmetic product or that are restricted. Substances for well defined applications have to pass a safety assessment prior to their listing in an Annex of the Directive. Either, if concerns arise or if manufacturers request for incorporation of an enzyme into one of the Annexes of the Cosmetic Directive, a safety assessment will be performed by the Scientific Committee for Cosmetic and Non Food Products (SCCNFP). The question of use of enzymes in cosmetic products is a new issue which has not yet been discussed and analysed in depth by the SCCNFP. According to AMFEP, primarily the potential for sensitization of the respiratory tract has to be considered when applying enzymes in personal care and cosmetics products.

Medicine products are subjected to Directive 65/65/EEC and Regulation (EEC) 2309/93. Medicinal products derived from a biotechnological process, have to be authorised by the European Agency for Evaluation of Medicinal Products (EMA) where a scientific evaluation of the product is carried out. Emphasis is put on the biotechnological production process from the genetic modification of the microorganisms to the specification of the product. Concerning toxicological properties, at present no set of safety tests can be described which are applicable to all different biotechnological produced product groups.

Table 22: Overview of EU harmonised legislation covering enzymes and bodies carrying out safety evaluation

Field of Application	NSN <sup>a</sup> (Directive 67/548/EEC)	Exemptions from NSN according to Directive 67/548/EEC (Quotations refer to Directive 92/32/EEC)	Sectoral Legislation	Safety Evaluation carried out by		Guidelines <sup>b</sup>	Reference in this report
				Mandatory	Voluntary		
Technical Enzymes	Y	n.a.	n.a.	nCA (NSN) <sup>a</sup>	n.a.	N	5.2
Food Enzymes – Additives	n.a.	If exclusively used as food additive according to Article 1 (2) d; MoD 5.3.	Directive 95/2/EC	SCF	n.a.	Y	5.3
Food Enzymes – Processing Aids	Y	Not fully clear if exempted from Article 1 (2)° d;  MoD 5.3.: exemption only for substances used exclusively as food or feed additives	n.a.	n.a.	SCF, JECFA	Y	5.3
Feed Enzymes	n.a.	If exclusively used as feed additive according to Article 1 (2) e; MoD 5.3.	Directive 70/524/EEC, Directive 87/153/EEC, Directive 94/40/EC, Directive 95/11/EC, (EC) 2200/2001, Directive 2001/79/EG	SCAN	n.a.	Y	5.3
Cosmetic Enzymes	n.a.	according to Article 1 (2) a;  MoD 5.4 : substances intended for use in a cosmetic are excepted from NSN if they are exclusively put on the market as cosmetic products in their final form, intended for the final user;	Directive 76/768/EEC	In certain cases performed by the SCCNFP but generally by the producer		Y	5.5
Enzymes for Medicinal Purposes	n.a.	according to Article 1 (2); 13 (1); MoD 5.2	Directive 65/65/EEC, Regulation (EEC) 2309/93, Directive 75/318/EEC	Performed by EMEA, if the product is derived from biotechnology		Y	5.6
Enzymes for R&D (commercial)	Y <sup>56</sup>	according to Article 13 (2): if less than 10 kg/a/manufacture or exclusively used for scientific R & D purposes or  if used for process-orientated R & D purposes (time-limited, not tonnage limit);	n.a.	nCA (NSN) <sup>a</sup>	n.a.	N	N.i.

Field of Application	NSN <sup>a</sup> (Directive 67/548/EEC)	Exemptions from NSN according to Directive 67/548/EEC (Quotations refer to Directive 92/32/EEC)	Sectoral Legislation	Safety Evaluation carried out by		Guidelines <sup>b</sup>	Reference in this report
				Mandatory	Voluntary		
Intermediates with limited exposure	Y	fall within the scope of Dir. 2001/59/EC (28 <sup>th</sup> ATP of Dir 67/548/EEC);  Substances manufactured and used in site or directly exported outside the Community costumes territory are not placed on the Community market and are therefore excepted from NSN (preamble of Dir. 92/32/EEC: ...any new substances placed on the market should be notified...); see also Article 2 (1) e; MoD 8	n.a.	nCA (NSN) <sup>a</sup>	n.a.	N	N.i.

<sup>a</sup> If not included in the EINECS and the annual tonnages exceeds >10 kg /yr/manufacturer or importer; <sup>b</sup> refers to guidelines which specify information/data requirements for enzymes; <sup>c</sup> "The Directive shall not apply to the following preparations in the finished state, intended for the final user: [...] d) foodstuffs" (Article 2, Directive 92/32/EEC). „foodstuff [...] must be interpreted as including food additives." (MoD 5.3): However, it is not quite clear, whether also processing aids are exempted as they are not mentioned in the MoD and as they are not additives per definition.. nCA ... national competent authorities; n.a. ... not applicable; NSN ... New Substance Notification according to Directive 67/548/EEC; Y ... Yes; MoD ... Manual of Decisions; SCF ... Scientific Committee on Food; SCCNFP ... Scientific Committee for Cosmetic and Non Food Products, EMEA ... European Agency for Evaluation of Medicinal Products.; N.i. ... not investigated.



## **6 HEALTH RISK OF ENZYMES REGARDING ALLERGOLOGICAL AND DERMATOLOGICAL ASPECTS**

### **6.1 General introduction**

Enzyme exposure mainly occurs from dust or liquid aerosols. The particles are deposited on the skin or on the mucous membranes of the respiratory tract. When an enzyme comes into contact with the respiratory tract or the skin, the body's immune system may be stimulated to produce antibodies resulting in respiratory allergy or contact urticaria, respectively. And because skin has a protein structure, enzymes which catalyse breakdown of proteins such as proteases are potential skin irritants. The question, whether enzymes do cause primary skin sensitisation or contact dermatitis directly, is still controversially discussed: denied by some articles (reviewed in PETERS et al., 1994; RODRIGUEZ et al., 1994; BOLAM et al., 1971; BANNAN et al., 1992), but supported by other publications (PODMORE et al., 1989; HERBST & MAIBACH, 1991; TUOMI, 2000).

Several common allergens from, e. g., house dust mites, storage mites, ragweed, pollens, *Alternaria*, cat dander, and bee venoms, are proteins with enzymatic activity (CHABANE et al., 1994). And if common antigens are enzymes by nature, classical natural (and artificial) enzymes may correspondingly represent antigens. Enzymes may also be more prone to cause sensitisation than other proteins because of their intrinsic capacity; e. g. the proteolytic function of many of these allergens has been proposed to be an important factor in the epithelial permeability and origin of allergy (ROBINSON et al., 1997; KAUFFMAN et al., 2000). It is thus not surprising, that a recent study from Finland confirmed that the currently used industrial enzymes are strong sensitisers (VANHANEN et al., 1997). Sensitisation may follow minute exposure; even office personnel in enzyme-processing factories may become sensitised. High prevalence of sensitisation was also reported from the Danish enzyme industry (JOHNSSEN et al., 1997), the baking industry (VANHANEN et al., 1996), and from many other heavily exposed occupations.

A discussion on the possibility and magnitude of medical problems caused by enzymes must consider the capacity of the individual enzyme to sensitise or irritate, its source and production method, its distribution, processing and stability, as well as time and extent of human exposure during production, industrial processing and consumption. Most medical reports come from the detergent industry. The highest percentage of exposed people nowadays seems to be affected in bakeries, but sensitisation may also occur in the animal feed industry, resulting in concerns for meat consumers. And only recently several reports have been published on allergic reactions in other occupations (pharmaceutical industry, at laboratory work, etc.) and even in consumers after dermal, mucosal, oral and parenteral (injection, infusion) exposure to enzyme drugs, enzymes in cosmetics, soaps, in soft lens fluids, in meat, and other products of daily life.

This article shall deal with published data regarding health problems associated with enzyme exposure, i. e. respiratory allergenicity and skin irritation potential, and further on how to possibly avoid them in the future.

### **6.2 Historical perspective, public awareness and concern**

The critical importance of controlling exposure, and the consequences of not doing so effectively, are illustrated by early experience when enzymes were first used in laundry detergents. Enzymes had been introduced in detergent products in 1963, and by 1969 80% of all laundry products contained enzymes. The detergent industry underestimated the risk of health effects in these early days (PETERS et al., 1994). Early detergent enzymes were

confectioned as powders and led to high airborne dust levels. As enzyme use increased, so did reports of respiratory illness at the workplace (FLINDT, 1969; LITTLE & DOLOVICH, 1973; SLAVIN & LEWIS, 1971; MCMURRAIN et al., 1970; WEILL et al., 1973; WEILL et al., 1974). Three separate studies at different manufacturers' plants (LITTLE & DOLOVICH, 1973; SLAVIN & LEWIS, 1971; WEILL et al., 1973) indicate that, by the early 1970's, for every 1,000 workers on average 400 were sensitised and 150 went on to develop respiratory symptoms. These results had important consequences in that external public concern arose; enzymes obtained the label of being dangerous substances for consumers and reports on hand eczema in laundry workers, that was attributed to enzymes, are still cited in modern American textbooks (RIETSCHEL & FOWLER, 1995) despite the fact, that a causal association nowadays seems quite unlikely and hardly reproducible.

In a second field of enzyme processing, the bakery industry, enzymes play an important role both for occupational respiratory as well as for skin problems. Bakers, being exposed to flour containing 2 groups of allergens, the protein flour itself and natural enzymes, as well as enzymes added during the processing of the dough, have the highest risk of developing occupational contact urticaria in a Finnish study (KANERVA et al., 1996). And enzymes were the 4<sup>th</sup> most common cause of occupational contact urticaria in this study. In a German investigation (BAUR et al., 1989), 23% of bakers suffering from workplace-related rhinitis and/or bronchial asthma were sensitised to industrial  $\alpha$ -amylase products obtained from *Aspergillus oryzae* cultures. Corresponding results were found in Switzerland (WÜTHRICH & BAUR, 1990), Italy (LARESE et al., 1993), and Spain (QUIRCE et al., 1992). Such reports again gave rise to public concern whether the addition of such products to food stuffs was safe for the consumer. However, the media's interest was based mostly on results from a study by SCHATA (1992), published only as a quarter page abstract which does not allow scientific evaluation. In this abstract, that was never published as a full paper, 58 out of 75 patients with a suspected food allergy and a positive skin intradermal test were orally exposed to  $\alpha$ -amylase and 45 of them reacted positive. 47 out of 58 became clinically better when avoiding bread and consuming food free of baking-additives. The authors concluded, „that  $\alpha$ -amylases in baking additives are a potential allergen even in the final product for specifically sensitised persons“.

Public awareness grew, encouraged by reports in both media and scientific literature. A dramatic rise of the percentage of allergic patients in the western world demands looking for specific reasons. The fact that classical allergens frequently are enzymes by their nature fuelled concerns regarding enzymes being dangerous products. And public discussion on the production of genetically modified plants and the introduction of enzymes produced by biotechnological methods boosted discussion on their safety for workers and consumers.

Allergy frequently also is an emotional problem, especially in people who feel sick – without knowing the reason – and suspect dangerous ingredients in the food to be the reason for this disease. The many reports on problems with enzymes seem to justify the fear, regarding that these biologically active substances exert their function in even minute amounts. And it is difficult to clear up the suspicion, if the proof of harmlessness is impossible to perform.

In addition, continuing negative reports in the lay media – being partially emotional but also substantiated by publications, that sensitisation still occurs despite encapsulation – from the detergent industry (CULLINAN et al., 2000) and still increasing numbers of bakers, that have to leave their job because of work-related problems (NIEUWENHUIJSEN et al., 1999), keep the discussion going on. And indeed, the vast variety of different enzymes, being produced from many different sources and used not only in many fields of daily life, but also as drugs, makes an analysis of their potential health problems – and how to deal with them – essential.

### 6.3 Medical terminology regarding enzyme exposure

Reports on medical problems that are still linked to enzyme exposure (including additives and contaminants) are numerous. It is important to first define the diseases, their underlying mechanisms, and the different technical terms that are used in the medical literature.

#### 6.3.1 Immunological terms

When an individual first encounters an antigen the cells of the immune system recognize this agent and either produce an immune reaction to it or become specifically tolerant depending on the circumstances. The immune reaction can take the form of cell-mediated immunity (delayed type or type IV reaction, the clinical correlate being allergic contact eczema) or may involve the production of antibodies directed towards the antigen (classically: immediate or type I hypersensitivity), the type of reaction being dependent on the way in which the antigen is presented to the lymphocytes. Many immune reactions however display both kinds of response. On second and subsequent encounters with the antigen the type of response is largely determined by the outcome of the first antigenic challenge, but the quantity and the quality of the response are different.

Whether a foreign substance leads to tolerance or sensitisation of any type (immediate or delayed) mostly depends on the type of agent; and in order to elucidate an immune response the agent must penetrate (or at least enter) the mucosal surface or the skin. There is hardly any specific literature regarding this topic:

- Enzymes modify skin antigens (KUMAR et al., 1983) and thereby may reveal previously hidden or produce new antigens; but the clinical relevance of this finding is unknown.
- ZENTNER et al. (1997) postulated that „the low molecular weight of proteolytic enzymes (30-30kDa) and their biologic activity might facilitate mucosal penetration more easily and thus – compared to amylase and lipase – permit an immune response and induction of allergic hypersensitivity”.

But whether a certain antigen in fact sensitises depends mostly on factors like molecular structure and binding capacities to certain reactive epitopes. The knowledge on relevance and conclusions for practical use are currently limited. Initial studies could demonstrate, that in certain models using different animal strains antigenic activity varies quite considerably among different enzymes (BARTON et al., 1988).

Allergic diseases, that have been reported to be caused by enzymes in sensitised individuals, are numerous and may be assigned as follows: Contact with enzyme dust or aerosols may cause immediate type allergic reactions such as conjunctivitis (of the eyes), rhinitis and asthma. Upon direct skin contact either contact urticaria (antibody mediated immediate type reaction) or allergic contact dermatitis (delayed type reaction) may follow; both terms are frequently not well differentiated and many literature reports are not absolutely conclusive; transitional reactions or even indeterminable reactions, simulating classical immune reactions seem quite likely.

There are however conditions such as *sensitisation* that urgently need to be differentiated – what is frequently not done in the literature nor in the clinical situation. Upon exposure, many people develop antibodies of different types: antigen-specific antibodies of the IgG-isotype do not correlate with disease activity but seem to be an indicator for exposure, i. e. detection of such antibodies does not signalise disease but just the amount of exposure (time and/or degree). In contrast, antigen-specific IgE-antibodies indicate allergic sensitisation, BUT NOT

allergic disease: this is an important difference, since in many clinical circumstances, where detection of IgE-antibodies is taken as an important parameter for allergic disease, this parameter is overvalued. A person that is sensitised (has detectable IgE-antibodies in his/her blood; or a positive skin prick test, see below) must not necessarily be sick (= be allergic). And whether this sensitisation will ever become relevant, i. e. the person will ever fall ill upon antigen exposure, is an open question that can only be answered statistically and depends on the specific antigen, the individuals constitution, the circumstances and other unknown and thus non-determinable reasons. But without sensitisation no allergic disease, and thus sensitisation is a first step to clinical allergy – although not a final one. Reports from the detergent, the baking and flour industry show that most exposed workers produce IgG-antibodies (MATSUMURA et al., 1994; MONEO et al., 1994), several develop IgE-antibodies (become sensitised) (VANHANEN et al., 2000a), but only a minority of them ever get sick – and thus have to leave their work-place. In contrast, in the production of car catalysts it is well known that workers sensitised to platinum should immediately leave their job as soon as antibodies are detected, since clinical disease (allergic asthma) can follow soon and may then persist even if further exposure is reduced (MERGET & SCHULTZE-WERNINGHAUS, 2000b).

SCHWEIGERT et al. (2000) classified the clinical effects to detergent enzymes into three grades (Table 23) Their experience showed, that asymptomatic, converted workers need not be removed from their work place. They were however placed under closer surveillance for the development of symptoms. Those with grade II or III symptoms had to be removed from the work area until their symptoms cleared and the source of exposure could be defined and eliminated.

Table 23: Grading of clinical effects (SCHWEIGERT et al., 2000)

Grade	Description
I	Sensitisation as determined by skin prick testing. No symptoms
II	Upper respiratory tract symptoms. Including conjunctivitis, rhinitis, and congestion. No lower respiratory tract symptoms
III	Lower respiratory tract symptoms. Including evidence of reversible obstructive airway changes

### 6.3.2 Allergic diseases, described in association with enzyme exposure

#### 6.3.2.1 Respiratory allergy

Respiratory allergy is, regarding medical text books and clinical practice, divided into diseases of the upper respiratory tract (i. e. nose) and the lower respiratory tract (i. e. bronchi/lung). Regarding this chapter, a division into these two categories seems not useful, since many persons suffer either from symptoms of both tracts or may initially start with problems of the upper and then continue to develop additional problems of the lower tract. The pathogenetical mechanisms, i. e. immediate type reactions, are identical.

Allergic rhin(oconjunctiv)itis is characterized by nasal itching, sneezing, watery rhinorrhoea, and nasal obstruction. Additional symptoms such as headache, impaired smell, and conjunctival symptoms may be associated. Patients with allergic rhinitis (hayfever) may suffer considerably as long as they are antigen-exposed (impaired quality of life parameters), and feel like suffering from a flu, but for weeks, months or even years.

Asthma bronchiale as defined by the World Health Organisation is a chronic condition characterised by recurrent bronchospasm, resulting from a tendency to develop reversible narrowing of the airways in response to various stimuli, and manifested by slowing of forced expiration which changes in severity, either spontaneously or as a result of therapy. This dis-



ease is not only associated with considerable morbidity, but also mortality. Despite of modern and effective treatment, or even because of this effective treatment, where many people rely too much on drug treatment and stop avoiding antigen exposure. In allergic asthma utmost priority should be given to antigen avoidance as complete as possible.

### 6.3.2.2 Skin allergy

Skin allergy is a frequently used term for contact allergy or allergic contact dermatitis. Regarding enzymes one should clearly differentiate two pathogenetically very different diseases, that may look quite similar, mimic each other in some situations, or even change their appearance from the one to the other form; i. e. classical allergic contact dermatitis (ACD) and immediate type contact urticaria (ACU) or protein contact dermatitis.

*Allergic contact dermatitis (ACD)* is an inflammation of the skin, that occurs as a result of antigen contact with external agents. ACD is normally the result of an immunological delayed-type reaction, clinically manifesting as eczema with considerable itching in the exposed areas following between 12 to 48 hours after skin contact with an allergen. The latter are usually low-molecular-weight chemicals that easily penetrate the skin and are presented by skin Langerhans cells to the immune system, resulting upon re-exposure in eczematous reactions. Typical antigens are used in various professional trades, such as dyes, household products or topically applied drugs and cosmetics.

Enzymes, being too large for penetrating normal skin, probably do not sensitise via this route and by these mechanisms, although there are a few case reports in the literature that seem to indicate this (PODMORE et al., 1989; HERBST & MAIBACH, 1991; TUOMI, 2000). But in all these studies, the way of sensitisation was not proven. Enzymes modify skin antigens (KUMAR et al., 1983), but whether this may contribute to skin sensitisation (delayed type reactions) is not proven. And indeed, the fact that those few reported persons with positive patch tests to non-proteolytic enzymes also have positive prick tests to the same enzymes may indicate „that the observed patch-test reactions in fact are IgE-mediated allergic reactions and not necessarily the classical type-IV reactions. This may also indicate that these enzymes are causative agents in developing contact urticaria, and that this urticaria may proceed to an allergic contact dermatitis (KANERVA et al., 2000a).

*Allergic contact urticaria (ACU)*, in contrast to ACD, is the result of an immunological immediate type reaction (IgE-antibodies), that clinically manifests as immediate itching, swelling and urticarial lesions appearing immediately after antigen contact. In certain clinical situations, the picture might switch within days to an eczema, characterized by itching and mimicking ACD. This is typical in patients with atopic dermatitis (protein contact dermatitis), but also occurs in non-atopic individuals. The antigens are, similar to those in rhinitis and asthma, high-molecular weight antigens such as proteins, and enzymes are frequently described to cause ACU. Contact urticaria is 'a new disease', that was first described only in the 1970s in fish mongers and vegetable merchants, that were handling such products over a prolonged period of time (HJORTH & ROED-PETERSEN, 1976).

In some case reports positive patch tests to non-proteolytic enzymes, i. e. cellulase, amylase, and xylanase (KANERVA & TARVAINEN, 1990) were demonstrated. It is worth noting that these persons also have positive prick tests to the same enzymes. This may indicate that the observed patch-test reactions in fact are IgE-mediated allergic reactions and not necessarily the classical type-IV reactions, at least in some cases.

Since IgE-antibodies play an important role, people that easily produce IgE-antibodies – so called atopics – are especially prone to develop allergic diseases. Lesions might develop in contact areas only, but they may also generalize and cause rhino/conjunctivitis, asthma, urticaria, and even anaphylactic shock. Thus, IgE-mediated asthma can occur via skin contact or via inhalation.

### 6.3.2.3 Food allergy

Food allergy is certainly one of the most controversial and difficult areas among allergic diseases. A major reason for this situation is the lack of universal agreement on definitions and diagnostic criteria. Major clinical symptoms are urticaria and provocation of atopic dermatitis, bronchospasm, anaphylactic shock, vomiting, colics, diarrhoea, bloating, etc. For most scientists, food allergy encompasses all possible immunological reactions to foods, not only the classical IgE-mediated responses.

### 6.3.2.4 Drug allergy

Drug allergy is not a major issue of this summary although several enzymes used as drugs have been reported to cause allergic reactions, from discomfort, pruritus, to urticaria and angioedema, serum sickness, bronchospasm, fever, arthralgias, myalgias, renal abnormalities to even fatal anaphylactic shock.

Enzymes such as aprotinin, chymopapain, streptokinase, trypsin and others are used for the treatment of several medical problems; a report on IgE-mediated allergic reaction to hyaluronidase in paediatric oncological patients may serve as example: Five out of 16 patients with tumors of the central nervous system (CNS) treated with hyaluronidase in addition to chemotherapeutic agents developed symptoms of immediate type allergic reaction to the hyaluronidase preparation from bovine testes (SZEPPFALUSI et al., 1997).

### 6.3.2.5 Test methods for allergy

Test methods for allergic disease: Diagnostic procedures should always be based on a detailed case history and the clinical manifestation. Dependent on the pathomechanism of the reaction and the quality of antigen, different steps have to follow (Table 24). Objective evaluation can be performed by skin tests and/or by serological allergen-specific IgE determination and/or by provocation tests.

Table 24. Summary on allergy test methods (for clinical routine)

Immediate type reaction (type I)	Delayed type reaction (type IV)
Skin prick test (skin intradermal test)	Patch testing (conventional)
Specific IgE-detection in serum	Skin“ patch test („stripped ng)
Specific provocation test (skin, nasal, bronchial, oral)	
Patch testing (modified technique)	

#### Immediate type reactions

For immediate type reactions such as hay fever, asthma and urticaria, skin prick testing with the suspected antigen (i. e. enzyme) in an appropriate concentration is the most direct approach to diagnosis. Skin testing, representing an in vivo-test method, is a fast, simple, cheap and straight forward test; but one has to be aware of the limitations (several reasons for false-negative or false-positive reactions), the need of control populations when dealing with non-standardized products, and the danger of severe and even life-threatening reactions.

Skin prick tests, when being performed as titration test, may allow to follow the exposed person and early detect sensitisation or progression of disease activity – skin prick tests are therefore frequently used to monitor enzyme-exposed persons.

These skin prick tests, when performed with suitable reagents, are most sensitive but not entirely specific indicators for identifying symptomatic workers.

For prick testing, commercial enzymes or non-commercial enzyme preparations can be used. For example, to reveal contact urticaria to  $\alpha$ -amylases, two bacterial  $\alpha$ -amylases and two fungal  $\alpha$ -amylases were used in a Finnish study (KANERVA et al., 1997b). Dry enzyme preparations were extracted in 0.1 M potassium phosphate buffer, pH 7.4 and diluted to the Coca solution (0.5% sodium chloride, 0.3% sodium hydrogen carbonate, 0.4% phenol) to achieve a protein concentration of 200  $\mu$ g/ml. A part of this solution (2.5 ml) was passed through a Millex-GV filter containing a 0.22- $\mu$ m membrane (Millipore Ltd) into a sterile vial containing 2.5 ml glycerol, yielding a final protein concentration of 100  $\mu$ g/ml. The Coca-glycerol solution served as a negative control. This description gives an example, how test preparations should be produced. In a routine work-up, preparations are frequently used as they are brought by the patient to the test institution, and tests are therefore frequently not only not standardized, but the results also non-reproducible.

In vitro-testing for antigen-specific antibodies of the IgE-isotype provides an important parameter. These tests are also quite specific but sometimes less sensitive than skin tests. In addition, commercial tests are hardly available (exceptions are papain and bromelain); self-prepared test reagents bear the likelihood of lacking reference values and standardization. Here again, reasons for false-negative (antibodies not detectable despite sensitisation or even clinical disease) and false-positive reactions (antibodies detectable, but not clinically relevant; i. e. sensitisation, see above) are frequent.

Bronchial provocation testing for asthma is not only time-consuming, but also tricky in performance and potentially dangerous in sensitised persons. In addition, many asthmatics suffering from severe bronchial hypersensitivity react non-specifically positive if e. g. flour is inhaled due of the flours irritating activity on the bronchial mucosa.

Regarding food allergy, even double-blind, placebo-controlled provocation procedures, often considered as 'gold standard' in diagnosis, is influenced by subjective evaluation and may require conditions not realised under experimental allergen procedures.

### **Delayed type reactions**

For delayed type reactions, patch testing on non-diseased skin is a reliable procedure. Since no commercial test substances are available, dilution series should be applied (e. g. 3.3%, 1% and 0.33%), in petrolatum and aqua. Regarding enzymes however, not the classical procedure leaving the substances for 48 occluded is appropriate, but a fractionated exposure time of 20 minutes, 1 hour, or 4 hours might be important. This is definitely true for ACU, but also for patients suffering from protein contact dermatitis. Dependent on the reactions after certain time-periods, not only the specific diagnosis but also the consequences might differ. Again, in patients suffering from ACU and even in cases of delayed protein contact dermatitis, positive skin prick tests and detection of IgE-antibodies might be the appropriate test procedure. In some patients, all these tests, patch tests and those relevant for immediate reactions, may cause positive results.

As controls, 20 non-exposed subjects (among them 10 atopics) should be tested using the same procedures, with negative results. Currently, there are scanty data on how to patch test with industrial enzymes. Optimal patch test concentrations need to be established for each enzyme, and controls are necessary.

### 6.3.3 Irritation (of skin and respiratory system) caused by enzyme exposure

Enzymes, being biologically highly active substances, do exert not only immune-mediated reactions but also effects, that are non-immune mediated.

#### 6.3.3.1 Respiratory tract irritation

Respiratory tract irritation is a frequent phenomenon caused by many particulate substances and is again dependent on the concentration (dusty environment), the product itself (irritative capacity) and the specific situation of the patient (many asthmatics suffer from bronchial hyperreactivity, where specific (= allergy) but also non-specific (= irritant) stimuli may cause inflammation).

#### 6.3.3.2 Skin irritation

Dermal irritation is the production of reversible tissue damage of the skin following the application of a test substance for up to 4 hrs (OECD Guideline 404, 2001 (EC method B.4)). Skin irritation may occur non-specifically due to dust exposure of sensitive/atopic skin or specifically due to the intrinsic properties of enzymes (proteases and lipases) by „digestion“ of the epidermis. The extent of damage is dependent on the specific activity of the substance (and co-substances, e. g. enzyme plus detergent), but also the time of exposure, and the condition of the skin (normal, damaged, sensitive/damage-prone). Factors like skin-care, cleaning procedures, climatic situation at the place of exposure (heat and humidity) may also play an important role.

#### 6.3.3.3 Test methods for irritation

Test methods for the irritative capacity of a substance are difficult to define and standardize.

Regarding intrinsic bronchial hyperreactivity, non-specific provocation tests are defined using non-specific stimuli such as cold air, histamine, codeine, methacholine or others and then perform a lung function test. The irritative capacity of certain occupational substances such as flour, isocyanates, acrylates or epoxides to the respiratory system, is however difficult to determine since well-defined and standardized procedures or recommendations are lacking.

Regarding skin irritation, the same problems do exist. For, among others, cosmetic ingredients and in general chemicals, the OECD Test Guidelines (2001) for „acute dermal irritation/corrosion“ and „acute eye irritation/corrosion“ have just become available in a revised version: Similarly to the testing for the sensitising capacity of a substance, decision trees for testing chemicals were established in the past. The updated version of Guideline 404 (adopted in 1981 and first revised in 1992) includes the recommendation of an integrated strategy, in that an *in vivo* test should be considered only, if a determination of irritancy or corrosivity cannot be made using structure activity relationship (SAR), *in vitro*, or other non-animal procedures (Figure 4). Human studies as last step should only follow the whole cascade, including animal studies, and only if necessary *and* ethically justified. The OECD method 404 corresponds to EC method B.4 which will be adapted accordingly.

In a well-advanced evaluation of a human patch test – as the last step of evaluation, if all previous steps were negative – for the identification and classification of a skin irritation potential, results with 29 test materials are presented; all of them being low molecular weight chemicals, with no comments on proteins in general or enzymes in particular (YORK et al., 1996). Follow-up studies still do not address this problem (ROBINSON et al., 2001; ROBINSON, 2002). It is quite unequivocally mentioned in the literature, that chemicals

should be tested undiluted in the human patch test (except when undiluted chemicals may be expected to be too damaging to human skin) (York et al., 1996). The OECD draft states, that for testing in animals „liquid substances are generally used undiluted (2001); regarding enzymes, enzyme concentrates and not enzyme preparations should be tested.

No specific test recommendations are established for the determination of the irritative capacity of enzymes in the literature, but testing may follow the OECD guidelines.

Step	Parameter	Finding	Conclusion
<b>1a</b>	existing human or animal experience	→ corrosive	→ classify as corrosive
	↓ not corrosive or no data		
<b>1b</b>	existing human or animal experience	→ irritant	→ classify as irritant
	↓ not irritant or no data		
<b>1c</b>	existing human or animal experience	→ not corrosive or irritant	→ no further testing
	↓ no data		
<b>2°</b>	structure-activity relationships or structure-property relationships	→ corrosive	→ classify as corrosive
	↓ not corrosive or no data		
<b>2b</b>	structure-activity relationships or structure-property relationships	→ irritant	→ classify as irritant
	↓ not irritating or no data		
<b>3</b>	pH with buffering	→ pH ≤ 2 or ≥ 11.5	→ classify as corrosive
	↓ not pH extreme or no data		
<b>4</b>	existing dermal data in animals indicate no need for animal testing	→ yes	→ possibly no further testing may be deemed corrosive/irritant
	↓ no indication or no data		
<b>5</b>	valid and accepted in vitro dermal corrosion test	→ positive response	→ classify as corrosive
	↓ negative response or no data		
	↓		

Step	Parameter	Finding	Conclusion
6	valid and accepted in vitro dermal irritation test	→ positive response	→ classify as irritant
	↓ negative response or no data		
7	↓ <i>in vivo</i> dermal corrosion test (1 animal)	→ corrosive response	→ classify as corrosive
	↓ negative response		
8	↓ <i>in vivo</i> dermal irritation test (3 animals in total)	→ irritant response	→ classify as irritant
	↓ negative response	→ no further testing	→ classify as irritant
9	↓ when it is ethical to perform human patch testing	→ irritant response	→ classify as irritant
	↓ not as above	→ non-irritant response	→ no further testing

Figure 4. Tiered testing and evaluation of dermal irritation potential (adapted from OECD, 2001)

#### 6.3.4 „Pseudoallergic reactions“

„Pseudoallergic reactions“ (i. e. reactions that clinically imitate allergic reactions) have mostly been reported when enzymes were taken as drugs. Symptoms were skin rashes, fever, arthralgias, myalgias, renal affections, bronchospasm, ... and others. The mechanisms may be heterogeneous and the importance for this chapter be a minor one.

#### 6.3.5 Risk-factors

Risk-factors for developing allergy: General risk factors for developing allergic symptoms upon contact with a potential allergen are atopic constitution, extent of exposure, and, according to some investigations, smoking (tobacco smoke stimulates IgE-synthesis – in animals – and might thus increase the chance of sensitisation). There is considerable discussion in the literature, whether persons with atopic constitution or smokers should be excluded from certain allergen-exposing, and thus potentially dangerous, professions, since evidence for smoking being in fact a risk problem (in humans) is only speculation and the atopic constitution frequently difficult to define. The extent of exposure to (inhaled) antigens however unequivocally correlates with the chance of a person to become sensitised; this is especially true for enzyme antigens. The amount of enzyme concentration in the air thus has to be kept as low as technically possible.

There are no reports in the literature on age or sex being specific risk factors.

Atopic disorders such as asthma and allergic rhinitis as well as drug allergy are apparently increased in patients infected with human immunodeficiency virus (HIV), but there is no obvious direct correlation between HIV infection and food allergy (TUBIOLO et al., 1997). Regarding enzyme allergy and HIV infection, there are no reported cases in the literature.

#### **6.4 Literature reports on medical problems regarding enzymes**

Literature reports regarding this topic are abundant, ranging from single case reports to case control studies, from retrospective reports („25 years ago I did observe ...”) to a nested case-control analysis for a cohort of new employees. In this report we try to include as many detailed information from the literature as essential and helpful, but also want to refer the quick reader to a selection of relevant publications from the different fields of enzyme exposure (Table 25).

Table 25: Selection of relevant articles from the literature (table adapted from VANHANEN, 2001)

Field of application and Reference	Protocol	Sensitisation	Symptoms
<b>Allergies to enzymes in detergent industry</b>			
SCHWEIGERT et al. (2000)	Review of allergies in the Procter & Gamble detergent industry	In 1984 – 1994, sensitisation for protease up to 10% and up to 5% for $\alpha$ -amylase	No new cases of occupational asthma among thousands of workers in North and Latin America since 1994
VANHANEN et al. (2000a)	Prevalence of enzyme sensitisation in detergent industry; cross sectional study	9/40 (22%) of the exposed workers were sensitised	All sensitised workers had symptoms at work; despite use of encapsulated products
CULLINAN et al. (2000)	Cross-sectional study in a modern detergent factory, 342 workers tested	26% sensitised; reactions towards all enzymes (protease, cellulase, $\alpha$ -amylase)	Work-related upper-respiratory tract (RT) symptoms, accompanied by sensitisation in 19% and lower RT symptoms in 16%
<b>Allergies to enzymes in bakeries</b>			
BRISMAN & BELIN (1991)	Cross-sectional study among 20 Swedish workers in a factory producing dough improvers	30% (6/20) to amylase	Rhinitis in 3 amylase-sensitised workers, verified by nasal challenge
VANHANEN et al. (1996)	Cross sectional study in four bakeries, one flour mill, and one crispbread factory; 365 workers tested	12 workers in bakeries (8%), 3 in flour mill (5%), and 4 in crispbread factory (3%) sensitised to enzymes; 12%, 5%, and 8% sensitised to flour	21 (14%) of workers in bakeries, 11 (18%) in flour mill, and 25 (17%) in crisp bread factory had symptoms at work; due to enzymes and/or flour
CULLINAN et al. (2001)	A nested case-control analysis for a cohort of 300 new employees in the UK industry; average period of follow-up 3.5 years	Incidence of sensitisation to amylase: 2.5 cases/100 py; to flour 2.2/100 py; positive exposure-sensitisation relationship	Incidence of 11.8/100 py for work-related eyes/nose symptoms; 4.1/ 100 py for chest symptoms; positive exposure-symptoms relationship
<b>Allergies in other industries – enzyme production</b>			
JOHNSEN et al. (1997)	Retrospective follow-up study of 1064 workers at Novo Nor-	36% with RAST value above detection limit of 0.5 SU and	8.8% developed enzyme allergy during the first 3y:



	disk A/S in Denmark during 1970-1992	8% >2 SU; sensitisation occurred to all tested enzymes: amylases, proteases, cellulases, lipases; smoking as risk factor for sensitisation, but not atopy	asthma in 5.3 %, rhinitis in 3.0%, urticaria in 0.6 %; prevalence of allergy declined by time: 13% in 1970-1979, 9.5% in 1980-1986, and 6.1% in 1987-1992
<b>Allergies in other industries – pharmaceutical</b>			
LOSADA et al. (1992)	83 workers from pharmaceutical industry exposed to powdered amylase	26 (31%) sensitised by SPT; exposure-response relationship by exposure assessment	20 out of 26 sensitised had symptoms of rhinitis and/or asthma
<b>Allergies in other industries – animal feed industry</b>			
DOEKES et al. (1999)	Cross-sectional study in a factory producing enzyme premixes for animal feed industry; 11 exposed workers studied	Four reacted definitely and four had a borderline reaction	Six had work-related respiratory symptoms; most of these were sensitised to phytase
VANHANEN et al. (2001)	Cross-sectional study in four factory producing enzyme premixes for animal feed industry; 218 workers studied, 140 were exposed	10 (7%) were sensitised to enzymes in the exposed group, none in the non-exposed group	Six of the 10 sensitised workers had respiratory symptoms at work, 2 of them especially in connection with exposure to enzymes
<b>Allergies in other industry – textile industry</b>			
KIM et al. (1999)	Single case report: a textile company worker using cellulase to remove fuzz from clothes	SPT and serum specific IgE positive	Asthma

In the following chapters, the publications will be selected and organized according to the disease symptom (e. g. chapter 6.4.1, respiratory allergy) and the different fields of exposure. Admittedly, not all the different reports are equally well performed and substantiated, but it seems inadmissible to make a correct and just judgement whether the workup performed and the conclusions drawn in a certain paper are relevant or not, substantiated by the facts, or valid regarding the test method and evaluation of the problem under investigation.

#### **6.4.1 Respiratory allergy (allergic rhinitis and asthma)**

Many reports on (occupational) asthma caused by inhalation of a variety of enzymes have been published in the past 30 years. Dusts of papain (OOSGOOG, 1945; BEECHER, 1951; MILNE & BRAND, 1975), trypsin (ZWEIMAN et al., 1966),  $\alpha$ -amylase (FLINDT, 1979), bromelain (GALLEGILLOS & RODRIGUEZ, 1978), derivatives of *Bacillus subtilis* (FLINDT, 1969; PEPYS et al., 1969), flaviastase (PAUWELS et al., 1978), pectinase (HARTMANN et al., 1983), pepsin (CARTIER et al., 1984), cellulase (LOSADA et al., 1985), and many others have caused increased concern. And the list of enzymes as a possible cause of asthma as well as patient numbers seem to still increase.

##### **Detergent industry**

The first adverse effects from handling detergent enzymes were reported in 1969 by Flindt (reported in FLINDT (1996)). He observed an epidemic among workers in a detergent powder factory in 1967: 28 patients had symptoms of the respiratory tract. Positive skin-prick tests and precipitating antibodies with detergent enzyme extracts indicated an immediate allergy (FLINDT, 1969). PEPYS et al. reported 1969 immunological findings in 3 selected patients referred from Flindt. Apart from reproducing the skin prick tests, they found immediate and late asthmatic reactions to inhalation tests with enzyme powder. Occupational asthma was also reported in the same year in the Swiss literature (WÜTHRICH & OTT, 1969). And general industry experience (PETERS & MACKENZIE, 1997) in the early 1970s was based on 3 studies, where sensitisation and respiratory symptoms were measured at 3 sites operated by different detergent producers. In total, the studies covered 1000 workers: 40% were sensitised and 15% exhibited respiratory symptoms. Several cases of enzyme allergy among consumers resulted in the removal of proteases from their products by most manufacturers until methods for enzyme encapsulation were developed and introduced in the 1970s (HOLE et al., 2000). Since then the problem seems to have decreased, because industry airborne dust levels were decreased, enzymes encapsulated, safe practices introduced, and older products replaced by antigenically distinct proteases. However, still numerous studies dealing with respiratory allergy in the detergent industry have been published; and continue to be published. And although detergent enzymes are currently unusual causes of occupational allergy (summarized by SCHWEIGERT et al., 2000), a recent study indicates that they may be more common than reported (VANHANEN et al., 2000a); and that the development of asthma continues to be a hazard (CULLINAN et al., 2000). Encapsulation alone is not the key to control exposure to enzymes (LISS et al., 1984) as shown in a modern British factory, which had exclusively used encapsulated enzymes; in this report, the rates of sensitisation to enzymes and occupational asthma were probably as high, or even higher, than within the industry before encapsulation. The authors suggested „that encapsulation alone is insufficient to prevent enzyme-induced allergy and asthma“. The introduction of new enzymes, such as bacterial amylases, and even more modern enzyme mixtures might increase the risk of allergy to amylase and associated asthma in the absence of appropriate preventive measures (HOLE et al., 2000). A recent prospective study reported 166 cases of asthma identified at five factories over a 20-year period (CATHCART et al., 1997). In this study, only 17 cases of occupational asthma were recorded in the latter 10 years, attributed to an effective control program – a programme that was based on meticulous reduction of exposure. But even in a recent study (VANHANEN et al., 2000a), 9 workers (22%) in a detergent factory were sensi-

tised in the exposed group of 40, whereas none were sensitised in the non-exposed group. Sensitisations occurred to proteases (esperase and maxapem), lipase, and cellulase. All the sensitised people had symptoms at work; all suffering from rhinitis and 1 from asthma.

Summarizing the data from the detergent industry leads to heterogeneous results: A high prevalence (22%) of sensitisation to enzymes was found in a „modern and clean“ factory in a recent study (VANHANEN et al., 2000a; see Table 26). By comparison, prevalences of 5% to 40% were reported in the detergent industry in the early 1970s (rev. in VANHANEN et al., 2000a). Later, SARLO et al. (1997b) reported prevalence of sensitisation to be 3.6% - 11.6% during a period of 6 years from 1986 to 1991. There are several reasons for these discrepancies, among them: enzymes with more potent catalytic activity were introduced, such as lipases and cellulase. And the selection processes for patients are not comparable in the different studies. In some, all exposed workers were investigated (VANHANEN et al., 2000a; see Table 26), in others it was shown that enzyme sensitisation and work-related respiratory symptoms were positively correlated with airborne enzyme exposure – that varies quite considerably within one factory being dependent on the specific work-place (CULLINAN et al., 2000; see Table 27).

Table 26: „Modern and clean“ detergent factory (VANHANEN et al., 2000a)

Exposure group	Enzyme test positive	Respiratory symptoms at work
Process workers (n = 40)	9 22%	19 47%
Office workers (n = 36)	0 0%	4 11 %

*Note: not all respiratory symptoms in a detergent factory are enzyme-related!*

Table 27: Respiratory problems in a modern detergent factory (CULLINAN et al., 2000)

Job		n (%) with positive enzyme skin-prick test		OR*	Upper respiratory symptoms		OR*	Asthma (work-related)		OR*
Packing-refilling	22	17	77%	144.0	14	64%	73.2	12	55%	50.5
Production	131	43	33%	21.0	33	25%	14.0	25	19%	10.1
Packing (other)	51	15	29%	17.7	10	20%	10.2	4	8%	3.6
Distribution	21	4	19%	10.3	3	14%	7.2	3	14%	7.3
Engineering	23	7	30%	19.4	6	26%	15.5	6	25%	15.8
Laboratory work	2	2	9%	3.6	0			2	7%	3.7
Other	26	1	4%	1.8	1	4%	1.8	1	4%	1.8
Office work	42	1	2%	1.0	1	2%	1.0	1	2%	1.0
<b>Total</b>	<b>342</b>	<b>90</b>	<b>26%</b>		<b>68</b>	<b>19%</b>		<b>54</b>	<b>16%</b>	

\* odds ratio.

## Biotechnical industry

Many enzymes have been described to cause respiratory allergy (TUOMI, 2000; see Table 28):  $\alpha$ -amylases, amyloglucosidase, and proteases in starch processing and baking, lipases, papain, trypsin, chymotrypsin, and proteases in the dairy industry, amylases, cellulases and

hemicellulases in the food industry, papain and pancreatic proteinase in the protein industry, cellulases, hemicellulases, proteases, amylases, and phytase in the animal feed industry, and chymotrypsin and trypsin in the pharmaceutical industry. And many of these also provoke skin symptoms. „No reports“ rather indicates „not tested“ or „allergic potential not proven“ than „safe – no problem“.

Table 28: Enzymes in biotechnical industry (TUOMI, 2000)

	Reported effects on	
	Skin	Airways
<b>Starch processing and baking</b>		
$\alpha$ -amylase	CA, CU	RA
amyloglucosidase		RA
glucose isomerase		
pullulanase		
B-amylase		
proteases	CA	RA
lipoxygenases		
phospholipase		
pentosanase		
<b>Dairy industry</b>		
rennet		
proteases	CA	RA
chymosin		
pepsin		
glucose isomerase		
lysozymes		
peroxidase		
catalase		
phosphatases		
lipases		RA
superoxide dismutase		
glucose oxidase		
papain	CU	RA
ficin		
trypsin	CU	RA
chymotrypsin		RA
sulfhydryl oxidase		
$\beta$ -1,4-galactosidases		
lactase		
<b>Food industry</b>		
pectinases		

amylases	CA, CU	RA
pectinesterase		
cellulases	CA, CU	RA
hemicellulases	CA, CU	RA
pectin glycosidase		
<b>Protein industry</b>		
bacterial proteases		
papain	CA	RA
pancreatic proteinase		RA
<b>Animal feed industry</b>		
cellulases	CA, CU	RA
hemicellulases	CA, CU	RA
proteases	CA	RA
amylases	CA, CU	RA
phytase	CU	RA
<b>Pharmaceutical industry</b>		
acylase		
chymotrypsin		RA
streptokinase		
trypsin	CU	RAU
urokinase		

Enzyme causing „contact allergy“ (CA), contact urticaria (CU), or respiratory allergy (RA)

### Pharmaceutical industry

The discrepancy on reports concerning workers and users is remarkable. Whereas production workers might be affected, reports on problems in users are almost completely missing. Two patients with asthma induced by occupational exposure to cellulase powder derived from *Aspergillus niger* are reported (LOSADA et al., 1985); cellulase, obtained from a variety of moulds, mainly *Trichoderma viride* and *A. niger* cultures, are used as an important member of combined digestive aids or for the removal or softening of unwanted cellulase e. g. in food preparations.

10 sensitised and 10 non-sensitised workers from a pharmaceutical factory who had been exposed to powdered trypsin, chymotrypsin, bromelain, papain, amylase, and lipase were investigated (ZENTNER et al., 1997). Skin test reactivity revealed multiple sensitisations to proteolytic enzymes, i. e. papain (9 out of 10), trypsin (8/10), chymotrypsin (8/10), and bromelain (7/10). It was hypothesized, that the low molecular weight of proteolytic enzymes (20-30kDa) and their biologic activity might facilitate mucosal penetration much more easily and thus – compared to amylase (3/10) and lipase (3/10) – permit an immune response and induction of allergic hypersensitivity more easily. Only half of the sensitised workers complained of work-related symptoms. This report, showing parallel sensitisation to trypsin and chymotrypsin, indicates an identical sensitisation potential. And the fact, that the exchange of four residues in the active site and two non-structural surface loops of trypsin gave the substrate sensitivity of chymotrypsin (HEDSTROM et al., 1992) further substantiates this assumption. The discrepancy in Table 28, showing no reports on contact urticaria for chymot-

rypsin, in contrast to trypsin, does reveal weaknesses in the literature: No report does not stand for „no sensitising potential“.

A multisensitised person developed an anaphylactic reaction following the intake of the seventh tablet of Ananase<sup>®</sup>, used as an antiinflammatory drug containing bromelain as the active ingredient (NETTIS et al., 2001). Sensitisation to bromelain usually follows inhalation, and not its ingestion (GAILHOFER et al., 1988), but cross-reactivity between bromelain and celery, papain, carrot, fennel, and cypress pollen, grass pollen and Compositae have been described. Therefore, the administration of drugs containing bromelain should be carefully evaluated in patients presenting allergic reactions to foods which may cross-react with such enzymes (NETTIS et al., 2001).

### **Laboratory work**

Several cases of rhinitis and conjunctivitis as well as asthma in laboratory workers exposed repeatedly to trypsin and chymotrypsin powders have been reported (ZWEIMAN et al., 1967; UNGER & UNGER, 1953; MACLAREN & ALADJEAU, 1957; HOWE et al., 1961). Again, not the long-term use of nebulized trypsin solution in asthmatic patients seems to be dangerous but the extraction of crude trypsin powder preparations from bovine and porcine pancreatic tissue (ZWEIMAN et al., 1967).

Allergy against pulverized or solubilized proteolytic enzymes used for immuno-histochemical or molecular biologic procedures (pronase E) has been reported only recently (KEMPF et al., 1999).

### **Bakers and baking industry**

Allergy to flour dust in bakers has been known since 1700. Bakers asthma is a major occupational disease; it has been estimated that 10% of unselected bakers may develop occupational asthma and that 20% of bakers apprentices develop skin sensitivity (HERXHEIMER, 1967). Problems are thought to be mainly caused by IgE-mediated sensitisation to cereal – mainly wheat, rye and barley – proteins. Many allergens have been identified in the water-soluble albumin fraction of flour protein, but also in the gluten fractions including globulin, gliadin, and glutenin (WALSH et al., 1985); mould spores of *Aspergillus* and *Alternaria* species, as well as grain mite allergens have been implicated as etiologies of bakers asthma (KLAUSTERMEYER et al., 1977). And recently enzymes were added to this long list. Enzymes and other flour additives are being increasingly used in bakeries to improve the properties of the baked products and accelerate the baking process. And parallel to this increased use, reports on skin and respiratory allergic symptoms were published.  $\alpha$ -amylase has long been known to cause occupational (bakers) asthma and rhinitis (BRISMAN & BELIN, 1991; BAUR et al., 1986; BIRNBAUM et al., 1988; QUIRCE et al., 1992; BLANCO CARMONA et al., 1991); SANDIFORD et al. (1994) have shown that beta-amylases are also allergenic. The onset of problems sometimes starts within a few months, sometimes only years after starting to work as baker (VALDIVIESO et al., 1994). The onset of nasal and respiratory symptoms (when sensitised) is within minutes of exposure to  $\alpha$ -amylase-treated flour dust and noticeable improvement while away from work during holidays points to the symptoms' occupational nature. BAUR et al. (1986) studied 118 bakers, 35 of whom had bronchial asthma, rhinitis and/or conjunctivitis after contact with flour. 12 individuals from the symptomatic group (34%) had positive IgE to  $\alpha$ -amylase, but none of the symptom-free group. In the meantime Asp o II has been shown to be a most important allergen in baking additives, inducing sensitisation in 16% of partially selected and in 32% of symptomatic bakers (BAUR et al., 1994b). Similar numbers on  $\alpha$ -amylase-sensitisation (30% in exposed bakers) have also been reported from other authors (BRISMAN & BELIN, 1991). In a Japanese study (MATSUMURA et al., 1994), 15.4% of workers handling mostly flour were sensitised to both baking additives,  $\alpha$ -amylase and papain, indicating that allergic symptoms in bakers do not result from wheat allergy alone, but rather from a combination of allergies to various materials. A British study suggests (NIEUWENHUIJSEN et al., 1999) that exposure to  $\alpha$ -amylase is

a considerable health risk in British bakeries and flour mills, dependent not so much on the exposure time but on peak concentrations. Thus, it is not so much the profession „baker“ itself, that is dangerous, but the specific factory, and in that factory the special places and functions, that cause high or low exposure. In this study, a direct relation was also found between exposure to  $\alpha$ -amylase and sensitisation to fungal  $\alpha$ -amylase.

Considerable attention was evoked by 2 studies on non-enzyme exposed millers, that were considered to be „negative controls“ for enzyme exposure. In one study from Sudan, FAKHRI (1992) found a high percentage of positive skin test responses to moulds in flour mill workers. In that study, *Aspergillus* sp. and *Rhizopus niger* were the predominant fungi in the wheat grains. And in a Spanish study (MONEO et al., 1994), 4.6% of a group of non  $\alpha$ -amylase-exposed millers had a positive skin test and 2.7% IgE antibodies. Most of these persons also suffered from clinical problems. The authors conclude, that millers may become sensitised to *Aspergillus oryzae* or other *A.* species even if never directly exposed (on purpose) to added enzymes.

In a recent extensive study from Finland (VANHANEN et al., 1996), 8% of the workers in bakeries (fungal  $\alpha$ -amylase and fungal proteases), 5% in the flour mill (fungal  $\alpha$ -amylase), and 3% in the crisp bread factory (cellulase, glucose oxidase) were skin test positive to enzymes; the corresponding percentages of positive reactions to flours were 12%, 5%, and 8%. Half of them suffered from allergic symptoms, such as cough, dyspnoea and rhinitis.

In summary, bakers and millers frequently develop asthma (and skin disease), that was formerly attributed mostly to extensive flour contact and flour sensitisation. Allergies to enzymes in bakeries have been reported since 1986 and enzymes have been proposed to be more potent sensitisers than cereal flours (BRISMAN & BELIN, 1991); this is however not the situation in all studies (VANHANEN et al., 1996; see Table 29).

The risk of asthma is less dependent on the cumulative dose of inhaled dust than on current exposure (at the time of falling ill) (BRISMAN et al., 2000), and these concentrations vary considerably regarding different bakeries and the special work-places in a certain bakery (NIEUWENHUIJSEN et al., 1999).

Table 29: Symptoms at work: test reactions in symptomatic workers (VANHANEN et al., 1996)

Positive skin prick test, workers with work related symptoms (n (%))					
Workplace	Total	Flours	Enzymes	Storage mites	Test negative
Bakeries	21	11 52%	5 24%	6 29%	6 29%
Flour mill	11	3 27%	1 10%	1 10%	6 55%
Crisp bread factory	25	6 24%	2 8%	3 12%	11 44%
Toal	57	20 35%	8 14%	10 18%	23 40%

## Miscellaneous

17 out of 33 workers who had been exposed to airborne papain at their work regularly developed asthmatic symptoms such as shortness of breath, wheezing, coughing, sneezing, rhinorrhea and conjunctival irritation upon contact with this proteolytic enzyme (BAUR et al., 1982). The incidence of IgE-mediated sensitisation was 34.5% in the screening group (i. e. non-selected but exposed workers). As 6 subjects developed clinically relevant hypersensitivity to common allergens during the time of papain exposure, it is suggested by the authors that airborne papain may constitute a triggering effect to further sensitisation. This is interesting as there is evidence for partial immunological cross-reactivity between papain and antigens of pollen, latex (BAUR et al., 1995b) and flour (BAUR, 1979).

Of special importance are reports, where patients develop occupational sensitisation and later on also may exert symptoms in private: WÜTHRICH reported in 1983 (HARTMANN et al., 1983) on 2 cases of sensitisation to pectinase, an enzyme used in the production of juices; one was a mechanic with direct exposure in a juice-producing factory, the other a secretary in the same factory, where the ventilation exhaust ended in front of her window. Patient 1 also had asthma after drinking wine, that may have contained pectinases. Pectinases were used for decades for this purpose, but problems had never been reported before.

### **Enzyme producing plant**

The allergy risk for workers is considerable. In a retrospective study covering the period 1970-1992, 8.8% of the workforce developed clinical enzyme allergy during the first 3 years of employment (JOHNSON et al., 1997), half of the cases occurred within the first 15 months of exposure. Sensitisation occurred to all types of enzymes handled in the plant, most often in production areas and laboratories. Ranking diagnoses of enzyme allergy by severity, the frequency of asthma was 5.3%, rhinitis 3%, and urticaria 0.6%. The risk declined during the period of investigation, probably due to improved industrial hygiene and training. But a risk remains.

Some studies report the risk of enzyme allergy in industries using enzymes (FLOOD et al., 1985; NEWHOUSE et al., 1970; JUNIPER et al., 1977; GILSON et al., 1976), especially in production of detergents with enzymes added. From these studies prevalences of 17% - 21% for skin tests were found, and FLOOD et al. (1985) estimate the incidence proportion of symptoms as 4.5%. These figures cover a period in which the formulation changed from powders to granulates. Since then coating of the granulates further reduced enzyme dust concentration (BRUCE et al., 1976). In certain detergent producing factories using enzymes, respiratory incidence throughout the 1990s have been in the range of 0 – 0.03% of the exposed population. This is a 700-fold reduction as compared with general detergent industry's experience of 15% in the early 1970s (PETERS & MACKENZIE, 1997).

In 2001, KANERVA & VANHANEN reported on an atopic person, a process worker in a chemical enzyme factory who developed continuous flu-like symptoms, consisting mainly of rhinitis and dyspnoea, that were work-related. The patient's prick test reactions to an industrial enzyme series was only positive to a *Bacillus subtilis*-derived detergent protease, but negative to all other enzymes. A skin provocation test revealed a wheal reaction within 20 min, a bronchial provocation test did not confirm occupational asthma but resulted in rhinoconjunctivitis.

### **Animal feed industry**

In this new area of enzyme applications (VANHANEN et al., 2001), sensitisation to enzymes was found in 10 out of 140 (7%) of the process workers – as compared to a prevalence of sensitisation of 22% of process workers in a „comparable“ detergent factory (VANHANEN et al., 2000a), and 20.6% in a group of workers „often exposed“ and 11.8% of workers „occasionally exposed“ in two enzyme producing plants (VANHANEN et al., 1997). Only 6 out of these 10 suffered from symptoms at work, mostly rhinitis, but dyspnoea only occasionally. 2 out of the 10 sensitised persons reacted merely to the protease Multifect P 3000, the other 8 reacted to several enzymes: 7 to cellulase, xylanase, or beta-glucanase, 5 to phytase, 3 to glucoamylase, and 1 to  $\alpha$ -amylase. 6 of the enzyme positive workers also reacted to environmental allergens, and 4 to flours.



## 6.4.2 Skin

### 6.4.2.1 Skin allergy (contact urticaria and contact allergy)

Since immediate type and delayed type reactions are frequently not clearly differentiated regarding the enzyme-allergy problem, they will be dealt with in one combined chapter here.

According to industry reports (PETERS & MACKENZIE, 1997; SCHWEIGERT et al., 2000), „extensive research confirms that enzymes do not cause skin sensitisation or contact dermatitis“. This recent message is however contrasted by many reports in the scientific literature (reviewed in KANERVA et al., 2000a) on the association of industrial enzymes and skin reactions. And enzymes were the fourth most common cause of occupational contact urticaria in a Finnish study (KANERVA et al., 1996). The authors of this report however qualify these numbers by saying that „the relatively high number of reported cases in Finland is due to the rather extensive enzyme industry, as well as the recent research interest in allergy caused by enzymes in Finland“ (KANERVA & VANHANEN, 2000b).

Several enzymes have been described to cause contact urticaria in many occupational (production of enzymes and occupational handling) and non-occupational situations ( $\alpha$ -amylases, glucoamylases, cellulase, papain, xylanase) (KANERVA & VANHANEN, 1999).  $\alpha$ -Amylase has long been known to produce skin symptoms (BRISMAN & BELIN, 1991; BAUR et al., 1986; BIRNBAUM et al., 1988; QUIRCE et al., 1992). Many cases of occupational dermatitis in bakers have been reported (MORREN et al., 1993; TARVAINEN et al., 1991a; VANHANEN et al., 1996).  $\alpha$ -amylase has caused contact urticaria and protein contact dermatitis in bakers (MORREN et al., 1993; SCHIRMER et al., 1987; TARVAINEN et al., 1991a). SCHIRMER et al. (1987) described one baker with dermatitis. He had positive skin-prick tests to  $\alpha$ -amylase and various bread improvers. The immediate test reaction to  $\alpha$ -amylase persisted for 48 hours and a patch test was also positive, as well as IgE detection to  $\alpha$ -amylase, malt and bread improvers. In a Finnish study, 12 workers in four bakeries, 3 in one flour mill and 4 in a bread factory were skin-prick positive to both  $\alpha$ -amylase and cellulase (VANHANEN et al., 1996). And a baker with whealing starting within minutes of direct skin contact with flour had to leave his job because of selective sensitisation to fungal but not bacterial  $\alpha$ -amylases (KANERVA et al., 1997b). The pathogenic reason for this discrepancy is unknown. In a cross-sectional study, BRISMAN & BELIN reported in 1991 on 29  $\alpha$ -amylase-exposed workers who had significantly more skin symptoms than the controls. In 1993, MORREN et al. reported on 32 consecutive bakers patch tested with  $\alpha$ -amylase. 7 had an immediate reaction, and two had a positive delayed skin test.  $\alpha$ -amylases have now proven to be „an important cause of skin reactions in bakers and should be tested routinely if a contact allergy is suspected“.

Although bakers probably represent the most heavily exposed occupational group (KANERVA et al., 1997a), enzymes may affect workers in several industries, such as paper (HYTÖNEN et al., 1994), textile and pharmaceutical industries, starch (KANERVA et al., 1998) and sugar production, alcohol and wine production, enzyme production (BAUR et al., 1982), farming, and industries in which workers come into contact with proteins, detergents, etc. A worker occupationally exposed to liquid proteases and amylases developed allergic contact urticaria and rhinoconjunctivitis after 1 year in his job; though the symptoms caused by industrial enzymes may be mild (KANERVA & VANHANEN, 1999), they were severe enough in this patient that he could not continue working with enzymes. In a recent summary on health problems in biotechnical workers, many different enzymes ( $\alpha$ -amylases (KANERVA et al., 1997b; SCHIRMER et al., 1987; TARVAINEN et al., 1991a; BRISMAN et al., 1991; MORREN et al., 1993; DEL POZO et al., 1995), amyloglucosidase (KANERVA & VANHANEN, 1999), proteases (DEL POZO et al., 1995; MCMURRAIN, 1970; SMITH et al., 1989; WÜTHRICH et al., 1971), papain (BAUR et al., 1982; BERNSTEIN et al., 1984; SANTUCCI et al., 1985; PODMORE et al., 1989; QUINONES et al., 1999; SOTO-MERA et al., 2000), trypsin, cellulases (KANERVA et al., 1997a; KANERVA & TARVAINEN, 1990;

TARVAINEN et al., 1991b; VANHANEN et al., 2000b; DEL POZO et al., 1995) and hemicellulases (KANERVA & TARVAINEN, 1990; TARVAINEN et al., 1991b), and phytase) used in many kinds of biotechnical industry (starch processing and baking, dairy-, food-, protein-, animal feed-, and pharmaceutical industry) have been described to cause contact allergy or contact urticaria (rev. in TUOMI, 2000; KANERVA & VANHANEN, 1999). Contact urticaria from papain has been reported by BAUR et al. (1982) in papain workers. Recently, localized urticaria in a technician working in a pathology laboratory after contact with pulverized proteinases has been reported (KEMPF et al., 1999). Two cosmeticians have been reported recently, who used papain tablets dissolved in water for removing adhesives and developed contact urticaria, rhinconjunctivitis, and asthma (SOTO-MERA et al., 2000). There is only one additional reference of papain allergy in cosmeticians in the literature (NIINIMÄKI et al., 1993), but the authors guess that „patients clearly perceive the symptoms from using this substance, avoid it, and never consult an allergist for this problem.“ And there is a single case report on an urticarial reaction to rennet in a cheese maker (KADLEC & HANSLIAN, 1970), where the enzyme casein is the major allergen for IgE mediated responses (STÖGER & WÜTHRICH, 1993).

Non-occupational exposure with skin symptoms has been reported in housewives and in contact-lens wearers (papain in cleansing solutions) (KANERVA et al., 1997a; SANTUCCI et al., 1985; PODMORE et al., 1989; HERBST & MAIBACH, 1991). Regarding the latter, proteases as contact lens cleaners, four different enzymes (subtilisin B > pancreatin, and subtilisin A > papain) caused differential effects dependent on enzyme, immunization system, and the kind of animal system employed (BARTON et al., 1988).

Whereas sensitisation is sometimes broad, i. e. to a battery of enzymes to which the respective person is exposed, some people react quite selective like a patient working on the distillation of ethanol who was just positive to fungal amylase but not to 11 other industrial enzymes (KANERVA et al., 1998).

When assessing allergy presumably caused by enzymes, it has to be noted that commercial enzyme products also contain traces of the microorganisms used for fermentation as well as preservatives, etc. These substances may be the actual cause of allergy in some cases. An argument that is mentioned frequently (KANERVA et al., 1997b), but has never been substantiated in the literature.

And in an article, that is often cited regarding the topics contact lens intolerance and enzyme allergy, out of „20 patients with contact lens intolerance, all suffering from the usual symptoms“, only one patient was allergic to papain, and for this single patient papain was recommended to be included in a „contact lens solution test battery“ (PODMORE et al., 1989).

#### **6.4.2.2 Irritant dermatitis**

Irritant dermatitis from pineapples, apparently from bromelain, was reported more than four decades ago. After the introduction of enzyme-containing washing powders, there was much concern about possible dermatological effects among users. These effects were regarded as irritative, caused by non-enzymatic ingredients or a combination of enzymatic and detergent action. In practice enzymes are thus supposed to be mild skin irritants in the workplace (PETERS & MACKENZIE, 1997). But early reports stating that „exposure to the concentrated enzyme in industry has caused dermatitis, allergic rhinitis, and asthma“ (WEILL et al., 1971) and the statement by BELIN et al. (1970) „that sensitisation to enzyme detergents can also occur in consumers of enzyme-containing washing powder and in every situation in which these enzymes are dust particles“ let American textbooks on Contact Dermatitis (RIETSCHEL & FOWLER, 1995) still repeat that „enzymes in detergents may rarely cause irritation and sensitisation“ (in consumers). In 1970, DUCKSBURY & DAVE reported a cross-sectional questionnaire study among home-helps; the prevalence of detergent-associated

dermatitis was 5% among this group. Patch tests with enzyme-containing detergents were negative. BOLAN concluded in 1970, after a study among housewives, that enzymatically active washing powders were not more irritant than conventional ones. In 1972, GÖTHE et al. reported on 50 workers exposed to enzymes during detergent production. Of these, 47% reported work-related skin symptoms, and the reactions were regarded as irritative; generalized urticaria upon skin contact, probably reflecting the contact urticaria syndrome, was reported in one case. Irritant dermatitis was also reported in Switzerland (WÜTHRICH et al., 1971), Finland (NIINIMÄKI & SAARI, 1978), Japan (OKAMOTO et al., 1972) and Denmark (ZACHARIAE et al., 1973). ZACHARIAE et al. (1973) reported 79 workers with skin symptoms and exposure in enzyme production, and 12 unexposed controls. Patch tests with subtilisin were negative. A high enzyme concentration was considered to cause irritant dermatitis, because skin has a proteinous structure, and proteases are thus potential skin irritants. Research has however shown, that the level of enzymes used in detergents does not cause skin irritation in consumers through direct exposure in hand laundry or by wearing clothes laundered by enzyme-containing products (summarized in SCHWEIGERT et al., 2000). In addition, ZETTERSTROM (1977) showed that even consumers with allergy to enzymes could use granulated product and sleep in bedding washed with granulated product without allergy symptoms.

Recent systematic literature reports on the irritative capacity of enzymes are however missing.

#### **6.4.2.3 Summary on skin problems regarding enzymes**

All enzymes may be supposed to be skin irritants, a feature that is probably due to their intrinsic nature. The extent of damage will mostly depend on the time and intensity of exposure, the nature of the enzyme (proteases !) and its concentration, the integrity of the skin, and cofactors such as additional detergent exposure; but controlled studies from the past two decades are missing.

Enzymes are also strong respiratory tract (and skin?) sensitizers and can cause and/or trigger skin problems; reports from non-occupational situations are however rare and usually easy to handle. Occupational problems seem frequent but are nowadays more or less focused on two occupations, that is the baking industry and even more to the enzyme production industry. Whereas symptoms are usually mild, they are sometimes bothering enough and hinder the patient to continue working with those enzymes. And in 4 cases of contact urticaria developing after 0.5 to 4 years of industrial exposure to the enzymes cellulase and xylanase, all patients later developed rhinitis and asthma (TARVAINEN et al., 1991a).

#### **6.4.3 Food allergy**

Microorganisms have been used for centuries in food processing operations (fermentation) such as the production of wine from grapes and souring of milk, but the use of isolated enzymes by the food industry has only become widespread in the last few decades (BATTERSHILL, 1993). Many enzymes that are (also) used in food such as papain and bromelain, trypsin, proteases from the skin yeast *Candida albicans*, from bacteria/subtilisins, fungal amylases, bacterial amylases, fungal hemicellulases, lipases, xylanases and cellulases are all examples of industrial enzymes known to induce allergic sensitization and respiratory occupational allergy; independent of origin or the methods of production. They all share the structural and biological properties that may cause sensitization when inhaled. In alleged contrast to this, „the Working group on Consumer allergy risk from enzyme residues in food“ (DAUVIRIN et al., 1998) came to the conclusion, that „from a scientific point of view there is no indication that enzyme residues in bread or in other foods may represent an un-

acceptable risk for consumers". And indeed, reports on enzyme sensitisation by foods are weak and in not a single case substantiated by the golden standard of food allergy testing, i. e. double blind placebo-controlled food challenge. Thus, recently two case reports by BAUR & CZUPPON (1995a) and KANNY & MONERET-VAUTRIN (1995) described respiratory symptoms in bakers with occupational allergy to fungal  $\alpha$ -amylase after ingestion of bread prepared with flour with and without fungal  $\alpha$ -amylase. The challenge procedure in BAUR'S case was single-blind, KANNY'S case was double-blind, and both cases had more pronounced symptoms after eating the bread with enzyme than the non-enzyme bread. This is similarly true for cases of suspected papain allergy (MANSFIELD & BOWERS, 1983) – a man developed periorbital edema on ingestion of papain-treated meat – as well as for those few other cases that are reported. People sensitised with common moulds, e. g. *Aspergillus fumigatus*, did not react to enzymes produced in related moulds, when they were challenged with bread containing enzymes that were produced in *A. oryzae* or *A. niger* (CULLINAN et al., 1997). LOSADA et al. (1992) challenged 5 patients with occupational allergy to fungal  $\alpha$ -amylase. 4 did not react, only 1 reacted with severe allergy symptoms. This patient had no history of reactions to bread. The challenge dose was 10 mg of native enzyme, which is a (too) high dose, equal to the amount added to 1 kg of flour before the mixing and the baking. Since 1989, it has been permissible in Finland to add cellulase, for example, to white bread dough to break up roughage. Four patients, allergic to cellulase, could eat white bread without any symptoms (TARVAINEN et al., 1991b). It has however also been reported recently, that allergens of enzymes may be preserved in bread and cause symptoms in sensitised individuals. And indeed, there are a few cases published where people with definite occupational respiratory allergy to flour and an additional sensitisation to  $\alpha$ -amylase developed allergic reactions after ingestion of enzyme containing foods (BAUR et al., 1994a; VIDAL & GONZALES QUINTELA, 1995; BAUR & CZUPPON, 1995a; KANNY & MONERET & VAUTRIN, 1995). But such patients are extremely rare; and almost all bakers with occupational asthma tolerate „their own bread“ without problems (BAUR et al., 1986; WÜTHRICH & BAUR, 1990; BAUR et al., 1994a). An explanation might be that heat inactivated  $\alpha$ -amylase loses its allergenicity to a quite considerable extent (BAUR et al., 1994a). Whether this will also hold true when more thermostable amylases and xylanases will be introduced remains an open question. But ingestion of enzymes seems to be less dangerous than inhalation.

In summary there are a few papers describing cases of allergy symptoms elicited by the ingestion of enzymes in people who have (pre-existing!) occupational allergy to enzymes. But they seem to be extremely rare. And indeed, none of the members of AMFEP appears to have any reports of sensitised employees who had experienced allergy symptoms in connection with ingestion of bread, and there are no reports of  $\alpha$ -amylase sensitised employees avoiding bread (DAUVRIN et al., 1998).

Persons that were consuming enzymes for medical purposes – healthy people, patients with chronic pancreatitis (DAUVRIN et al., 1998) or lung diseases (LUCARELLI et al., 1994) and as digestive aids – hardly ever reported on problems (DAUVRIN et al., 1998). And if they do, sensitisation is usually not initiated by enzyme intake but by inhalation or through cross-reactivity with common food allergens; this might have happened in a person that suffered from a severe adverse reaction after ingestion of raw celery and 4 weeks later did not tolerate a bromelain-containing drug (NETTIS et al., 2000). Anaphylaxis has followed the use of chymotrypsin for the treatment of epididymitis (LIEBOWITZ & RITTER, 1969), and the use of chymopapain for the treatment of herniated lumbar discs (BRUNO et al., 1984).

DEL POZO et al. (1995) described a patient who developed generalized dermatitis after taking digestive tablets containing amylase, protease, and cellulase enzymes. Type-IV hypersensitivity was believed to be involved. However, only some people who have allergic contact dermatitis develop systemic reactions after ingesting the allergen (MENNE & HJORTH,

1982). Therefore, patients with type-IV hypersensitivity may be able to eat products containing the allergen.

After carefully reviewing the literature the author of this summary agrees with the Working group (DAUVRIN et al., 1998) that „enzyme residues in bread or other foods do not represent any unacceptable risk to consumers“ (this might not always be true for drugs containing enzymes). And indeed, patients with insufficient enzyme production of the pancreas take industrial enzymes in doses 100,000 - 1 million times higher than the amounts found in food – again without problems (DAUVRIN et al., 1998). Moreover, the potential cross-reactivity between plant proteases such as papain and bromelain, and plant-derived foods, latex and pollen, may serve as an indicator of broad sensitisation (DIEZ-GOMEZ et al., 1999); but the clinical relevance of this finding remains to be elucidated (BAUR et al., 1995b).

Impurities in commercial enzymes for food production, such as traces of the microorganisms used for fermentation as well as preservatives or contaminating microbes and other traces of the growth material, are suspected to be the actual cause of allergy in some cases (PARIZA & FOSTER, 1983; TIIKKAINEN et al., 1996; KANERVA et al., 1997b). And even other enzymes produced by the microbes may contaminate (VANHANEN et al., 1994); but although frequently suspected (TARVAINEN et al., 1991b; TIIKKAINEN et al., 1996), there are no reports in the literature that would substantiate this potential risk.

Although enzymes produced by genetically modified organisms (GMO's) have been on the market in many countries for many years, there are no reports in the medical literature regarding specific problems caused by these enzymes. In fact, enzyme producers have not experienced any difference in allergenicity of these enzymes as compared to traditional extracted or fermented enzymes (DAUVRIN et al., 1998). They appear to have the same sensitising potential and are capable of sensitising exposed employees at the same rate as traditional enzymes (DAUVRIN et al., 1998).

#### **6.4.4 Drug allergy**

There are several reports in the literature, where patients reacted to enzyme-drugs (see section 6.4.1).

#### **6.4.5 Summary**

Enzymes can cause allergy, the main problems being allergies of the respiratory tract and, probably secondary, of the skin. Several reports document this, most of them indicating more an occupational problem than one that is affecting consumers.

The available documentation in the medical literature reflects the way in which the toxic effects of environmental factors are described: first, case reports; then cross-sectional studies. Unfortunately, studies on enzymes with a longitudinal epidemiological design are rare (BRISMAN, 1994).

Enzymes seem to be potent sensitisers in concentrated form, and thus sensitise quite frequently in the production factory, but due to the limited number of exposed people only few persons are affected – and not necessarily clinically diseased. In contrast, in bread producing factories, exposure rates are high and enzymes have become a major cause of occupational disease.

The medical literature also reflects personal interests of scientists. Most reports come from just a few clusters of reports from Germany, Switzerland, Scandinavia, and England, and mostly single case reports were published in other countries. With the exception of Finland, where there is „a relatively high number of reported cases due to the rather extensive en-

zyme industry, as well as the recent research interest in allergy caused by enzymes in Finland" (KANERVA & VANHANEN, 2000b).

And finally, several literature reports, especially older ones, seem to be based on weak data or badly defined test methods, but can not be refuted solely because of suspicion.

## **6.5 Discussion on potentially new enzymes**

Any chemical substance may represent a potential allergen. The introduction of new types of enzymes thus poses a potential new risk. New sources of enzymes, enzymes from organisms which were hitherto difficult to handle or non-culturable, genetic engineering techniques and other developments may on the one hand lead to the introduction of new allergens, new (and non-suspected) sources of allergens or exposure to higher concentrations of allergens.

Whereas literature reports frequently do not give detailed descriptions on the source of enzymes and purity of the preparations, that caused a problem, one fact is unequivocal: There are *currently* no reports on specific problems caused by enzymes, that were produced using genetically modified organisms; they appear to have the same sensitising potential and are capable of sensitising exposed employees at the same rate as traditional enzymes (DAUVIRIN et al., 1998). But special attention is mandatory: Changes in the amino acid sequence of enzymes, their structure, or properties such as thermostability might change the allergenicity. Thus, in the assessment of the allergenicity of „new enzymes“, the characteristics of the novel proteins must be evaluated in light of their similarities to known enzymes, food, and environmental allergens. But evaluation should not only be restricted to the rules established for the assessment of the allergenic potential of foods derived from genetically modified crop plants (METCALFE et al., 1996; see Figure 5), since that decision tree was developed for ingested but not inhaled proteins. Testing for the allergenic potential of new enzymes should thus follow the regulations as suggested for any new substance (see 1.6).

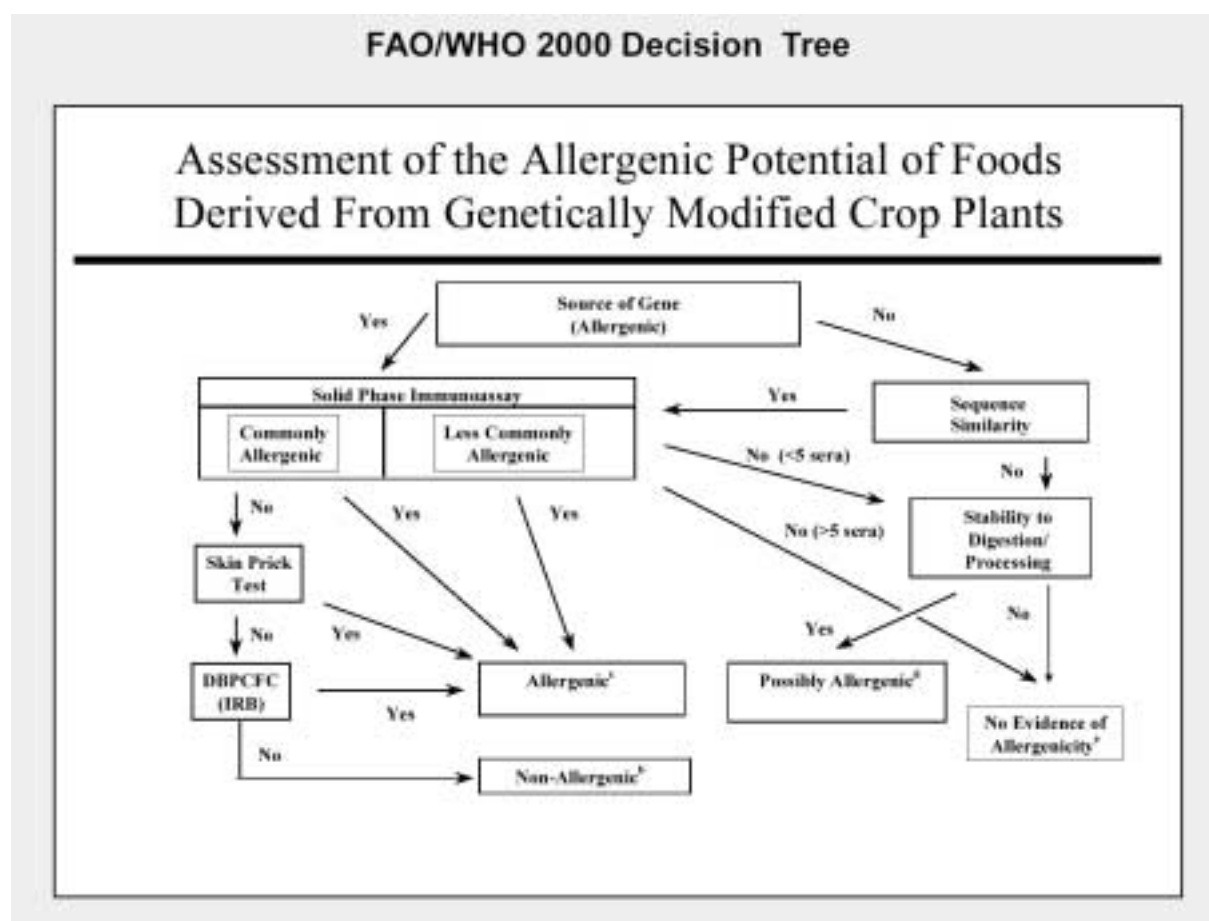


Figure 5: FAO/WHO 2000 Decision Tree

Footnotes to Figure 2 (FAO/WHO, 2001)

- a) The figure was adapted from decision-tree approach developed by International Food Biotechnology Council and Allergy and Immunology Institute of the International Life Sciences Institute (METCALFE et al., 1996).
- b) The combination of tests involving allergic human subjects or blood serum from such subjects would provide a high level of confidence that no major allergens were transferred. The only remaining uncertainty would be the likelihood of a minor allergen affecting a small percentage of the population allergic to the source material.
- c) Any positive results obtained in tests involving allergic human subjects or blood serum from such subjects would provide a high level of confidence that the novel protein was a potential allergen. Foods containing such novel proteins would need to be labelled to protect allergic consumers.
- d) A novel protein with either no sequence similarity to known allergens or derived from a less commonly allergenic source with no evidence of binding of IgE from the blood serum of a few allergic individuals (<5), but that is stable to digestion and processing should be considered a possible allergen. Further evaluation would be necessary to address this uncertainty. The nature would be determined on a case-by-case basis.
- e) A novel protein with no sequence similarity to known allergens and that was not stable to digestion and processing would have no evidence of allergenicity. Similarly, a novel protein expressed by a gene obtained from a less commonly allergenic source and demonstrated to have no binding with IgE from the blood serum of a small number of allergic individuals (>5 but <14) provides no evidence of allergenicity. Stability testing may be included in these cases. However, the level of confidence based on only two decision criteria is modest. The Consultation suggested that other criteria should also be considered such as the level of expression of the novel protein.

## 6.6 Safety evaluation of enzymes

### 6.6.1 Allergenicity

Any enzyme, representing an existing or a „new“ product, being produced either by conventional or new technologies, theoretically should be tested for its allergenic potential like any other new chemical before it is placed on the market.

The criteria for classification of skin- and airway-sensitising substances in the work and general environment were defined only recently (SCHNUCH et al., 2002; FLYVHOLM et al., 1997). In brief, a substance may be termed being a skin sensitiser (causing contact allergy)

- if studies in at least two independent centres report significant numbers of clinically relevant sensitisations, i. e. association of symptoms and exposure, or
- if epidemiological studies reveal an association of sensitisation and exposure, or
- if case reports from more than one patient from at least 2 independent centres present clinically relevant sensitisation, or
- if at least one positive animal study is published, performed according to relevant test methods without using adjuvants, or
- if at least two not so well documented positive animal studies, one of them performed without adjuvant, showed sensitisation.

A substance may be termed being a proven airway sensitiser (inhalant allergy – i. e. type I allergy, IgE-mediated)

- If studies or case reports document specific hypersensitivity of the upper airways or the lung, that indicate an immunologic pathomechanism, and stem from more than one patient and from at least two independent centres.
- An association of exposure and symptoms or functional incapacibilities of the upper or lower airways must be proven.

Investigations show that certain enzymes may bear a different potential, dependent on the source – example: sensitisation to fungal but not bacterial  $\alpha$ -amylases in a baker (KANERVA et al., 1997b) – and their employment – example: occupational allergy to fungal  $\alpha$ -amylases without relevance for ingested products containing  $\alpha$ -amylases (LOSADA et al., 1992). Therefore both, the intrinsic capacity of a certain enzyme to sensitise and the relevance of this capacity, being also dependent on the type of exposure, have to be determined.

The safety evaluation of enzymes used in food processing nowadays includes several steps (Table 30). And based upon a no-effect level derived from in vitro and animal feeding studies it may be concluded that enzymes are safe for the intended use; if all the essential studies are being performed (JENSEN & EIGTVED, 1990).

*Table 30: Safety evaluation of enzymes used in food processing*

- |   |
|---|
| <ul style="list-style-type: none"> <li>- Safety assessment of the production strain</li> <li>- Identity and quality of raw materials used in the fermentation</li> <li>- Processing techniques used in recovery</li> <li>- Identity and quality of the stabilizers, diluents or formulation aids</li> <li>- Toxicology studies on enzyme concentrate</li> <li>- Determination on safety margin</li> </ul> |
|---|



Regarding however the allergic potential that an enzyme may exert during production or its employment, additional tests are necessary. In analogy to common chemicals, the Buehler test with guinea pigs was used in some studies (BUEHLER 1965). This model – like many other animal models – was developed and is validated for the potential of chemicals to cause skin sensitisation (contact allergy) and not for enzymes. It seems evident that proteins are able to cause an immune response through the formation of B-cell mediated antibodies but it has not been assessed to what extent a T-cell mediated type IV reaction is involved. The model is therefore considered not suitable to predict the potential of enzyme preparations to cause allergic contact dermatitis in man (AIS/AMFEP 1995, BERG 1995) and one should therefore rely on human experience.

Some authors conclude (BERGMAN & BROADMEADOW, 1997), that the experience of the last 25 years has not indicated any clinical evidence that enzyme preparations are the cause of sensitisation by skin contact in man, despite considerable employee and consumer monitoring and clinical studies (GOETHE et al., 1972; RODRIGUEZ et al., 1994). In a study with a *Thermomyces lanuginosus* xylanase enzyme (SP 628) and the *Aspergillus aculeatus* xylanase enzyme (SP 578) (BERGMAN & BROADMEADOW, 1997), repeated occluded dermal applications of the retail concentration of Xylanase SP 628 caused hypersensitivity in guinea pigs, whereas in Xylanase SP 578 the incidence of significant responses was not negative but below the EEC limit value of 15%. The authors do not regard both these enzymes as skin-sensitisers and assume that both can be applied without a risk to human health. This assumption may be correct – or not, but the authors seem to ignore, that most enzymes do have an inhalative sensitisation potential, that might become relevant in workers handling these substances – as clinical evidence has shown in thousands of affected workers.

In an (excellent!) safety evaluation of  $\beta$ -glucanase derived from *Trichoderma reesei* (COENEN et al., 1995), a strong skin-sensitising potential of this enzyme was detected using a MAGNUSSON & KLIGMAN (1969) guinea pig model. According to the EEC criteria (91/325/EEC) the tested product should be labelled as a skin sensitiser (DAAMEN, 1994). The data indicate, according to the authors (COENEN et al., 1995), that „there is a potential risk for the workers in the poultry feed industry when handling the preparation. The workers should take precautions to avoid inhalation and skin contact“. In the feed compounding industries, this substance is added to barley-based poultry diets in concentrations of approximately 100ppm (0.01%, w/w). According to the EEC directive on dangerous preparations and substances, preparations containing less than 1% of a sensitiser should not be labelled (BOTHAM et al., 1991). The risks of sensitisation are considered to be acceptable when taking into consideration (a) the strong sensitising potential of the original substance by epidermal exposure, (b) the normal occupational health precautions taken in the manufacture and use of enzymes, (c) the extremely low exposure of workers in the feed-compounding industry who take due precautions and (d) the fact that strong sensitisers may be used safely if there is a short period of contact between a low concentration and a normal skin (BOTHAM et al., 1991). And indeed, the sensitising potential for humans can be judged from test results in guinea pigs only by taking into account the probable human exposure. This exposure may be influenced by several variables, such as the nature of formulation of the enzyme product and the frequency and duration of exposure (BOTHAM et al., 1991).

These detailed presentations of two – controversial ? – studies should highlight the current problem regarding studies on the sensitisation potential: Studies on this potential of (low molecular weight) chemicals should be performed in the guinea pig according to OECD 406 and EEC 92/69/EEC B6. This is a test for the prediction of contact allergy. And positive results may or may not be relevant, depending on the time of skin exposure, concentration and formulation of the substance, and many other factors. But there is no recommended test for (high molecular weight) proteins such as enzymes, that normally do not induce contact allergy (type IV reactions) but may cause immediate type, IgE mediated type I reactions. One author (BERGMAN & BROADMEADOW, 1997) regards the enzymes under investigation not

as skin-sensitiser despite a positive Buehler test, the other (COENEN et al., 1995) concludes from positive Magnusson-Kligman tests (and other test systems) that „the enzyme is safe for use in feed of the intended target species; that, however, some occupational health precautions should be taken to avoid skin contact and inhalation, *as is the case for almost all enzyme proteins*“.

And indeed, test systems were only recently introduced to study the capability of enzymes to induce antibody responses: SARLO et al. (2000) studied the influence of MHC background on the antibody response to detergent enzymes in the mouse intranasal test. The authors could demonstrate, that different enzymes induced antibody responses to a different extent, and this reaction was dependent on many factors. The hybrid strain BDF1 ranks enzymes the same as the guinea pig, which in turn correlates with sensitisation in occupationally exposed humans. The ranking was based upon the dose of enzyme needed to give one-half maximal IgG1 antibody response (ED-50) where Termamyl is more potent than Alcalase, which is equipotent to Savinase. And BARTON et al. (1988) could demonstrate that four different proteases caused differential effects dependent on the immunization system, the kind of animal and the enzyme itself (subtilisin B > pancreatin, and subtilisin A > papain).

Animal models were developed to evaluate all new enzymes for their potential to induce the formation of allergic antibodies recently. In the guinea-pig intratracheal test (GPIT) animals were exposed to defined protein levels of enzyme once/week for 10-12 weeks (RITZ et al., 1993). Sera were collected at selected time-points and the amount of enzyme-specific IgG1 homocytotropic antibody measured by either passive cutaneous anaphylaxis test or by ELISA. A reference or benchmark enzyme, Alcalase (subtilisin), was always included in the test. And in 1996, ROBINSON et al. developed a mouse model as an alternative to the guinea-pig. Intranasal exposure of BDF1 mice to enzymes induced a dose-dependent and enzyme specific IgG1 antibody response after the exposure. Extension of the dosing regimen led to the generation of enzyme specific IgE antibodies.

But: many questions remain, a generally accepted system to predict allergenicity of enzyme preparations is not available, and key questions remain unanswered like: the clinical relevance of current animal and in vitro test systems, the comparability of in vitro and in vivo assays, and probably quite important regarding the hazard of enzymes: how relevant is the dose level versus duration of exposure? e. g. high dose acute or subchronic exposure vs. low dose chronic exposure (CAVAGNARO, 1995). In addition, co-factors such as surfactants present in detergent products have been reported to exert adjuvant activity (MARKHAM & WILKIE, 1976), that make literature data difficult to compare.

Evidence is abundant, that many (all?) enzymes can induce immediate type reactions in man upon relevant exposure. Since neither safe limits for exposure time nor concentration could be defined yet for any enzyme, any exposure should be kept as limited as possible. And in fact, while in previous reports a „safe“ exposure limit of 60 ng/m<sup>3</sup> has been recommended (ACGIH, 1980), many studies have shown that sensitisation and symptomatic allergy may occur at much lower levels. Even for job titles with a relatively low exposure to fungal  $\alpha$ -amylase of less than 2-5 ng/m<sup>3</sup>, and in some cases even in a concentration below the detection level of the assay (0.3-0.5 ng/m<sup>3</sup>), a clearly enhanced risk of sensitisation was demonstrated (DOEKES et al., 1998). The authors conclude, that, „if a safe occupational exposure limit for fungal  $\alpha$ -amylase can be defined, it should be around or below the detection limit of the assay, and far below the previously recommended value for enzyme dust (60 ng/m<sup>3</sup>)“. And this may not only be true for fungal  $\alpha$ -amylase but for all enzymes – at least until safe limits are proven for a certain preparation.

The low molecular weight of proteolytic enzymes (20-20kDa) and their biologic activity might facilitate mucosal penetration more easily and thus – compared to amylase and lipase –

permit an immune response and induction of allergic hypersensitivity more likely (ZENTNER et al., 1997). Such comparison studies are however currently missing.

Negative tests for skin sensitisation should be taken with care; in the safety evaluation of a lipase expressed in *Aspergillus oryzae* (GREENOUGH et al., 1996), in the first epicutaneous challenge positive responses were seen in three of 20 test animals; for several reasons it was concluded „that sensitisation had not been shown with the lipase“. And the summary saying „that the lipase appears safe for consumers in the given applications, and requires no special occupational health precautions in manufacture“ surprises, since there „was evidence for mild skin irritation“.

Regarding new enzymes, a decision tree approach for the evaluation of their allergenicity is mandatory in a similar way as it has become obligatory for genetically modified foods. This includes (FAO/WHO report, 2001)

- animal models
- amino acid sequence comparison with known allergens
- in vitro and in vivo tests in allergic and non-allergic individuals
- testing for physico-chemical characteristics (common food allergens are resistant to proteolysis, stability to digestion in the gastrointestinal tract, have a tendency to be abundant, are often glycosylated, and be resistant to food processing or cooking (METCALFE et al., 1996)
- adequate post marketing surveillance.

Highly allergenic proteins are often expressed at relatively high levels. However, allergens can sensitise susceptible individuals at less than milligram levels, possibly at less than microgram levels (SORVA et al., 1994; JARVINEN et al., 1999). The elicitation of objective symptoms in already sensitised individuals can also occur at low levels of exposure, but has not been documented below 500 micrograms (RANCE & DUTAU, 1997; HOURIHANE et al., 1997). It is therefore not possible to define a level of expression below which a protein can be considered safe from the allergenicity point of view. Thus, currently level of expression cannot be incorporated into the assessment of the allergenicity of genetically modified foods (FAO/WHO report 2001).

EU regulation mandates labelling a substance being a sensitiser if certain criteria are fulfilled:

R42 sensitisation via inhalation possible,

- if from experience a substance or product may cause specific sensitivity at the respiratory tract
- if positive results from animal tests do exist.

At least one of the criteria is probably fulfilled for every enzyme available on the market – if adequate testing was performed.

R43 sensitisation via skin contact possible,

- if from experience a substance or product may cause specific sensitivity in a considerable proportion of exposed persons via skin contact
- if positive results from animal tests do exist.

Following previous arguments, there is no hard evidence that skin contact with any enzyme does cause contact allergy (a delayed-type hypersensitivity reaction) in man; animal skin sensitisation models may/should thus be considered not suitable, since they are developed to predict the potential of enzyme preparations to cause allergic contact dermatitis

(AIS/AMFEP 1995; BERG 1995); thus no valid data exist to justify labelling enzymes with R43.

In summary, any enzyme preparation, independent of its source (conventional or by using GMO's or any other „new“ source) should be labelled as inhalant/type I-sensitiser (R42) and accordingly handled – until the opposite is proven with unequivocal experiments.

Approval of new enzymes requires consideration of a series of toxicological and multigenerational animal studies, depending on the degree of risk involved (DENNER et al., 1983). Regarding contact allergy, no currently available (animal or human) tests should be applied; concerning inhalant allergy, again either labelling R42 should be performed, if not appropriate test methods (SCHWEIGERT et al., 2000) prove safety. Additionally, monitoring of exposed workers and post-marketing surveillance studies should be regularly performed.

### **6.6.2 Skin irritation**

In several well performed safety evaluations, some enzymes such as lipase expressed in *Aspergillus oryzae* (GREENOUGH et al., 1996) were shown to be skin irritants in the animal models used. Several others like  $\beta$ -glucanase derived from *Trichoderma reesei* (COENEN et al., 1995), the *Thermomyces lanuginosus* xylanase enzyme and the *Aspergillus aculeatus* xylanase enzyme (BERGMAN & BROADMEADOW, 1997), and a *Bacillus acidopullulyticus* pullulanase (STAVNSBJERG et al., 1986) were non-irritating to skin and did not produce eye injury in rabbits in those concentrations tested. The same is true for quite a number of other enzymes, but negative studies were only performed using incompletely delineated assays (MODDERMAN FOLEY, 1995), or in rabbits (STAVNSBJERG et al., 1986).

Despite some negative test results, an intrinsic capacity of enzymes like proteases, lipases and others to irritate skin is quite likely. This is especially true for wet working places like bakeries and laundries, where the skin of workers is additionally burdened by exposure to water and many other irritants.

Thus again, any enzyme concentrate should be handled as if it were an irritant, until the opposite is proven.

Skin (and eye) contact when handling enzyme preparations should be avoided!

## **6.7 Safety measures currently performed or suggested**

### **6.7.1 Safety evaluation of enzymes**

#### **6.7.1.1 Allergens**

The regulator should define and validate the test systems to be used, in vitro and in vivo (animal studies). Currently, negative skin-responses associated with repetitive, low-dose exposure to industrial chemicals and consumer products all too often are not accurately predicted by the required assays. Predictive assays generally require data on the dose-response relationship for prudent and optimal interpretation. There are many critical variables which, when data are available, can facilitate more robust and sound information (Table 31). Currently several steps of testing are in use for the evaluation of the allergenic potential of chemicals, but all these studies may predict a potential for the induction of contact allergy by chemicals only (Table 32). None is capable of exactly predicting immediate type reactions; and numerous artefacts may lead to either false-positive or false-negative results.

*Table 31: Critical variables for the comparability of predictive tests*

- Effect of species as related to man (as compared to animals)
- Vehicle effects (may be 100-fold)
- Dose-response relationships ( $\mu\text{g}/\text{cm}^2$ ; linear, flat, or inverse)
- Anatomic site (more completely studies in man than in animals)
- Duration of occlusion (more completely studies in man than in animals)
- Experimentally damaged skin (may, or may not, increase bioavailability)

*Table 32: Evaluation of a chemical to induce contact allergy*

- Quantitative structure-activity relationships
- Guinea pig sensitisation tests, open epicutaneous tests, Buehler test, Freund's complete adjuvant test, optimisation test, split adjuvant test, guinea pig maximization test
- Human sensitisation assays, repeat insult patch test, human maximization tests, etc.

Regarding predictive assays for evaluating the ability of enzymes to produce immunological, immediate type I allergic reactions, test methods are scarce, controversial, and of limited clinical relevance, hardly standardized and exclusively based on animal models.

The recently published mouse intranasal test (SARLO et al., 2000), developed to assess the immunogenic potential of detergent enzymes, uses inbred mouse strains of different haplotypes, hybrid and congenic strains. Certain strains ranked enzymes the same as the guinea pig, which in turn correlates with sensitisation in occupationally exposed humans.

Studies in mice have also demonstrated, that enzymatically active papain preferentially induces an IgG1 response and results in mast cell degranulation, both features typical of an allergic reaction. In contrast, inactive papain desensitised mice to subsequent challenge by active enzyme (CHAMBERS et al., 1998). It is only the recently developed guinea-pig intratracheal test (RITZ et al., 1993) and the mouse-intranasal exposure test (ROBINSON et al., 1996), that seem to produce reproducible results in the respective systems.

These examples may demonstrate, that progress is being made by adapting techniques for the predictive study of enzymes to induce or elicit immediate type reactions in animals. The question on the relevance for man remains to be answered. And time does not seem ready to recommend certain test methods routinely for such evaluations yet.

In addition, the potential for interaction among different enzymes with an effect on sensitisation (SARLO et al., 1997b) as well as adjuvant factors important for an increased immune response (MARKHAM & WILKIE, 1976) remain to be uniformly defined.

### 6.7.1.2 Skin irritation

Numerous in vitro assays for irritation exist. BASON et al. (1991) summarize these assays and offer guidelines as to their potential validation.

Regarding irritation tests in animals and humans, many variables of the chosen test procedure (e. g., vehicle, type of application and concentration tested) may modify the intensity of the response. Selection of subjects tested may also influence the outcome. Differences in intensity of responses has been linked to differences in age, sex, and race.

Tests nowadays should be performed according to EC method B.4 (OECD guideline 404), Acute Dermal Irritation/Corrosion, and EC method B.5 (OECD 405), Acute Eye Irritation/Corrosion, being based on considerations regarding existing human and animal data, structure activity relationship, results from in vitro and ex vivo tests that are generally accepted for purposes of hazard or risk assessment, as well as defined animal in vivo tests (OECD, 2001).

An adjustment for high molecular weight substances such as enzymes should be performed, evaluated, and standardized. This has not been worked out for the time being in systematic studies; thus EC methods B.4 and B.5 may currently well be termed inappropriate for such substances such as enzymes. But they may as well lead to correct negative or positive results.

### **6.7.2 Workers protection**

Occupational asthma and allergy associated with the use of enzymes was first reported soon after the introduction of alkaline and heat stable proteolytic enzymes into detergent products in the 1960s (rev. in SCHWEIGERT et al., 2000). But despite encapsulation of the enzymes and improved hygiene at manufacturing sites, the development of asthma has continued to be a hazard (HOLE et al., 2000; CULLINAN et al., 2000). It was only recently that effective control programs in reducing exposure, based on a surveillance programme to identify and correct excess exposures before symptomatic illness occurs, that numbers of new cases have declined. This approach includes coating of enzymes, compliance with operational guidelines, introduction of exposure guidelines for airborne enzyme proteins, reduction of peak exposures, training, medical monitoring, commitment and changes of behaviour, surveillance systems, etc. (PETERS & MACKENZIE, 1997); some of these essentials shall be highlighted in more detail.

#### **6.7.2.1 Clean workplace**

Manufacturing sites of enzymes should adhere to operational guidelines that reflect daily safe work practices for controlling dust and enzyme exposure. These include: no visible dust, no recurring spills and temporary repairs, no gross skin contact and the treatment of empty containers as if full of enzyme products. These guidelines are met by engineering controls to minimize dust and aerosol generation during normal operation and peak exposure situations, product formulations that minimize dust and aerosol generation (encapsulation by inert materials), limits on the level of enzyme in detergent product, air monitoring systems to verify adherence to exposure guidelines and employee education and training to minimize personal exposure. Ventilation systems must adequately filter and not recirculate particulate matters. Companies must fully comply with OSHA standards.

In the baking industry, where the use of enzymes is likely to continue and increase, less dusty preparations, such as granules or oil suspensions, should be introduced. Exhaust hoods and local ventilation systems especially in the dusty phases, like the weighing processes, and on the dough pots are needed (JAUHAINEN et al., 1993). Replacement of fungal  $\alpha$ -amylase by bacterial  $\alpha$ -amylase should be considered, since no reactions were found to the latter, indicating different antigenic characteristics, which were confirmed by immunoblotting and RAST inhibition (VANHANEN et al., 1996). Measuring wheat flour specific IgG antibodies as an index of allergen exposure may help monitor this exposure, since there was a tendency for IgE and IgG1 levels to decrease after the installation of an air purifier and the introduction of other protective measures (MATSUMURA et al., 1994).

### 6.7.2.2 Personal safety measures

Workers handling enzymes should use protective clothing and eye protection. Water should be easily accessible in case spillage or splashing occurs. Adequate protective breathing equipment should be used if dust or aerosols are encountered. Skin exposed to enzymes should be washed immediately, and eyewash and shower stations must be convenient and fully functional. If enzyme is inadvertently swallowed, adequate rinsing and water should be given, and medical advice should be sought immediately. Plant and laboratory practices should emphasize safety and good housekeeping.

Enzymes can be allergenic, depending on the susceptibility of individuals (see 1.7.2.5). Adequate precautions to protect the individual include optimal skin care, where skin contact cannot be avoided as in bakeries, since eczematous skin is prone to become sensitised more easily.

### 6.7.2.3 Air-exposure monitoring

Despite enzyme encapsulation and modern process techniques in the detergent industry, there still seems to be a risk of allergy. Exposure levels may vary widely, probably from  $>10\mu\text{g}/\text{m}^3$  at sites of primary production and packaging of purified preparations, to less than  $1\text{ ng}/\text{m}^3$  where enzyme preparations are finally used, like in bakeries.

Air sampling may be performed by static samplers, distributed at different working places, or by individual sampling, where workers wear a personal sampler for a whole shift (NIEUWENHUIJSEN et al., 1999).

Enzyme concentration measurements at worksites have mainly focused on protease production and its use in the detergent industry (rev. in VANHANEN et al., 1996). It is not known which concentrations are capable of sensitising workers, but it is probable that concentrations below the threshold limit value (TLV) proposed by the American Conference of Governmental Industrial Hygienists of  $60\text{ ng}/\text{m}^3$  can sensitise (ACGIH), or at least cause symptoms in sensitised workers. This guideline was based on the protein content and activity of enzyme receipt materials, estimation of exposure levels during the late 1960s and measurement of enzyme levels in facilities that had eliminated asthma and allergy symptoms. The detergent industry moved to levels lower than the ACGIH TLV due to the suspected potentiation of allergy to enzymes by detergent matrix. Thus some detergent companies use an internal occupational exposure guideline of  $15\text{ ng protein}/\text{m}^3$  (SCHWEIGERT et al., 2000).

Protease concentrations were generally  $< 20\text{ ng}/\text{m}^3$  in a Finish detergent factory (VANHANEN et al., 2000a), but occasional peak values up to  $80\text{ ng}/\text{m}^3$  were detected in the packing and maintenance tasks, and high values of  $> 1\mu\text{g}/\text{m}^3$  in the mixing area.

In a study on fungal  $\alpha$ -amylase allergy in bakery workers, a dose-response relation between the job title-related mean exposure level – assessed by personal airborne dust sampling combined with a sensitive enzyme immunoassay for  $\alpha$ -amylase – and both sensitisation and the presence of respiratory symptoms were evaluated (DOEKES et al., 1998). Even for job titles with a relatively low exposure of less than  $2\text{--}5\text{ ng}/\text{m}^3$ , and in many cases a concentration below the detection level of the assay ( $0.3\text{--}0.5\text{ ng}/\text{m}^3$ ), a clearly enhanced risk of sensitisation was demonstrated. The authors conclude, that a safe occupational exposure limit for fungal  $\alpha$ -amylase should be around or below the detection limit of the assay, and far below the previously recommended value for enzyme dust. And the authors point to the importance of differences between

- assay methods for  $\alpha$ -amylase in filter extracts
- air sampling procedures – type of filter and sampling head, flow, personal or stationary air sampling, etc.

- extraction procedures – which buffer, with or without detergents, at which temperature, etc.
- storage of filter after sampling, and of extracts: duration, at which temperature, in which type of vial, etc.

Personal sampling revealed that a small proportion of workers in bakeries are exposed to  $\alpha$ -amylase at concentrations that result in high rates of sensitisation; and exposure to  $\alpha$ -amylase showed only a moderate correlation with concentrations of dust in these workers (NIEUWENHUIJSEN et al., 1999).

Finland specifies no hygienic limit values for occupational exposure to any enzyme. The occupational limit of exposure to organic dust is 5 mg/m<sup>3</sup> (eight hour average) and 10 mg/m<sup>3</sup> (15 minute average) (VANHANEN et al., 1996). Total measured dust concentrations in bakeries were from 0.1 to 18 mg/m<sup>3</sup> (thus exceeding the limits by far), with highest values in dough making areas.  $\alpha$ -amylase generally followed the total dust concentrations and reached the highest values of < 6.6 µg/m<sup>3</sup> in the same areas. Cellulase and xylanase varied with concentrations < 180 ng/m<sup>3</sup> and < 40 ng/m<sup>3</sup>, respectively, in the flour mill and the crispbread factory. No cellulase, but concentrations of 1-200 ng/m<sup>3</sup> xylanase, were found in the bakeries, probably indicating the natural xylanase activity of wheat.

In the animal feed industry (VANHANEN et al., 2001), enzyme concentrations in the air varied greatly: xylanase from less than 0.8 ng/m<sup>3</sup> up to 16 ng/m<sup>3</sup>,  $\alpha$ -amylase from less than 20 ng/m<sup>3</sup> up to 200 ng/m<sup>3</sup>, and protease from less than 0.4 ng/m<sup>3</sup> up to 2900 ng/m<sup>3</sup>. Exposure to enzymes does not uniformly follow exposure to total dust: added enzymes are not present in the grain when it is received, and their concentration is low in the end product. Between these stages, exposure to enzymes is possible in the adding of premixes and other process phases.

Several studies point to the importance of the assay method: Some methods are based on the enzyme activity; thus only active enzyme is measured, omitting inactive but potentially sensitising enzyme proteins. In addition, methods may not be specific for the origin of the enzyme activity, as would be an immunological assay: colorimetric assays for proteases may detect microbial enzymes as well as proteolytic activity of the grain dust (VANHANEN et al., 2001). Flour, especially wheat, contains natural  $\alpha$ -amylase (SANDIFORD et al., 1994). But the extent to which the measured  $\alpha$ -amylase in air is due to flours cannot be assessed with colorimetric methods.

Contamination exerts another problem when controlling dust exposure in bakeries. The industrial grades of enzymes are poorly purified (VANHANEN et al., 1996), they contain varying amounts of other enzymes produced by the micro-organism in question. Thus  $\alpha$ -amylase, for example, may contain proteases, and cellulases xylanases, and vice versa. This may cause unexpected sensitisations and cross reactions.

Several studies have clearly demonstrated, that not so much the amount of exposure over time, but exposure peaks exceeding the mean concentrations, do represent the major problem for sensitisation (CATHCART et al., 1997). Since however the exposure levels are usually mean values for job titles and production facilities, it is not clear whether sensitisation in a „medium exposure“ population was due to this mean or a cumulative dose of allergen, or to repeated short peak exposures, e. g. of 10-100 ng/m<sup>3</sup> or more, which most probably occur with some frequency. It thus might be speculated that time-weighted average levels of more than 5-10 ng/m<sup>3</sup> could be „safe“, if such peak exposures can be effectively prevented. However, as long as no experimental or observational data are available to resolve this question, it seems reasonable to use time-weighted average values as the basis for any exposure limit (DOEKES et al., 1998).

As shown in recent reports by the industry, it seems practicable to reduce enzyme concentrations far below the TLV (CATHCART et al., 1997). The control of occasional peak expo-



tures, which are probably important in inducing sensitisation, remains a principal challenge (VANHANEN et al., 2000a).

Indirectly controlling total exposure over time by monitoring the patients antigen-specific IgG antibodies may at least in the baking industry be a feasible task (MATSUMURA et al., 1994).

#### 6.7.2.4 Workers surveillance programmes

In several studies extensive monitoring programs of exposed persons for the development of sensitisation or disease was performed. Sensitisation represents the first step of progression towards the development of occupational asthma, but sensitisation does not necessarily progress to asthma. For the definition of clinical enzyme allergy, all the following criteria need to be met (in brief): A formal diagnosis of asthma, rhinitis or urticaria; symptoms related to exposure to enzymes; and a positive skin test (skin prick test, SPT), and/or positive IgE antibodies in blood, and/or a positive workplace challenge to enzymes (JOHNSEN et al., 1997).

The sensitivity and specificity (for enzyme allergy in the detergent industry) are considered higher for the SPT vis a vis the RAST testing method (SCHWEIGERT et al., 2000); whereas specificity seems to be similar for both, the sensitivity of the SPT ranges from 75 to 89% compared to the RAST where sensitivity ranges from 57 to 86%. But despite the literature supporting SPT testing as superior to serological tests, there is evidence demonstrating the use of serological tests as an effective alternative (POULSEN et al., 1995).

In an extensive monitoring programme in an enzyme producing plant, JOHNSEN et al. (1997) could show that sensitisation occurred to all types of enzymes handled in the plant, most often in production areas and laboratories; 8.8% developed clinical enzyme allergy during the first three years of employment. The frequency of sensitisation, expressed as significant RAST values, was 8%. Ranking diagnoses of enzyme allergy by severity, the frequency of asthma was 5.3%, rhinitis 3.0%, and urticaria 0.6%. Half of the cases occurred within the first 15 months of exposure.

In a cross-sectional study conducted in a detergent factory (VANHANEN et al., 2000a), 22% workers were sensitised to enzymes in the exposed group of 40, whereas none was sensitised in the non-exposed group. All the sensitised people had symptoms at work; all had rhinitis and one had asthma – despite the use of encapsulated enzyme concentrations.

Surveillance programmes in the detergent industry (SCHWEIGERT et al., 2000) to identify and correct excess exposures before symptomatic illness occurs have shown, that asymptomatic, converted workers (skin or blood test positive, but not ill) need not be removed from the work environment. They should be placed under closer surveillance for the development of symptoms. Those with Grade II (symptoms of upper respiratory tract) or III (asthma) symptoms are removed from the work area until their symptoms are cleared and the source of exposure defined and eliminated. The experience has shown that symptoms among skin test-positive workers are a function of exposure – and not of disease.

In a pharmaceutical factory, multiple IgE-mediated sensitisations to the enzymes as demonstrated by positive skin tests and IgE results, occurred despite protective measures (ZENTNER et al., 1997), but only half of the sensitised workers complained of work-related symptoms, although there was no obvious difference in duration or intensity of exposure. The authors conclude, that measurement of enzyme-specific IgG and IgE might serve as a sensitive tool to control for exposure and allergic sensitisation, respectively, to these enzymes.

In a study in British bakeries and flour mills (NIEUWENHUIJSEN et al., 1999), workers were asked to fill out questionnaires on work related symptoms, and they were skin prick tested to assess sensitisation. They found a direct correlation between exposure to  $\alpha$ -amylase and sensitisation to fungal  $\alpha$ -amylase.

Surveillance programmes also have to take into consideration, that sensitisations and disease may only partially be caused by the supposedly most potent allergens, in that in a study performed in the baking industry (VANHANEN et al., 1996), beside sensitisation to enzymes, reactions to storage mites and flours might be even more frequent. And in a Japanese study, „allergic symptoms in a bakery resulted not from wheat flour allergy alone, but rather also from enzymes in baking additives“ (MATSUMURA et al., 1994).

In a study on bakers, DOEKES et al. (1998) show that a positive outcome of a specific skin test or IgE test as such may be regarded as a negative health effect, since allergen-specific sensitisation is strongly associated with respiratory health problems. They do however point out, that this association is not absolute, and may differ per allergen, and possibly also for different populations. For example, they found IgE sensitisation to pig urinary proteins in 5-7% of Dutch pig farmers, but without a demonstrable association with respiratory health parameters (DOEKES et al., 1997). In contrast, IgE sensitisation to rodents, particularly rats, is very strongly associated with self-reported symptomatic rat allergy (HOLLANDER et al., 1996). And in workers exposed to platinum (MERGET et al., 2000a), the duration of exposure in high exposure areas in spite of work-related symptoms determines prognosis: early cessation of exposure after the occurrence of symptoms results in complete recovery in most subjects, whereas remaining at the work-place for a prolonged time may cause permanent asthma. The authors postulate that secondary prevention programs should become mandatory for employees with exposure to potent occupational inhaled allergens – what enzymes definitely are.

Enzymes are potent allergens, and exposure may cause sensitisation (7% in the animal feed industry, 13-30% in the baking industry, 22% in a detergent factory, and 36% in an enzyme producing plant (rev. in VANHANEN et al., 2001). Detection of sensitisation by SPT or in vitro (RAST) indicates exposure; this has different consequences in different work places. In some fields of occupational type I allergy (platinum asthma), sensitisation should lead to immediate removal of the worker from the work place, until the source can be detected and eliminated (MERGET et al., 2000a). In others (pig farmers), sensitisation frequently does not bear any consequence (DOEKES et al., 1997), whereas IgE-response to rat dander is strongly related to problems (HOLLANDER et al., 1996). Regarding enzymes, the consequences may differ from enzyme to enzyme and sometimes be dependent on amount of exposure. Nevertheless, regular screening of those workers potentially exposed to these allergens seems mandatory, whether by SPT or RAST may be discussed. The goal is to identify those workers whose immunological status has changed before symptoms occur (SCHWEIGERT et al., 2000). Such programmes also provide a means to measure the impact of primary prevention efforts. The workforce should be tested annually except when the process changes (e. g. introduction of a new enzyme, major equipment change) which would warrant more frequent monitoring. In the event of any new cases, increasing sensitisation rates or unexpected progress of disease, the work area should be assessed for excess exposure.

#### **6.7.2.5 Handling of workers predisposed for allergy**

Pre-employment examinations in order to exclude persons predisposed for sensitisation or allergy seems attractive in order to reduce problems. One however first has to define the criteria for such persons. Surprisingly, in employees exposed to the complex salts of platinum, smoking, but not atopy (predisposed people for developing type I allergies) was a predictive factor for sensitisation.

Regarding occupational enzyme exposure, there are several studies concentrating on smoking habits, atopy, and sex, that lead to contradictory results:

Smoking at the time of engagement or investigation was found to be an independent risk factor for clinical allergy in some studies in enzyme production (OR = 2.3, JOHNSEN et al.,

1997), but was *not* found to be a risk factor in others: a detergent factory (CULLINAN et al., 2000; VANHANEN et al., 2000a), and bakeries (VANHANEN et al., 1996; even decreased risk: NIEUWENHUIJSEN et al., 1999).

Atopic predisposition was found to be an independent risk factor for clinical allergy in some studies: detergent industry (PEPYS et al., 1969), pharmaceutical factory (ZENTNER et al., 1997), animal feed industry (VANHANEN et al., 2001), and bakeries (VANHANEN et al., 1996; NIEUWENHUIJSEN et al., 1999), whereas it was *not* in some others: Enzyme production (JOHNSEN et al., 1997) and a detergent factory (CULLINAN et al., 2000; VANHANEN et al., 2000a).

Male or female sex was not associated with any outcomes in a modern detergent factory (CULLINAN et al., 2000), but an increased incidence of asthma in male bakers, and not in women, was reported (BRISMAN & JÄRVHOLM, 1995).

Since enzyme-exposed non-atopic workers are also at risk (VANHANEN et al., 2001) to develop allergy, and since all data seem quite contradictory, pre-employment selection based on atopy, smoking habits or sex seems problematic. In addition, the effect of various selection mechanisms can hardly be excluded in any study. Study populations represent survivor populations and usually no records are available about the leavers. Thus atopy should not be construed as a justification for excluding workers at recruitment since the predictive value of atopy is far too low to justify such a practice and the exclusion would have small impact. Also, in several jurisdictions discrimination based on a physiological characteristic of an individual is forbidden unless the employer can demonstrate that the characteristic interferes with that persons ability to perform work *and* the employer cannot remedy the situation without undue hardship (ANFIELD, 1992). The workplace can be safe for atopics by systematically controlling exposure to enzymes to very low levels (except in bakeries!?). Consequently, in preventing sensitisation and clinical allergy, the focus has to be at controlling exposure, informing the workers, and monitoring their health.

In summary, a multicause analysis of sensitisation leads to the following problems (PETERS & MACKENZIE, 1997):

- lack of knowledge: risk and protection
- unsafe methods: check all practices
- not enforcing safe practices: teaching, training, enforcing
- engineering
- inadequate personal protective equipment
- inadequate inspection and maintenance
- inadequate reward and feed back.

Tertiary methods for prevention of occupational asthma include recovery from illness and reintegration into the workforce once symptomatic illness occurs. This third method of prevention, while obviously important, is recognized as a failure of the other methods of prevention (SCHWEIGERT et al., 2000).

#### **6.7.2.6 Summary on the preventive measures regarding workers handling enzymes**

The current high prevalences of enzyme sensitisation and clinical allergy in workers handling enzymes emphasize the need for preventive measures, that are established and enforced by the regulator. The major points of prevention were stressed already by FLINDT in 1969 (e. g. enclosure of processes, proper storing and cleaning methods, and sufficient personal protective equipment), but problems persist. Guidelines for the safe use of enzymes have been

published by the enzyme producers (AMFEP 1994) and the detergent industry (GILSON et al., 1976; SCHWEIGERT et al., 2000), but they must be observed and probably improved.

Information is the fundamental basis of prevention (VANTANEN, 2001). First, employers, employees and occupational health professionals must be aware of the sensitising properties of enzymes used at the workplace and the symptoms and diseases they can cause. Material safety data sheets should be available on substances containing enzymes, and the sensitising properties of the substances should be indicated in them – if the absolute safety is not unequivocally proven.

An industrial hygiene programme to minimize exposure is mandatory (VANTANEN, 2001). The experience from the detergent industry and the demonstrated exposure-response relationships prove that lowering exposure levels is beneficial. The change from powdered proteases to less dusty preparations proved to be beneficial. This same change could be made in the baking industry, where paste and liquid baking additives are already available. Technical solutions to lower flour and enzyme exposure have been developed, thus introduction of local exhaust and a local air supply at a workstation resulted in a reduction of up to 99.8% in the dust concentration in the breathing zones of the workers (HEINONEN et al., 1996; ENBOM & SÄÄMÄNEN, 1998). Personal protective equipment can be used to supplement other measures for short periods in the dustiest phases. The enclosure of processes and adequate ventilation help lower exposure. System failures of machinery cause high peak exposures and should be minimized. Good work practice includes the careful handling of enzymes.

Periodic health checks at occupational health units should be mandatory, the most important measure in this respect being the supply of information. Periodical testing for sensitisation (skin prick test or IgE detection) as a biological monitoring tool for controlling industrial hygiene conditions may help detect pitfalls and poor air monitoring methods in a facility as well as early signs of clinical disease (SCHWEIGERT et al., 2000).

Air sampling can be used to determine the general exposure conditions and to ensure the effect of industrial hygiene improvements in the workplace. However, short duration peak exposures might not be detected, and those seem quite common and are important in eliciting sensitisation (PETERS et al., 2001).

Threshold limit values (TLV) were set for different enzymes. The TLV for subtilisin (60 ng/m<sup>3</sup>) (ACGIH) in the detergent industry however was shown that it cannot serve as the NOAEL (no observable adverse effect level) for proteases or other enzymes. But even a shift to 15 ng/m<sup>3</sup> for proteases (SCHWEIGERT et al., 2000) proved not to be absolutely safe. In the baking industry, fungal  $\alpha$ -amylase has been shown to cause sensitisation in the low ng/m<sup>3</sup> range (HOUBA et al., 1997), in comparison to wheat flour in the mg/m<sup>3</sup> range (HOUBA et al., 1998). Data on animal experiments confirm the varying potency of different enzymes to elicit sensitisation. A bacterial amylase, Termamyl<sup>®</sup>, was found to be three to ten times as potent as a protease, subtilisin (SARLO et al., 1997a). Thus it is probable that each enzyme is different as to its allergenic potency to humans. Moreover, multiple exposure to enzymes may modify the response, as indicated by animal experiments: proteolytic enzymes in a mixture enhanced antibody responses to other enzymes in guinea pigs (SARLO et al., 1997a).

Setting a TLV requires that a valid method of monitoring the substance in the workplace air be available to users. The literature shows the need to keep at least protease and amylase levels lower than 60 ng/m<sup>3</sup>. Immunologic methods with very low detection limits are available, but there is still a need for the methods to be standardized. And the development of new enzyme structures by the use of protein engineering creates a need for constant development of new antibodies and assays. Moreover, the technical problem of monitoring short peak exposures remain.

### 6.7.3 Consumer protection

Safety studies describe some enzymes, on the basis of toxicological studies, *as safe for the enzyme production worker and the consumer* (STAVNSBJERG et al., 1986; KONDO et al., 1994; BERGMANN & BROADMEADOW, 1997); these are extensive and careful studies, but studies without any relevant allergy tests.

A handful of consumer cases of allergic responses owing to exposure to enzymes in laundry products used in Sweden were described many years ago (SCHWEIGERT et al., 2000). Nowadays it is convincingly proven, that even people that are occupationally sensitised to enzymes can privately use enzyme containing laundry products, wear garments, sleep in bedding washed with these detergents and remain symptom free. And the NATIONAL RESEARCH COUNCIL concluded already a long time ago, that the risk for developing allergy to enzymes used in laundry detergents was very low and that these products were safe (1971).

Regarding enzyme residues in bread/food, there is currently sufficient evidence, that many cases of perceived allergy to enzymes may be attributed to insufficient diagnostic procedures employed by members of the medical profession. And there are currently no convincing cases in the literature, where persons were sensitised to enzymes by ingestion of enzyme containing food. But in theory, such sensitisation may occur (DAUVIRIN et al., 1998), and thorough surveillance mechanisms should be instituted, if new enzymes (with unusual characteristics) are introduced into food production.

These demands for surveillance programmes are essential for all kinds of enzymes, being produced conventionally or with new techniques, and for all fields, where they are introduced; in order not to miss any new problems *and* for reassurance of the consumers / public opinion.

The importance of such programmes has recently been documented, when enzyme-containing personal cleansing products (soaps) were being considered for the consumer market. The potential for inhalation exposure to the enzyme during use of this new product while showering was evaluated in a clinical trial (KELLING et al., 1998). After 6 months of at-home product use, 4 out of 61 test subjects had positive skin prick tests and were IgE-positive in serum; none had become sick. The likelihood of both induction of an immunologic response and subsequent elicitation of allergy symptoms in a small but significant fraction of the user population was identified as being high. This finding resulted in the decision to halt further development of this prototype.

In contrast to this negative example, combined studies from Korea and Japan on the effects of protease used in dishwashing liquids indicated, that 0.005% and 0.02% protease caused no adverse dermatological complications to the subjects after even prolonged use (LEE et al., 2002).

Such post-market surveillance is necessary, being both a tool for risk assessment as well as for risk management. And it corresponds to the social demand, especially in such delicate fields like biotechnology and food (FAO/WHO, 2001). New allergens are constantly appearing and it is not easy to provide a simple, universal and definite answer to the question: „what makes an allergen an allergen?“ (AAS, 1978). It is an outstanding challenge to understand why a common glycoprotein which is an innocuous antigen is also an allergen for some groups of people, or why it may suddenly or progressively become a much more potent allergen than usual.

## 6.8 Industry's arguments regarding testing of enzyme concentrates

### 6.8.1 AMFEP position<sup>57</sup>

It is correct that „after three decades of industrial uses of enzyme preparations the only critical toxicological endpoint is their ability to cause inhalation sensitisation“ (quotation mark indicates original citation). But skin and eye irritation should not be neglected in workers.

And it is not correct at all what the next paragraph states: „Isolated cases of allergy alleged from occupational exposure are reported but those incidences happened only from production sites not meeting current enzyme industry engineering and monitoring standards.“

First of all, its not only isolated cases: Its a considerable proportion of workers in the detergent industry (CULLINAN et al., 2000), in bakeries (SANDER et al., 1998), enzyme production plants (JOHNSEN et al., 1997), animal feed industry (DOEKES et al, 1999), and others. And its also true for modern factories, where modern standards for enzyme handling were observed (CULLINAN et al., 2000).

Regarding enzymes for personal care products (for consumers!), where the paper states, that „specified testing and pre-market approval is not required“, the argumentation is not substantiated by facts: in one of the hitherto few attempts to introduce enzyme-containing personal cleansing products (KELLING et al., 1998), it was only by specified pre-market testing that the problems became evident.

The selection of „relevant publications“ is somehow strange: 2 out of 4 are from the industry (SCHWEIGERT et al., 2000; SORENSEN et al., 1998); both signal that the problems are gone. The two other ones from CULLINAN et al. (2000) and VANHANEN et al. (2000a) convey a different opinion: Vanhanen concludes his article saying that „despite the use of encapsulated enzyme preparations, high enzyme concentrations in workplace air are possible, resulting in a higher risk of sensitisation than expected“.

### 6.8.2 Position of detergent enzyme producers<sup>58</sup>

Regarding skin irritation, it is concluded „that the majority of the enzymes is categorized as non-irritating to the skin“ (quotation marks indicate original citation). That's an opinion that might be vulnerable: all enzymes may well be supposed to be irritants due to their intrinsic activity; this capacity is dependent on the type of enzyme, its concentration, and time and intensity of exposure.

The industry construes that:

- Subtilisin products are labelled with R38.
- Cellulases and lipases demonstrate the lack of irritation.
- Testing new enzyme preparations on a case-by-case basis
- in *in-vitro* evaluation may be considered
- Not a problem concerning consumers.

In direct response to these arguments / comments / statements:

- Subtilisin products are labelled with R38: what is the evidence for this, and why not label all proteases, or further, even all enzymes? Proteases may crack skin proteins, and li-

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<sup>57</sup> AMFEP Document, 27 February 2002, p. 8

<sup>58</sup> Position of detergent enzyme producers concerning the adequacy of toxicological data for the safety assessment of detergent enzymes (2002), see also section 13.4 (Annex).

pases skin lipids, both being essential for an intact skin barrier. The potential to irritate is immanent for all enzymes.

- Cellulases and Lipases *may* demonstrate the lack of irritation in experiments available to the industry; if so, these data should be published. GREENOUGH et al. (1996) summarized their studies saying that there was evidence of mild skin irritation with a lipase artificially expressed in *Aspergillus oryzae*. And there are similar reports from other enzymes.
- Case-by-case-testing: The subject here are not enzyme preparations, but, regarding enzyme registration, enzyme concentrates. Thus testing on a case-to-case basis does not suffice, any new enzyme has to be tested according to the rules.
- *In-vitro* evaluation: this is in accordance with OECD guidelines (Figure 4, step 6); but first, a „valid and accepted“ *in vitro* dermal irritation test must be established, evaluated, and agreed upon.
- Consumers safety: might currently be true; regarding „new“ enzymes, with different properties: can we be sure without testing?

Regarding eye irritation, the situation is similar.

And if the industry wants to re-evaluate current EU irritation classification of subtilisins (currently R38), based on „results from the AMFEP project on *in-vitro* alternatives“, one might also wish such tests for all enzymes, existing and new.

Concerning skin sensitisation, „the validity of the skin sensitisation assay for enzymes may be questioned by some regulatory agencies“; and any kind of testing may currently be unnecessary indeed – but only regarding the detergent industry.

Regarding inhalation sensitisation, the industry's position seems to be divided; whereas in this paper „the enzyme producers do not recommend animal studies for this purpose“, some others do perform such quite successfully (SCHWEIGERT et al., 2000). And when the industry states that „workers are well protected by current occupational exposures based on the established ADCIH threshold limit values (TLV) of 60 ng/m<sup>3</sup> for the benchmark enzyme subtilisin“, not everybody does agree (VANHANEN et al., 2000a). And it seems practicable to reduce enzyme concentrations far below the TLV, as show in recent reports by the industry (CATHCART et al., 1997). If „the industry assures that worker safety has been achieved“: this opinion just is not reflected in the scientific medical literature.

## 6.9 Open questions and problems

What makes an allergen an allergen? We do have only limited knowledge now.

What are the relevant allergens in the different fields? Asp o II ( $\alpha$ -amylase from *Aspergillus oryzae*) has been isolated and denominated as an important allergen in baking additives (BAUR et al., 1994b). Molecular biology and biochemical techniques have significantly advanced the knowledge of allergens derived from plant foods, and the understanding of the underlying mechanisms is rapidly growing (BREITENEDER & EBNER, 2000). We have learned to better understand phenomena like cross-reactivity of IgE antibodies, e. g. recognizing epitopes of latex allergens and papain (BAUR et al., 1995b). And we do have better models for the prediction of allergenicity than we had previously (BARTON et al., 1987); but we have to learn how to apply them appropriately. It is inadmissible to conclude from an invalid experimental design, that a certain product is safe; if one does not do the relevant tests. Thus it is difficult to understand how researchers can „conclude that no safety concerns were identified“ if they don't do allergy test for enzymes (COENEN & AUGHTON, 1998). It is also important that investigations are not prevented, illnesses and their incidence not suppressed, and the relevant results published in the scientific literature (FLINDT, 1996).

As the use of enzymes expands into new areas, there is also potential for exposure to the general population which differs from the current situation. And if enzymes are developed for cosmetic and other consumer product uses, it becomes imperative to understand how people can be exposed to enzymes, the level of exposure and the risk for sensitisation. Continued research on understanding how enzymes act as allergens, linked with an understanding of how individuals become sensitised to enzymes is important to the continued control of occupational and non-occupational disease caused by enzymes (SCHWEIGERT et al., 2000). And where we believe to know, we have to sometimes start from scratch: the striking decrease in the occurrence of protease-induced occupational asthma in the detergent industry has been attributed to enzyme encapsulation. British authors recently reported an outbreak of asthma, at least equal in size to the numbers reported in the 60s, in a modern European factory which has exclusively used encapsulated enzymes (CULLINAN et al., 2000). A survey revealed that enzyme sensitisation and work-related respiratory symptoms were positively correlated with airborne enzyme exposure. The authors suggest that encapsulation alone is insufficient to prevent enzyme-induced allergy and asthma; and did not find the real reason for this outbreak.

## **6.10 Summary**

Enzymes are potential allergens. In addition, some may act as skin and eye irritants. But they have a good record of occupational health and safety, if the users keep to the handling precautions, avoid the formation of dust and aerosol sprays – by using liquids, encapsulates, or immobilized preparations – and avoid direct contact with the skin or eyes.

Enzymes are also supposed to be safe for the consumer, as long as microbial enzymes are obtained from non-pathogenic and non-toxinogenic microorganisms grown on raw materials that do not contain compounds hazardous to human health.

And genetically engineered microorganisms can be used under the same conditions of containment, and the same security rules apply as for equivalent, naturally occurring microorganisms. Provided an enzyme is produced by a harmless host, the contained use of recombinant microorganisms does not warrant any special provisions concerning production conditions, worker protection, environmental assessment, field monitoring, or product approval.

Guidelines for the safety assessment of enzymes are established. Allergy is a potential problem intrinsic for all enzymes; and allergy is not only a potential, but also an emotional hazard for the consumer. Guidelines should therefore give detailed recommendations how to deal with the allergenicity of enzymes; what they currently not really do (BATTERSHILL, 1993).

## **6.11 Essential knowledge on enzymes with regard to enzyme notification / registration**

Studies have clearly shown, that the structure of an enzyme protein is determined by its origin. Thus the antigens of fungal  $\alpha$ -amylase do not cross-react with the antigens of bacterial  $\alpha$ -amylase in immunoblotting (VANHANEN et al., 1994). This phenomenon is important with respect to diagnostics and not only has a bearing on the future use of immunologic methods for measuring enzyme exposure, but even more for the classification and labelling of an enzyme regarding its properties.



### 6.11.1 Irritation

Any enzyme may irritate the skin due to its intrinsic properties; the capacity depends on type and source of enzyme, time period and intensity of exposure, concentration and other factors.

Concerning consumers, potential irritating properties are not a problem today since the concentrations of enzymes in end products are toxicologically insignificant.

Regarding occupational exposure, a pragmatic procedure should be chosen:

- It should be proved that the relevant enzyme is not irritating using the appropriate testing method.
- An *in vitro* evaluation should be established in order to avoid animal studies as possible. The validity of negative test results, obtained in that evaluation, should be scrutinized in exposure tests in animals.
- Current industry position, „to assess the need for a dermal irritation assay in case of a new enzyme preparation on a case-by-case basis depending on the type of enzymes and their respective concentrations in the preparations“, seems tempting. But this system does not work now, and it will not work in the future. There are too many enzymes on the market that irritate – but are not labelled accordingly.
- Restricting testing to specific groups – supposing that the others such as lipases „are safe“ – seems inappropriate: not the affiliation to a supposedly safe group, but the specific characteristics of an enzyme itself determines whether it does irritate or not.
- Restricting testing to a certain concentration of enzyme in a preparation again seems tempting, but it is not manageable – chemicals are irritating by nature, or they are not; although it is a question of concentration. But classification and labelling has to be done irrespective of the concentration used at certain work-places.

### 6.11.2 Sensitisation/allergenicity

#### 6.11.2.1 Skin sensitisation (contact allergy)

High-molecular weight proteins such as enzymes are no skin sensitisers. There has not been a case of allergic contact dermatitis in both workers and consumers since the introduction of detergent enzymes in the mid 60s despite repeated and frequent exposures. And all those cases reported in the industry from different professions do not represent cases of contact allergy (delayed type reactions), but patch-test positive immediate type reactions that are mediated by a different mechanism. Testing for skin sensitisation, as it was conducted in the past in guinea pigs, thus should be avoided. And there is no other animal model that has been validated for assessing proteins as contact skin sensitisers; a fact recognized by US EPA (Subdivision M).

In conclusion: currently produced enzymes need not be tested for any skin sensitising potential. If the introduction of new methods leads to the development of essentially „new“ enzymes, one might have to reconsider this point of view.

#### 6.11.2.2 Inhalation sensitisation

Enzymes, being non-endogenous proteins, are potential, and frequently potent, inhalation sensitisers. It seems quite likely, that any enzyme may be able to sensitise, if concentration and exposure time are sufficiently high and long enough. And in fact, all enzyme prepara-

tions will produce positive responses in those animal model assays established (Position of the detergent enzyme producers).

Again a pragmatic approach should thus be chosen regarding occupational exposure:

- All enzymes should be classified and labelled with the Risk-phrase R42 „May cause sensitisation by inhalation“ unless a certain enzyme proves not to do.
- Since predictive (to humans) inhalation sensitisation assays have never been established although several studies have been carried out in various animal species using many different routes of exposure since the early 70s, such evidence (of safety) is currently difficult (if not impossible) to obtain.
- Since the established ACGIH threshold limit values of 60 ng/m<sup>3</sup> for the benchmark enzyme subtilisin in the detergent industry does not well protect exposed workers – in contrast to the current industry position – safety measures have to be intensified; and responsibility of the industry for workers health reinforced. This is even more true for other professions such as bakers.
- The industry's „trusting that development of new sensitisation assays in suitable animal and/or in *in vitro* models might become a valuable futuristic tool in the development and screening of low or non allergenic enzymes“ should be substantiated; as long as such models do not exist, no new enzymes should be released - at least not without labelling them as being a sensitiser.
- „The industry assures that worker safety has been achieved“ (Position of detergent enzyme producers) through several different measures. But reality looks different: as long as up to 20% and more of the exposed workers still become sensitised, this assurance seems implausible.

Before enzyme allergy can be effectively prevented, sufficient information about the health hazards of enzyme use must become available to employers, employees, and occupational health personnel. Only then can the actions needed to minimize exposure be taken (VANHANEN, 2001). And as long as the opposite is not proven, enzymes should be labelled as skin irritants and inhalant sensitisers.

## 7 TOXIC PROPERTIES OF ENZYME CONCENTRATES

### 7.1 Introduction

This chapter mainly addresses the potential toxic properties of enzyme concentrates resulting from acute, sub-acute and chronic exposure. Possible adverse effects such as sensitisation via dermal and inhalation route as well as skin and eye irritation are discussed in chapter 6 and are only mentioned here to preserve coherence. The main question to be asked is: What data are required to describe the toxic properties of an enzyme concentrate (i. e. the fermentation product)? To approach this question the scientific literature (safety evaluations, review articles) and industry position papers were screened and also testing requirements in relevant EU regulations were considered.<sup>59</sup>

Table 33 provides a survey of basic toxicity testing requirements in different areas of EU legislation. Sectoral regulations become operative instead of chemical regulation depending on whether the enzyme preparation is used in feeding-stuffs, food or cosmetics. Therein the safety concerns may distinguish between exposure to consumers, workers and to the environment (including target organisms). While exposure is depending on the particular enzyme application or, if the production process is concerned, toxicological properties are primarily affected by changes in the composition of the enzyme concentrate,<sup>60</sup> i. e. by the active enzyme as such and any of the by-products and contaminants present in the fermentation batch.

The percentage of active (pure) enzyme in enzyme concentrates ranges between 2% and 70% of the dry matter. Thus, the amount of by-products present in enzyme concentrate could be considerably high. It is therefore widely accepted that toxicity testing should be done on the enzyme concentrate used in practice and not on a somehow highly purified enzyme, since notable toxicity may arise from impurities or side products of the fermentation process, while on the contrary no relevant acute or systemic toxicity was ascribed to the enzyme protein, so far. Usually, risks arising from additives are not taken into consideration.

*Table 33: Toxicity testing requirements or recommendations based on EU chemical and sectoral regulation*

Regulation	Industrial Enzymes	Food enzymes	Feed enzymes	Enzymes in Cosmetics
Source	Directive 67/548/EEC (Notification Base Set requirements for a production volume > 1 t /y*)	Guidelines for the presentation of data on food enzymes (SCF, 1992)	Directive 87/153/EEC	Notes of guidance for Testing cosmetic Ingredients (SCCNFP, 2000)

<sup>59</sup> Reports of toxicological evaluations on food additive enzymes prepared by the FAO/WHO Expert Committee on Food Additives were also screened. Beginning with 1972, toxicological studies were examined focussing on acute and repeated dose toxicity studies (duration 2 weeks up to 2 years; test animals are mainly rats, dogs and rabbits), mutagenicity and teratogenicity testing. Further endpoints found were studies on reproduction, immune response, pathogenicity, skin and eye irritation. These reports generally confirm the findings of the screened scientific literature. Since they are lacking a detailed specification of the testing methods (e. g. OECD methods), they are not considered further, but this cannot be seen as an appraisal of their quality.

<sup>60</sup> It is important to stipulate a persistent definition about what the „enzyme“ consists of in practice. This chapter follows the terminology outlined in a position paper of AMFEP, which defines an enzyme concentrate as the active enzyme and including any by-products and contaminants, but excluding additives such as preservatives or stabilisers.

Regulation	Industrial Enzymes	Food enzymes	Feed enzymes	Enzymes in Cosmetics
Acute Toxicity	Required	Not required	Not required	Safety of a cosmetic ingredient has to be confirmed by the producer (dossier). Sub-acute toxicity is thought to be essential.
Subacute Toxicity	Toxicity test on rodents (28d)	Toxicity test on rats (90d)	Toxicity test on rats (90d)	
Mutagenicity/ Genotoxicity	In vitro bacterial mutagenicity In vitro non bacterial mutagenicity (= in vitro chromosome aberration)	Bacterial mutagenicity In vitro chromosome aberration	Bacterial mutagenicity In vitro chromosome aberration	
Others	Irritation, Sensitisation**	-	No chronic and carcinogenicity testing required	

*\*...according to AMFEP information only 5-10 enzyme types actually pass the level of 10 t/y. Since reporting being on enzyme concentrate, 10 t/y corresponds to approx. 0.5-5 t/y on enzyme protein basis. \*\*... more detailed testing requirements are listed in Table 40.*

## 7.2 Safety evaluation of enzymes in scientific literature

A screening of recent scientific literature concerning safety evaluation and toxicity testing on industrial enzyme preparations was carried out. The articles resulting from this search were examined and used to compile a survey of testing methods and specify testing strategies of enzymes. The studies are generally investigating the toxic potential of enzyme concentrates. Table 34 lists the enzymes evaluated in literature. While only one study could be found which deals with an enzyme used in feeding-stuff, 16 studies assess enzyme in food or food processing.

*Table 34: Safety evaluations of enzymes found in literature. The number of evaluation studies are given for the corresponding catalytic enzyme activity*

Enzyme activity	Author(s)	Number*
Phosphodiesterase	KONDO et al. (2001)	1
Xylanase	PICO et al. (1999) BERGMANN & BROADMEADOW (1997)	2
Lipase	GREENOUGH et al. (1996); KONDO et al. (1994) BROADMEADOW et al. (1994); COENEN et al. (1997), FLOOD & KONDO (2001)	5
Lactase	COENEN et al. (2000)	1
Tannase	LANE et al. (1997)	1
Aminopeptidase	COENEN & AUGHTON (1998)	1
Glutaminase	OHSHITA et al. (2000)	1
Pectinesterase	LISSAU et al. (1998)	1
Acetolactate Decarboxylase	DE BOER et al. (1993)	1
Pullulanase	MODDERMAN & FOLEY (1995); STAVNSBJERG et al. (1986)	2
$\beta$ -Glucanase	COENEN et al. (1995)	1 (feed)

\*... If not mentioned otherwise the assessments are referring to enzymes used in food.

### 7.2.1 Characterisation of the test batch

Enzyme batches used in toxicity testing are normally samples from the production process including submerge fermentation, recovery steps (precipitation, filtration, evaporation, drying) prior to formulation. Sometimes several single fermentation batches are pooled to a test batch. From a toxicological view and as mentioned above it is essential to include process contaminants or fermentation side products in the toxicity testing. Since there are very rarely statements about additives (STAVNSBJERG et al., 1986; DE BOER et al., 1993) or diluents, it can be assumed that they are not present in the test batch. Table 35 shows the frequency of parameters used to specify the test batch. Therefore, enzyme main activity and TOS (Total organic substance) are most frequently used to describe the test batch.

Table 35: Frequency of physico-chemical and biological parameters specifying test batches

Application	Food	Feed
Enzyme main activity (units/w)	14	1
Enzyme side activity (units/w)	3	-
Molecular weight (kDa)	1	-
Isoelectric point	1	-
Particle size	-	1
pH (solution)	1	1
TOS *	11	1
Dry matter (105°) (%)		
Water content (%)	12	1
Ash content; inorganic material (%)	9	1
N <sub>Protein</sub> (%)	11	1
Fats (%)	4	-
Carbohydrates (%)	7	1
Contamination – heavy metals	9	-
Contamination – toxins	7	1
Stability	4	
Determination – antibiotic activity/antimicrobial activity	6	1
Total microbial/viable/plate count (CFU/g)	8	1
Determination: Production strain	3	1
Determination: Microorganisms (e. g. E coli, Salmonella, coliform bacteria, (CFU/g)	10	1
Homology of amino acid sequence to allergenic proteins**	1	-
Total number of studies	16	1

\*... TOS is defined as  $100\% - (A + W + D) \%$  where A is the Ash content, W is the water content and D is the diluent content. \*\*... In the study (PICO et. al., 1999) the amino acid sequence of the enzyme was compared to a database of known allergens.

### 7.2.2 Safety of production strain

Production organisms should normally be absent or inactivated in the enzyme preparation due to downstream processing. In some cases their absence is confirmed by determining antimicrobial activity of the test batch (Table 35). Table 36 shows the frequency of statements made about the safety of the production strain. In several cases recombinant production organisms are used. However, references to details of genetic engineering (identity of insertions and vectors) are rarely made but in most cases the identity of the donor organism is stated. Several studies evaluate the pathogenicity of the production strain by in vivo testing on mice or rats. The injection of the microorganism into the test species followed by scarification and histopathological evaluation seems to be a customary testing strategy as well.

Table 36: Frequency of specification referring to production (host) or donor organisms in safety evaluation studies

Application	Food	Feed
Identity of donor organism	11	-
Confirmation of the safe use of the production organism (literature review, history of safe use,...)	9	1
Pathogenicity studies	7	-
Total number of studies	16	1

### 7.2.3 Hazard assessment – Testing strategies and methods

Toxicological endpoints found in these studies are acute toxicity, repeated dose toxicity (subacute/subchronic studies over an exposure period of 14, 28 or 90 days) and mutagenicity testing (usually in vitro). Further, there are a few long term studies on teratogenicity and chronic toxicity. Table 37 reveals that for food application purposes testing was predominantly done by a rodent repeated dose study (14, 28 or 90 days) together with mutagenicity short term in vitro tests (gene mutation in bacteria; chromosomal aberration). Acute toxicity testing and testing of the irritating and sensitising potential is less frequent; teratogenicity studies and ecotoxicological testing are rarely done. Usually the methods applied are declared to fulfil OECD standard methods criteria.

Table 37: Frequency of toxicity testing methods in safety evaluation studies

Application	Food	Feed	OECD* method
Acute oral toxicity	6	1	401
Acute inhalation toxicity	4	1	403
Repeated dose/subacute oral toxicity (14/28 d)	10	1	407
Repeated dose /subchronic oral toxicity (90 d)**	13	1	408
Long term chronic toxicity over one year	1	-	-
Mutagenicity: Salmonella typhimurium reverse mutation assay, E coli reverse mutation assay (i. e. Ames Test resp. in vitro bacterial test)	14	1	471, 472
Mutagenicity: In vitro mammalian cytogenetic test (i. e. chromosome aberration test resp. cytogenetic assay resp. in vitro non-bacterial test)	10	1	473
Mutagenicity: In vivo bone marrow micronucleus and chro-	3	-	-

Application	Food	Feed	OECD* method
mosome aberration test			
Teratogenicity study	2	-	-
Skin and eye irritation	7	1	404, 405
Skin sensitisation	3	1	406
Total number of studies	16	1	

\*... or methods which seems to be equivalent according to the test description; \*\*... Tests with 91 days (13 wk) duration are also described.

The testing results provide no evidences for relevant toxic properties concerning acute, sub-acute and sub-chronic toxicity and mutagenicity testing of the enzymes.<sup>61</sup>

#### 7.2.4 Safety evaluation and confirmation

The risk assessment (i. e. safety evaluation) of a substance for human health in principle comprises a comparison of the concentration level to which the consumer is exposed or likely to be exposed (Estimated Daily Intake, EDI) with concentration levels at which no toxic effects are likely to occur (No Observed Adverse Effect Level, NOAEL). The NOAEL/EDI ratio gives an indication, whether the exposition to a substance is likely to present a risk to human health. For most food additives a ratio exceeding 100 is normally assumed to present a sufficient safety (margin of safety). In case of toxicity studies on enzymes the corresponding NOAEL value is calculated from the repeated dose (subacute or subchronic) oral toxicity studies. The exposure is estimated from the enzyme concentration in the final food product (average human consumption). For standardisation normally TOS contents are used. Table 38 shows that this procedure together with a final safety confirmation is applied in almost any study.

Table 38: Elements of safety evaluation

Application	Food	Feed
Calculated NOAEL	14	1
EDI	14	1
Calculated margin of safety (safety factor, MoS)	14	1
Safety confirmation*	14	1
Total number of studies	16	1

\*... Phrases used: „can be considered safe for“, „safe for use as“, „possesses no discernible risk“, „no reasons for safety concerns“.

All of the calculated margins of safety (NOAEL/EDI ratio) were found to exceed the traditional 100 safety factor indicating that there are no safety concerns from a toxicological point of view.

<sup>61</sup> In the safety evaluation of the feed enzyme a positive skin sensitising potential was reported.

### 7.3 Possible generation of toxins or biological active contaminants in enzyme production

In enzyme technology, it is widely accepted that the major concerns arising from enzyme products result from fermentation-derived toxic metabolites in the final enzyme product rather than from the enzyme itself. Two review articles provide a critical and in depth discussion of this issue and are therefore discussed in the following.

The first review article (RASMUSSEN & SKOVGAARD, 1996) addresses the question what factors are influencing toxicological hazards of commercial enzyme products. Although restricted to enzyme food application, conclusions may partly be extended to other areas of enzyme applications.

The production of enzyme preparations is said to be mainly influenced by two factors: The first one is the pheno- and genotype characteristic of the production strain, the second is the technology applied during fermentation and subsequent downstream processing including the composition of the fermentation media. According to the authors *„one of the most important factors in safety assurance of enzyme preparations is a correct identification of the microorganism used. This apply first of all to the name of the organism used according to accepted taxonomy. It is equally important that an in-plant system of traceability and quality assurance of the culture in use compared with the approved stock culture exists to safeguard against drift in taxonomy, mutations or selections of strains with unwanted abilities”* (p. 3).

Shortcomings in identification are due to the use of different taxonomic systems with classifications primarily based on morphology studies without further identification. It is stated that *„in bacteria the identification is well developed but reclassification constantly takes place. Identification systems has mainly relied on biochemical tests to determine phenotypic characteristics and to „identify” the isolates. This is however in many cases insufficient to give information about the ability of the organism to produce toxins of different nature. For this purpose phylogenetically based systems of identification is needed...To secure that the culture actually used in the fermentation process is identical to the original stock culture it may be necessary to have the stock characterised not only by conventional identification technique but also by using DNA fingerprinting system or DNA sequencing. On top of this in-plant quality assurance system should be to secure that the correct strain is used in each batch fermentation”* (p. 4 - 5).

Changes in conditions and technical parameters such as substrate, pH, temperature or purification process may affect the nature and the amount of metabolic by-products including toxins produced by the source strains. According to the authors, it cannot be excluded, that microorganisms not known to be harmful in food application, under different conditions could turn out to be toxin producers when used as a source organism for the manufacturing process. This means that the conditions of enzyme production should be well defined, even when using species formerly assumed to be safe in use.

*„The rapid developments in mycotoxin detection and the fact that new ones constantly are added to those already known justify reappraisal of the safety of the strains used for enzyme preparation. Many of the strains have been approved long before a number of mycotoxins known today have been discovered”* (p. 7).

*„The strain improvement is a commercial necessity, but makes it impossible to carry out a safety assessment based on toxicological data from one strain of a species only since one strain is not representative for all microorganisms belonging to the species. The same species of a bacteria may contain strains with great differences in ability to propagate at different water activities. This also underlines the importance of and need for an in plant quality assurance system by which the strain in use in the fermentation is fully traceable to the stock culture or parent culture as mentioned earlier”* (p. 8).



Generally analytical methods and detection limits for microbial toxins vary considerably between different toxins and laboratories and it is difficult to analyse enzyme concentrates as they are very complex mixtures. The number of possible toxins is quite large and further increasing as indicated by the known list of mycotoxins and toxic metabolites. Therefore in vivo tests as well as in vitro studies have to be performed. Enzyme concentrates are supposed not to contain viable cells from the microbial source organism. However, some microorganisms are able to produce highly toxic components even when their number is far below the intended level of organism used for the fermentation process. Depending on the purification process toxins produced by contaminating organisms might pass on to the final preparation. Therefore the risks can only be estimated using toxicological investigations of the end-product.

The authors finally conclude that *„a safety assessment only relying on the identification of a source organism on species level alone will give a false impression of safety. The same argument applies to changes in the production technology apart from the change of species e. g. change of the nutritional composition of the culture broth. Consequently, in general a safety documentation has to include toxicity tests performed on microbially derived enzyme products. The toxicity tests should include a 90-days oral toxicity test in a rodent species and two short term test (i. e. a test for gene mutations in bacteria and a test for chromosomal aberrations). From a toxicological point of view it is important to perform a toxicological testing procedure on each specific enzyme preparation produced from a microbial source. Exemptions from toxicological requirements or acceptance of a reduced toxicological testing i. e. in vitro test/cell culture assays have to be few and justified in each case. However, for enzymes of very high purity and specificity the full toxicity testing may not be needed. This has to be decided on a case-by-case basis”* (p 12).

In the second review article (PARIZA & JOHNSON, 2001) the authors state, that the toxigenic and pathogenic potential of the production strain (source organism) remains the primary consideration in evaluating food enzyme safety. According to the toxigenic potential both bacteria and filamentous fungi are able to produce oral toxins, which have been extensively studied and provide the basis for testing new bacterial isolates for toxigenic potential. The oral toxins produced by filamentous fungi are referred to as mycotoxins. Most of them are acutely toxic and most of them also induce chronic toxicity (e. g. cancer). Chemical tests have been developed for the more important known mycotoxins, which can measure low levels that would not elicit an acute response.

The pathogenic potential is linked to viable cells. Since food enzyme preparations rarely contain viable production organism and since no human pathogens are used, this case is unlikely a risk for consumers but important for worker safety. In evaluating pathogenicity one has to distinguish between the effect resulting from the microbe itself and the host response to the microbe (e. g. septic shock due to injection of dead bacteria). Accordingly, simply injecting microorganisms into animals is not an appropriate way to assess potential pathogenicity.

*„Thoroughly characterised non-pathogenic, non-toxigenic microbial strains, particularly those with a history of safe use in food enzyme manufacture are logical candidates for generating a safe strain lineage, through which improved strains may be derived via genetic modification either by traditional (chemically or UV induced mutagenesis) or rDNA strain improvement strategies. DNA sequence data coupled with the phenotypic analyses permit an accurate assessment of the taxonomy of donor and production organisms. Hence using traditional and modern molecular techniques, it is now possible to precisely determine the degree of relationships of microorganisms used in food enzyme production”* (p. 178).

Since enzyme function may be changed by intentionally altering the amino acid sequence (e. g. protein engineering) the question arises if such modifications might also affect the

safety of an otherwise safe enzyme. To deal with this – as the authors call it – „theoretical risk”, a decision tree approach was presented to update previous enzyme safety evaluation mechanisms used in the US and to keep up with the advances in enzymology.

As the authors state, it is unlikely that changes which improve upon desired enzyme function will result in the creation of a toxin protein. This small theoretical risk can be assessed by conducting limited toxicological tests on engineered enzymes.

*„We anticipate that when a manufacturer synthesizes a series of products through protein engineering, inserting the engineered gene into the same host with the same vector system and demonstrating through appropriate toxicological testing that each product is safe, there will come a point after which further testing of additional similar products should be considered redundant and unnecessary. The point at which this may occur would be established by independent experts on a case-by-case basis. These conclusions should be reassessed on a regular basis, as the body of knowledge from such testing grows” (p 182).*

The authors further state, that the results of numerous mutagenicity tests on enzyme concentrates support the conclusion that testing enzyme preparations from traditional and genetically modified microorganisms for mutagenicity is no more necessary in the course of safety evaluation, since testing has failed to reveal the presence of a single mutagen or clastogen that would not have been detected using a decision tree approach described by authors (PARIZA & FOSTER, 1983) and the International Food Biotechnology Council IFBC (1990). Therefore fifty seven out of sixty three chromosome aberration assays were negative and six false positive, similar results were found with bacterial mutagenesis tests. Therefore, these tests can be substituted by analytical chemistry and limited animal tests.

Finally the authors present an enzyme safety evaluation concept including a decision tree which is also considering enzymes from GM (protein engineering). Relevant decision criteria within this concept are stated in Table 39. Criteria are set up for both the properties of the production strain and for the enzyme concentrate.

*Table 39: Part of the decision tree of PARIZA & JOHNSON (2001) for food enzyme safety evaluation pertaining recombinant microorganism.*

Criteria	Comments and Explanations
– Is the production strain genetically modified?	Modification either by rDNA technologies or traditional methods (UV, chemically)
– In case of modification by rDNA techniques: – Does the expressed product have a history of safe use? – Is the product free of transferable antibiotic resistance gene DNA? – Is all introduced DNA well characterised and safe? – Is the introduced DNA randomly integrated into the chromosome?	To have a history of safe use, enzymes have to be derived from a safe lineage of previously tested enzymes expressed in the same host using the same modification system.  Antibiotic resistance genes are commonly used in the genetic construction of enzyme production strains to identify, select, and stabilise cells carrying introduced DNA.  DNA has to be free of attributes that would render it unsafe for constructing microorganisms producing food grade products.  Introduced DNA refers to all DNA introduced into the production organism, including vector and other sequences incorporated during genetic construction.
– Is the production strain sufficiently well characterised to conclude that unintended pleiotropic effects will not arise? – Is the production strain derived	Pleiotropic effects may result in synthesis of toxins or unsafe metabolites.  In determining safe strain lineage the host organism, all of the introduced DNA and the methods used to genetically modify the host should be considered.

Criteria	Comments and Explanations
<p>from a safe lineage?</p> <ul style="list-style-type: none"> <li>– Is the organism non-pathogenic?</li> </ul>	<p>Relevant to consumers only if the preparation contains living cells.</p>
<ul style="list-style-type: none"> <li>– Is the test article free of antibiotics?</li> <li>– Is the test article free of oral toxins known to be produced by other members of the same species?</li> <li>– Are the amounts of such toxins in the test article below levels of concern?</li> </ul>	<p>Test article refers to the enzyme-containing material that is actually tested. It may differ from the commercial enzyme preparation that it is devoid of preservatives or stabilisers.</p> <p>The process for producing the test article should be representative of the process used for the final enzyme product. The test article is often produced using the production process, stopping before the final purification and formulation steps.</p> <p>Toxins of concern are those active via the oral route.</p> <p>New enzymes should be analysed for toxins that might be reasonably expected, using chemical, biochemical or biological methods. For example, all test material from mould sources should be assayed for mycotoxins that are known to be synthesised by closely related species (aflatoxins, zearalenone, T-2 toxin, ochratoxin A, sterigmatocystin analysis).</p>
<ul style="list-style-type: none"> <li>– Is the NOAEL for the test article in appropriate oral studies sufficiently high to ensure safety?</li> </ul>	<p>Ordinarily a NOAEL is derived from long-term feeding studies, but acute oral toxicity testing is also an option, since with rare exception the only toxins known to be produced by bacteria are proteins or peptides which are acute toxins.</p> <p>The choice of the test is done by a case-by-case basis between:</p> <ul style="list-style-type: none"> <li>– Acute oral toxicity test (OECD 401): Dose at least greater than 100-fold daily human intake; standardisation on TOS.</li> <li>– Repeated-dose oral study (14-91 days) preferably on rat.</li> </ul> <p>The NOAEL should provide at least a 100-fold margin of safety for human consumption (calculation uses standard methods)</p>

## 7.4 Industry position on toxicity testing for enzyme safety assessment

### 7.4.1 Position paper of detergent enzyme producers<sup>62</sup>

Since detergent enzymes are subject to Directive 67/548/EEC (dangerous substances) and Directive 1999/45/EC (dangerous preparations), this recent position paper directly addresses notification requirements and other aspects of chemical regulation in relation to enzymes as well. Therefore, sections relevant to toxicological data requirements are outlined in the following.

<sup>62</sup> In March 2002, AMFEP provided a position paper outlining the detergent enzyme producers position on toxicological data requirements for the safety assessment of detergent enzymes (*Position of Detergent Enzyme Producers Concerning the Adequacy of Toxicological Data for the Safety Assessment of Detergent Enzymes*). This position paper is a response to concerns raised by the German Federal Institute for Consumer Health Protection and Veterinary Medicine and Federal Environmental Protection Agency.

Enzymes from existing safe strain lines, considered as substantially equivalent to enzymes previously tested, e. g. new subtilisin proteases, are generally subject to analytical product characterisation and limited in vitro screening comparing to known enzymes. Detergent enzyme producers accept the pre-classification of enzymes to be respiratory sensitiser (Xn; R42-“harmful; may cause sensitisation by inhalation”) and consequently, no need is seen for inhalation sensitisation assay. Present labelling (i. e. 16 types of enzymes in Annex I of Directive 67/548/EEC) is accepted and voluntarily extended to enzymes not yet listed in the Annex I. For the classification of enzyme preparations concentration limit rules apply according to Dangerous Preparations Directive 1999/45/EC. The position paper lists two cases which require toxicological assays:

- If there is no EINECS and no ELINCS entry of the enzyme, a new substance notification is necessary. If the production volume exceeds 1t/y (enzyme protein or TOS), data at least according to the Base Set requirements of Directive 67/548/EEC are required.
- In the position paper it is stated, that „enzymes listed on EINECS but not previously marketed are generally subject to analytical product characterisation and limited in vitro/in vivo testing in order to investigate the acute toxicity and ecotoxicity as a basis for risk assessment”.<sup>63</sup> This has to be seen as an internal self commitment of the detergent enzyme producers. The particular tests performed are chosen on a case-by-case basis and may comprise: Analytical-chemical characterisation, protein and immunochemistry, in vitro or in vivo toxicology (e. g. acute oral and skin or eye irritation), ecotoxicity and specific detergent manufacturers’ needs (e. g. allergenicity test) or non-EU authority requirements (e. g. Ames Test for import to Japan)

The paper further outlines the industry position on specific toxicological endpoints and refers to the need of generating toxicological studies for detergent enzymes. This position is briefly summarized in Table 40 and compared with the notification testing requirements according to Annex VII of Directive 67/548/EEC.

*Table 40: Need for toxicological endpoints – comparison between notification requirements for industrial enzymes and position of the detergent industry*

Toxicological Endpoint		New substance notification requirements (Base Set)	Detergent enzyme producers position paper
Acute Toxicity	Oral LD50	Required	Only needed for enzymes that differ completely from types presently used.
	Inhalation LD50	Depends on exposure and substance properties	No endpoint of interest for detergent enzymes.
	Dermal LD50		Not necessary, since enzymes are not absorbed through skin.
Irritation	Skin	Required	Self commitment for protease (subtilisin) labelling R38. New enzyme preparations should be assessed case by case.
	Eye		Self commitment for protease (subtilisin) labelling R36. No additional testing is necessary.

<sup>63</sup> The industry points out, that new enzyme molecules belonging to the same IUB class as existing enzymes on EINECS do not require notification for detergent application in the EU.

Toxicological Endpoint		New substance notification requirements (Base Set)	Detergent enzyme producers position paper
Sensitisation	Skin	Required	Skin sensitisation tests are not considered useful, since the molecular weight makes absorption through the skin unlikely.
	Inhalation	-	It is well known that inhalation sensitisation is a critical toxicological endpoint. No animal studies are recommended.
Mutagenicity	In vitro	Required	Genotoxicity assays are presently performed but no longer warranted. Tests are only required when enzyme type and/or production organism is new or not well defined.
	In vivo	If in vitro tests give positive results	
Subacute /Subchronic Toxicity	28d or 90d animal studies	Required	Assaying the toxicity after repeated exposure is unessential, since enzymes are digested or unable to be absorbed (dermal). Repeated inhalation exposure should be excluded because of the known sensitising properties.
Reproduction Toxicity	-	Not required	Reproductive toxicity assays should not be conducted, since enzymes are biodegraded in the gastrointestinal tract resulting in negligible bioavailability. They further do not penetrate skin or mucous membranes and are not related to any known endocrine disruptor.
Chronic Toxicity /Carcinogenicity	Long term animal studies	Not required	Carcinogenicity assay should not be considered, since there is absolutely no indication for carcinogenic properties neither by genotoxic nor epigenetic mechanisms.
Toxicokinetics	-	No test required but assessment of toxicokinetic behaviour is required	Since enzymes are not absorbed via skin, mucous membranes or gastrointestinal tract due to molecular weight and particle size, investigating the kinetics of enzymes is not scientifically valid.
Immunotoxicity	-	Not required, if there are no indications in the 28 d repeated dose study	There are no obvious or suspected mechanisms of action regarding possible adverse effects of enzymes on the immune system beside inhalation sensitisation (Type I hypersensitivity).

#### 7.4.2 Regulatory aspects of microbial food enzymes<sup>64</sup>

A precise definition of the taxonomy of the production strain should be given together with a literature review for reports of use in food, toxigenicity and pathogenicity associated with the species. On the basis of this information AMFEP established criteria to develop a list of microorganisms that can be recognised as safe sources for microbial food enzymes. If the production strain is obtained by genetic engineering, a description of the donor strain should be included. Information about composition of the enzyme concentrate (protein content, carbohydrates, fat, ash, water and diluent) should be given. Purity specifications should be documented, the requirements should correspond to JECFA recommendations (see also section 5.3.4) and limits (metals, mycotoxins, antibacterial activity, coliforms, *E. coli*, salmonella, total viable count). Proof of non-toxigenicity of the production organism may be provided by extensive animal feeding trials of the enzyme preparation. Evidence of the non-pathogenicity of the production strain should be provided either by literature or by tests of the strain in rodents.

### 7.5 Factors affecting the toxicological properties of enzyme concentrates

It is widely accepted that apart from well known allergic and irritative risks (e. g. in case of proteases) pure industrial enzyme proteins normally do not cause significant acute and systemic (chronic) toxic effects. At present, the main concerns are arising from toxic by-products or contaminants of the fermentation process, which may remain in the final product. As previously mentioned it has to be considered that toxic by-products or contaminants may be produced from the source organisms (production strains) and therefore, changes in the production process may influence the ability of the organisms to produce harmful metabolites. Factors affecting the ability of production organisms are discussed in the following, since they may affect extent and frequency of toxicity testing requirements<sup>65</sup>.

#### 7.5.1 Toxigenic and pathogenic potential of production organisms

While the pathogenic potential (ability to induce infectious diseases) of microorganisms is linked to the presence of viable cells, the toxigenic potential enables them to produce toxic compounds (e. g. mycotoxins) which could remain in the final product. These capabilities are therefore primarily considered in the course of safety evaluation. The toxigenic and pathogenic potential can be determined by investigating pheno- and genotype characteristics of the production strain used. A strategy to cope with this problem is to use „safe“ microorganism, i. e. to confirm that the production strain is non-toxigenic or non-pathogenic.

In order to ensure the safety of production strain the microorganism has to be thoroughly described and taxonomically identified. Shortcomings in the identification occur partly due to progress and changes in the taxonomy. Proposals are made to supplement conventional identification techniques using DNA fingerprint system or DNA sequencing as well as verifying the identity in case of doubt via independent recognised laboratories (Guidelines for the Presentation of Data on Food Enzymes, SCF 1992).

In safety evaluations of food enzymes the safety confirmation of production microorganism is largely based on long-term experience of history of safe use. The SCAN Guidelines on the assessment of feed enzymes yet state that strains of „*microorganism belonging to a taxo-*

<sup>64</sup> AMFEP (1992)

<sup>65</sup> In contrast to the considerations presented in this section possible hazardous properties of the enzyme itself (e. g. inhalation sensitisation, irritation) are normally be dealt with by labelling and by limitation or avoidance of exposure (e. g. encapsulation of detergent enzymes).

*nomie group which includes members known to be capable of the production of toxins or other virulence factors, should be subject to appropriate tests to demonstrate at a molecular level the absence of any cause of concern. In each case, the absence of a functional gene encoding the toxin(s) or other virulence factors should be established.*" (Opinion of the Scientific Committee on Animal Nutrition on the Revision of the Guidelines for the Assessment of Additives in Animal Nutrition; Part II, 1999, p. 7).

It is further required, that the production strain is proofed to be stable. Manufacturers are therefore recommended to use source cultures from recognised national and international reference collections and samples of production cultures should be deposited with reference numbers (BATTERSHILL, 1993).

Microorganisms used in the production of enzymes can be wild type strains isolated from natural sources but they are rather „variants" derived either directly from wild type organism via selective serial culture, mutagenesis or genetic engineering or from other variants. The safety confirmation has thus to include details about the genetic modifications (i. e. host organism, identity of insertions and vectors, procedure used for genetic modification). Table 39 presents a decision tree approach recently proposed in the literature, which allows a preliminary check of safety of a GMM strain prior to toxicological testing of the enzyme product.

### **7.5.2 Stability of the manufacturing process and in-process control**

The ability of microorganisms to produce toxins may quantitatively and qualitatively depend on the fermentation conditions (e. g. media composition, pH, temperature). Therefore, the risk remains that a production strain experienced to be safe under certain fermentation conditions may turn out to produce toxins under different conditions. Consequently, the fermentation process has to be thoroughly described and any changes have to be documented. Routine quality assurance checks are recommended (BATTERSHILL, 1993) to monitor changes in the production process. This includes verification of the culture (morphology, identity, purity, stability), the growth media (composition), the operational parameters (pH, aeration, temperature) and to ensure hygienic control during enzyme recovery. These measures also form part of Good Manufacturing Practice (GMP) as an in-plant quality assurance system and serve to minimise risks arising from the manufacture process. It is obvious that clear criteria and recommendations for the production process together with a transparent documentation and a monitoring and surveillance system are necessary to guarantee the effectiveness of in-plant quality control.

## **7.6 Specifications and toxicity testing requirements necessary to describe the toxic properties of enzyme concentrates**

### **7.6.1 Discussing specification requirements**

Toxicological relevant information is primarily gathered from direct toxicity testing. Specifications on the

- production organism (i. e. source or host organism),
- genetic modification (donor organism, inserted DNA, vector), and the
- manufacturing process (e. g. fermentation conditions, downstream processing)

provide (indirect) evidences on toxicity, since the formation of toxic substances could be influenced by changes in either of these. These evidences could be important for guiding toxicity testing.

In order to provide information on these aspects a set of data has to be generated including for example product strain characterisation, enzyme classification, production method description, fermentation monitoring and product specifications. Compliance of the whole manufacturing process with these specifications is essential to guarantee that a test batch used for toxicity testing represents the manufacturing process and has therefore regularly to be checked.

Physico-chemical and biological parameters of the enzyme concentrate in food application as exemplified in Table 35 comprising heavy metal content, total viable count as well as analysing the enzyme activity, TOS and contaminating toxins and antibiotics of the enzyme concentrate. Analytical methods are well established and could provide useful tools for detecting known toxins and otherwise harmful or undesirable substances. However, these methods cannot ensure that all possibly unknown – or simply unexpected – toxins or metabolites can be detected. As discussed above, changes in the manufacturing process might induce the formation of toxic metabolites not experienced before.

In vivo and in vitro toxicity testing is thus essential and has to be applied as soon as there is a significant change in the specified manufacturing process (including production strain, traditional or genetic modification, fermentation) or if a new process is to be established.<sup>66</sup>

This approach is also valid, if genetic engineering techniques are applied, as long as these techniques lead to changes in the genetic information of the production organism or the amino acid sequence of the protein which might presumably affect the overall safety. Although rather unlikely changes in the functional characteristics of enzymes might lead to new properties (e. g. changing pH optimum, increasing thermal stability, reduced requirements for cofactors, improved stability against chemical oxidation) also induce toxigenic effects (PARIZA & JOHNSON, 2001). More likely, using genetic engineering techniques one might have to face unintended and unpredictable pleiotropic effects of DNA recombination. These pleiotropic effects could give raise to the increased amount of known toxins or the formation of new toxins. However, it cannot be concluded, that these techniques are demanding a different or new approach of toxicity testing or overall safety evaluation of enzymes now or in the foreseeable future.

### 7.6.2 Discussing toxicity testing endpoints

In this section toxicological endpoints are discussed in the light of their relevance for enzyme testing. Regulatory requirements and recommendations as outlined in Table 33, and scientific literature and industry position papers as outlined in Table 38 are considered:

#### Acute Oral Toxicity

This endpoint is not required in food and feed enzyme regulations, however sometimes forms part of the safety evaluation of food enzymes in the scientific literature (Table 37). New chemical substance notification (Base Set) requires testing using the „standard“ route of exposure. PARIZA & JOHNSON (2001) proposed an acute oral toxicity test according to OECD 401 for food enzymes safety evaluation. It is however emphasised that the test is not intended to establish an LD<sub>50</sub> but to „*determine the safety of bacterial enzymes, since with rare exceptions the only toxins known to be produced by bacteria are proteins or peptides (enterotoxins and certain neurotoxins) which are acute toxins that are produced by only a few bacterial species*“. According to the detergent enzyme industry position,<sup>67</sup> that oral toxicity

<sup>66</sup> According to the SCF guidelines for evaluating food enzymes only *Saccharomyces cerevisiae* is presently exempted from in vivo testing requirements.

<sup>67</sup> Citations are referring to the position paper of the detergent enzyme manufacturing industry (see also section 13.4 Annex).



testing is „only needed for enzymes, that differ completely from the types currently used and should not be considered as essential for safety assessment due to human reasons.”

### **Acute Inhalation/Dermal Toxicity<sup>68</sup>**

There are no requirements in food enzyme testing (SCF; 1992). Yet in four out of sixteen food enzyme safety evaluations acute inhalation studies are included, all giving negative results: One lipase used in detergent and baking industry, one xylanase used in baking industry and two pullulanase used as a food processing aid. In feed enzyme testing one (out of one) safety evaluation includes testing of this endpoint: The enzyme type is a beta-glucanase for poultry diets. According to feed enzyme testing requirements (Directive 87/153/EEC) the dermal and inhalation route has to be checked to enable a judgement about risks in handling. This obviously does not automatically include a testing, since the SCAN states in their guideline document: *„Enzyme and microbial additives will be regarded as respiratory sensitisers (R42) unless convincing evidence to the contrary is provided. As such, measures should be taken to minimise the inhalation exposure of workers and inhalation toxicity studies will thus not be required”* (Opinion of the Scientific Committee on Animal Nutrition on the Revision of the Guidelines for the Assessment of Additives in Animal Nutrition; Part II, 1999, p. 19). New chemical substance notification (Base Set) requires testing of either the inhalation or the dermal route additionally to the „standard” oral route. The detergent enzyme industry considers both endpoints of no relevance for their enzymes, since enzymes are not absorbed via dermal route (large molecular weight) and inhalation studies would result in procedural errors as they are not suitable for protein testing. A further argument given is, that the main concern of enzymes, their potential respiratory allergenicity, cannot be evaluated in the acute inhalation testing.

### **Subacute/Subchronic Toxicity**

This endpoint is required both in food and feed regulation (oral route, 90 day test on rodent species) and forms a core element of the safety evaluation of food enzymes in the scientific literature (Table 37). New Chemical Substance Notification (Base Set) requires testing (28 day, normally oral route). Test results are generally used to determine the „No observed adverse effect level” (NOAEL) and subsequently the „Margin of Safety” (MoS) as outlined previously in this chapter. PARIZA & JOHNSON (2001) recommend repeated dose oral studies (14 - 91 days) for food enzymes preferably on rats to detect toxicity associated with known microbial toxins active via the oral route, a similar recommendation (oral, 90 days on rats) is given by RASMUSSEN & SKOVGAARD (1996). The detergent industry considers repeated dose toxicity testing (14, 28, or 90 days) to be unessential, since enzymes are expected not to produce systemic toxicity, since they are susceptible to destruction by digestive enzymes. Further dermal application is assumed not to result in any significant effect, because the large enzyme molecules cannot be absorbed in large amount. Inhalation exposure should generally be precluded due to the enzymes known sensitising properties after inhalation exposure.

### **Mutagenicity/Genotoxicity**

Testing is required in food and feed regulation as well. The standard test battery comprises a combination of an in vitro bacterial mutagenicity and an in vitro chromosome aberration test. New Chemical Substance Notification (Base Set) requires a combination of an in vitro bacterial and a non bacterial mutagenicity test. In vitro mutagenicity testing is frequently done in safety evaluation of food enzymes, in vivo genotoxicity testing is however rare. PARIZA & JOHNSON claim that a *„genotoxicity testing of enzyme preparations from traditional and ge-*

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<sup>68</sup> Sensitising and irritative properties of enzymes are comprehensively discussed in chapter 6.

*netically modified microorganisms is unnecessary for safety evaluation*" (2001: 183)<sup>69</sup>. Consequently, they do not foresee such tests in their food enzyme safety evaluation strategy (Table 39). Contrary to this position RASMUSSEN & SKOVGAARD (1996) recommend a bacterial together with chromosome aberration test to be essential for food enzyme preparations. The detergent enzyme industry believes that mutagenicity tests for enzyme preparation are no longer justifiable, since an extensive data base already exists and due to a documented lack of genotoxic effects with enzyme preparations both in bacterial and mammalian cells. Therefore, mutagenicity assays should only be required if the enzyme type and/or the production organism is new or not well defined.

### Reproduction Toxicity

Testing is not required in feed and food regulation. In two out of sixteen screened food safety evaluations teratogenicity studies (rodents) are performed, however there are no reasons given for implementation (STAVNSBJERG et al., 1986; DE BOER et al., 1993). No other hints for a need of reproductive toxicity studies are found in the remaining scientific literature. New Chemical Substance Notification (Base Set) does not require testing. The detergent enzyme industry considers reproduction toxicity testing to be unnecessary, since there is no bioavailability due to gastrointestinal degradation, no significant skin and mucous membrane penetration and no known endocrine disruptor activity.

### Chronic Toxicity/Carcinogenicity

Testing is not required in feed and food regulation. In one out of sixteen screened food safety evaluations a long term chronic toxicity test is performed, however there are no reasons given for carrying out this test (OHSHITA et al., 2000). New Chemical Substance Notification (Base Set) does not require testing. Detergent industry considers carcinogenicity testing unnecessary, since there are no indications for genotoxic or epigenetic mechanisms.

## 7.7 Conclusions and recommendations

This chapter is not based on a systematic data base, since appropriate and detailed testing data could not been obtained from the industry (AMFEP). The conclusions therefore largely base on scientific literature (safety evaluations, review articles). A position paper from the detergent enzyme industry and testing requirements in the present chemical, food and feed regulation are also taken into consideration. Accordingly, the following conclusions can be drawn:

- Referring to the toxicological endpoints<sup>70</sup> acute, sub-acute and sub-chronic toxicity and mutagenicity testing, carried out in the scientific literature does not reveal any toxic potential of enzymes used in food or feed application. This statement yet has to be seen against the background of the limited available data base, especially considering feed enzyme application (Table 33).
- There is no clear consensus about what is necessary for enzyme toxicity testing regarding EU regulation, scientific literature and (detergent) enzyme industry position.
- For enzymes in food and feed application a test battery could be identified, which forms a commonly applied test set in enzyme toxicity testing (Table 33). This consists of a repeated dose (subchronic) oral toxicity test on rodents together with a set of two mutagenicity tests (one in vitro bacterial and one in vitro non bacterial test). Although this test

<sup>69</sup> As mentioned this conclusion is justified as follows: in vitro genotoxicity testing has failed to reveal the presence of a single mutagen or clastogen that would not have been detected using the more comprehensive decision tree approach according to PARIZA & FORSTER (1983) and International Food Biotechnology Council (1990).

<sup>70</sup> So far, this statement is not including the irritative and sensitising properties.

battery is well established and extensively used, notable objections are made. PARIZA & JOHNSON (2001) argue, that mutagenicity testing has failed to reveal the presence of a single mutagen or clastogen. So far, mutagenicity testing led to either negative or false positive results. In view of the detergent enzyme industry repeated dose and mutagenicity testing is no longer required. According to repeated dose testing it is justified, that enzyme proteins are digested and therefore cannot be accumulated in the body. This argument does, however, not take into account side products or contaminants (i. e. toxins). Referring to mutagenicity testing the industry argues that there is an extensive database showing lack of genotoxic effects with enzyme preparations in both bacterial and mammalian systems. A need for testing is therefore only recommended in case of new enzyme types and/or production organisms.

The following toxicological endpoints seems to be of limited relevance for enzymes:

- Acute oral toxicity testing is not recommended in sectoral regulation, it is, however, as recommended by Pariza & Johnson (2001) of relevance in few cases of food enzyme testing if the production organism is suspected to produce enterotoxins. It is the detergent enzyme industry position, that acute toxicity testing is of relevance only in case of new enzyme types. It is therefore considered by the authors that testing should be limited to well defined cases.
- Acute toxicity testing via dermal route does not seem to be necessary, since enzymes molecules are too large to be absorbed by the skin. Only in case of cosmetic application a testing may be considered.

The following toxicological endpoints seem to be of no or little relevance for enzymes:

- Acute toxicity testing via inhalation route seems to be of little relevance, since the present testing methods may give procedural errors (industry position). Moreover enzymes are known to be sensitizers (R42; „May cause sensitization by inhalation”).
- Reproductive toxicity testing is required depending on the outcome of mutagenicity study for chemical notification purposes. There are few cases of teratogenicity testing in food safety evaluation literature, however the motivation for carrying out these tests is not revealed. This is also true for chronic (cancerogenicity) testing. It is assumed that these endpoints are not relevant as long as mutagenicity testing is constantly negative. According to PARIZA & JOHNSON (2001) there are strong indications that this is the case, but validation on a broad data base is outstanding.
- There is no evidence, that immunotoxicity and toxicokinetic evaluations are of relevance for enzyme testing except in medicinal application.

It is assumed, that enzymes produced by GMM demand no substantially new toxicity testing requirements provided that the consequences of these modifications are considered prior to the testing and are used to guide the toxicity testing. No evidence could be found which indicates that the present and foreseeable improvement of certain enzyme properties (e. g. changing pH optimum or increasing thermal stability by „protein engineering”) will demand new or altered toxicity testing requirements.

Therefore the following recommendations are given:

- Prior to toxicity testing information/data should be compiled on the enzyme concentrate and the manufacturing process and regarding to the production organism, the conditions for fermentation and downstream processing and the composition of the enzyme concentrate. Genetic modification (and the donor organism) should thereby also be taken into account. This should preferably be standardised using, for example a decision tree approach/check list as proposed by PARIZA & JOHNSON (2001) (see Table 39). Emphasis should be put on this information, since this may help to reduce animal toxicity testing requirements.

- Since no consensus for toxicity testing methods could be identified, a general discussion process should be initiated, for example addressing the needs and value of mutagenicity testing requirements. According to the AMFEP list on toxicity testing (see Annex, section 13.1.2) hundreds of toxicity tests were carried out and toxicity data as well as testing experience accumulated within the industry (including enzymes from GMM and enzymes with altered protein structure). None of these testing reports were made available to the project team. However, this data could form a valuable basis for discussions to refine and/or reduce testing requirements, e. g. to perform acute toxicity testing only in certain cases (e. g. risk for production of bacterial toxins). Therefore, this data should be collected and systematically investigated.
- From a scientific point of view, toxicity testing requirements for new substances should be flexible. This should depend on a critical, standardised and comprehensive evaluation of the manufacturing process safety (e. g. modification in production strain, fermentation, downstream processing; composition of enzyme concentrate) and (expected) exposure route(s). A reduction of testing requirements may be considered on a case-by-case basis, if the evaluation reveals no significant changes in the process compared to a process already described and confirmed to be safe. This mainly addresses the need for acute, sub-chronic and mutagenicity testing. Testing requirements should however be extended depending on the expected exposure routes and application modes. This is also true for new types of enzymes or new production organisms. Referring to toxicology the authors of this report assume that these adaptations can be well done within the scope of an amended Substance Directive 67/548/EEC and that there is no need for creating a separate or new (sectoral) regulation.

## 8 ENVIRONMENTAL CONSIDERATIONS

An intense search for scientific literature was performed, but only resulted in very few publications some of which are probably not up-to-date. Detailed information on potential environmental problems caused by enzymes were expected to be provided by the enzyme-manufacturing industry. But, as cooperation with industry could not be achieved due to reasons explained previously, we concentrated our investigations on the information available in scientific literature.

The following chapter gives therefore a very limited overview on the results of ecotoxicological and degradation testing on enzymes. The potential risks and ecological problems resulting from the release of enzymes and enzyme preparations into the environment are discussed on a general basis.

Subsequently, potential alterations of ecotoxicological properties and degradation in enzymes manufactured by modern biotechnology (e. g. enzymes from GMOs and structurally modified enzymes) are discussed in section 8.2.2.

### 8.1 Testing of ecotoxicity and degradation on enzymes – review of scientific literature

We identified and investigated publications on ecotoxicity testing in four enzymes/enzyme products – a lipase, a phenol-oxidising enzyme and two proteolytic enzymes – and three publications on biodegradability of enzymes/enzyme products. Two studies testing enzymes produced by a GMO were available.

#### 8.1.1 Characterisation of the test batch

Table 41 and Table 42 include a short description of the test batch as far as it was indicated in the respective studies.

Lipase (GREENOUGH et al., 1996): Tests on ecotoxicity and biodegradation were performed on this enzyme. The test batches were produced in the same manner as in production scale. The production organism is *Aspergillus oryzae* which was modified to host the lipase gene from *Humicola lanuginosa*.

The following characteristics of the lipase test batches were indicated: enzyme activity (defined as the activity (LU) of 1 g of pure enzyme protein),  $N_{\text{total}}$ ,  $N_{\text{protein}}$ , % water and % total organic substance TOS. Ecotoxicity tests were performed using a test batch containing 27.0 respectively 43.8 % TOS of the enzyme.

Laccase (NICNAS, 1998): Ecotoxicity testing was performed on this enzyme. The production organism (*Aspergillus oryzae*) was genetically modified to host the laccase gene from *Polyporus pinsitus*.

In the test batch, the active enzyme protein made up 3.5 % of TOS. The test batch contained 6.6 % TOS, 93 % water and 0.4 % ash.

Savinase (NICNAS, 1993): The production organism and process for the enzyme was not indicated in the report. Tests on ecotoxicity and biodegradation were performed on this enzyme. The test batch used is not characterised in the assessment report. Enzyme preparations and physical and chemical properties of proteinases are described on a very general basis.

Maxatase P (MANN, 1971): is a proteolytic enzyme. Besides protease, it contains a small amount of alpha-amylase and 15 % sodium sulfate. There is no description of the pro-

duction process or production organism neither a more detailed composition was given. The enzyme activity is indicated in delft units (D.U.) per gram. Maxatase P has its optimal activity within a pH-range of 6 - 8. Tests were performed at these pH-range. Enzyme activity decreases at a temperature beyond 50°C.

SG-2331 (SWISHER, 1969): is a commercial product used in detergents. Only tests on biodegradation were performed on this enzyme. It is a composite containing max. 10 % protease, inert protein and polysaccharide produced from a strain of *Bacillus subtilis*.

### 8.1.2 Acute toxicity on aquatic organisms

Table 41 gives an overview on the tested species, the test methodology used and the most important results of the tests including the EC/LD/LC<sub>50</sub> values.

Lipase (GREENOUGH et al., 1996): All tests performed according to OECD Guidelines. Three taxonomic groups (fish, daphnia, algae) were tested. No adverse effects were detected in fish and daphnia at the tested concentration. Growth inhibition on algae revealed an EC<sub>50</sub> (72h)<sub>growth</sub> of 97 mg/l. The NOEC was calculated to be 40 mg/l (based on nominal concentrations).

Laccase (NICNAS, 1998): All tests were performed according to OECD Guidelines. No adverse effects were observed in either fish or daphnia studies (LC<sub>50</sub> resp. EC<sub>50</sub> > 100 mg/l). Laccase is non-toxic to sewage microorganisms.

During the algal study, the growth rate was reduced about 23 % compared to the control. Microscopic investigations of the test media at the end of the test revealed no abnormalities of the algal cells. A limit test was conducted using a nominal concentration of 100 mg/l. The measured concentration was ca. 75 % of the nominal. The EC<sub>50</sub> for algae is indicated to be > 100 mg/l based on nominal concentrations

Comment: In general, an inhibition of 10-15 % is accepted as statistical variation. However, the observed reduction of growth rate of 23 % indicate that there is a slight effect on algae.

Savinase (NICNAS, 1993): Tests on the substance were performed according to OECD and EU Guidelines. The only tested species was fish. Savinase is considered to be practically non-toxic to fish, while the inactivated enzyme was found to be even less toxic to the test species (heat treatment at 90°C). Bioaccumulation of the enzyme is not expected given its high water solubility, high molecular weight and high biodegradation potential.

Maxatase P (MANN, 1971): Tests were not performed according to standard methods. The test conditions were not described in detail and are indicated in Table 41 as far as mentioned in the study. Acute toxicity tests were performed on a variety of species, mainly fish including early development stages. In order to render results comparable, the authors indicated LD<sub>50</sub> values for an exposure period of 24 hours for all tests.

Maxatase P had adverse effects on water organisms. The most sensitive organisms were fish eggs and larvae. The LD<sub>50</sub> (24 h) for eggs of aquarium fish (*Rivulus cylindraceus*) and trout fry amounts to approximately 1 or 5 mg/l of Maxatase P. Fully grown fish can endure relatively higher concentrations. The detrimental effect of Maxatase P examined increases with increasing salinity.

Comment: Testing was not performed according to standard test methods. The exposure period in fish tests was 24 hours at the most, while OECD guideline 203 recommends an exposure period of 96 hours. Taking this into account, the results give an indication that the tested enzyme had an effect on fish, especially on early life stages. The most frequent observations were: death of fish, altered swimming behaviour, decreasing egg hatching rates, die-off of eggs, infestation of eggs with fungi. However, the extent of the effect on aquatic organisms can not be assessed from this study.

### 8.1.3 Ready biodegradability of enzymes

Lipase (GREENOUGH et al., 1996): Two biodegradation tests were performed. The first test (recording dissolved organic carbon) revealed that the substance is readily biodegradable (99 % in 28 days; criteria for 10-day-window were fulfilled).

In the second test (closed bottle test), degradation rates after 28 days were > 70 %, but the 10-day-window was not fulfilled. Therefore, the authors considered the material to be not readily biodegradable.

Comment from study authors: In general, there are no „false-positive results“ in biodegradation tests, however „false-negative“ could be found. Despite the criteria for the 10-day-window were not fulfilled in the closed bottle test, the degradation rates after the test period were definitely above 70 %. Taking also into account the readily biodegradation revealed in the first test, lipase should be considered as ready biodegradable.

Savinase (NICNAS, 1993): In the closed bottle manometric respirometry test, degradation was 41 % after 22 days, which is within the limits observed for readily biodegradable compounds. These results were supported by the measurement of dissolved organic carbon (85 % loss after 22 days). According to the test method, this is an indicator for ready biodegradability.

SG-2331 (SWISHER, 1969): The test revealed an entire degradation of the product SG-2331 and a complete destruction of the enzyme activity within a 24-hour period. Thus, SWISHER concluded that the bacteria of the activated sludge utilized both the active and the inert components of the product as food to an extent comparable with glucose and nutrient broth.

### 8.1.4 Conclusions drawn in the publications

In the investigated studies, both the environmental effects and the estimated release / exposure was taken into account for the assessment of risk. In principle, all authors concluded that the tested enzyme / enzyme product was unlikely to present a risk to the aquatic environment due to the inherent properties of enzymes:

- low or very low aquatic toxicity of enzymes / enzyme products
- ready biodegradability of enzymes / enzyme products

and due to the low predicted concentration of the enzyme in the environment. The reasons given are summarised below.

- the enzymes / enzyme products decompose at higher temperatures (e. g. detergent enzymes during washing)
- small amount will be released into the environment, as the products contain very small quantities of active enzymes and the sewage water is diluted to a high extent in rivers, lakes and oceans
- the environmental concentration of the enzyme is magnitudes lower than acute aquatic toxicity levels.

Lipase (GREENOUGH et al., 1996): No adverse effects were found on fish and daphnia. But, testing revealed a slight inhibition of algal growth. The enzyme is considered to be readily biodegradable.

Laccase (NICNAS, 1998): The ecotoxicity data indicate that the substance is practically non-toxic to fish, daphnia, and sewage microorganisms. Despite a slight observed effect on

algal growth rate, NICNAS (1998) conclude that the enzyme is non-toxic to algae. Degradation data were not reported in this study.

The predicted environmental concentration was estimated to be 1.8 ppb of laccase TOS (taking into account the discharge from a large textile plant, the discharge from the sewerage treatment plant and a 10-fold dilution in receiving waters). This concentration is four orders of magnitude lower than the lowest NOEC observed for fish, daphnia or algae. Thus, the authors do not expect that the discharge of laccase from textile plants is hazardous to aquatic organisms.

**Savinase (NICNAS, 1993):** NICNAS concluded that proteinases are unlikely to present a hazard to the environment at any stage of their use, whether when formulated into laundry detergents or when consumers drain the laundry detergent from washing machines to the sewer.

A predicted environmental concentration of proteinases in sewage water throughout Australia was assessed to be 0.05 ppm. This assessed concentration would swiftly be reduced to insignificant levels by biodegradation in sewage treatment plants and dilution in rivers, lakes and oceans which act as receiving waters to nearly all treatment plants in Australia. The likely environmental concentration of proteinases are at least 3 orders of magnitude lower than acute aquatic toxicity levels.

**Maxatase P (MANN, 1971):** Ecotoxicity testing on Maxatase P revealed adverse effects on water organisms, whereas the most sensitive organisms were fish eggs and larvae.

However, MANN (1971) reckons that under normal conditions proteolytic enzymes of detergents will have no detrimental effect, as the enzymes are to a great part denatured due to the heating of the detergent during the washing process. Subsequently, only a comparatively small quantity gets into household waters. The authors reckon that the remainder will be decomposed in the purification plants (Biodegradation data was not indicated in the study). Detergents contain proteolytic enzymes in very small amounts. When getting into drains, they are diluted to a high degree.

**SG-2331 (SWISHER, 1969):** Detergent enzymes will enter the general environment via domestic sewage. Assuming an enzyme level of 1 % in the detergent formulation, the resulting enzyme concentration in domestic sewage would be around 1 mg/l order of magnitude. The enzyme concentrations used in this degradation test were considerably higher (20, 30 or 300 mg/l). It seems unlikely to the author that the use of detergent enzymes would cause environmental reactions either from direct action or from second order effects. This statement is based on biodegradation testing and the assumption that similar enzymes occur at comparable levels in nature. Ecotoxicity testing is not reported in this study.



Table 41: Acute toxicity testing of enzymes on aquatic organisms - overview of scientific literature

Test substance	Information on test substance	Production organism	Test organism	Test methodology conditions	Results	Reference
<b>Lipase</b> used in food and detergent industry	27.0 % TOS in test batch	Aspergillus oryzae (modified to host the Lipase gene from <i>Humicola lanuginosa</i> )	Daphnia magna	OECD 1981a 24-hr exposure period, static conditions; concentration of test substance: 1 g/l; 5 individuals per test tube (3 replicates);	EC <sub>50</sub> (24h) > 1 g/l significant loss of enzyme activity during 24-hr exposure (76 % enzyme activity after 24 hour exposure)	GREENOUGH et al. (1996)
	27.0 % TOS in test batch		Cyprinus carpio (carp)	OECD 1981e 96-hr exposure period, semi-static conditions; concentration of test substance: 1 g/l; 10 individuals per test vessel;	LC <sub>50</sub> (96h) > 1 g/l no significant loss of enzyme activity during 48-hr exposure	
	43.8 % TOS in test batch		Scenedesmus subspicatus	OECD 1981c 72-hr exposure period; five concentrations (10-160 mg/l) each in triplicate;	EC <sub>50</sub> (72h) <sub>growth</sub> 97 mg/l EC <sub>50</sub> (24-72h) <sub>growth rate</sub> 99 mg/l NOEL 40 mg/l The results are based on nominal concentrations.	
<b>Laccase</b> a phenol-oxidising enzyme used in textile industry	TOS made up 6.6 % of the test batch (active enzyme was 3.5 % of TOS)	Aspergillus oryzae (genetically modified to host the laccase gene from <i>Polyporus pinsitus</i> )	Oncorhynchus mykiss (rainbow trout)	OECD 203	LC <sub>50</sub> > 100 mg/l*	NICNAS (1998)
			Daphnia magna	OECD 202, Part 1	EC <sub>50</sub> > 100 mg/l* NOEC > 100 mg/l	

Test substance	Information on test substance	Production organism	Test organism	Test methodology conditions	Results	Reference
			Selenastrum capricornutum	OECD 201	EC <sub>50</sub> (72h) <sub>growth</sub> >100 mg/l* EC <sub>50</sub> (0-72h) <sub>growth rate</sub> > 100 mg/l NOEC < 100 mg/l During the test a 23 % reduction in the growth rate was observed compared to the control.	
			aerobic waste water bacteria	OECD 209 (respiration inhibition)	EC <sub>50</sub> (3h) > 3200 mg/l	
<b>Savinase</b> is the commercial name for a proteolytic enzyme used in the detergent industry	n. i.	n. i.	Brachydanio rerio (zebra fish)	OECD TG 203 semi-static	LC <sub>50</sub> (96h) 200-400 mg/l LC <sub>50</sub> (96h) > 1000 mg/l (for Savinase inactivated at 90°C)	NICNAS (1993)
<b>Maxatase P</b>	mainly consists of protease, a small amount of alpha-amylase and 15 % sodium sulfate; not indicated in more detail;	n.. i.	Lebistes reticulatus	n. i. in fresh water	LD <sub>50</sub> (24h) 25 mg/l	MANN (1971)
			Salmo gairdneri (trout)	n. i. in fresh water	LD <sub>50</sub> (24h) 15 mg/l in young fish LD <sub>50</sub> (24h) 5 mg/l in eggs and larvae	
			Rivulus cylindraceus	n. i. in fresh water	LD <sub>50</sub> (24h) 1 mg/l in eggs	

Test substance	Information on test substance	Production organism	Test organism	Test methodology conditions	Results	Reference
			Anguilla anguilla (eel)	n. i. tested in sea water (26.9 ‰ salt content), brackish water (13.7 ‰) and fresh water;	LD <sub>50</sub> (24h) 7.5 mg/l (sea water) LD <sub>50</sub> (24h) 20 mg/l (brackish water) LD <sub>50</sub> (24h) 30 mg/l (fresh water)	
			Gammarus salinus	n. i. in brackish water (10.46 ‰ salt content)	LD <sub>50</sub> (24h) 200 mg/l	
			Tubifex sp.	n. i. in fresh water	LD <sub>50</sub> (24h) 50 mg/l	

*n. i. ... not indicated, TOS... Total Organic Substance (i. e. 100% - (%water + %ash), that means that the active enzyme and the impurities are expressed together.*

*\*... A limit test was conducted using nominal concentration of 100 mg/l. The results are based on nominal concentrations. The measured concentrations were around 75 % of the nominal.*

Table 42: Overview on biodegradability testing in enzymes – overview of scientific literature

Test substance	Information on test substance	Production organism	Test methodology/conditions	Results	Reference
<b>Lipase</b> used in food and detergent industry	27.0 % TOS in test batch	Aspergillus oryzae  (modified to host the lipase gene from <i>Humicola lanuginosa</i> )	OECD 1981f (recording dissolved organic carbon (DOC)); concentration of test material: 250 mg (in duplicates);	99 % biodegradation within 28 days; the pass level of 70 % was reached within 10 days;  The substance can be termed as readily biodegradable.	GREENOUGH et al. (1996)
	27.0 % TOS in test batch		closed bottle test (measuring oxygen demand); concentrations of test material: 5 and 25 mg/l;	73 % after 28 days (at 5 mg/l) 78 % after 28 days (at 25 mg/l)  Criteria for 10-day-window <sup>#</sup> were not fulfilled, therefore the material is not ready biodegradable.  The tested substance appeared not to be toxic to the microbial activity present in the medium.	
<b>Savinase</b> is the commercial name for a proteolytic enzyme used in the detergent industry	n. i.	n. i.	closed bottle manometric respirometry test (according to Annex 5 of Directive 79/831/EEC*)	20 % degradation after 5 days 38 % after 15 days 41 % after 22 days;  These values are within the limits observed for ready biodegradable compounds (according to 79/831/EEC).	NICNAS (1993)
				85 % loss of DOC after 22 days;  According to 79/831/EEC this indicates ready biodegradability.	

Test substance	Information on test substance	Production organism	Test methodology/conditions	Results	Reference
<b>SG-2331</b> commercial product used in detergents	containing max. 10 % protease, the remainder is inert protein and polysaccharide;	Bacillus subtilis	semicontinuous activated sludge test; die-away test (according to conditions of the Soap and Detergent Association, 1965)  enzyme concentrations: 20, 30 or 300 mg/l;	complete destruction of protease activity within 24 hours (300 mg/l tested) and entire degradation of substance;  fully degradation of enzyme within 1 to 2 days (20, 30 mg/l tested);	SWISHER (1969)

*n. i. ... not indicated; #... Criteria for 10-day-window: Biodegradation of at least 60 % has to be reached within 10 days of biodegradation exceeding 10 %.*

*\*... Methods for the determination of ecotoxicity. 5.2. Degradation. Manometric Respirometry. – DGXI/283/82, Rev.6.*

## 8.2 Discussion of environmental effects of enzymes

### 8.2.1 Environmental effects and degradation – test results

Test were conducted on species of different trophic levels: primary producers (algae), primary consumers (daphnia) and secondary consumers (fish), as well as on destruents (microorganisms).

Only very few study reports were available. Four studies on ecotoxicity including two on enzymes produced by GMO's and three studies on degradation testing. The detailed results were presented in sections 8.1.2 and 8.1.3.) and are summarised in the following:

- Fish:** GREENOUGH et al. (1996), NICNAS (1993 and 1998) identified no adverse effects of the tested enzymes on fish.
- Whereas, results obtained by Mann (1971) give an indication that the tested proteolytic enzyme had adverse effects in several fish species investigated, especially on early life stages. The adverse effects of the substance increased with increasing salinity.
- Daphnia:** There were no adverse effects of the investigated enzymes on the water flea (*Daphnia magna*) (GREENOUGH et al. (1996) and NICNAS (1998)).
- Algae:** A slight inhibition in algal growth was observed (GREENOUGH et al., 1996 and NICNAS, 1998).
- Bacteria:** The performed test indicate that the tested enzyme was non-toxic to sewage microorganisms (NICNAS, 1998).
- Biodegradation:** All available studies indicate that enzymes / enzyme products were readily biodegradable. Bacteria of the activated sludge utilized both the active enzyme as well as the inert components of the enzyme product as food (SWISHER, 1969).

Taking into account the ready biodegradability of the tested enzymes and the low effects on aquatic organisms, the enzymes would not be classified as dangerous to the environment according to Annex VI of Directive 67/548/EEC. The environmental exposure of enzymes is expected to be low. On the basis of the investigated studies, enzymes seem not to present a risk to the aquatic environment.

The following chapter shall highlight the problem of altered properties of enzymes produced by means of modern biotechnology.

### 8.2.2 Potential alteration of ecotoxicological properties and degradation in enzymes manufactured by modern biotechnology

The application of new technologies in enzyme manufacturing has lead to an improvement of the production strain, the exploitation of new enzymes, the improvement of enzyme properties and the development of new enzyme properties. One major goal to improve enzyme properties is to increase enzyme stability. Stability to pH and temperature are important factors for e. g. detergent enzymes.

Due to genetic engineering, enzymes from extremophiles having different properties compared to those marketed so far, could be made accessible and exploitable. This includes for example enzymes with increased thermal stability (thermophilic, extreme thermophilic enzymes) or with high specific activity at low and moderate temperatures (psychrophilic enzymes). Also enzymes with higher stability at higher / lower pH or high salt concentrations would be accessible.

Applying protein engineering could yield in enzymes with improved properties and/or with properties not found or accessible in nature (discussed in detail in section 3.4).

Techniques for chemical modifications of enzymes focus on increased enzyme stability and activity. The production of enzymes containing synthetic amino acids *in vivo* might also be technically feasible in the near future, resulting in proteins with properties that natural proteins do not possess. Applying this technique might also result in enzymes without a natural counterpart.

The investigated test reports indicate that enzymes may have an adverse effect on fish and a slight effect on algae. However, data on ecotoxicological effects of enzymes derived from exotic sources or chemically modified enzymes with improved properties have not been available.

For the effect assessment, the ready biodegradability of enzymes is a crucial factor. The investigated test reports show that the tested enzymes are ready biodegradable. Enzymes with improved stability could possibly be more resistant to biodegradation. Also protein engineering might decrease biodegradability of enzymes as a non-intended effect. Nevertheless, this decreased biodegradability might not necessarily be of relevance for environmental safety. This conclusion has, however, to be based on results from extensive biodegradability studies. Though, data on biodegradation of enzymes with such properties have not been available to the project team. Therefore, it is inadmissible to conclude if modified enzyme properties have any significant impact on the ready biodegradability. This should be proven on the basis of data.

The quantity of active enzymes released into the environment after industrial application might increase due to lower degradation or denaturation, e. g. during processing at elevated temperatures. This is of relevance if the enzymes are released in significant quantities (e. g. detergent enzymes). Due to the widened field of application of enzymes, exposure is considered to increase in the near future.

Concluding, it cannot scientifically be proven that, based on the few data available, enzymes in general, and in particular those derived by certain techniques of modern biotechnology, do not present a risk to the aquatic environment. In particular, this concerns enzymes with altered properties (e. g. higher thermal stability) which might have decrease biodegradability.

### 8.2.3 Industry position on ecotoxicity and degradation of enzymes

In the context of the *Voluntary Commitment* signed between detergent enzyme producers and German authorities, detergent enzyme producers submitted their position concerning the adequacy of toxicological data for the safety assessment of detergent enzymes<sup>71</sup>. Original citations are printed in italic letters followed by comments made by the authors of this study.

#### Biodegradation:

*Numerous studies have demonstrated that enzymes from all major classes (subtilisins, amylases, cellulases, lipases, etc.) are ready biodegradable. This is expected considering their globular protein structure.*

*Ready biodegradability tests with enzymes from GMO's including PE-variants have not shown any different characteristics compared to naturally occurring or wild type GMO enzymes. This would also be expected, since these techniques do not change the general globular protein structure.*

*On this basis, the detergent enzyme producers conclude that enzymes, as a group of substances, are ready biodegradable. Therefore, testing of enzyme variants and currently used enzymes from new microbial sources will therefore normally not be performed. New classes of enzymes will be tested, if warranted.*

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<sup>71</sup> Position of detergent enzyme producers concerning the adequacy of toxicological data for the safety assessment of detergent enzymes.

Comment: These studies have not been made available to the project team. Therefore, they could not been taken into account for the assessment of environmental risks of enzymes in the context of this study.

Due to genetic engineering, enzymes from extremophiles having considerably different properties compared to those marketed so far, could be made accessible and exploitable (e. g. enzymes with increased thermal stability).

The question was raised if these enzymes differ significantly in their degradation behaviour. However, no data were available to scientifically prove if enzymes with modified properties differ in degradation.

#### Acute toxicity to aquatic organisms:

The potential of acute toxicity effects of detergent enzymes on aquatic organisms are not critical due to their general biodegradability characteristics. For the majority of enzyme concentrates, the aquatic median concentrations are in the range of 100 to 1000 mg/l suggesting that detergent enzymes would not do harm to the environment.

*Effective aquatic median concentration at less than 100 mg/l may occur with proteolytic enzymes, such as Subtilisins due to their particular enzymatic function. However, it should be noted that a substantial inactivation of enzyme activity takes place during and following wash before discharge to waste water streams.*

In our opinion, this should be proved for enzymes with modified properties, e. g. for enzymes with increased thermal stability.

*In conclusion, the acute toxicity to aquatic organisms is generally very low for enzymes. Acute toxicity tests with enzymes from GMO's including PE-variants have not shown any different characteristics compared to naturally occurring or wild type GMO enzymes. This would also be expected, since these techniques are not used to change the general catalytic function of the enzyme, i. e. they will belong to the same class. Testing of enzyme variants and from new microbial sources will therefore normally not be performed but generally limited to new classes of enzymes.*

Comment: These studies have not been made available to the project team. Therefore, they could not been taken into account for the assessment of environmental risks of enzymes in the context of this study.

*The combination of readily biodegradability and slight to moderate aquatic toxicity means that detergent enzymes are not harmful to the environment.*

Adverse effects and biodegradation of enzymes should be proved on the basis of testing data and not on general statements. This is essential in the case of enzymes derived by means of modern biotechnology, especially for enzymes with modified properties e. g. stability to higher temperature.

### **8.3 Summary and conclusions**

The investigated studies revealed that enzymes seem unlikely to be dangerous to the aquatic environment due to their ready biodegradability and the low effects on aquatic organisms observed. However, enzymes derived from of new technologies might have increased stability and therefore altered biodegradability. This assumption could not be verified as no data on biodegradability of enzymes with increased stability was available to the study authors.

The environmental exposure of enzymes is expected to be low. But due to a widened field of applications and possibly decreased biodegradability, exposure might increase in the future.

Thus, on the basis of the few data available, it cannot scientifically be proved that enzymes derived by techniques of modern biotechnology, do not present any risk to the aquatic environment.



Therefore, the ready biodegradability of enzymes with increased stability (e. g. with higher stability to temperature or pH) should be proved. It is suggested, only to perform a biodegradation test in the case the enzyme has „unusual stability”. Decision criteria for „unusual stability” have to be set taking into account already existing information. Thus, data on enzymes with increased stability possibly available within industry should be collected and investigated.

Enzymes are normally biodegradable and show low effects on aquatic organisms. Thus, it is suggested to only perform acute toxicity tests on aquatic organisms in case, the enzyme is not readily biodegradable. Then, the most sensitive species should be tested. The need for ecotoxicity testing and the testing organism should be decided by the authorities taking into account all information that can possibly be made available by industry in the near future.



## 9 PARAMETERS APPLICABLE FOR THE DESCRIPTION OF ENZYMES

In terms of describing enzymes there are three different perspectives to look at enzyme products (also outlined in section 3.4):

- (i) enzymes as active compounds characterised by their molecular structure or by their function;
- (ii) enzyme concentrates containing the active component (i. e. the enzyme) plus any impurities resulting from the fermentation broth and the subsequent purification steps;
- (iii) the enzyme preparation as the ready-to-sell product intended either as an intermediate product or for application by end-users – additives serving as stabilising, granulation, colouring, coating and other aids/agents.

In case of (i), parameters used for describing enzymes in scientific practice will be presented (section 9.1). The relation of enzyme structure and enzyme function and the question of similarity between enzymes will be discussed in more detail. Information available for (ii) and (iii) (section 9.2) as well as the parameters used in regulatory practice will be described (section 9.3). Different national/international regulatory frameworks (e. g. food, feed, technical enzymes, other) will be taken into account. Thereby, the challenges for any new regulatory notification scheme in characterising and distinguishing between enzymes should become more clear.

### 9.1 Enzymes as the active compound

Enzymes are the catalysts of biological systems; nearly all known enzymes are proteins.<sup>72</sup> The structural units of proteins are amino acids. Basically, all proteins in all species from bacteria to humans are constructed from the same set of twenty amino acids. The side chains of these amino acids differ in size, shape, charge, hydrogen-bonding capacity, and chemical reactivity. They can be grouped as follows:

- aliphatic side chains – glycine, alanine, valine, leucine, isoleucine, and proline
- hydroxyl aliphatic side chains – serine and threonine
- aromatic side chains – phenylalanine, tyrosine, and tryptophan
- basic side chains – lysine, arginine, and histidine
- acidic side chains – aspartic acid and glutamic acid
- amide side chains – asparagine and glutamine
- sulfur side chains – cysteine and methionine.

Almost all enzymes are made consist of more than 100 amino acid residues, which gives them a mass greater than 10 kDa and a diameter of more than 25 Å. The active site of an enzyme is the region that binds the substrate(s) (and the prosthetic group, if any) and contains the residues that directly participate in the catalytic reaction. These residues are called the catalytic group. The active site comprises a relatively small part of the total volume of an enzyme. Most of the amino acid residues in an enzyme are not in contact with the substrate.

The theoretically possible arrangements of 20 amino acids within a polypeptide of length 100 amino acids or more will result in a nearly infinite multitude of different molecules. Practically, 3-dimensional structure seems to be more robust than amino acid sequence: the approximate 10,000 protein structures, which are now discovered and maintained in the protein data

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<sup>72</sup> The discovery of catalytically active RNA molecules – so-called ribozymes – however, indicates that proteins do not have an absolute monopoly on catalysis.

bank (PDB) (BERMAN et al., 2000) adopt only some 500 different folds (three-dimensional conformations).

Enzymes share some properties with polymers; both are macromolecules consisting of a (defined) set of elements (often arranged in linear sequence) giving rise to complex three-dimensional structures. Polymers usually consist of only one or few different elements which are often arranged in regular patterns. Polymers are therefore characterised by the proportions of the respective elements, average (or range of) length of chains, and branching degree. This kind of description is sufficient as the exact order and the exact place of a particular element usually does not matter in polymers. In contrast, the exact order and nature of amino acids determines the three-dimensional structure of proteins which in turn is important for their properties.

Enzymes can, therefore, be described by the amino acid sequence and the three-dimensional conformation. Whereas chemical substances are traditionally described by their molecular structure, enzymes, in contrast, are usually characterised by functional parameters.

### 9.1.1 Function-related properties of enzymes

For historical reasons, enzymes are defined by their function/role within the biological system (either procaryotic or eucaryotic cells). This is reflected in nomenclature (parameters N1 - N5 of Table 43). Enzyme ligand interactions (parameters E1 - E9 of Table 43) refer to the specificity of an enzyme: they indicate if the enzyme is tailored (by nature/evolution or via biotechnology) to be highly specific for a single substrate or if it is able to react on a broad range of related or even completely different substrates. The need for ligands are indicators of mechanisms controlling enzyme activity. Functional parameters (parameters F1 - F7 of Table 43), such as pH and temperature optima and ranges, as well as kinetic parameters (specific activity, turnover number) may be regarded as a „functional fingerprint“ of the enzyme. Furthermore, they are important with regard to application conditions. This is also true in the case of other molecular properties, e. g. stability aspects (parameters M1 - M6 of Table 43).

*Table 43: Function-related parameters of enzymes used in the scientific enzyme database BRENDA (<http://brenda.bc.uni-koeln.de>). The code (letters and numbers) refers to the structure of BRENDA (see Table 64, Annex).*

No.	Nomenclature	Description
N1	EC Number	The EC Number is given by the IUBMB (International Union of Biochemistry and Molecular Biology). Classes of enzymes and subclasses are defined according to the reaction catalysed. An EC Number is composed of four numbers divided by a dot. For example the alcohol dehydrogenase has the EC Number 1.1.1.1. The term „IUB number“ is often used as synonym for „EC number“.
N2	Recommended Name <sup>a</sup>	Name given by the IUBMB.
N3	Systematic Name <sup>a</sup>	Name given by the IUBMB.
N4	Enzyme Names <sup>a</sup>	Names given by the IUBMB or found in other databases, literature references, abbreviations, and cross references to other enzymes.
N5	Synonyms <sup>a</sup>	Name found in other databases, literature references, abbreviations, and cross references to other enzymes.
N6	CAS Registry Number	The majority of enzymes has a single Chemical Abstracts Service (CAS) number. Some have no number at all, some have two

No.	Nomenclature	Description
		or more numbers. Sometimes two enzymes share a common number.
N7	Reaction <sup>b</sup>	The reaction is defined by the IUBMB. The commentary in BRENDA gives information on the mechanism, the stereochemistry, or on the thermodynamic data of the reaction.
N8	Reaction Type <sup>c</sup>	According to the enzyme class a type of reaction can be attributed. This can be oxidation, reduction, elimination, addition or a reaction name.
N9	Organism	The systematic name or the common name of the organism.
<b>Enzyme-Ligand Interactions</b>		
E1	Ligands	Ligands are defined as follows: „If it is possible or convenient to regard part of a polyatomic molecular entity as central, then the atoms, groups or molecules bound to that part are called ligands“. Ligands of enzymes act e. g. as inhibitors, activators, co-substrates, substrates, or products.
E2	Substrates and Products	All natural or synthetic substrates and products are listed (not in stoichiometric quantities). Information of the reversibility of the reaction is given.
E3	Substrates	All natural or synthetic substrates are listed.
E4	Products	All natural or synthetic products are listed.
E5	Natural Substrates	The substrate which is used in the normal metabolism of the cell. It is only given when it is mentioned in the literature.
E6	Cofactors	All compounds which act as true cofactors: Many enzymes require the presence of an additional, non-protein, cofactor. Some of these are metal ions such as $\text{Zn}^{2+}$ , $\text{Cu}^{2+}$ , $\text{Mn}^{2+}$ , $\text{K}^+$ , and $\text{Na}^+$ . Some cofactors are small organic molecules called coenzymes (e. g. vitamins, sugars, lipids). Coenzymes may be covalently bound to the protein part (called the apoenzyme) of enzymes as a prosthetic group. Others bind more loosely and, in fact, may bind only transiently to the enzyme as it performs its catalytic act.
E7	Metals/Ions	This field lists all ions or salts that have activating effects, or are closely bound to the enzyme.
E8	Inhibitors	Compounds found to be inhibitory are listed.
E9	Activating Compounds	All Compounds are given that have activating effects except metal ions or cofactors.
<b>Parameters related to the catalytic activity (functional parameters)</b>		
F1	$K_m$ Value	The Michaelis constant, or $K_m$ value, is defined as the substrate concentration which gives rise to a velocity equal to half of the maximal velocity; the $K_m$ gives as an approximate measure information about the enzyme-substrate affinity: a high $K_m$ indicates a low affinity, and vice versa. The unit of this value is mM for a given substrate.
F2	Turnover Number ( $k_{\text{cat}}$ )	The turnover number is defined as the number of molecules of a substrate that are transformed per minute by a single enzyme molecule when the enzyme is working at its maximum. The turnover number ( $k_{\text{cat}}$ ) is given in the unit 1/min. for a specified substrate and is generally in the range of $10^3$ to $10^7$ per second.
F3	Specific Activity	The specific activity is the velocity of the enzyme catalysed reaction (usually $V_{\text{max}}$ ) for the desired enzyme divided by the total amount of protein in the sample. The unit of this value is micro-mol/min/mg of protein. As the purification scheme progresses, the specific activity should increase. In a pure sample, the specific activity will reach a fundamental maximum, which is propor-

No.	Nomenclature	Description
		tional to $k_{cat}$ .
F4	pH Optimum	Different enzymes have different pH optima, where the enzyme is most active, depending on the organism and environment they have evolved in.
F5	pH Range	pH range in which the enzyme is active.
F6	Temperature Optimum	Different enzymes have different optimum temperatures, where the enzyme is most active, depending on the organism and environment they have evolved in.
F7	Temperature Range	Temperature range in which the enzyme is active
<b>Molecular Properties</b>		
M1	pH Stability	Either a range or a single value at which the enzyme is stable.
M2	Temperature Stability	Either a range or a single value at which the enzyme is stable.
M3	General Stability	This field summarises general information on stability, e. g. the addition of stabilising compounds. <sup>d</sup>
M4	Organic Solvent Stability	The stability in presence of organic solvents is described.
M5	Oxidation Stability	Stability in presence of oxidating agents e. g. $O_2$ , $H_2O_2$ . <sup>e</sup>
M6	Storage Stability	Storage conditions and reported stability or loss of activity during storage.

<sup>a</sup> Example: For the enzyme with the EC Number 3.2.1.20 the recommended name is *alpha-glucosidase*, the systematic name is *Alpha-D-glucoside glucohydrolase*, and synonyms are *Alpha-1,4-glucosidase*, *Alpha-D-glucosidase*, *Alpha-glucopyranosidase*, *Alpha-glucoside hydrolase*, *Glucoinvertase*, *Glucosidoinvertase*, *Glucosidosucrase*, *Maltase*, *Maltase-glucoamylase*. <sup>b</sup> Reaction of e. g. *alpha-Amylase*: Polysaccharide containing alpha-(1-4)-linked glucose units +  $H_2O$  = maltooligosaccharides; endohydrolysis of 1,4-alpha-D-glucosidic linkages in polysaccharides containing three or more 1,4-alpha-linked D-glucose units. <sup>c</sup> Reaction type of e. g. *alpha-Amylase*: O-Glycosyl bond hydrolysis (endohydrolysis). <sup>d</sup> Some examples: *Bovine serum albumin* stabilises; Stabilisation by ethanol, glycerol, serum albumin; High protein concentrations stabilise. <sup>e</sup> Some examples:  $t_{1/2}$  in presence of  $O_2$  at room temperature: 65 min; DTT is absolutely necessary for stabilisation; Autooxidation, reactivation by glutathione; Photooxidation inactivates catalase-peroxidase.

In the following, parameters presented in Table 43 (pertaining to enzyme-ligand interactions, functional parameters and molecular properties) are discussed in more detail. Special attention shall be drawn on their aptitude with respect to identification and characterisation.

### 9.1.1.1 Enzyme-Ligand interactions

#### Substrate/Product spectrum (E2)

This category gives an overview on the substances the enzyme is able to act on. The substrate/product spectrum indicates the degree of „specialisation“ of an enzyme. An observed shift in the spectrum may either be caused by changes in reaction conditions (e. g. pH, temperature, presence of activating agents, inhibitors) and/or indicate structural variation.

An example shall illustrate, that minor structural variations may have an effect on the observed substrate/product spectrum: „Inositol monophosphatase (EC 3.1.3.25) in hyperthermophilic archaea, the sequence of which is substantially homologous to that of human inositol monophosphatase, exhibits inositol monophosphatase activity but with substrate specificity that is broader than those of bacterial and eukaryotic inositol monophosphatases and it can also act as a fructose bisphosphatase“ (JOHNSON et al., 2001).

#### Natural substrate (E3)

Natural substrate (E3) provides information on the enzyme function in its natural source. The question remains, if the enzyme's function is always fully understood. Examples from extre-

mophiles (see JOHNSON et al., 2001) for inositol monophosphatase in hyperthermophilic media) show that analogies do not always lead to the correct conclusions. Very often, industrial enzymes do not act on their natural substrates. For these reasons, information on the natural substrate alone does not help very much to identify and characterise a particular enzyme.

### Metals/Ions (E7), Inhibitors (E8)

The difference of two (similar) enzymes in type or stringency of need of either *cofactors* or *metals* (ions, salts) or sensitivity towards *inhibitors* indicates structural and/or functional variation.

Enzyme inhibitors are substances which inactivate the enzyme by changing the shape of the enzyme molecule or blocking the active site. Competitive inhibitors block the active site by „competing“ with the substrate. These competitive inhibitors resemble the shape of the substrate and fit into the active site. Non-competitive inhibitors change the shape of the enzyme by binding to the molecule at a site other than the active site.

Type and spectrum of inhibitors are characteristic for an individual enzyme and may even serve to elucidate structural and maybe functional differences hidden by sequence homology and three-dimensional similarity as is illustrated in the following (JOHNSON et al., 2001):

The MJ0109 gene product from the thermophilic archaeon *Methanococcus jannaschii*, first identified by its high sequence homology to inositol monophosphatases (IMPase; EC 3.1.3.25), was confirmed to be an efficient IMPase. In contrast to mammalian and other bacterial IMPases, the MJ0109 enzyme is not inhibited by low concentrations of  $\text{Li}^+$ , even though sequence alignment programs suggest that residues corresponding to the ligands of the human enzyme exist. The determination of the 3-dimensional structure revealed that the overall fold, as expected, is similar to that of the mammalian enzyme, but the details suggest a closer relationship to fructose 1,6-bisphosphatases (FBPase). Three complexes of the MJ0109 protein with substrate and/or product and inhibitory as well as activating metal ions suggest that the phosphatase mechanism is a three-metal ion assisted catalysis which is in variance with that proposed previously for the human inositol monophosphatase. Subsequently, the MJ0109 protein was characterised by in vitro experiments to be a bifunctional enzyme that can function as both an IMPase and FBPase.

#### 9.1.1.2 Parameters related to the catalytic activity

##### $K_m$ value (F1)

The Michaelis-Menten constant  $K_m$  measures enzyme/substrate affinity and equals the substrate concentration at half the maximal velocity  $V_{\max}$ .  $K_m$  is independent of enzyme concentration and is a true characteristic of the enzyme under defined conditions of temperature, pH etc. and thus can be used to identify a particular enzyme protein. A low  $K_m$  indicates a strong enzyme/substrate affinity. This can be a useful parameter in specifying and characterising an enzyme of industrial importance in e. g. a patent (GODFREY & WEST, 1996: chap. 6.1, 494 - 495). The  $K_m$  values of enzymes varies widely, but for most industrially used enzymes they are in the range  $10^{-1}$  to  $10^{-5}$  M when acting on biotechnological important substrates under normal conditions. The ratio  $k_{\text{cat}}/K_m$  is a measure of enzyme specificity. The kinetic parameters may be influenced by the assay conditions (see below). The enhancement of catalytic activity through structural modification is reflected by an increase of the kinetic parameters.

Normally linearised forms of the Michaelis-Menten equation are used: the two most common methods are the Lineweaver-Burk and the Eadie-Hofstee method (for more details see e. g. ENGEL, 1996a).

##### Activity (F3)

The activity of an enzyme is stated in units. One unit is the amount of enzyme which will hydrolyse one  $\mu\text{mole}$  of substrate per minute under given conditions of pH, buffer composition

and temperature. The number of units in an enzyme preparation is determined from a progress curve of the reaction (Figure 6).

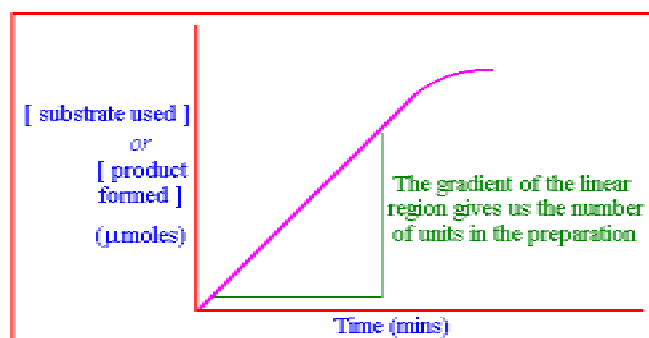


Figure 6: Determination of enzyme activity in units

In order to measure the activity of an enzyme, it is necessary to have a suitable substrate. This, although apparently obvious, is not always easy to achieve. Certain enzymes such as proteases are notoriously difficult to classify and it is often impossible to know what the „natural“ substrate is for, e. g. an extracellular fungal protease. Such enzymes frequently have a broad specificity and many different protein substrates can be used.

The level of enzyme activity is clearly of major interest when the enzyme is to be used in a process. For this reason, enzymes are usually marketed in terms of activity rather than weight.

The specific activity (e. g.  $\text{U kg}^{-1}$ ) is therefore a parameter of interest. However, a major problem with these definitions of activity is the rather vague notion of „standard conditions“. These are referring to optimal conditions, especially with regard to pH, ionic strength, temperature, substrate concentration and the presence and concentration of cofactors and co-enzymes. However, these „optimal“ conditions vary both between laboratories and between suppliers. They are also depending on the particular enzyme application.

A common problem in industrial enzymology is how to compare enzyme preparations. The manufacturer supplies enzymes activity by specifying the activity units. The problem is that there do not exist unit definitions internationally agreed and relevant for industry. As a result of these difficulties manufacturers usually use their own activity units which will differ among the manufacturers and depending on the particular application.

There are essentially two ways around this problem:

- (1) To assay the enzymes to be compared using one standard assay and substrate. The problem with this approach is that the assay chosen might have little relevance for the application and thereby for the manufacturer. It does, however, allow the quantitative comparison of the enzymes.
- (2) To develop an assay based on the particular application and evaluate comparative performance. It can be difficult to set up a „model“ application for lab-scale evaluation that accurately simulates the real application.

## Assay methods

Depending on the type of enzyme and on the respective substrates, there exist different analytical methods to determine enzyme activity. AMFEP and FEFANA (AMFEP/FEFANA Position Paper annex (August 1999)) describe the following 6 analytical methods for enzyme assays:

### (1) Reducing sugar release

These assays use either a normal or chemically modified substrate of the enzyme in question. The enzyme is incubated for a predetermined period under fixed conditions of pH, tem-



perature and substrate concentration, with the substrate in question. The products of the reaction are reducing sugars, which are determined colorimetrically against a standard of the particular sugar. Enzyme activity is then normally expressed in units of activity per gram or ml as mmole product produced/minute. It is widely used, but sensitive to the influence of metal ion concentrations (pre-mixes) and substrate variations.

#### (2) Colorimetric assays (I)

These assays use a chemically similar molecule to that of the enzyme's normal substrate to which has been linked a molecule that on release generates a soluble colour. By incubating the enzyme with this chemically modified substrate as above, the coloured compound is released and may be measured spectrophotometrically against a standard curve. Enzyme activity is expressed relative to a known enzyme standard. This assay does not differentiate between endo and exo activity patterns but is normally regarded as being more indicative of exo rather than endo activity. Recently, standard substrates in tablet form have become available and these are now widely used by the industry.

The main drawback to such methods is the cost of the substrate and that chemical modification of the substrate may alter the ability of the enzyme to hydrolyse the substrate. As these substrates are normally water insoluble, they are not suitable for auto analysers.

#### (3) Colorimetric assays (II)

In these assays a simple substrate is produced in which a cologenic molecule is chemically bound to a sub-unit of the enzyme's normal substrate. One example is PNPG in which para nitro phenyl is linked to galactose to form an analogue of the normal lactase substrate, lactose (Glucose-Galactose). Enzyme hydrolysis of the molecule liberates the PNP, which can be determined following a chemical reaction against a standard curve of PNP.

These assays are commonly used in biochemical laboratories but are not well suited to feed enzyme assays where the enzyme's substrate is normally of high molecular weight.

#### (4) Viscometric assays

This type of assay is based upon the enzyme's ability to reduce the viscosity of a standard substrate solution (controlled pH, temperature etc.). Both chemically modified (e. g. Carboxy Methyl Cellulose for Cellulase) and unmodified (e. g. extracted wheat arabinoxylan for xylanases) substrate are used. Enzyme activity is not absolute but normally calculated against an enzyme standard assayed at the same time.

While these assays are attractive as viscosity reduction is a major part of the enzyme's in vivo effect they have also weaknesses. Chemically modified substrate may not react in the same way as „natural“ substrate and the modification can block enzyme access. The „natural“ substrate have to be extracted and this procedure also modifies them to a certain extent. Also, large batches of substrate are needed (or at least a guarantee of supply) and have to be produced under semi-industrial conditions.

#### (5) Immunological methods

Several immunological methods including ELISA and immuno-gel diffusion have been developed for enzyme assay. In each case they are based on a reaction between an enzyme and an antibody raised against it. A second reaction (ELISA) or staining (gel diffusion) is then used to quantify the enzyme level against standard levels of added enzyme.

These methods are extremely sensitive and can detect very low levels of enzyme protein. The main drawback is the requirement for specific anti sera against each enzyme product and the fact that many anti sera will react with inactive enzyme protein. As antibodies are specific for the protein used to prepare them, there will not be any cross over between enzymes of the same type from different producers. There is also the question of the sensitivity of using laboratory animals for this type of purpose.

#### (6) Gel Diffusion methods

These methods incorporate the enzyme substrate into a gel poured into petri dishes. After solidifying, wells are cut in the gel and enzyme solutions added (test and standards). After a

period of incubation a zone of hydrolysis can be seen around the well, whose radius is proportional to the enzyme concentration. In some cases a further reagent may be added to show up the zone of hydrolysis.

These methods while simple, are slower than the above chemical and viscometric methods as they require overnight incubation. Their accuracy is slightly lower than other non-diffusion based methods as it depends on the evenness of the zone of diffusion. They have the advantage that they are simple to operate and do not require sophisticated apparatus which would not normally be found in e. g. a feed laboratory. Recently, optical methods of measuring the diffusion zone have been introduced which while improving accuracy increase dramatically the cost of establishing this type of assay method.

Usually, assay conditions include the following: temperature or temperature range in which the enzyme is stable and has high activity, pH, buffer and its concentration, concentrations of the substrates and coenzymes, required cofactors such as metal ions, structure stabilisers and substances that prevent degradation. As mentioned above, assay conditions and units of activity published by industry for one particular enzyme (e. g. in product information sheets) vary a lot among manufacturers, thus complicating/preventing comparison of different products. AMFEP (2002.02.27/PE) has provided the project team with an overview of general methods of analysis for enzymes mentioned in guidelines (Table 44).

Table 44: Enzymes for which assay methods are mentioned in guidelines.

JECFA Compendium Source: <a href="http://apps3.fao.org/jecfa/additive_specs/foodad-q.jsp">http://apps3.fao.org/jecfa/additive_specs/foodad-q.jsp</a>	<u>alpha-Acetolactate Decarboxylase from Bacillus brevis expressed in Bacillus subtilis</u>
	Amyloglucosidase from Aspergillus niger, var.
	Avian Pepsin
	Carbohydrase from Aspergillus niger, var.
	Carbohydrase from Saccharomyces species (only a reference is given)
	Cellulase from Penicillium funiculosum
	Hemicellulase from Aspergillus niger, var.
	Lipase from Animal
	Lipase from Aspergillus oryzae, var.
	Malt Carbohydrase
	Maltogenic Amylase from Bacillus stearothermophilus expressed in Bacillus subtilis
	Pectinase from Aspergillus niger, var.
	Pepsin from Hog Stomach
	Trypsin
JECFA Guide to Specifications, Food and Nutrition Paper 5, Rev. 2 (1991)	Alpha-amylase, bacterial
	Alpha-amylase, fungal
	Alpha-amylase, malt
	Catalase
	Cellulase
	Glucoamylase
	Beta-Glucanase
	Glucose Isomerase
	Glucose Oxidase
	Hemicellulase
	Milk Clotting activity
	Protease, Viscometer
	Proteolytic activity, bacterial

	Proteolytic activity, fungal (HUT)
	Proteolytic activity, fungal (SAP)
	Proteolytic activity, plant
	Pullulanase
Food Chemical Codex, 4th edition	Acid phosphatase from <i>Aspergillus niger</i>
	Alpha-Amylase from <i>Aspergillus niger</i> , <i>Aspergillus oryzae</i> , <i>Rhizopus oryzae</i> , barley malt
	Alpha-Amylase from <i>Bacillus subtilis</i> , <i>Bacillus licheniformis</i> , <i>Bacillus stearothermophilus</i>
	Catalase from <i>Aspergillus niger</i> , <i>Micrococcus lysodeikticus</i> , bovine liver
	Cellulase
	Chymotrypsin from porcine or bovine pancreas
	Diastase from barley malt and other enzyme preparations
	Alpha-Galactosidase from <i>Aspergillus niger</i>
	Beta-Glucanase from <i>Aspergillus niger</i> and <i>Bacillus subtilis</i>
	Glucoamylase from <i>Aspergillus niger</i> , <i>Aspergillus oryzae</i> , <i>Rhizopus oryzae</i>
	Glucose isomerase
	Glucose oxidase from <i>Aspergillus niger</i>
	Beta-D-Glucosidase from <i>Aspergillus niger</i> , <i>Trichoderma longibrachiatum</i>
	Hemicellulase from <i>Aspergillus niger</i>
	Invertase from <i>Saccharomyces</i> sp ( <i>Kluyveromyces</i> )
	Lactase (neutral) from <i>Kluyveromyces marxianus</i> var. <i>lactis</i> , <i>Saccharomyces</i> sp.
	Lactase (acid) from <i>Aspergillus oryzae</i>
	Lipase from microbial sources and pancreatic tissues
	Lipase/esterase from forestomach sources
	Maltogenic amylase from <i>Bacillus subtilis</i> containing a <i>Bacillus stearothermophilus</i> gene
	Milk-clotting activity from animal or microbial sources
	Pancreatin
	Pepsin from porcine or other animal sources
	Phospholipase A2 from porcine pancreas
	Phytase
	Plant proteolytic activity (papain, ficin and bromelain)
	Proteolytic activity, bacterial (PC) from <i>Bacillus subtilis</i> , <i>Bacillus licheniformis</i>
	Proteolytic activity, fungal (HUT) from <i>Aspergillus oryzae</i> , <i>Aspergillus niger</i>
	Proteolytic activity, fungal (SAP) from <i>Aspergillus oryzae</i> , <i>Aspergillus niger</i>
	Pullulanase from <i>Bacillus acidopullulyticus</i>
	Trypsin from porcine or bovine pancreas

### pH optimum, pH range, Temperature optimum, Temperature range (F4 - F7)

Optimum conditions can be determined in terms of enzyme activity and in terms of enzyme stability: Enzymes may be tailored for increased stability and/or increased activity by structural modifications, also in the active site thus eventually even change the substrate specificity. Optima are thus reflecting the structure of a given enzyme. pH and temperature activity

can be determined as curves.<sup>73</sup> The optima profiles can thus be regarded as the fingerprints of the enzyme. Since enzymes are often stabilised by the presence of their substrates and temperature may affect the observed pH optima (et vice versa), it is important to specify the (concentration, pH or temperature) conditions in detail.

When considering the effects of temperature on the activity and stability of an enzyme, it must be remembered that they are time-dependent. Increasing the temperature will result in an increase in activity but also in an increase in temperature-induced denaturation.

Experimental curves defining the optimum temperature for activity and/or stability are usually determined over a short time scale (Figure 7).

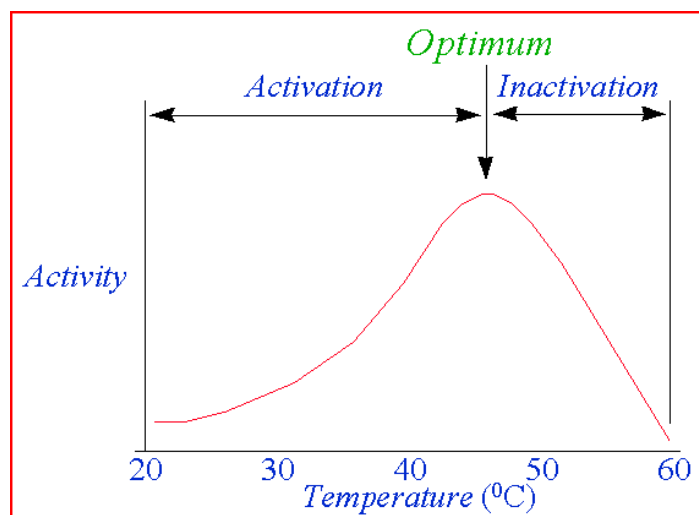


Figure 7: Temperature Optimum. Source: <http://www.fst.rdg.ac.uk/courses/fs560/topic6/t6c/t6c.htm>

This may not adequately represent the situation in an industrial application, where the enzyme might be expected to operate for extended periods of time. A process might be more productive at a temperature which results in fairly rapid inactivation of the enzyme.

This is an important point; regulatory authorities frequently require that no detectable enzyme activity remains in the product. If a soluble enzyme is used in a process, it is frequently desirable to operate the process at the highest temperature that the activity optimum will allow and for the enzyme to be inactivated over time. If this process were to be operated with an immobilised enzyme, the goal would probably be to maximise stability of the enzyme at a cost of operating efficiency.

Another important factor to be taken into consideration when determining the activity and stability optima is substrate concentration. Experimental activity optima curves are usually determined at fairly dilute substrate concentrations and stability optima curves frequently are determined in the absence of substrate. Industrial processes, on the other hand, operate at high substrate concentrations to allow greater productivity. Many enzymes are stabilised by the presence of high concentrations of substrate which can result in a broadening of the stability optimum under industrial conditions.

An appropriate approach of comparing the stability of industrial enzymes might be to determine the stability half life under process conditions. This is the time taken for the enzyme activity to decay to half its initial value. This parameter can be used as a quantitative measure of stability to perform enzyme comparisons and to determine the effect of process variables on the stability of the enzyme.

<sup>73</sup> As pH effect is more specific than temperature effects, any irregularities or tendencies to dual optima peaks in pH curves can be taken as an indication that the enzyme preparation under test probably contains more than one enzyme protein of similar catalytic effect (GODFREY & WEST, 1996: chap. 6.2, 508).

### 9.1.1.3 Molecular properties

#### Stability (M1 - M6)

The catalytic site represents a relatively small portion of the protein molecule. The primary role of the greater part of the enzyme molecule is to maintain the catalytic site of the protein in a limited range of configurations so that it is able to bind to substrates and get the catalytic reaction to work. An additional role of the non-catalytic area of the protein is to bind other substances which could influence the catalytic activity. This means that physical conditions can affect the enzyme on two levels, at the whole protein level and on the micro-environmental level of the active site. Stability and activity curves are thus usually not the same: stability optima are often broader than activity optima. Furthermore, preparations of the same stated specific activity may differ with respect to stability and may be capable of very different total catalytic productivity (this is the total substrate converted to product during the lifetime of the catalyst, under specified conditions). Conditions for maximum initial activity are not necessarily those for maximum stability. These factors have to be considered for choosing the most efficient catalyst for a particular purpose.

### 9.1.2 Structure-related properties of enzymes

Experimental methods to determine biomolecular structures were developed at a later stage than experimental observations of enzymatic activity. Thus, amino acid sequence, post-translational modifications and three-dimensional conformation of many enzymes used in industrial applications, have not been known for a long time. With the emergence of genetic engineering, methods to explore the structure of proteins have become available. Some of them, especially the determination of the amino acid sequence on the basis of the nucleotide sequence, nowadays are applied on a routine basis. Table 45 gives an overview on the structural parameters taken into account by protein or enzyme databases.

*Table 45: Structure-related parameters of enzymes used in the enzyme database BRENDA. The code (letters and numbers) refers to the structure of BRENDA (see Table 64).*

No.	Enzyme Structure	Description
S1	Sequence	Accession Number or Sequence ID from the databases SwissProt or TREMBL (SwissProt is a protein sequence data bank consisting of sequence entries, see also Table 64; TREMBL is a computer-annotated supplement of Swiss-Prot that contains all the translations of EMBL (European Molecular Biology Laboratory) nucleotide sequence entries not yet integrated in SwissProt).
S2	PDB	PDB identification code; directly linked to the PDB database (the Protein Data Bank PDB is the single worldwide repository for the processing and distribution of 3-D biological macromolecular structure data..
S3	Molecular Weight	This field gives the molecular weight of the holoenzyme.
S4	Subunits	The tertiary structure of the active enzyme is described. It can be active as a monomer, a dimer, trimer and so on. The stoichiometry of the subunits is given.
S5	Crystallization	References are cited which describe the procedure of crystallization or the X-ray structure of an enzyme from a given organism.
S6	Posttranslational Modification	Described as: side chain modification, glycoprotein, glycolipoprotein, lipoprotein, phospholipoprotein, proteolytic modification, ribonucleoprotein, sialoprotein, no modification, no glycoprotein, no monosaccharides, no phospholipoprotein.
	Molecular Properties	

No.	Enzyme Structure	Description
M9	Engineering	The properties of modified proteins are described (amino acid exchanges or deletion).

As postulated by C.B. Anfinsen in the 1960s, all information needed to determine a protein structure is found in the amino acid sequence. Usually one particular arrangement has a lower energy than the others and is therefore the most stable one – the native conformation. Parts of the sequence far apart in linear space may be close together in order to result in proper protein function and folding of the chain brings these residues together.

Categorisations of protein structures are based upon the observation that globular proteins could be organised in a structural hierarchy.

- Regular secondary structures, e. g.  $\alpha$ -helices and  $\beta$ -strands, are formed: structures with consecutive amino acid residues adopting similar backbone conformations.
- Tertiary structure is then formed by packing secondary structural elements into one or several compact globular units called domains. The rigid framework formed by secondary structures is the best-defined part of a protein structure. The three basic supersecondary structural motifs,  $\alpha$ -hairpin,  $\beta$ -hairpin, and  $\beta\alpha\beta$ -unit are frequently found in so-called superfolds, suggesting that there is a high degree of correlation between the simplicity of secondary structural arrangement and the capacity of the fold (ZHANG & DELISI, 2001).
- Some proteins contain several polypeptide chains arranged in a quaternary structure.

Relevant methods for determining enzyme structure are described in the following.

### Sequencing (S1)

#### Determination of the amino acid sequence on the basis of DNA sequencing

Nowadays, DNA sequencing provides an appropriate method to determine the amino acid sequence of a protein on an automated and low cost basis. However, the corresponding enzyme gene must be available.

#### N-terminal protein sequence analysis

Prior to sequencing peptides it is necessary to eliminate disulfide bonds within peptides and between peptides.<sup>74</sup> Edman degradation allows for additional amino acid sequence to be obtained from the N-terminus inward. Using this method it is possible to obtain the entire sequence of peptides: The entire sequence of reactions can be repeated over and over to obtain the sequences of the peptide. This process has subsequently been automated to allow rapid and efficient sequencing of even extremely small quantities of peptide. Due to the limitations of the Edman degradation technique, peptides longer than around 50 residues can not be sequenced completely. The ability to obtain peptides of this length, from proteins of greater length, is facilitated by the use of enzymes, endopeptidases, that cleave at specific sites within the primary sequence of proteins. The resultant smaller peptides can be chromatographically separated and subjected to Edman degradation sequencing reactions.

#### Molecular weight determination (S3)

The accurate molecular weight of a peptide or a protein provides information on e. g.:

- definition of the purity of the peptide/protein
- confirmation of the protein sequence
- control of the structure of recombinant proteins

<sup>74</sup> The most common treatments are to use either 2-mercaptoethanol or dithiothreitol followed by iodoacetic acid in order to alkylate the free sulfhydryls.

- characterisation of genetic variants or mutant proteins in which there is an amino acid substitution
- definition of the proteolytic processing of the protein, both at the N- and C-termini
- identification of post-translational maturation events or chemically induced changes
- identification of the glycosylation pattern of glycoproteins.

The accurate molecular weight of peptides and proteins is determined by electrospray (ESMS) and MALDI-TOF mass spectrometry analysis of crude or HPLC purified samples. Pre-treatment of the samples and/or reverse-phase HPLC purification can also be performed. Identification of modified components or contaminating species is also provided.

### **Determination of the three-dimensional structure (S4 - S5)**

In order to determine the three-dimensional enzyme structure a number of different techniques can be employed, each offering different advantages and limitations. X-ray crystallography and nuclear magnetic resonance (NMR) are the main source of data for determination of protein structure at the molecular level. Techniques such as neutron diffraction also play a role in examining the interaction between protein and solvent (WESS, 1998).

#### X-ray crystallography

X-ray crystallography is a technique used to analyse the structure of proteins to atomic detail. The use of x-rays is optimal since the wavelength of the radiation is comparable with the length of a covalent bond. The crystallisation of macromolecules is not a trivial task and can take months of experiments to find favourable conditions. „The determination of protein structure by x-ray crystallography is becoming more widespread and molecular replacement phase determination can facilitate the structure of closely related structures to be solved in a matter of weeks. Technical progress is greatly extending the range of structural problems that can be tackled and the accuracy and efficiency with which structures can be solved. However, the determination of protein structure has not yet become a routine task and still remains an arduous work that requires dedication and intense computational resources (WESS, 1998).

#### Nuclear magnetic resonance (NMR)

Until 1984, x-ray diffraction remained the only method to determine protein structures to atomic resolution. At present, NMR remains the second most widely used method for protein structure determination. So far, NMR is limited to molecules of less than 30,000 M<sub>r</sub>. NMR is however of great use since the structures can be analysed in solution and in relatively low concentration. This obviates the need for lengthy crystallisation trials (WESS, 1998).

### **Posttranslational modifications (S6)**

Posttranslational modifications of proteins can be identified by comparing the accurate mass of a modified protein or peptide, as determined by mass spectrometry, with the predicted mass based on its amino acid sequence. Any discrepancies may correspond to posttranslational modifications. The nature of the change is deduced from a knowledge of the molecular masses of common posttranslational modifications. Glycoforms, e. g., which may vary by only one or two sugar groups, may be easily detected by mass spectrometry (MS) (ENGEL, 1996b).

## **9.1.3 From structure to function – investigating „similar“ enzymes**

### **9.1.3.1 Structure-function relationship**

Enzyme function is determined by its protein structure. A closer look at the relationship between enzyme structure and enzyme function will, however, elucidate the complex, sometimes puzzling and contradictory, nature of this relationship. The peculiarities of the structure-function relationship are particularly important to keep in mind when evaluating whether enzymes with differences in amino acid sequence and/or posttranslational modifications are

equivalent with respect to functional properties. Figure 8 illustrates the possible implications of differences in primary structure to enzyme function: different primary structures may result in the similar tertiary structures (same folds), and different tertiary structures (folds) may result in same catalytic function.

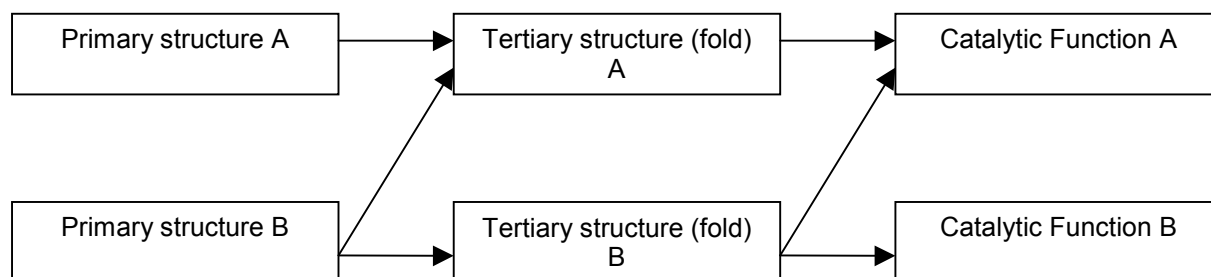


Figure 8: Structure-function relationships.

The extent of differences between the amino acid sequence of enzyme A and enzyme B is not always a reliable indicator, whether either tertiary structure or function are altered or not, since for enzymes the location of the modification is relevant. There is a significant difference, whether amino acid insertions take place at or near the active site or somewhere else in the molecule.

Since a particular function can be fulfilled in more than one way, *different proteins acquired the ability to serve similar functions* (functional convergence). There are numerous examples of independently evolved enzymes that catalyse the same reactions. For instance, the ability to hydrolyse the peptide bond has evolved many times: there are the sulfhydryl proteases, metalloproteases, aspartyl proteases, serine proteases, and some others (RAWLING et al., 1993). Moreover, serine proteases evolved on at least three different occasions, as evidenced by the subtilisin, trypsin and  $\alpha$ - $\beta$  type enzymes (OLLIS et al., 1992).

One of the major surprises to emerge from early X-ray structure determination of proteins was the observation that in two different serine proteases *the side-chains of the catalytic triad were gathered together into the same geometry from completely different folds and sequence arrangements* (mechanistic convergence) (KRAUT, 1977).

$\alpha$ - and  $\beta$ -segments often occur alternately in a protein sequence and fold into  $\alpha$ - $\beta$  barrels. *Their three-dimensional structures are all remarkably similar, but usually no detectable sequence homology exists.* Many of them might have descended from a common ancestor, but the great diversity of function could reflect a general „structural convergence“ to the same structure, either as a result of the stability of these barrels or their ease of formation.

It is evident that it is the tertiary structure that determines the function. The relationship between amino acid sequence and tertiary structure is still not well established. However it is much better defined than the relationship between amino acid sequence and function of the protein. The same fold is often seen in apparently different homologous families with different functions. In contrast, a variety of biochemical functions can be performed by proteins with the same fold or even by members of a single homologous family. GERLT & BABBITT (2000) draw the attention to an important, possibly insufficiently acknowledged problem of functional alignment, namely that „*homologous proteins need not catalyse the same chemical reaction*“.

Even in cases where the functional „identification is well specified by homology to highly conserved consensus sequence,<sup>75</sup> there are several examples where *highly similar sequences*

<sup>75</sup> A minimum nucleotide sequence found to be highly homologous (although not necessarily identical) in different genes that is associated with a specific function.



*have completely different functions.* A well-known example is the structural protein eye-lens crystalline and the metabolic enzyme glutathione S-transferase (TOMAREV et al., 1992), both of which have sequence and structural similarity but differ in function. Another example comes from protein engineering experiments: Lactate dehydrogenase was converted into malate dehydrogenase by changing only a single amino acid (WILKS et al., 1988). (The impacts of protein engineering techniques are discussed in more detail in section 3.4).

GERLT & BABBITT (2000) described an example of a mechanistically diverse family, where different oxidation reactions occur without significant sequence divergence: „An oleate hydroxylase from *Lesquerella fendleri* shares 81% sequence identity with an oleate desaturase from *Arabidopsis thaliana* but 71% identity with an oleate hydroxylase from *Ricinus communis*. These facts suggest that divergence of function is facile and may have occurred many times in the specification of higher plants. Noting that seven residues were conserved in oleate desaturases from multiple species but diverged in hydroxylases, Sommerville and co-workers (see ref. 9 in GERLT & BABBITT, 2000) constructed libraries of mutants in which these conserved residues were replaced. Interestingly, as few as four substitutions were able to convert an oleate 12-desaturase into a hydroxylase; in the opposite direction, six substitutions converted a hydroxylase into a desaturase (both enzymes have the same substrate: oleate).“ These findings illustrate that *mechanistic diversity does not require a large significant divergence in sequence*, and underscore that high levels of sequence identity do not „guarantee“ the same enzymatic function.

### 9.1.3.2 Homology and similarity

This section discusses the different meanings when enzymes are considered to be homologous or similar. First, one needs to clarify the confusion arising by facultative use of the terms homology, similarity and analogy when comparing proteins in the scientific literature.

Proteins are usually compared to each other on the level of nucleotide sequence or on the level of the deduced amino acid sequence. As the amino acid sequence contains the information relevant for protein function the analysis mostly focuses on protein primary structure. Sometimes also secondary and tertiary structural properties are considered.

Homologous sequences are termed *homologs* and this term may be applied to both genes and proteins. Homologs look similar to each other and appear to share common ancestry but they may or may not display the same activity. *Heterologs* differ both in origin (no similarity in amino acid sequence) and activity. *Analogs* have common activity but not common origin (no sequence homology).

*Similarity* is a quantitative term that defines the degree of sequence matches between two compared sequences. If aligned sequences contain 28 matches out of 39 possible, the degree of similarity is then 28/39 or 72%.

*Similarity and Homology:* When two sequences (nucleic acid or protein) are similar over a short stretch or in a global way, it does not necessarily mean that they will share common functionalities. On the other hand, when two sequences are homologous then they share common functional identities somewhere along their sequences. A very high level of similarity between two or more sequences is a strong indicator of homology between those sequences.

*Global versus Local:* Global similarities consider the entire length of the sequences being compared and a quantitative „similarity score“ is assigned. Initial attempts at creating algorithms for similarity searches focused mainly on global similarities (NEEDELMAN & WUNSCH, 1970). Global algorithms are not usually sensitive for highly diverged sequences with some local similarities within them. Sequence similarities can be better analysed with local similarity algorithms which, in general, assign a total similarity score for two sequences based on a summation of local similarity scores.

In comparative sequence analysis either two or more sequences can be compared to each other or nucleotide/protein sequence databases are searched starting from a given sequence

and yielding a set of possibly homologous proteins. In the early days of database scanning, the computer time required to execute the scan was a major consideration. Today, the ready availability of cheap, high performance computers means that computer resources are rarely a limiting factor; it is currently feasible to perform protein database scans in a few hours on a personal computer using dynamic programming algorithms. The algorithms FASTA (PEARSON & LIPMAN, 1988) and BLAST (ALTSCHUL et al., 1990) are commonly used approximations.

### Factors affecting the percentage of similarity

The percentage of similarity between proteins calculated by using computer alignment programs is highly affected by scoring schemes and alignment methods as described in detail by Geoffrey J. Barton in STERNBERG (1996).

Scoring is depending on identity or alternatively the degree of similarity between amino acids found. E. g. chemical similarity scoring schemes aim at giving greater weight to the alignment of amino acids with similar physico-chemical properties. This is desirable since major changes in amino acid type could reduce the ability of the protein to perform its biological role. Long experience with scoring schemes based on observed substitutions suggests that they are superior to simple identity, genetic code, or intuitive physico-chemical property schemes.

Given a scoring scheme, the next problem is how to compare the sequences, decide how similar they are and generate an alignment. The results might be different depending on the „window length“ applied and on the insertions or deletions allowed.

For protein sequences, the most commonly used local alignment algorithm that allows gaps is that described by SMITH & WATERMAN (1981).

Furthermore, several factors can be calculated from sequence alignments that provides additional information on the sequence homology, e. g. standard deviation and percentage of identity (on a given part (length) of sequence).

When an alignment of two or more sequences is made, the implication is that the equivalent residues are performing similar structural roles in the native folded protein. The best judge of alignment accuracy is thus obtained by comparing alignments resulting from sequence comparison with those derived from protein three dimensional structures.

Determining homology on the basis of three-dimensional structure is much more complicated. The methods available are based on alignment of secondary structure elements as well as alignment of intra- and inter-molecular atomic distances.

### 9.1.3.3 Conclusions

From these findings, it can be concluded that a particular enzyme function could be fulfilled by different protein structures and similar structures could give rise to different enzyme functions. The replacement of merely one amino acid residue might switch the catalytic type of an enzyme. This question is closely related with homology as overall homology, e. g. on the basis of amino acid sequence, this does not necessarily imply that the resulting enzymes are also closely related in function.

Thus, designating enzymes as homologous as it is frequently done in the scientific and sometimes also regulatory context, this does not necessarily imply that they are identical in primary structure or in tertiary structure. Even if they are highly similar, as shown by a high percentage number, this does not necessarily imply that they are identical in catalytic function. The validity of the percentage number is very limited and one has to consider the particular algorithm applied and the parameters used e. g. overall or local homology, scoring scheme, alignment methods, indels (insertions/deletions), window size, taking into account tertiary structure homology.

Thus, in order to make this numbers more meaningful, one has to consider additional parameters which should also be documented in the course of homology studies.

- Algorithm(s)
- Substitution matrix: All modern search programs use substitution matrices. The choice of substitution matrix can greatly affect search results; therefore it is imperative to document which matrix (or matrices) were used in searching and aligning.
- Gap penalty. For algorithms which use gap penalties (such as FASTA), it is critical to state the gap penalty used.
- Name of database. Explicit specification of the database used, not only by type (nucleotide, protein, sequence).
- Version of database. Databases are changing very rapidly, much faster than the publication cycle and frequently faster than local system administrators can handle. It is therefore critical to state the version of the database used. If searching a constantly updated database, then the date of the last search should be stated.

Unfortunately, many published sequence comparisons do not meet these requirements.

#### **9.1.4 From gene to protein – a road without forks?**

In order to understand what influences the properties of proteins one has to make the picture outlined in section 9.1.3 even more complex: the structural considerations have to be expanded to structural features of enzymes not encoded in the respective gene and – interconnected to each other – to structural variability of enzymes derived from a given nucleotide sequence.

The way from the DNA-sequence to the protein in its biologically native form comprises several processing steps. Variation at any stage may lead to altered structural and functional properties of the final enzyme. The resulting enzyme variants may thus differ with respect to identity, activity, stability, health and environmental properties. A closer look at the single processing steps („from gene to protein“) shall highlight possible implications on function and identity.

##### **9.1.4.1 From gene to amino acid sequence**

In principle, the DNA sequence is translated into the corresponding amino acid sequence without any modifications or variations (one gene-one protein hypothesis). Though – under certain circumstances – multiple protein products may arise from a single gene by a number of mechanisms (POLY, 1997):

- Transcriptional modification via alternate transcription: more than one pre-mRNA is transcribed.
- Posttranscriptional modification: different mRNAs are produced from a single pre-mRNA.
- Alternate translation: more than one polypeptide is translated from one pre-mRNA.
- Cotranslational modification: amino acids of the polypeptide are modified before release from polysome.
- Posttranslational modification: polypeptide is modified after release from polysome (will be dealt with in the following chapter).

##### **9.1.4.2 Posttranslational modifications**

Many proteins require posttranslational modification by specific enzymes to become functional. The following posttranslational modifications on residues of the amino acid chain may take place:

- formation of N-pyrrolidone carboxyl,
- disulfide bridge formation (oxidation/reduction of the sulfhydryl group of cysteine),

- C-terminal amidation, deamidation (Asn to Asp or Gln to Glu),
- N-methylation, oxidation of Met or Tryp,
- proteolysis of peptide bond,
- N-formylation, acetylation (of a lysine  $\epsilon$ -amino group or the N-terminal amino acid),
- phosphorylation,
- carbamylation,
- N-myristoylation,
- conjugation,
- polymerisation and dissociation,
- glycosylation.

Many post-translational modifications are indicated by local sequence motifs, some are characteristically or uniquely associated with defined protein homology.

Prokaryotic expression systems were part of the early repertoire of molecular biology. The usability of these systems were however limited, especially if expressing eucaryotic proteins. The expression of a eucaryotic protein in a prokaryotic system imposes a number of detrimental constraints on the eucaryotic gene product. Among the two most critical are improper protein folding and assembly, and the lack of posttranslational modification, principally glycosylation and phosphorylation. Prokaryotic systems do not possess all the appropriate protein synthesising machinery to produce a structural and/or catalytically functional eucaryotic protein. To tackle this hurdle, eucaryotic cells are used as expressions systems.

As mentioned above, posttranslational processing of proteins is different in prokaryotic and eucaryotic cells. In prokaryotic organism posttranslational processing except proteolytic cleavage rather rarely takes place. In eukaryotic cells almost all kind of posttranslational processing takes place. If a particular organism is capable of a particular posttranslational processing mechanism depends on the phylogenetic group or species of organism. Moreover the extend of posttranslational modification – if this happens at all – depends on the particular protein.

Most production organisms used in enzyme manufacturing so far are of prokaryotic origin. However the number of eukaryotic production strains is also increasing. Especially due to the availability of enzymes from almost every sources more and more enzymes could be produced by means of heterologous expression in either prokaryotic or eukaryotic hosts.

Variations in either type or amount of posttranslational modifications may accompany homologous as well as heterologous expression: in the case of homologous expression the posttranslational machinery may result in uncompleted products due to overexpression. In the case of heterologous expression the posttranslational machinery of the host is responsible for the resulting posttranslational modifications of the product and may therefore not be capable of processing the protein correctly. Incorrect posttranslational processing could, however, affect the properties of an enzyme.

The most important posttranslational modifications with potential implications on technical, health, and environmental properties and thereby on substance identity are:

- Proteolytic processing, which means peptidase cleavage of terminal residues: If proteolytic processing does not take place in the right order, this will possibly result in protein structure different from the mature protein and/or eventually the protein remaining within the cell membranes.
- Glycosylation: The glycosylation profile of a protein is specific to both the host organism and the culture conditions. The oligosaccharide part of a glycoprotein is responsible for important and specific biological features such as: immunogenicity, solubility, recognition, protection from proteolytic attack, induction and maintenance of the protein conformation in a biologically active form. Thus, the modification of the structure of the glycan chains can lead to a modification of the activity of the glycoprotein (MEYNIAL-SALLES & COMBES, 1996).

Example: The effect of different posttranslational glycosylation patterns was demonstrated in a case study on Lipolase®, an enzyme used in detergents (KÜNG – BIOTECH + UMWELT, 1999). The Lipase gene of *Humicola lanuginosa* is expressed in *Aspergillus oryzae*. The amino acid sequence of the cloned *Humicola* lipase remains unchanged even though the properties of the enzyme were altered compared to the wild type: the enzyme is more stable also at high temperatures and the resistance to alkaline *Bacillus* proteases (Esperase®) within the temperature range of 40 - 55 °C is increased, therefore the proteolytic degradation is reduced. The authors attributed these effects mainly to the different glycosylation patterns in source and host organism.

Example: Experiments with controlled site-selective glycosylation (via site-directed mutagenesis combined with chemical modification; DAVIS et al. 2000; LLOYD et al., 2000) have shown that amount and even the type of enzyme activity may change due to the differences in the glycosylation pattern: The model protein subtilisin *Bacillus lentus* SBL does not contain a natural cysteine and is not naturally glycosylated and thus represents a suitable model for homogeneous glycoproteins. The authors created 48 different chemically modified mutants of the *Bacillus* strain in order to study precise glycan structure-catalytic activity relationships. The resulting  $k_{cat}/k_m$  ratios varied in the range of 1.1 fold-higher to 7-fold lower than wild type. Subsequently the authors (LLOYD et al., 2000) found out that the site-selective glycosylation of subtilisin *Bacillus lentus* at key positions within the active site causes dramatic increases in esterase activity combined with a lowered amidase activity (which is used to tailor the *Bacillus* strain for use in peptide ligation reactions).

Most of the posttranslational modifications can be detected because of their changes in mass (see section 9.1.2) or due to altered charge resulting in altered migration of the polypeptide in a gel matrix. There exist prediction tools for protein homology domain-associated post-translational modifications in e. g. the RESID database (post-translational modification database; <http://pir.georgetown.edu/pirwww/dbinfo/resid.html>).

### 9.1.4.3 Three-dimensional conformation (fold)

Empirical observations support the notion that structure is more robust than sequence (see ref. 13 - 16 in ZHANG & DELISI, 2001). Over time, some nucleotide/amino acid sequences diverged so much that no significant resemblance remains, even though the overall shape of the protein has hardly changed. Clearly, there are several ways of maintaining similar three-dimensional structures with different arrangements of 20 amino acids. Proteins that have diverged beyond significant sequence similarity still retain the three-dimensional fold of their ancestors.

One assumes that in contrast to the number of sequences the number of folds is finite.

Almost 12,000 protein structures are presently compiled in the protein data bank (PDB) (BERMAN et al., 2000) comprising over 25,000 domains (THORNTON et al., 2000). Of the 2,159 structures determined in 1999, only 8% adopted novel folds (THORNTON et al., 2000). All data support the hypothesis that there is a limited number of folds (protein domains with a distinct back-bone topology) despite the requirement for a vast array of different functions.

Although it is expected that the number of sequence families (all proteins in a sequence *family* have similar sequences) in nature is probably orders of magnitude larger than the number in the PDB, many of the families that are currently not represented will turn out to have folds that are already known. The number of unknown/new folds is estimated/deduced (ZHANG & DELISI, 2001) to be only twice the number of folds currently known.

## 9.2 Enzyme concentrates and enzyme preparations

As mentioned in section 3.4 a characterisation of enzyme products does not only take into account enzymes as the active compounds (characterised by their molecular structure or by function, see section 9.1). In the following, parameters applicable for the characterisation of

enzyme concentrates (comprising the active component (i. e. the enzyme) plus any impurities resulting from fermentation and purification steps) and final enzyme preparations (enzyme concentrate plus additives) will be outlined. As almost all industrial enzymes are manufactured from and as most regulations are focusing on microorganisms, animal and plant sources are not considered in this section.

### 9.2.1 Enzyme concentrate

Basically, there are three different fermentation products in use containing enzyme activity:

- (i) The crude microbial fermentation product: The enzymes as well as the entire medium are recovered, complete with other metabolites and fermentation substances. This includes the solids and solubles associated with the fermentation (e. g. *Aspergillus niger* fermentation product).
- (ii) A more purified enzyme source consists of only the soluble portion of the fermentation product, from which the solids have been separated (e. g. *Aspergillus niger* fermentation solubles).
- (iii) Relatively pure enzyme product results from extracting and purifying the solubles (e. g. *Bacillus subtilis* fermentation extract).

Thus, depending on the succeeding purification steps, the enzyme concentrate resulting from the fermentation does not only contain the active enzyme protein, but also left-overs from the fermentation, extraction solvents, etc. As has been illustrated in section 3.4: the active enzyme protein in the enzyme concentrate makes up 25 - 75% for technical, food, and feed enzymes, and 50 - 100% personal care, therapeutic and analytical/diagnostic applications. The rest (0- 75%) consists of impurities.

The nature and percentage of impurities depends on

- the choice of source material (production organism and media)
- the fermentation process itself
- the subsequent purification steps.

The impurities of an enzyme concentrate, thus, vary to a certain extent due to changing operational parameters. Particular impurities are regarded as contaminants:

- microorganisms, spores
- toxins and antibiotica
- toxic metals, and
- DNA.

Contaminants that are harmful to human health and the environment are of major concern. Thus, a careful selection of the production organism as well as monitoring of the production process to ensure hygienic conditions are crucial to avoid contaminants in the resulting fermentation product.

#### Production organism

Production microorganisms are not only selected for their capability of producing the desired enzyme. The organism must also be non-toxicogenic, i. e. it should not produce any toxins harmful to the consumer or the environment, and non-pathogenic, to ensure the safety of the workers in the enzyme production factory. In the case of the production of toxins, the production of the toxic metabolites by fungi (mycotoxins) is of main concern. Other possible toxins are enterotoxins, neurotoxins, and antibiotics. In practice, the range of organisms commercially used in enzyme production is rather limited (see chapter 4).

Usually, a particular strain is selected and/or modified for enhanced properties (e. g. higher enzyme yield). Even strains which underwent considerable genetic rearrangements resulting in multiple changes of physiological properties either induced by mutagenesis or by genetic

engineering techniques are not regarded to constitute a new species. Thus, the accurate description as well as the safety assessment of a production strain are important.

### **Media**

A suitable medium for the production of enzymes by fermentation consists of a carbon source, a nitrogen source, minerals, and water. The carbon source may consist of sugars, alcohols, and organic acids. Nitrogen is often provided by animal or vegetable proteins, but amino acids, peptides, nitrates, and ammonia may also be suitable. Minerals are mainly added as salts. Media used for fermentation have to keep to certain conditions of use: raw materials used for fermentation should be of high quality and free of toxic contaminants. Raw materials are usually sterilised before use. If the enzyme is intended to be used in e. g. food, all ingredients (including media) have to be of food-grade as well.

### **Production process**

The fermentation process is monitored during the entire period of fermentation. The control of the production process shall be based on a well defined control program according to a recognised quality system e. g. GMP. This system includes the control and checking/adjustment of the equipment, sampling and analysing, and the use of analytical results for process control. All the control procedures are well documented during manufacturing. Purity and identity of the organism are to be regularly checked during this period.

Contamination with foreign (pathogenic) organisms is also checked: e. g. Salmonella, Shigella, E. coli, Listeria, Campylobacter, Clostridium perfringens. For application in food, limits for the presence of coliforms as well as the total viable count are set. The enzyme concentrate should also be analysed for the presence of toxic metals such as arsenic, cadmium, lead, mercury.

#### **9.2.1.1 Conclusion**

Since the impurities resulting from fermentation and purification are highly complex and variable, the enzyme concentrate is best described by the production strain and the production process itself. The absence or level of total viable count, known pathogenic microorganisms and toxins, as well as heavy metals have to be checked if required.

#### **9.2.2 Enzyme Preparation**

The enzyme concentrate is formulated to the final enzyme product – the enzyme preparation. Additives serve as stabiliser, preservatives, granulation, coating, and (de)colouring aids (see section 3.4). Material safety data sheets (MSDS), product information and technical leaflets describe the composition and properties of a marketed enzyme preparation. MSDS (an example is given in Table 66;Annex) provide information on

- Identification of the Preparation and the Company
- Information on Ingredients
- Physical and Chemical Properties
- Stability and Reactivity
- Toxicological Information, Ecological Information
- Hazards Identification, First Aid Measures, Fire-fighting Measures, Accidental Release Measures
- Handling and Storage, Exposure Controls/Personal Protection
- Disposal Considerations, Transport Information, Regulatory Information, Other Information

Typical product sheets or technical leaflets usually contain some or all of the information stated in Table 46.

Table 46: Information covered by product sheets and technical leaflets

Product description	Enzyme type
	Production organism (e. g. a <i>Bacillus lentis</i> production strain)
	protein engineered
	physical form
Composition	Principal components (in %)
Product specifications	Other enzymatic activities
	Appearance, Colour
	pH
	Dry matter
	Bulk density
	Viscosity
	Electrical conductivity
	Average particle size
	Solubility
Characteristics	Activity
	Assay conditions
	pH optimum and profile
	Temperature optimum and profile
	Temperature and storage stability
Application	Use level
	Dosing
Microbiological specifications	Bacterial count/g
	Moulds/g
	Salmonellae (e. g. neg. in 25 g)
	Enterobacteriaceae/g
Environmental analysis	Heavy metals (as Pb)
	Arsenic (as As)
Handling precautions	
Packaging	
Production method	e. g.: inoculation, fermentation, filtration, conditioning, packing
Regulatory status	

### 9.3 Comparison of regulatory practice

An overview on the regulatory practice for technical, feed, food and other enzymes has been given in chapter 5. A comparison of the regulatory practice with respect to parameters applicable for the description of enzymes shall reveal how existing regulatory frameworks deal with

- parameters for enzyme identification,
- information required for enzymes from GMM,
- differentiation between new and existing substances,
- information required on the production organism,



- requirements for the production process, and
- additives and other ingredients.

### 9.3.1 Parameters for enzyme identification

For notification of technical enzymes, Canada was the only investigated country that explicitly requested specific information on substances with catalytic activity (section 5.2.8). For food enzymes, only the FDA/GRAS guidelines (section 5.3.6) define parameters obligatory for enzyme identification. Requirements in other regulations are restricted to systematic name, trade name, synonyms, CAS and IUB number. Table 47 gives an overview on the particular parameters requested by the Canadian authority and by the FDA .

*Table 47: Parameters for enzyme identification as requested by the Canadian authority for notification of technical enzymes, and by the FDA/GRAS guidelines for food enzymes*

Canada	FDA/GRAS
chemical name	systematic names
trade name, synonyms	common and/or trade names
CAS No	CAS Registry Number
IUB number (equals „EC number“)	EC numbers (equals „IUB number“)
gram molecular weight	molecular weight (if available or necessary to support the enzyme identity.)
purity, impurities, additives, stabilizers	N.s.
identification of the production organism (synonyms, common names, the source and history)	N.s.
concentration of the viable production organism	N.s.
description of the separation method	N.s.
a description of all known catalytic functions	enzymatic function, mode of action, other major enzymatic activities
known substrate specificity for each known catalytic function	substrate specificity
N.s.	isoelectric point (if available or necessary to support the enzyme identity)
optimum pH and temperature for the most appropriate substrate(s)	temperature and pH (if such characteristics are relevant for the intended use of the enzyme preparation.
the catalytic constants $K_M$ and $K_{cat}$ , as well as conditions under which they were measured.	kinetic properties (if available or necessary to support the enzyme identity)
N.s.	inorganic ions (if such characteristics are relevant for the intended use of the enzyme preparation.
known cofactors necessary for enzymatic activity	N.s.
N.s.	units
the activity per unit weight of the final product.	Specific activity (if available or necessary to support the enzyme identity.

*N.s. ... not specified.*

### 9.3.2 Information required for enzymes from GMM

In the case of recombinant enzymes from GMM, special requirements exist for application in food in FDA/GRAS and JECFA guidelines (described in Table 48).

Table 48: Information required for food enzymes from GMM

FDA/GRAS <sup>a</sup>	JECFA <sup>b</sup>
Description of permanent or transient structural modifications (by chemical or genetic engineering)	N.s.
Comparative analysis (If an enzyme normally produced in one organism is expressed in another organism through molecular cloning, especially in case of gene transfer to genetically distant organisms):	N.s.
molecular weight	molecular weight
isoelectric point	isoelectric point
gel-migration, chromatographic (or similar) properties	N.s.
enzymatic activity	substrate specificity
N.s.	activity as a function of pH and temperature
kinetic parameters	reaction kinetics
Amino acid composition	amino acid composition
Amino sugar composition	N.s.
Amino acid sequence (full or partial)	amino acid sequence
N.s.	a peptide map
N.s.	DNA base sequence coding for the enzyme

<sup>a</sup> FDA 1993; <sup>b</sup> JECFA 1999. N.s. ... not specified.

### 9.3.3 Differentiation between new and existing substances

For technical enzymes, in all investigated countries, a distinction is made between substances that are included in a chemical inventory and that may therefore be used without any need for a notification procedure (section 5.2.10). As enzymes are identified by their catalytic properties, in general this parameter is applied to differentiate between new and existing substances.

In the USA, any information or characteristics that helps to identify the enzyme is taken into account, in case any information has been submitted.

In Canada, the „two percent rule“ is applied for proteins. A protein that is not on the DSL can be considered to be substantially equivalent to a protein that is listed on the DSL (section 5.2.8). Proteins that are substantially equivalent are not subjected to the new substances notification regulation. A protein is considered substantially equivalent if:

- the function of the protein has not been changed from the protein listed on the DSL; and
- (a) the protein has 98% amino acid sequence homology with the listed protein, based on amino acid or DNA sequence; or (b) the protein is 98% identical to the listed protein based on all of the following items:
  - molecular weight
  - isoelectric point
  - amino acid composition

- peptide map
- N-terminal sequence.

### 9.3.4 Information required on the production organism

For technical enzymes, in Canada certain information on the production organism as well as on the produced substances has to be submitted: identification of the production organism by synonyms, common names, source and history, description of any genetic modification, sources of genetic material (in the case the microorganism is genetically modified). In the other countries investigated, no information on the production organism is required for notification of a substance (section 5.2.10).

For enzymes used as additives in feeding-stuff, the SCAN guidelines (SCAN, Guidelines for the Assessment of Additives in Feeding-stuffs (22 October 1999), section 5.3) require information for enzymes produced using recombinant DNA technology:

- The biological origin of each declared enzyme activity or micro-organism should be given.
- All microorganisms, whether used as a product or as a producer strain, should be deposited in an internationally recognised culture collection (preferably in the European Union) and maintained by the culture collection for the authorised life of the additive. Evidence of deposition in the form of a certificate of deposition from the culture collection, which should specify the accession number and name under which the strain is held, must be provided.
- In addition, all relevant morphological, physiological, and molecular characteristics necessary to identify the strain and confirm its genetic stability should be described.
- The donor organism should be identified and described, with particular reference to any characteristics likely to cause concern.

For application in food, SCF, FDA, JECFA, AMFEP, and the French Order also provide precise requirements for source materials (indication of sources (animal, plant or microbial, including GMM), GMM donor organisms, the vectors used, and DNA introduced (see also Table 62, Annex).

AMFEP/FEFANA<sup>76</sup> suggests, that the original safety assessment for a production strain should comprise the following set of input:

- Taxonomic identification, verified by a well-known, independent laboratory.
- A literature survey summarising all known and published data on the safety aspects of the microorganism; a characterisation of the strain from a toxicological and pathogenic point of view (if the literature survey points to areas of possible concern).
- A toxicological and physicochemical characterisation of the fermentation product (the enzyme concentrate).

According to AMFEP/FEFANA (Position Paper, August 1999), the notification of a strain modification (of an approved feed enzyme) should follow the route outlined in Figure 9. The original safety analysis (which led to the authorisation of the enzyme for the first time) is reviewed. If the review points to specific areas of concern relative to the toxicological or physicochemical characterisation of the enzyme product, the producer will undertake an experimental program. If the experimental program uncovers new information, the enzyme manufacturer collects all relevant data and submits a new application to the rapporteur Member State. Otherwise the rapporteur Member State for the original additive dossier is notified by the enzyme producer of the strain change and of the conclusion of the review/program. Furthermore, the enzyme producer shall deposit the new strain in a culture collection.

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<sup>76</sup> AMFEP/FEFANA Position Paper, August 1999, Safety assessment and notification policy of a modified production strain of an approved feed enzyme (which has been modified by classical or recombinant DNA techniques).

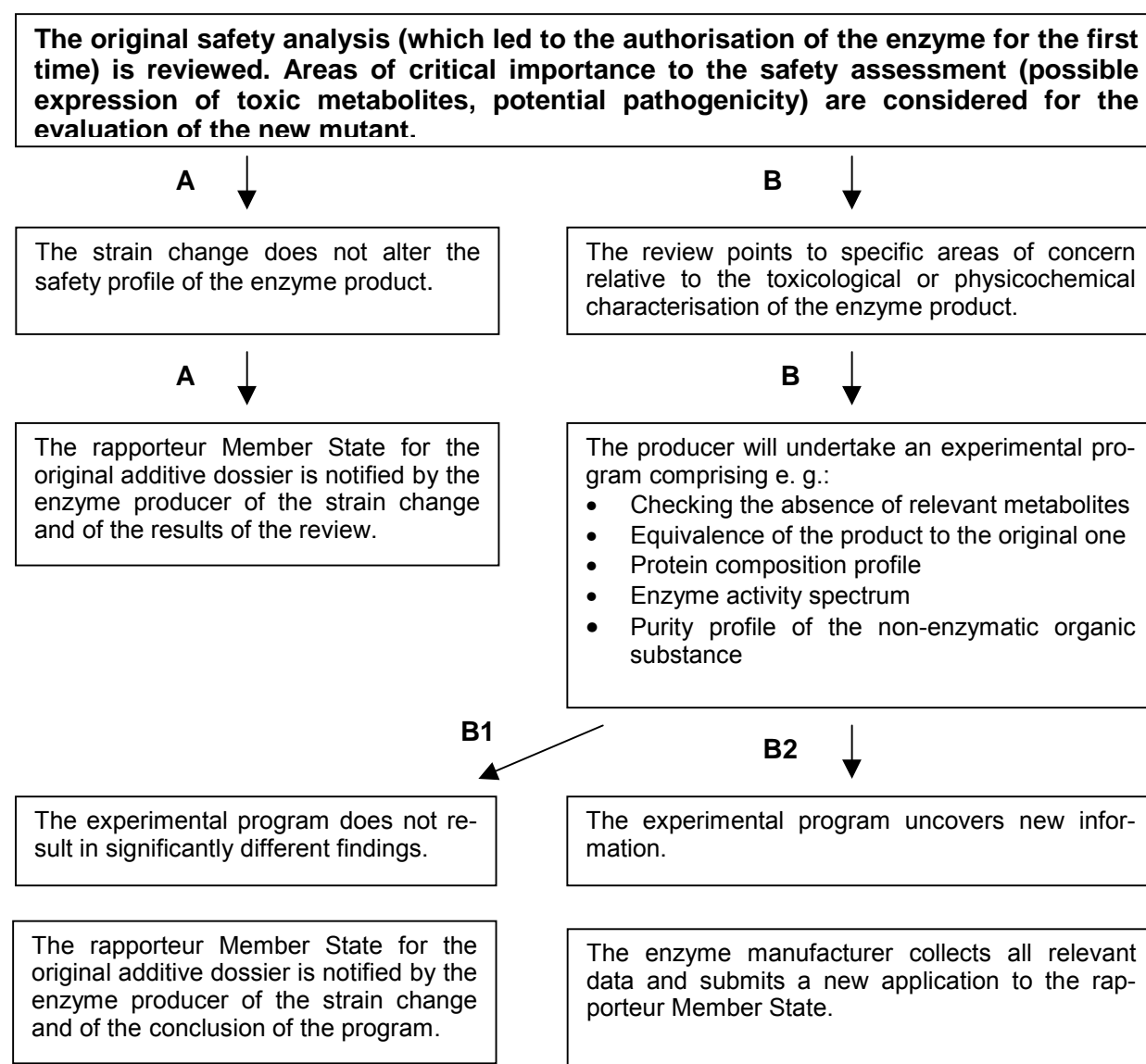


Figure 9: Notification of a strain modification, scheme proposed by AMFEP/FEFANA) on safety assessment and notification policy of a modified production strain of an approved feed enzyme which has been modified by classical or recombinant DNA techniques (AMFEP/FEFANA Position Paper, August 1999).

### 9.3.5 Requirements for the production process

Requirements on the production and purification process are defined for enzymes used as additives in feeding-stuffs as well as for enzymes used in food applications (Table 49).

Table 49: Requirements on production and purification process

Feed enzymes	Food enzymes	
SCAN <sup>a</sup>	SCF <sup>b</sup>	FDA/GRAS <sup>c</sup>
<ul style="list-style-type: none"> <li>– culture medium</li> <li>– fermentation conditions</li> <li>– downstream processing</li> <li>– batch to batch variation</li> <li>– quality control procedures</li> </ul>	In case of MO: <ul style="list-style-type: none"> <li>– information on fermentation media</li> <li>– information on fermentation conditions</li> </ul>	Production and purification process should be thoroughly described.  In case of GMM: <ul style="list-style-type: none"> <li>– fermentation process</li> <li>– fermentation medium</li> </ul>

Feed enzymes	Food enzymes	
SCAN <sup>a</sup>	SCF <sup>b</sup>	FDA/GRAS <sup>c</sup>
<ul style="list-style-type: none"> <li>– methods to monitor genetic drift</li> <li>– protocol used for routine screening of production batches for contaminants and impurities</li> <li>– flow chart describing the production and purification process.</li> </ul>	Information on purification procedure	<ul style="list-style-type: none"> <li>– Isolation of the enzyme including all physical and chemical treatment</li> </ul>

<sup>a</sup> Guidelines for the Assessment of Additives in Feeding-stuffs (22 October 1999); <sup>b</sup> SCF 1992; <sup>c</sup> FDA 1993.

### 9.3.6 Additives and other ingredients

In general, all regulations require, that the composition (purity, impurities, additives) and the methods of detection and determination are specified.

Regarding the regulation of chemicals, the composition of the notifiable substance is requested including purity, impurities, stabilisers, additives) in all investigated countries.

For food applications, AMFEP (1992) recommends, that the typical composition of a commercial enzyme preparation, such as content of protein, carbohydrates, fat, ash, water and diluents should be given. Identity and quality of stabilisers, standardizers, preservatives, and formulating agents should be given.

This is in line with the FDA/GRAS guidelines which request

- Content of proteins and nucleic acids, carbohydrates, fats, total solids, and ash measured for at least five representative batches
- Percentage of enzyme protein in enzyme preparation
- TOS.

Moreover, the SCF and JECFA guidelines for food applications required, that additives and processing aids must be substances that are acceptable for the relevant food uses of the enzyme preparations concerned, or substances which are insoluble in food and removed from the food material after processing.

## 9.4 Summary and conclusion

Most parameters used in scientific practice for the description of enzymes focus on the (i) enzyme as the active compound. Parameters used in the description of the (ii) enzyme concentrate, i. e. the active component (i. e. the enzyme) plus any impurities resulting from fermentation and subsequent purification steps are largely used in industrial and regulatory contexts. This is also true for (iii) the enzyme preparation as the ready-to-sell product intended to be used either as an intermediate or for application by end-users and which also contains additives as stabilising, granulation, colouring, coating and other aids/agents.

Enzymes as active compounds could be characterised by their function as well as by their molecular structure. Due to historical reasons enzymes are identified and grouped according to their catalytic activity as reflected by IUB nomenclature (E.C. number). However, for identifying and distinguishing enzymes the information on the catalytic type has to be supplemented by additional parameters. The more functional parameters are used the more precise and the less ambiguous the identification will be. Nevertheless, to unambiguously identify/distinguish enzymes the primary structure plus information on posttranslational modification has to be specified.

The relationship of enzyme structure and enzyme function is not well understood so far. On one hand, a particular enzyme function can be fulfilled by different protein structures. On the other hand, similar structures can give rise to different enzyme functions. The replacement of amino acids could result in no detectable effect, the exchange of merely one amino acid residue might also switch the catalytic type of an enzyme. Functional properties of enzymes can therefore not reliably be deduced from enzyme structure and vice versa. Thus, functional parameters are also relevant for the description of enzymes.

If characterisation of enzymes aim at providing a basis for the decision, whether two enzymes are identical in a chemical sense according to Directive 67/548/EEC either the primary sequence and posttranslational modification has to be revealed. In scientific literature and also in regulatory documents identity is often replaced by „similarity“ in order to describe the relation between to enzymes or as a decision criterion.

Similarity between enzymes largely refers to homology, measured as a percentage of identity/similarity in the order of amino acids. However, a high percentage number in homology does not necessarily imply that two enzymes are also closely related in function. Homology does in general not reflect the location and nature of differences. Furthermore, the particular algorithm applied and the parameters set have to be considered e. g. overall or local homology, scoring scheme, alignment methods, indels (insertions/deletions), window size, might heavily affect the resulting percentage. The Canadian two-percent rule for distinguishing enzymes in terms of notification does not consider these problems. Neither the number nor the nature and location of differences both of which are crucial for differences in function are thereby considered. Therefore and because this threshold limit cannot be substantiated by scientific arguments this rule is not regarded as sensible (at least as long as no additional parameters are considered).

As enzymes are not produced as just active compounds, a characterisation has to be extended to by-products or impurities comprising 30 to 98% of a final enzyme concentrate. The nature of these impurities resulting from fermentation and purification are highly complex and variable. Quantitative and qualitative analysis of these by-products is technically feasible but not practicable and sensible because of efforts and variability. Thus, the enzyme concentrate is usually characterised by describing essential operational determinants namely, the production strain and the production process itself. Changes in one of these determinants will lead to changes in the range and content of by-products. Furthermore, the absence or level of total viable count, known pathogenic microorganisms, known toxins, as well as heavy metals are routinely estimated or verified if required by legislation.

The enzyme preparation is described by specifying the content of protein, carbohydrates, fat, ash, water and diluents as well as the identity, quality and range of stabilisers, standardizers, preservatives, and formulating agents which are added to the enzyme concentrate.

Regulatory practice for describing technical, feed, food and other enzymes largely makes use of the parameters described above, thereby focussing on parameters for enzyme identification, information required on the production organism, requirements for the production process, and additives and other ingredients used.

Furthermore, additional information is often required for enzymes from GMM often including a description of the genetic modification and information on functional and structural aspects of the enzyme (including amino acid sequence). FDA is furthermore requesting a comparative analysis of the enzyme from GMM and from wildtype. AMFEP/FEFANA are proposing a decision tree approach to decide which data are required in case of GMM.

In general, information/data on these parameters is used in regulatory contexts to identify and to distinguish enzymes and to provide indirect evidence (direct in case of tests for pathogens, heavy metals etc.) for safety properties and thereby also for handling requirements. Information requested in the provisions and guidelines is pertaining to rather similar aspects. However, extent and details of requirements differ considerably (for information on regulations covered by this study see chapter 5). Nevertheless, the data requirements specified in these provisions and guidelines, as well as guidelines published by industry provide a useful basis for compiling a set of parameters which can be used for enzyme description.

## 10 REGULATION OF ENZYMES – POSSIBLE IMPACTS OF DIFFERENT APPROACHES IN ENZYME IDENTIFICATION

### 10.1 Introduction

A notification/registration scheme suitable for technical enzymes has to meet the following demands:

- (i) Identification – parameters have to be defined to ensure substance identification.
- (ii) Characterisation – properties relevant for human and environment have to be recorded in order to ensure safety during production, handling, application, and disposal.

Parameters applicable for the description of enzymes – as discussed in section 9 – are either related to *functional* or *structural properties of enzymes*. For historical reasons, the description of enzymes is based on their catalytic activity, which is also reflected by IUB nomenclature/E.C. number. Functional parameters, describing reaction and specificity, kinetics and physical factors are therefore well established in the description of enzymes. Conventional chemical substances, in contrast, are usually specified by structural parameters such as molecular weight, molecular formula and three-dimensional conformations.

A future notification/registration scheme applicable to enzymes largely depends on the parameters used for the description of enzymes: whether enzymes are distinguished by functional or by structural parameters may have a tremendous impact on regulatory practice (notification/registration requirements), efforts for applicants and competent authorities. Furthermore, also safety issues may be considerably affected.

In this section, these impacts will be illustrated and discussed with the help of two scenarios. In order to facilitate the analysis and to illustrate the impacts more clearly the two fictitious scenarios constitutes somewhat extreme options. In both scenarios a full notification (including safety testing) is dependent on whether an enzyme can be regarded as identical to an existing one. For simplicity reasons no further parameters are used to distinguish between enzymes.

- (1) Scenario 1 outlines a fictitious notification/registration system which is distinguishing enzymes solely according to their catalytic type. This approach is presently used in regulatory practice and corresponds to the traditional way of enzyme description.
- (2) Scenario 2 outlines a fictitious notification/registration system which is distinguishing enzymes according to their chemical structure. This approach reflects the definition of substance in directive 67/548/EEC and corresponds to the idea of chemical legislation in the EU.

Although the enzyme can be regarded as the active compound, commercially available enzymes are produced as *enzyme concentrates*. The enzyme concentrate resulting from the fermentation and subsequent purification steps does not only contain the active enzyme protein, but includes different kind of impurities as well. These impurities often account for a larger portion than the active compound itself, namely 30 - 98% (w/w on basis of dry matter) in technical, food, and feed enzyme concentrates (see also section 3.4) and vary in type and percentage: the particular compounds present are depending on the source organism used, the operational parameters of the fermentation process as well as the downstream processing applied.

Thus, in addition to identification and characterisation of the enzyme as the *active substance*, parameters applicable for characterisation of enzyme concentrate have to be implemented. These aspects will also be considered in the scenarios.

The enzyme concentrate is then, in a subsequent step, formulated to the final ready-for-use *enzyme preparation*. Types and amounts of additives are depending on the particular application and on customers demands. However, enzyme preparations are regarded as prepa-

rations according to the definition in Directive 1999/45/EC and are therefore not considered in this study.

Each scenario is described and investigated for the ability to distinguish between enzymes and for the ambiguities remaining, for possible impacts on regulatory practice, and for possible impacts of safety issues.

## 10.2 Scenario 1: Enzymes distinguished by catalytic activity

### 10.2.1 Description of the approach

Enzymes are identified and distinguished by their catalytic type which is reflected by the IUB (E.C.) number<sup>77</sup>. Structural parameters are not taken into account.

Example: E.C. 3.2.1.1; common name:  $\alpha$ -amylase; reaction: endohydrolysis of 1,4- $\alpha$ -D-glucosidic linkages in polysaccharides containing three or more 1,4- $\alpha$ -linked D-glucose units.

In contrast to the existence of several thousand different enzyme classes (IUB numbers), the 186 enzymes listed by AMFEP (Table 54, Annex) correspond to as few as 47 different E.C. classes.

This approach largely corresponds to the present system in use. Enzymes are listed in the EINECS by their catalytic activity. However, the EINECS listings do not include the E.C. number and also not uniformly registered enzymes. EINECS entries are either generic (e. g. dehydrogenase), and/or sometimes complementary information on natural substrate and (natural) source organism is given.

Examples: dehydrogenase, EINECS 2329074, CAS 9035-82-9; alcohol dehydrogenase, EINECS 2328704, CAS 9031-72-5; Elastase (pig pancreas), EINECS 2844536, CAS 39445-21-1.

The praxis of EINECS (as illustrated by this example) implies that the enzyme spectrum covered by a single entry is not homogeneous: a particular EINECS entry may

- include a broad range of enzymes performing different reactions (e. g. more than 500 matches for „dehydrogenase“, when searching the IUB enzyme list)
- refer not only to the specific reaction catalysed (e. g. E.C. 3.4.21.36, pancreatic elastase), but also on e. g. source organism.

### 10.2.2 Differentiation between enzymes

This approach implies that a great variability of functional and structural properties is covered by a single E.C. number. Enzymes from different organisms with differing enzyme-ligand interactions (substrates and products, cofactors, metals/ions, inhibitors, activating agents), differing parameters relating to the catalytic activity ( $K_m$ ,  $k_{cat}$ , specific activity, pH and temperature ranges, stability properties) as well as different amino acid sequence, posttranslational modifications and 3D-structure are covered by one entry/notification.

Neither the possibly different nature of enzymes found in extremophiles, nor enzymes produced by GMO's and/or resulting from protein engineering are taken into consideration.

### 10.2.3 Impacts on safety issues

Since a variety of enzymes differing in functional and structural aspects is covered by one particular IUB number, only one member of this particular enzyme class has to be subjected

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<sup>77</sup>Enzymes belonging to the same IUB type (having the same E.C. number) do catalyse the same reaction.



to safety testing. The possible impacts of this approach on human health and the environment will be described.

*Allergic properties* of enzymes are related to either linear or three-dimensional conformation of the macromolecule. A characterisation of enzymes via catalytic type does not consider any differences in amino acid sequence (plus any post-translational modifications) which might account for allergic reactions.

However, in practice this might not be regarded as a problem as long as the following conditions are in place: (i) no validated and agreed test system are available to test for inhalative sensitisation, (ii) enzymes are not regarded as skin sensitizers; (iii) industry is voluntarily classifying, labelling and handling all enzymes as inhalative sensitising agents.

A characterisation of enzymes based on catalytic type alone, however, will make it very difficult if not impossible to introduce a post-marketing surveillance of allergic properties of a particular enzyme (preparation).

Toxicological and ecotoxicological properties of enzymes are depending on the type of reaction catalysed, the enzyme activity and maybe on additional functional parameters<sup>78</sup>. However, especially in case of toxic properties the impurities present in the enzyme concentrate becomes relevant. Thereby, the production organism, production process, and its modifications constitutes important information for the toxic properties.

Consequently, characterisation of enzymes solely based on catalytic type will not be sufficient to ensure toxic and ecotoxic safety of extremozymes or of structurally modified enzymes. This approach will also fail to ensure safety regarding the accompanying impurities (e. g. mycotoxins). Any modification in either enzyme, production organism or production process may induce changes in (eco)toxicological properties.

With respect to handling of enzymes, not only the catalytic type but additional functional parameters, such as pH and temperature optima and ranges, and information on stability and storage conditions is relevant. For instance the recommendation „handling in cold environments” might be insufficient if enzymes from psychrophilic organisms are considered.

#### 10.2.4 Impacts on regulatory practice

In principle, this identification approach would provide a clear and simple tool to distinguish between „existing” and „new” enzymes. As this system is based on main catalytic activity ambiguities might arise from distinguishing between main and side activities. However, side activities are said to be magnitudes of about  $10^5$  lower than main activities. If enzymes are solely distinguished and notified by their IUB type only a small number of different enzymes would be expected to be notified. At present, 47 IUB different types are included in the AMFEP list comprising 186 enzymes. However, theoretically several thousands of enzymes differing in E.C. number are existing and described in scientific literature. Practically the range of IUB numbers is not expected to increase dramatically as industrial applications is focussing on a small number of catalytic types.

#### 10.2.5 Conclusions

As a consequence, identifying enzymes basing on catalytic activity, no distinction is made between enzymes, e. g. between wildtype organism and GMM, between natural mesophilic enzymes, extremozymes and structurally altered enzymes.

As a possible consequence, safety data for relevant handling, for the toxic potential and for environmental effects would not be reported.

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<sup>78</sup> Possibly toxic effects due to unspecific binding to other proteins or part of cells are not considered in the context of regulating technical enzymes as they are mainly an issue if enzymes are injected (venoms, medical applications) or could enter the body by passing a somehow damaged a skin.

This approach would only be useful if relevant toxic, ecotoxic, allergic or handling properties can be attributed to the enzyme alone and thereby to IUB based functional differentiation (which is not the case), or if relevant toxic, ecotoxic, allergic or handling properties can be generalised for all enzymes including enzyme concentrates (which is not the case) or if toxic, ecotoxic, allergic or handling properties are negligible (this cannot be verified on the grounds of the small data base in case of toxicity and ecotoxicity available to the project team).<sup>79</sup>

## 10.3 Scenario 2: Enzymes distinguished by chemical structure

### 10.3.1 Description of the approach

In contrast to the approach described above, enzymes could also be identified and distinguished by structural parameters (primary, secondary, tertiary, and quaternary structure, posttranslational modifications) as outlined in section 9.1.2.

In principle, most information, that uniquely defines an enzyme is encoded in the amino acid sequence. Sequence information and posttranslational modifications (e. g. glycosylation) determine, how the enzyme folds in 3-dimensional space. *Thus, enzymes which are identical in structure will also be identical in function.*

### 10.3.2 Differentiation between enzymes

Provided that enzymes are properly described by their amino acid sequence *and* by information on posttranslational modifications (and if relevant also on quaternary structure), almost no ambiguities will appear for distinguishing enzymes. However in contrast to scenario 1, the efforts for characterising the enzymes are much higher.

If not identity but similarity (e. g. homology) is introduced as a basis of distinguishing between enzymes one has to provide criteria that enables to differentiate between „equivalent“ and „not equivalent“. Otherwise, the decision has to be made on a case-by-case basis taking into account additional information on both candidates. The Canadian chemical legislation specified 98% sequence homology as a „threshold limit“. However, this approach does not take into account

- (i) the different methods and operational parameters set to measure homology (discussed in section 9.1.3.2) which will heavily affect the resulting percentage and
- (ii) the probably huge differences in impacts on enzyme properties (discussed in section 9.1.4). The substitution/deletion/insertion of a few amino acids might have no negligible or a heavy impact on enzyme properties.<sup>80</sup>

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<sup>79</sup> Due to theoretical considerations the availability of a larger database, which would confirm the safety of enzymes could only lead to the conclusion that enzymes might *not be expected* to be harmful substances (except sensitising properties). As enzymes are known which are toxic to humans (venoms, toxicity factors of certain pathogens) and as any regulation has to consider that also enzymes not used before might be marketed, the conclusion could not lead to a card blanche for all enzymes in general. Furthermore, the impurities present in the enzyme concentrate have to be considered. Due to similar considerations enzymes produced from microorganisms with a long history of safe use and which are not-pathogenic, non-toxicogenic (and maybe non-sporulating) might result in impurities which are potentially less dangerous. However, as productions strains are constantly subjected to genetic rearrangements (either by mutagenesis or by genetic engineering techniques), they might remain a residual risk of adverse pleiotropic affects, i. e. unintended and unpredictable secondary effects leading e. g. to the formation of a (new) toxin which will pass on to the enzyme concentrate.

<sup>80</sup> The findings in section 9.1.2 illustrate that mechanistic diversity does not require a large significant divergence in sequence, and underscore that high levels of sequence identity do not automatically indicate the same enzymatic function. Furthermore, presently no reliable prediction methods are available, which are able to derive the three-dimensional conformation from sequence information alone (*ab initio*). There do not exist methods which are able to deduce functional characteristics from structure, as well.

As this example illustrates, it might not be easily possible to base a concept of similarity on the simple and clear-cut criteria. 98% homology as the sole criterion does not seem to be scientifically sound and useful.

### 10.3.3 Impacts on human health and the environment

As mentioned above, enzymes which are identical in structure will also be identical in function. In contrast, this identity in structure and enzymatic function does not necessarily mean to be identical in terms of safety.

It is true that the allergenic potential of enzymes is depending on structure. At present, there exist data bases, which allow to scan the sequence of a particular enzyme under investigation for linear epitopes.<sup>81</sup> Information on enzyme structure may therefore provide indirect evidence for sensitising properties of an enzyme.

However, in practice this might not be regarded as an advantage as long as the following conditions are in place: (i) no validated and agreed test system are available to test for inhalative sensitisation, (ii) enzymes are not regarded as skin sensitisers; (iii) industry is voluntarily classifying, labelling and handling all enzymes as inhalative sensitising agents.

As every enzyme differing in structure/function will be regarded as „new“, (eco)toxicological properties will have to be tested of each enzyme variant. This will ensure that no safety related impacts resulting from changing of the enzyme itself – whatever it would be – would remain undetected. However, possible toxic properties are mainly caused by the impurities present in the enzyme concentrate. These impurities might significantly differ as it will e. g. be possible to use different hosts and manufacturing conditions for producing the same enzyme (basing on the same gene; and assumed that posttranslational modification is not relevant).

In case if similarity (homology) is introduced as a basis for differentiation, the impacts of a particular threshold limit (e. g. 98%) might depend on particular nature of the differences. Furthermore, the objections regarding to toxicity mentioned above can also be made.

### 10.3.4 Impacts on regulatory practice

As a consequence of this approach (identification based on structure), enzymes could be identified/distinguished unambiguously. However, efforts for applicants are much higher. For enzymes from GMM, the amino acid sequence is generally known, even if it might be considered as confidential information. Information on posttranslational modification might be available to a lesser extent. In contrast, no sequence information might be available on enzymes not originating from GMM. Assuming that more than 100 enzymes (out of 186 enzymes in the AMFEP list) are not produced from GMM this might pertain all of these enzymes. Nevertheless, using modern molecular genetic techniques the cloning and sequencing of an enzyme gene starting off with a given enzyme protein could in principle be done and might become a more and more routine approach.

A further consequence of this approach is that *any modification* – in the amino acid sequence (e. g. also an exchange of a single amino acid), or differences in the posttranslational modification pattern – will imply that an enzyme has to be regarded as „new“. This will pose a heavy burden on both industry (e. g. determination of structural data, safety testing and) and the competent authorities.

Furthermore, the number of enzymes which have to be notified as new substances will be much higher compared to scenario 1. At present, 186 enzymes are included in the AMFEP list. Most of these enzymes are assumed to be structurally different. Assumed that, one particular entry in the AMFEP list might cover more than one structural variant (MAURER,

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<sup>81</sup> For structural epitopes no database has been developed so far.

EIGTVED pers. communication), the number of „different“ enzymes according to structure might be about 400 or so. This is about 10 fold of the number of enzymes notified in scenario 1. Furthermore, it might be expected, that the number of variants produced from GMM will dramatically increase in the near future thereby leading to a constant stream of notifications.

If similarity/homology is taken into consideration to differentiate between enzymes, appropriate methods for uniformly measuring homology remain to be defined. Furthermore, the principle objections against this approach raised in scenario 1 are still true.

### 10.3.5 Conclusion

If enzymes are distinguished by their structure, structural and functional variability is fully considered and enzymes could be unambiguously identified. However, the efforts for both the applicant and the competent authority are much higher if each enzyme variant, resulting either from nature or from genetic engineering, has to be notified. Nevertheless, this burden might be justifiable if considerable negative impacts to man and the environment might be expected and/or if the safety level can be increased. However, on the basis of present knowledge, enzymes are neither regarded as highly dangerous substances of high priority nor advantages for safety of enzymes become evident. In contrast, as toxicological and probably also ecotoxicological considerations are focusing on impurities in the enzyme concentrate, this structure-based concept would have to be supplemented by information on the by-products.

A structural based distinction based on similarity as is experienced by the Canadian chemical regulation which has already established a threshold limit of 98% overall homology on amino acid sequence does not provide advantages. The exact extent of homology is, however, difficult to justify: the exact location and nature of amino acid mutation/insertion/deletion(s) is regarded as being more important than the sheer number in terms of function.

Functional parameters cannot reliably be deduced from structure. Highly homologous amino acid sequences may have completely different functions; homologous amino acid sequences and folds may show other (additional) activities; and structurally unrelated enzymes may serve similar functions (as described in section 9.1.2).

## 10.4 Summary

Enzymes are presently identified by their catalytic activity. Applying this system leads especially in the context of EINECS to several problems discussed in section 5.2.2. From these problems the idea arose to base enzyme identification on structural characteristics, as differences in enzyme properties do have to depend on differences in enzyme structure. At first glance, distinguishing enzymes by structure (primary structure; posttranslational modification) in chemical legislation seems to provide criteria for clear and almost unambiguous distinctions between enzyme molecules. If, however, this implies full notification requirements for enzymes differing e. g. in a few amino acids, this would put a considerable burden on both the manufacturer and the regulator.

Nevertheless, this burden might be justifiable if considerable negative impacts to man and the environment might be expected and/or if the safety level can be significantly increased. However, on the basis of present knowledge, enzymes are neither regarded as highly dangerous substances of high priority nor advantages for safety of enzymes become evident. In contrast, as toxicological, and probably also ecotoxicological considerations are focusing on impurities in the enzyme concentrate, this structure-based concept would have, therefore, to be supplemented by information on these impurities. Unfortunately, enzyme concentrates are still complex mixtures. The composition of by-products in enzyme concentrates in terms of quality and quantity is extremely variable and depending on the organisms, the media and the conditions applied during fermentation and subsequent downstream processing. Even impurities of enzyme concentrates containing the same particular enzyme from the same

particular production strain might differ in an unpredictable way if some parameters in the enzyme manufacturing process are shifted or media compounds are changed.

Consequently, the structure based concept (scenario 2) might end up in similar difficulties as the activity based concept (scenario 1). Additional criteria have to be taken into account to distinguish between enzyme concentrates (this is discussed in further detail in chapter 11, see also Table 50).

According to the nature of these criteria, decisions whether an enzyme concentrate could be regarded as same compared to another one might have to be made on a case to case basis.

As qualitative or quantitative data will be not available indirect evidences (description of and experience with the production strain, description of fermentation conditions and downstream processing) have to be gathered and known toxin producers have to be excluded from use in enzyme manufacturing.



## 11 REGULATION OF ENZYMES – OUTLOOK AND RECOMMENDATIONS

### 11.1 Introduction

This chapter aims at drawing conclusions and deriving recommendations on the basis of the relevant findings described and discussed in detail in the preceding parts of the study. As the application of new technologies in enzyme manufacturing, especially genetic engineering techniques, is considered an important motive for commissioning this study the first section (11.2) shows relevant trends and outlines possible impacts on the present regulatory system and on safety issues triggered by the use of genetic engineering techniques. The second part (11.3) of this chapter comprises a synopsis of considerations relevant for outlining any registration system applicable for technical enzymes. The third part (11.4) finally provides some inputs for further discussions on enzyme regulations by describing some cornerstones of a future registration system. According to the overall scope of the study sections 11.3 and 11.4 are pertaining enzymes in general but are frequently referring to the application of genetic engineering and enzymes with unusual properties.

### 11.2 Possible impacts of recent developments in enzyme manufacturing on regulatory and safety issues

On the basis of recent trends in enzyme manufacturing (discussed in section 3.4) possible impacts in presence and near future on regulatory and safety issues are discussed and questions are raised. This section is thereby clearly focussing on genetic engineering and related techniques.

#### 11.2.1 General trends in enzyme manufacturing

As a result of the increasing application of genetic engineering techniques in enzyme manufacturing, regulatory measures have to be aware of some trends:

- The number and variety of enzymes on the market will rapidly increase
- Most of the newly introduced enzymes will be manufactured by genetic engineering
- The number of enzymes with altered properties (protein engineering) will rapidly increase
- Enzymes will be altered in order to modulate almost any property of industrial importance (activity, stability, co-factor dependency, substrate spectrum etc.)<sup>82</sup>
- Alterations of enzyme structure will affect more amino acids and larger regions of proteins
- Enzymes might be placed on the market which do not resemble any natural enzyme<sup>83</sup>
- The number of enzymes with „unusual“ properties (compared to enzymes available so far) will increase, especially due to enzymes from extremophiles
- Enzymes will be used in an increasing number of industrial and non-industrial areas concerning many aspects of human life; thereby exposition to enzymes might increase.

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<sup>82</sup> In an extreme case, the functional identity of enzymes can be altered.

<sup>83</sup> A non-natural enzyme is understood as follows: It is not obvious that a particular structurally modified enzyme could in fact be found in nature. Even if industry is using techniques mimicing natural processes and even if the variety of enzymes in nature is huge the application of these techniques does not necessarily lead to enzymes which are already present in nature. In fact, normally neither the presence nor the absence of natural counterparts can be verified. However, the presence of a possibly non-natural enzyme does not present a hazard per se.

### 11.2.2 Variety of enzymes covered by a particular EINECS entry increases

Using EINECS as decision basis for determining notification duty, the number and range of structurally and functionally different enzymes covered by a particular EINECS entry (e. g. „Proteinase“ EINECS No. 232-642-4) will significantly increase due to the availability of enzymes from organisms not exploitable before and to enzymes with altered protein structure. Furthermore, enzymes with „new“ or unusual properties, e. g. extremozymes, which are being introduced for the first time on the market, will also be covered.

### 11.2.3 Difficulties to distinguish between „existing“ and „new“ enzymes will increase

According to the nature of EINECS entries and because of the lack of criteria to distinguish between existing and new enzymes, the increase in the variety of enzymes will aggravate the problems on determining between existing and new enzymes. If an enzyme is understood as a substance solely defined by its catalytic activity type (e. g. defined by IUB-number) numerous structural variants both natural and non-natural<sup>84</sup>, exhibiting different properties will be covered. This problem is even more aggravated by the fact that some enzymes could be considered as „less familiar“<sup>85</sup> (even if they are not considered as new substances according to the present chemical regulation) because of their exotic sources and unusual properties, e. g. extremozymes.

In order to decide whether an enzyme is subjected to notification or not, it has to be checked if the particular enzyme is listed either in EINECS or in ELINCS. In EINECS, enzymes are registered as functional classes covering a huge variety of enzymes. Enzymes are listed on EINECS both as generic entries without further specification (like the source organism) and specific entries indicating the substrate or the source organism. Whether an enzyme is covered by a particular EINECS entry or not depends on the interpretation of EINECS entries. Problems arising from interpretation of EINECS have been discussed at several Technical and Scientific Meetings (TSM) and at CA Meetings (see also section 5.2.1).

One possible interpretation is that all enzyme variants exhibiting e. g. protease activity, regardless of their properties and if they are of natural or non-natural sources, are covered by the EINECS (see also section 5.2.2). However, more detailed information is given in the notification dossier. Thereby the range of enzymes covered by this notification is considerably more narrow. However, as no amino acid sequence is given in the notification, the ELINCS entry will still cover structurally different variants of the enzyme, e. g. resulting from protein engineering techniques.

### 11.2.4 Potential impacts on the purity of enzyme preparations

The purity of enzyme concentrates/enzyme preparations is discussed in section 3.4.1 of this study. It is quite obvious that due to heterologous expression of enzyme genes in well known microbial hosts, the risks of potentially harmful by-products can be decreased. However, a (theoretical) risk of so far unknown and potentially harmful by-products might appear as a non-intended pleiotropic effect if a enzyme manufacturing process is altered – regardless whether the production strain is e. g. improved by classical mutagenesis or by genetic engineering or either downstream or fermentation conditions are altered.

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<sup>84</sup> See footnote on previous page.

<sup>85</sup> Industry has gathered considerable experiences in the manufacturing and application of enzymes from a rather limited number of mesophilic microorganisms for decades. These microorganisms and enzymes are regarded as more familiar than those enzymes which become accessible due to the use of genetic engineering in the last decade. Thus, „experience“ does not refer e. g. to knowledge of the amino acid sequence but on long periods of manufacturing and use under various technical conditions.



### **11.2.5 Potential impacts for human health**

Many „conventional“ enzymes bear a considerable inhalant sensitizing potential. When humans as consumers and/or workers and animals get into contact with particular enzymes for the first time (extremozymes, „non-natural“ enzymes), this might be of relevance for health considerations e. g. as there is almost no experience regarding to allergic properties in case of extremozymes. Although extremozymes do not seem to have completely different primary and tertiary structures, there are of course differences. Furthermore, as these enzymes often are of unusual high stability, this might affect the magnitude of exposition. E. g. enzymes could more easily pass the intestine without being fully degraded or denatured.

Both extent and frequency of altering protein structures has dramatically increased, it is therefore more likely that these alteration will – also in a non-intended way – affect potential allergenic properties. However, even if allergenic properties might be changed, these changes could also decrease allergenic properties or the changes might be of no relevance for consumer and/or workers.

### **11.2.6 Potential impacts for the environment**

Due to their ready biodegradability and the low observed effects on aquatic organisms, enzymes seem do not seem to be dangerous to the environment (chapter 8 in this study). However, enzymes with unusual stability (extremozymes, protein engineered variants) could be more resistant to biological degradation following release to the environment. Also protein engineering might decrease biodegradability of enzymes as a non-intended effect. The amount of active enzymes present in the environment is presently expected to be low but might also increase because less enzymes might be degraded or denatured e. g. during processing at elevated temperatures.

### **11.2.7 Potential impacts for handling of enzymes**

As enzymes usually exhibit no or less activity at low temperature, „handling in cold environments“ is a widespread suggestion for workers in modern enzyme manufacturing companies (according to KANERVA & TARVAINEN, 1990). Psychrophilic enzymes e. g. proteases and lipases could be more catalytically active on the skin or mucous membrane of workers even in cold environment thereby affecting their health.

### **11.2.8 Relevance of changing enzyme properties**

In principle the questions mentioned above have to be agreed. Handling of enzymes, health and environmental properties of enzymes might be affected as an unintended effect if protein structures are altered. Furthermore, enzymes from extremophiles exhibiting unusual properties especially in terms of stability might also exhibit different allergenic properties or may behave differently in toxigenicity tests. However, the question remains if these changes will be of practical relevance for human health or the environment.

## **11.3 Considerations important for a registration system suitable for technical enzymes**

### **11.3.1 Scope of the considerations**

The considerations and recommendations outlined in the following have to be seen in the context of the present chemical regulation but can also be considered in any horizontal legislation on enzymes. Such a regulation covers all kind of enzymes above a certain tonnage

threshold except those subjected to sectoral regulations<sup>86</sup> (feed enzymes as food additives, enzymes in cosmetics, enzymes in medicinal products).

Enzymes used as processing aids in food are presently not regulated by harmonised EU legislation. It therefore remains still unclear if this category of enzymes is actually exempted from Directive 67/548/EEC.<sup>87</sup> Having in mind the precautionary principle, there is no reason to distinguish between additives and processing aids, this points to a demand for action. As is reflected e. g. by the SCOOP task 7.4. of DG SANCO (see also section 5.3), the European Commission seems to be well aware about this fact. As long as no harmonised legislation on enzymes as processing aids is in place, these enzymes are only subjected to mandatory safety evaluation except in Denmark and France. However, the authors of this report are assuming that this category of enzymes will have to be covered by sectoral legislation.

Therefore, the recommendations outlined in the following only apply to horizontal regulatory approach without the need to consider special exposition routes such as daily intake, skin application, injection etc.

### 11.3.2 EINECS and enzymes

As discussed elsewhere in this report (section 5.2.2) EINECS is not regarded as sensible nor as applicable for enzymes. This is due to the nature of enzyme entries itself and to the differences between these entries as well. Both led to a situation where some enzymes are subjected to notification and others are not, mainly based on historical reasons (the way the EINECS was created in the early eighties).

Therefore, as far as enzymes are concerned, EINECS should be replaced by a more enzyme specific inventory or database. However, this might include a registration of enzymes covered by EINECS so far.

### 11.3.3 Applicability of the present approach of „existing” and „new” to enzymes

The authors of this study are aware, that the approach of „new” and „existing” chemicals will not be utilized any more within the REACH system. However, the differentiation between a registered and a non-registered substance will be of importance in the new system.

As is the case in chemical regulation, regulatory authorities are usually demanding precise definitions (e. g. chemical structure) to clearly and unambiguously distinguish between existing and new substances. The more precise these definitions are, the more clear it will be, whether a substance can be considered as new or not and the less negotiations between the industry and competent authorities are needed. Furthermore, clear-cut definitions are of considerable importance in case it is up to national competent authorities to authorise particular substances. Less precise definitions will lead to case-by-case decisions which might differ among national competent authorities.

In order to establish such criteria, one has to consider scientific knowledge; adequate measuring methods must be available. However, one has to consider the efforts necessary for both the applicant and the authorities and the possible benefit for safety aspects as well. As can be understood from the discussion in the preceding chapters (especially chapter 10) establishing clear-cut criteria is only possible for enzymes as the active compounds (distinction by structure). However, these criteria will lead to a considerable burden for applicants and authorities and do not take into account the by-products present especially in microbial enzyme concentrates which are very important in terms of possible toxic properties. But en-

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<sup>86</sup> Presently, exempted in Article 1(2) of Directive 67/548/EEC.

<sup>87</sup> According to AMFEP at least „the classification, labelling and packaging requirements apply for all enzyme preparations marketed by Amfep members in the EU. In this context the Dangerous Preparation Directives (88/379/EEC and, from 30/07/2002, 1999/45/EC) and Material Safety Data Sheet Directive (91/155/EEC) should also be complied with.” (AMFEP 2001, Draft).

zymes are produced as enzyme concentrates consisting up to 98% of by-products and not just as theoretically pure enzymes. These by-products in enzyme concentrate considerably vary in terms of quantity and nature – not only between different enzymes or source organisms, but also affected by different operational conditions in the fermentation and purification process, by genetic engineering or different media charges and may even vary from batch to batch. Therefore, the establishment of criteria covering this aspect is far more difficult.

An enzyme registration system has therefore to be adopted to the unique properties of enzymes and that means to refrain from a system based on the clear-cut distinction between „existing” and „new”. In practice, enzymes would more often be regarded as more or less „familiar” than clearly as „new”.

In order to establish and run a registration system, one has to establish an inventory or database including enzymes which are already registered. In case of enzymes, a registration system thus has to include parameters and data providing indirect evidence e. g. for toxicity and pathogenicity. These parameters/data can only be described qualitatively (e. g. taxonomy and description of host/source organisms) and/or have to be evaluated on a case-to-case-basis (e. g. description of fermentation and purification process).

An extensive review on the regulation of enzymes in the context of chemical and sectoral legislation (food, feed, cosmetic and medical products) in the EU and other countries (chapter ) failed to provide any clear-cut criteria for enzymes. In contrast, in the context of sectoral regulations enzymes are usually evaluated on a case-by-case basis. An enzyme can be regarded as being „similar” or „equivalent” (USA, Canada) and thereby covered by an already listed substance if the manufacturer can justify this by providing data and information on the enzyme.

The notification system of Canada tried to introduce „quantitative” criteria with the „two-percent-rule”<sup>88</sup>. On the one hand, a substitution, of two percent of amino acids (e. g. substitution of six amino acids within a chain length of 300 amino acids) may result in an enzyme without any relevant difference in enzyme properties. On the other hand, the substitution of just one amino acid may have considerable impact as shown in section 9.1.3. Thus, the two-percent rule (for amino acid homology) is not based on sound science and might fail in practice. Applying the two-percent rule to a set of parameters such as molecular weight, isoelectric point, amino acid composition, peptide map, N-terminal sequence will again lead to a case-by-case consideration in practice.

In the opinion of the authors of this study distinguishing between enzymes can therefore not be achieved solely on the basis of clear-cut criteria and will have to include case-by-case decision (see 11.4).

#### 11.3.4 Calculation of tonnage

For any registration system depending on tonnage, the way the tonnage is calculated is not just simply an academic question. Whether tonnages are calculated on the basis of enzyme preparations (final product, including additives), enzyme concentrate (without additives), pure active enzyme or TOS will lead to significant differences of magnitudes. Active enzyme usually forms 0.5-50% of the enzyme preparation, TOS comprises 2 to 50% (for details see section 3.4). For instance the overall amount of detergent enzymes used in Austria was calculated to be about 420 tons (enzyme preparation) which corresponds to about 26 tons of pure enzymes (SPÖK et al., 1998).

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<sup>88</sup> An enzymes is considered substantially equivalent if the function of the protein has not been changed from the protein listed on the DSL; and the protein has 98% amino acid sequence homology with the listed protein, based on amino acid or DNA sequence, or the protein is 98% identical to the listed protein based on all of the following items: molecular weight, isoelectric point, amino acid composition, peptide map, N-terminal sequence (see also section 5.2.8).

Although AMFEP does recommend to calculate and report tonnages on the basis of enzyme concentrate (AMFEP, 2002, see also Annex, section 13.3), it is not quite clear if all companies are complying with this recommendation (EIGTVED, pers. comm.).

Consequently, the calculation method of enzyme tonnages has to be harmonised. As an enzyme preparation has to be regarded as a preparation in the understanding of directive 99/45/EC, the calculation of an enzyme as a substance has presumably to be based on either enzyme concentrate or TOS. Furthermore, it has to be considered that the overall tonnage of a particular enzyme produced by one company might be lower than those of many bulk chemicals.

### 11.3.5 Parameters for describing / distinguishing enzymes

Parameters for characterising and distinguishing enzymes are thoroughly discussed in chapter 9. Unambiguously distinguishing between enzymes on a structural basis is in principle technically feasible but is not regarded as practicable as a basis for distinguishing enzymes (chapter 10). Unambiguously distinguishing between enzymes concentrates is not feasible in practice. Nevertheless, a wide range of parameters is available to characterise enzymes/enzymes concentrates. Most of these data are anyhow available within companies.

Table 50 summarises the principal applicability of parameters available for enzyme description and also those presently required in case of a new substance notification according to Directive 67/548/EEC.<sup>89</sup>

*Table 50: Information/data provisions as required for new substance notification (NSN), safety evaluation of food enzymes (Food) and authorisation for enzyme use in feeding-stuff (Feed) and applicability for technical enzymes. Information regarded as very important is designated „obligatory”, information regarded as useful for further characterisation is designated „relevant”, information/Data provisions not applicable or not sensible for technical enzymes or of limited relevance are designated accordingly.*

Type of Information	NSN*	Food **	Feed***	Applicable for Technical Enzymes
1) Pure Enzyme				
Enzyme Nomenclature				
EC (IUB) Number	Y	Y	Y	Regarded as obligatory
Recommended Name	Y		N	Regarded as relevant
Systematic Name	Y	Y	N	Regarded as relevant
Synonyms	Y	Y	N	Regarded as relevant
CAS Registry Number	Y	Y	Y	Regarded as relevant
Reaction	N	Y	N	Regarded as relevant
Reaction Type	N	Y	Y (most important reaction type)	Regarded as relevant
Enzyme-Ligand Interactions				
Substrates	N	Y	N	Regarded as relevant
Products	N	N	N	Regarded as relevant
Natural Substrates	N	N	N	Limited relevance, since industrial enzymes usually do not act on their natural substrates
Cofactors	N	N	N	Regarded as relevant
Metals/Ions	N	Y	N	Regarded as relevant, if metals or ions

<sup>89</sup> These list should be read in that sense that each parameter is regarded as being indispensable to characterise a particular enzyme.

Type of Information	NSN*	Food **	Feed***	Applicable for Technical Enzymes
				are cofactors or activating compounds
Inhibitors	N	N	N	Regarded as relevant
Activating Compounds	N	N	N	Regarded as relevant
Parameters related to the catalytic activity				
K <sub>m</sub> Value	N	Y <sup>##</sup>	N	Limited relevance, because K <sub>m</sub> value is dependent on assay conditions which could not easily be standardised.
Turnover Number (k <sub>cat</sub> )	N	Y <sup>##</sup>	N	Limited relevance, see K <sub>m</sub> value.
Specific Activity	Y <sup>#</sup>	Y	Y (tested with pure substances)	Limited relevance, see K <sub>m</sub> value.
pH Optimum	N	Y <sup>##</sup>	Y	Regarded as relevant
pH Range	N	Y <sup>##</sup>	N	Regarded as relevant
Temperature Optimum	N	Y <sup>##</sup>	Y	Regarded as obligatory
Temperature Range	N	Y <sup>##</sup>	N	Regarded as obligatory
Isoelectric point	N	Y	N	Regarded as relevant
pH Stability	N	N	N	Regarded as relevant
Temperature Stability	N	N	N	Regarded as relevant
General Stability	N	N	N	Regarded as relevant
Organic Solvent Stability	N	N	N	Limited relevance (for handling)
Oxidation Stability	N	N	N	Limited relevance (for handling)
Storage Stability	N	N	N	Regarded as relevant
Side activities	N	Y	N	Regarded as relevant; Description of any known side activities of the particular enzyme protein.
Molecular Properties				
Melting point	Y	N	N	Not appropriate for enzyme identification or handling information
Boiling point	Y	N	N	Not appropriate for enzyme identification or handling information
Water solubility	Y	N	N	Not appropriate for enzyme identification or handling information
Molecular Weight	Y	Y	N	Regarded as relevant
Amino acid composition	N	Y <sup>###</sup>	N	Regarded as relevant if no sequence data are available
Peptide map	N	Y <sup>###</sup>	N	Regarded as relevant
Nucleotide and/or amino acid sequence database entry number	N	N	N	Regarded as relevant; Sequence Accession Number or Sequence ID from the databases
(Deduced) amino acid sequence	N	Y <sup>###</sup>	N	Regarded as relevant
PDB Protein Data Bank (3-D data)	N	N	N	Regarded as relevant
Subunits	N	N	N	Regarded as relevant if enzyme consists of more than one subunit (number and type of subunit(s) might affect activity)
Crystallisation	N	N	N	Limited relevance
Posttranslational Modifica-	N	N	N	Regarded as relevant

Type of Information	NSN*	Food **	Feed***	Applicable for Technical Enzymes
tion				
Protein / Chemical Engineering	N	Y	N	Regarded as obligatory; Description of the modification (number, localisation and effect of amino acids replacements, deletions, insertions) and technical effect; description of chemical modification and technical effect
Vector	N	Y <sup>###</sup>	N	Description has to be included in „Protein/chemical engineering”
Introduced DNA	N	Y <sup>###</sup>	N	Description has to be included in „Protein/chemical engineering”
2) Pertaining the Enzyme concentrate				
TOS		Y	Y	Regarded as relevant
Physical state (solid, liquid etc.)	Y	N	N	Regarded as relevant for handling
Other enzyme activities present	N	N	N	Regarded as relevant; Description of enzyme activities and importance for technical effect.
3) Microbiology, Hygiene				
Host (production) organism	N	Y	Y	Regarded as obligatory; Genus, species and unambiguous identification number of the strain including all information on adverse effects to human health and the environment (pathogenicity and toxicity)
Source Organism	N	Y <sup>###</sup>	Y	Regarded as obligatory; Genus, species and unambiguous identification number of the strain.
Total Viable Count	N	Y	N	Regarded as obligatory for worker protection
Threshold Limits for Salmonella, etc.	N	Y	N	Regarded as obligatory for worker protection
Antibiotic activity	N	Y	Y	Regarded as relevant
Viable production organism	N		Y	Regarded as relevant with respect to hygienic conditions
DNA from antibiotic resistance genes	N	Y <sup>###</sup>	N	Regarded as obligatory if antibiotic resistance genes are introduced into the GMM.
Known Toxins	N	Y	Y	Regarded as obligatory for worker protection
Heavy metals	N	Y	Y	Regarded as relevant
4) Further information				
Description of Production Process	N	Y	Y	Regarded as obligatory

\*... Requirements for new substance notification (NSN) according to Directive 67/548/EEC; \*\*... Recommendations for food enzyme safety evaluation accumulated from JEFCA and SCF Guidelines; \*\*\*... Requirements for feed enzyme authorisation according to Directive 87/153/EEC; # Not explicitly requested in chemicals legislation, but considered as essential information for the identification of an enzyme; ## not clearly defined in the guidelines (e. g. „temperature”, „pH”, „kinetic parameters”); ### in case of enzymes from GMM.

### 11.3.6 Testing potential hazards of enzyme products

#### 11.3.6.1 Sensitising properties

Enzymes can cause allergy as is substantiated by many case studies and surveys in the literature. The main problems being allergic symptoms of the respiratory tract and, probably secondary, of the skin. Almost all of these reports indicate more an occupational problem than one that is frequently affecting consumers. Enzymes seem to be potent inhalation sensitizers in concentrated form, and thus sensitize frequently in the production factory, but due to the limited number of exposed people only few persons are affected. In contrast, in bread producing factories, exposure rates are high and enzymes have become a major cause (concern) of occupational disease.

#### Testing

Currently, adverse skin-responses associated with repetitive, low-dose exposure to industrial chemicals and consumer products all too often are not accurately predicted by the required assays. There are currently several tests in use for the evaluation of the allergenic potential of chemicals but all these tests predict the potential for the induction of contact allergy (guinea pig sensitization tests, open epicutaneous tests, Buehler test, Freund's complete adjuvant test, optimisation test, split adjuvant test, guinea pig maximization test, human sensitization assays, repeat insult patch test, human maximization tests, etc.). None is capable of exactly predicting immediate type reactions to enzymes; and numerous artefacts may lead to either false-positive or false-negative results.

In addition, the potential for interaction among different enzymes with an effect on sensitisation (SARLO et al., 1997) as well as adjuvant factors important for an increased immune response have also to be considered.

At present, no validated test method exists to determine the risk for sensitisation via the inhalative route. Thus, no recommendations can be given for test methods that can be routinely used for the evaluation of sensitising properties of enzymes to the respiratory tract and the skin.

In summary, any enzyme preparation, independent of its source (conventional or by using GMOs or any other „new“ source) should be labelled as sensitizer (R42) and accordingly handled – until the opposite is proven with unequivocal experiments (see also section 11.3.4).

#### 11.3.6.2 Irritative Properties

All enzymes may be potential skin irritants, a feature that is probably due to their intrinsic nature. The extent of damage is mostly dependent on the time and intensity of exposure, the nature of the enzyme (proteases !) and its concentration, the integrity of the skin, and cofactors such as additional detergent exposure. Occupational problems seem frequent but are nowadays more or less focused on two occupations, that is the baking industry and to the enzyme production industry. Whereas symptoms are usually mild, they are sometimes bothering enough and hinder the patient to continue working with those enzymes.

#### Testing

Test methods for the irritative capacity of a substance are difficult to define and standardise. Regarding enzymes and the respiratory tract, no specific methods are validated; however, occupational exposure by – enzyme-containing dust – has to be kept at the technically lowest possible level, since enzymes might not only irritate the mucosal surfaces, but the resulting inflammation may trigger sensitization.

The skin irritation potential can be determined applying OECD guidelines 404 and 405 regarding testing for dermal and eye irritation. (These test methods correspond to EU test methods B.4 and B.5). The test methods have been revised recently suggesting a tiered testing and evaluation strategy. The factors considered in the testing strategy include

- evaluation of existing human and animal data regarding the respective enzyme
- analysis of structure activity relationship
- physicochemical properties and chemical reactivity
- dermal toxicity
- results from *in vitro* or *ex vivo* tests
- *in vivo* test in rabbits.

### Measures for workers protection

This approach includes coating of enzymes or working with liquid enzymes („dust“-reduction), compliance with operational guidelines, introduction of exposure guidelines for airborne enzyme proteins, reduction of peak exposures, training, medical monitoring, commitment and changes of behaviour, surveillance systems, etc.

Skin contact and inhalation should be avoided, as good as technically possible.

### Measures for consumer protection (post-market surveillance)

As the use of enzymes expands into new areas, there is also potential for exposure to the general population which differs from the current situation. As enzymes are developed for cosmetic and other consumer product uses, it becomes imperative to understand how people can be exposed to enzymes, the level of exposure and the risk for sensitisation. Continued research on understanding how enzymes act as allergens, linked with a continued understanding of how individuals become sensitised to enzymes is important to the continued control of occupational and non-occupational disease caused by enzymes.

#### 11.3.6.3 Other toxic properties

Regarding the toxicological endpoints acute, sub-acute and sub-chronic toxicity and mutagenicity, the scientific literature does not reveal any toxic potential of enzymes used in food or feed application. This conclusion has to be qualified due to the limited available data base, focussing almost exclusively on food enzymes. This conclusion might therefore not necessarily be applicable to technical enzymes which might be of a lower grade of purity. According to the AMFEP list on toxicity testing (see annex, section 13.1.2) hundreds of toxicity tests (including technical enzymes) were carried out so far and toxicity data as well as testing experience accumulated within the industry (including enzymes from GMM and enzymes with altered protein structure). None of these testing reports were made available to the project team. However, this data might form a valuable basis for discussions to refine and/or reduce testing requirements e. g. to perform acute toxicity testing only in certain cases (e. g. risk for production of bacterial toxins). Therefore, this data should be collected and systematically investigated.

### Testing

Concerning toxicity testing on enzymes, requirements and recommendations in EU legislation, scientific literature, and (detergent) enzyme industry position do not correspond to each other, so that a consensus could be outlined.

Acute oral toxicity testing seems to be of limited relevance for enzymes. Reproductive toxicity testing, chronic (cancerogenicity) testing as well as immunotoxicity and toxicokinetic evaluations seems to be of little or no relevance for industrial enzymes.

Toxicity endpoints of relevance are repeated dose toxicity (oral) and mutagenicity.

A test set could be identified, which forms a kind of standard testing in food/feed enzyme toxicity testing. The test set consists of a repeated dose (subchronic) oral toxicity test on rodents together with a set of two mutagenicity tests (one *in vitro* bacterial and one *in vitro* non bacterial test). Although testing is well established and extensively used, notable objections are made from industry and in literature, arguing mainly that mutagenicity testing failed to reveal any positive results and therefore is no longer required.



It could not be found, that enzymes produced by GMM demand new or altered toxicity testing requirements provided that these modifications are considered prior to testing and are used to guide the toxicity testing. No evidence could be found indicating that the present and foreseeable improvement of certain enzyme properties (e. g. changing pH optimum or increasing thermal stability by „protein engineering“) will demand new or altered toxicity testing requirements.

Therefore the following recommendations are given:

- Prior to toxicity testing information/data should be compiled on the enzyme concentrate and the manufacturing process and regarding to the production organism, the conditions for fermentation and downstream processing and the composition of the enzyme concentrate. Genetic modification (and the donor organism) should thereby also be taken into account. This should preferably be standardised using for example a decision tree approach/check list as proposed by PARIZA and JOHNSON (2001; Table 39). Emphasis should be put on this information, since this may help to reduce animal toxicity testing requirements.
- Since no consensus for toxicity testing methods could be identified, a general discussion process should be initiated for example addressing the needs and value of mutagenicity testing requirements. Toxicity data and testing experience available within industry (including enzymes from GMM and enzymes with altered protein structure) should be collected and systematically investigated and consequently form a basis for discussions to refine and/or reduce testing requirements.

From a scientific point of view, the New Substance Notification according to Directive 67/548/EEC (Base Set) should be flexible in its toxicity testing requirements. These requirements should depend on a critical, standardised and comprehensive evaluation of the manufacturing process safety (i. e. production organism, fermentation, downstream processing, composition of enzyme concentrate). A reduction of testing requirements should be considered on a case-by-case basis, if the evaluation reveals no significant changes in the process compared to a process already described and confirmed to be safe. This mainly addresses the need for acute, subchronic and mutagenicity testing. Testing requirements should however be extended depending on the expected exposure routes and application modes. This is also true for new types of enzymes or new production organisms.

#### **11.3.6.4 Environmental properties**

Literature studies revealed that enzymes seem unlikely to present a risk to the aquatic environment due to their ready biodegradability and the low effects on aquatic organisms observed. The environmental exposure of enzymes is expected to be low. However, on the basis of the very few and partly outdated data available, it cannot be concluded that enzymes derived from new technologies do not present any risk to the aquatic environment.

Therefore, information on the biodegradability of enzymes with modified properties (e. g. with higher stability to temperature or pH) is a pre-requisite to assess the risk of enzymes derived from new technologies. The ecotoxicological properties of the enzyme product might be related to toxic by-products produced by the production organism. Anyway, information on the production organism, any known adverse environmental effects of the production organism and a description of the production process should be submitted.

#### **Testing**

Prior to discuss a testing strategy on ecotoxicological effects and biodegradability of enzymes, data already available within industry should be collected and investigated. This especially concerns data on enzymes with increased stability derived by means of new technologies. This data might form a valuable basis for discussions on the requirement to perform ready biodegradability testing on enzymes with improved stability and on establishing

criteria to define „unusual” stability”. The ready biodegradability of a „new” enzyme should be tested only in the case, the enzyme has an „unusual” stability”.

It is proposed to perform acute toxicity tests on aquatic organisms only in the case the enzyme is not readily biodegradable. Then, only one acute toxicity test should be performed on the most sensitive species. However, the decision for the need of ecotoxicity testing and on the testing organism has to be taken by competent authorities taking into account all information that can be made available by industry in the near future.

Presently, for notification purposes of chemicals, tests for ready biodegradability are performed according to EC C.4 A-F<sup>90</sup> (OECD 301 A-F). The potential of acute toxic effects on aquatic organisms is tested on fish (EC C.1, OECD 203), Daphnia (EC C.2, OECD 202) and algae (EC C.3, OECD 201).

*Table 51: Testing required for new substance notification (NSN), safety evaluation of food enzymes (Food) and authorisation for enzyme use in feeding-stuff (Feed). Relevance of toxicological endpoint for and applicability of routine testing methods to technical enzymes.*

Toxicological Endpoints (in parenthesis: methods according to Annex V of 67/548/EEC)		Base Set	Considered as relevant endpoint for enzyme / applicability of available methods for testing of enzymes	Conclusion based on
Health related properties				
Acute toxicity oral (B.1)		Y (two routes)	Limited relevance	feed enzyme authorisation, guidelines for presentation of data on food and feed enzymes, published safety evaluations of food and feed enzymes (16) in scientific literature, review articles and detergent enzyme producers position paper.
Acute toxicity dermal (B.3)				
Acute toxicity inhalative (B.2)				
Repeated dose toxicity (28 d on rodents) (B.7)		Y	Oral toxicity test on rodents (sub acute)	
Mutagenicity	In vitro bacteriological (B.13; B.14)	Y <sup>a</sup>	Both tests could be part of a standard toxicity testing of enzymes	
	In vitro non-bacteriological test (B.10)			
	In vivo <sup>a</sup>			
Reproduction toxicity (B.34)		N	Little or no relevance	
Toxicokinetics		N <sup>c</sup>		
Cancerogenicity (long term animal studies)		N	Not relevant	
Immunotoxicity		N <sup>b</sup>	Not relevant	
Irritating	Acute dermal irritation/corrosion“ (EC B.4)	Y	Regarded as necessary. Testing according to OECD guideline 404 and 405	clinical experience, voluntary labelling of subtilisin with (R38), reports in the scientific literature, published specific safety evaluations
	Acute eye irritation/corrosion” (EC B.5)	Y		
		Irritating to the respiratory system*	N	Well-defined and standardised procedures or recommendations are lacking.
Skin sensitization (B.6)		Y	No hard evidence, that enzymes cause primary skin allergy (i. e. contact allergy). Regarding contact urticaria no test is capable of exactly predicting immediate type reactions to enzymes; numerous artefacts may cause false-	controversial data in the literature; generally indicating elicitation but not sensitization of skin allergy upon cutaneous contact

<sup>90</sup> Annex V of Directive 67/548/EEC.

<b>Toxicological Endpoints</b> (in parenthesis: methods according to Annex V of 67/548/EEC)	<b>Base Set</b>	<b>Considered as relevant endpoint for enzyme / applicability of available methods for testing of enzymes</b>	<b>Conclusion based on</b>
		positive or false-negative results.	
Sensitization by inhalation	N	Regarded as necessary. However, no validated test method exists	unequivocal problem, based on clinical experience and extensive literature data
<b>Environmental Properties</b>			
Biodegradation (C.4 A-F)	Y	Possibly relevant for enzymes with increased stability: prior to testing, there is need for further information on enzymes from new technologies	literature review on technical enzymes, but few data and not up-to-date
Acute toxicity on organisms (fish, Daphnia, algae) (B.1 – B.3);	Y	Only relevant in the case the enzymes is not ready biodegradable; prior to testing, there is need for further information on enzymes from new technologies	literature review on technical enzymes, but few data and not up-to-date
Respiration inhibition on bacteria (C.11)	Y	Not relevant	literature review
Adsorption/desorption (C.18; C.19)	Y	Not relevant	

<sup>a</sup> Several test methods are available. *In vivo* mutagenicity test is only required if *in vitro* tests give positive results. <sup>b</sup> Not required if there are no indications in the 28 d repeated dose study. <sup>c</sup> No test required but an assessment of the toxicokinetic behaviour of a substance required Base Set ... Testing required for base-set notification according to Directive 67/548/EEC are marked with an Y (yes).

#### 11.3.6.5 Substance definition and substance to test

As the definition of substances in Directive 67/548/EEC also covers „*any additive necessary to preserve the stability of the products and any impurity deriving from the process used*“<sup>91</sup> enzyme concentrates *and* added stabilisers could be seen as the substance in the regulatory sense. However, stabilisers might be present in the enzyme concentrate and might also be added during formulation. In case of enzymes it should therefore be ensured that substance definition is identical with what industry regarded as enzyme concentrate and what is used in safety testing.

Generally, the enzyme concentrate should be used as test substance in order to cover the safety of both enzyme and other components derived from the fermentation. The enzyme concentrate is the substances gained from the fermentation process after purification but before the addition of additives. The description of the production process and indication of the production organisms and its modifications are crucial, as they influence the by-products present in the enzyme concentrate.

### 11.4 Framework proposal for a future approach of enzyme registration within the EU chemicals regulation - Recommendations

On the basis of the preceding considerations (section 11.3) a possible framework of an enzyme registration system is outlined in the following. The scope of this outline is thereby restricted to technical enzymes in the context of chemical legislation (section 11.3.1).

<sup>91</sup> [...] „substances“ means chemical elements and their compounds in the natural state or obtained by any production process, *including any additive necessary to preserve the stability of the products and any impurity deriving from the process used* [...] Council Directive 92/32/EEC, Article 2, 1, a.

The present decision criteria whether an enzyme has to be notified are mainly a matter of history and are neither regarded as sensible nor as sufficient for enzyme notification. Neither is EINECS regarded a suitable inventory nor does the concept of notification ask for clear-cut distinctions between existing and new substances applicable to enzymes. Furthermore, data and information requirements for chemicals are only partly applicable for enzymes. Enzymes exhibit considerably different – sometimes unique – properties compared to low-molecular weight chemicals normally dealt with in chemical legislation. Enzymes are high-molecular weight molecules of complex structure. Enzyme structures are not only of high natural variability but have also been subjected to modifications by mutagenesis and genetic engineering techniques in the last decade. Enzymes are furthermore usually not manufactured as more or less pure substances, but are accompanied by by-products which are complex and highly variable mixtures. This mixture cannot be totally characterised in terms of quantity and quality. However these by-products are relevant - at least for toxicity.

Considering this, enzymes pose problems regarding the requirements of the present chemical legislation. To adapt both the registration/notification approach and information requirements and in order to keep up with technical progress it is necessary to consider novel approaches for enzyme registration / notification.

In February 2001, a „Strategy for a future Chemicals Policy” (Commission White Paper; EUROPEAN COMMISSION 2001) has been published. There, the lack of knowledge about the impact of many chemicals on human health and the environment is identified as a cause for concern. In order to achieve the objectives of the White Paper, the Precautionary Principle is fundamental.

In the SLIM report<sup>92</sup>, it is stated that the notification requirements included in Directive 67/548/EEC are not appropriate for the notification of enzymes. In particular, the testing methods from Annex V are not suitable for these substances. Therefore, it is recommended, that particular areas should be explored, for which more appropriate testing methods should be developed, such as for the evaluation of enzymes (recommendation 38).

Enzymes were also mentioned within the working group „Testing, Registration and Evaluation” in the follow-up of the White Paper. The final proposal was that they will be required to be registered under REACH but there was a possibility for a variation in the information package. Modified data requirements are needed for enzymes, given their specific properties.

### **Enzyme database and registration**

All enzymes manufactured or imported in the EU should be subjected to a reporting. All data provided by manufacturers during the reporting period should be included in a central database. Waivers from reporting full data sets might be possible if manufacturer or importer provide evidence that an enzyme is covered by another entry (see below). During the reporting period all available data from safety testing carried out by the applicant or by independent laboratories – including data from testing done in the context of sectoral regulations (if an enzyme is also used in food, feed, cosmetic or medical application) should also be provided. For the registration of enzymes (this may correspond to present notification procedures) data from safety testing as described in section 11.4.4 should be provided.

### **Core parameter/data and additional parameter/data**

A set of data requirements has to be established including characterisation of the enzyme, enzyme concentrate, and data on toxicological, ecotoxicological properties. This list of requirements could include „core data/parameter” and „additional data/parameter”. Core data/parameter are regarded as essential and have to be specified in any case, additional data/parameter may not have to be provided if the applicant provides convincing evidence that they are not necessary. Suggestions for core data/parameter are made in sections 11.4.3 and 11.4.4. Additional parameter can be taken from Table 50 (marked as „relevant”).

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<sup>92</sup> Final Report of the SLIM Phase IV Team on Dangerous Substances (Directive 67/548/EEC) (EUROPEAN COMMISSION 2000).

A final list of core data/parameters has to be based on sound science and has to be discussed in detail. In general, industry should report all available data on their enzymes. The more data are available on the individual enzymes the better the basis for case-by-case decisions. Authorities that are evaluating the data on enzymes should have the right to request further information/data from the manufacturer in order to assist in case-by-case decisions (see below).

### **Reporting/registration approach**

Enzymes should have to be registered providing all data/parameters while leaving out additional data/parameters if the applicant provides convincing evidence that they are unnecessary. Enzymes should be reported/registered by IUB type (indicating the catalytic activity) but equivalence of enzymes has to be justified using core and additional parameters/data. Parameters relevant for this decision are described below.

Considering the fact that enzyme manufacturing processes and also enzymes itself are constantly subjected to improvements, it is neither regarded as feasible nor as useful if each minor change in the production process would lead to full registration requirements. On the other hand such changes might be important with respect to functional and safety properties. Therefore, a flexible approach might be more useful.

### **Proposed approach to differentiate between already registered and non-registered enzymes**

#### ***Registration required in any case***

An enzyme is regarded as „significantly different“ (new) if certain changes appear in the core data/parameters, e. g.

- if IUB-number is not present in the database
- if a new production organism (species level) is used
- if the source organisms or closely related microorganism are known to be pathogenic or toxic

#### ***Registration to be considered by authorities on a case-by-case basis***

An enzyme might be considered as „significantly different“ (new) on a case-by-case basis if changes are involving e. g.

- a new production organism (strain level) is used<sup>93</sup>
- a new source organisms is used (if non-pathogenic and not-toxic)
- major changes in production process
- temperature optimum/range or pH optimum/range is changed so that handling, or inactivation techniques might need to be changed
- enzymes with „unusual“ properties, e. g. enhanced stability, are introduced

In these cases the manufacturer should supply additional data (including safety data) to prove that no relevant changes are to be expected. This could ideally be done by a comparative analysis using the already registered organisms as conventional counterparts (in case of changes in the production process: before and after alterations are introduced). If the competent authorities agree the additional data are introduced into the central database.

The use of well-experienced production strains is regarded as crucial for the safety of by-products. Thus, a list of production strains, that are recognised as safe, should be generated, possibly based on an already established list such as microorganisms regarded as GRAS (section 5.3.6). Changes in the production organism within this list might not lead to a case-

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<sup>93</sup> Regarding production strains one could think about the introduction of a list of production strains that are recognised as safe. Changes in the production organism within this list might not lead to a case-by-case evaluation but might require company surveillance.

by-case evaluation but might require company monitoring. Whether this list should be based on the species level or on the strain level should be clarified in further research work.

#### ***Process surveillance by the company***

The manufacturer should be obliged to monitor toxicity, ecotoxicity and biodegradability if changes in the enzyme manufacturing process occur, e. g. concerning

- protein engineering
- genes from pathogenic or toxigenic sources are introduced
- genetic constructs are altered
- minor changes in the production process
- specific activity is increased
- side activities (of both the enzyme concentrate and the enzyme itself) are altered.

Changes as mentioned above and results of subsequent testing carried out in the course of monitoring should be made available at the own initiative of the manufacturer or importer to the competent authorities who will include this information into the central database.

A detailed list of core and additional parameters as well as detailed criteria what changes in the production process are regarded as „major” should be defined in an discussion process between experts and stakeholders including industry, national and EU authorities. An initial proposal of a set of core parameters is given in 11.4.4. The decision criteria shall be workable and clear and should as far as necessary give guidance in case-by-case decisions of authorities. Experience from sectoral regulations (e. g. feed enzyme authorisation) should be integrated.

#### **11.4.1 Foundations for further adaption to technical progress**

Industry appears to have gained a lot of experience during the last decades by routinely investigating the potential harmful properties of enzymes. However, the majority of these data are still not available for systematic evaluation so far. Furthermore, genetic engineering will make available new catalytic types of enzymes and enzymes from exotic sources harbouring special properties (e. g. thermostability). The (eco)toxic properties of such enzymes or of protein engineered variants may be affected to an extent that has to be considered for regulatory purposes (even if there is almost no empirical evidence so far).

Therefore, the data collected during registration of enzymes could be subjected to systematic evaluation at a later stage. Provided that this database includes all relevant data, the assumptions on which safety testing and safety precaution measures are based could be verified by experience. Such evaluations could also serve to adapt enzyme regulation to the technical progress.

#### **11.4.2 Applying the precautionary approach**

Presently, no validated and generally accepted test methods exist to determine the risk for sensitisation via the inhalative route. However, industry voluntarily and generally classifies, labels and handles currently all enzymes as sensitizers – Xn; R 42 – „May cause sensitisation by inhalation”. As long as no validated test methods are available, this seems to be a feasible approach which is in compliance with the precautionary principle.

#### **11.4.3 Information requirements for enzymes – suggestion for core parameters**

If an enzyme is registered, a set of information should be submitted. This set should consist of information/data regarded as „core data/parameters” and is suggested to include the following information:

- E.C. (IUB) number
- Temperature optimum and range
- Host (production) organism (thoroughly taxonomically characterised by an independent laboratory and along with the results of a literature search)
- Source organism (at least the results of an extensive literature search have to be provided)
- Total viable count and/or
- Absence or acceptable level of Salmonella, etc
- Absence or acceptable level of known toxins
- Description of production process.
- Toxicity testing (as described under 11.4.4)

In order to assist the authorities in case-by-case decisions, the set of core data might be larger in defined cases. To give an example: In case of enzymes from GMM, information on the genetic construct used, the genes introduced and information whether the enzyme is posttranslationally modified in wildtype/production strain; the absence or tolerable level of antibiotic resistance genes or activity in the preparation should be requested. In case of protein engineering, the data should include amino acid sequence information on the wildtype and the variant. In case of extremozymes one might also consider an extended set of parameters for functional / structural characterisation of the enzymes.

#### **11.4.4 Testing requirements for enzymes (core parameter)**

Enzymes are low-volume products and normally biodegradable. With the exception of the sensitizing and irritating properties, enzymes used so far do not exhibit toxic or ecotoxic properties that raise serious concern. However, as novel enzymes become available and novel production organisms may be used a set of toxicity and ecotoxicity testing should be carried out. If testing will be depending on tonnages, the tonnage limits should be applicable for enzymes and calculated in a standardised way (section 11.3.4). The enzyme concentrate should be the substance to test in order to cover the safety of both enzyme and other components derived from the fermentation. The enzyme concentrate is the substances gained from the fermentation process after purification but before the addition of additives.

##### **Toxic properties**

At present, no validated test method exists to determine sensitisation via the inhalative route. No recommendations can be given regarding appropriate test methods. As there is indication, that enzymes have the potential to cause sensitisation of the respiratory tract, we propose to generally label enzymes with R42 „May cause sensitisation via inhalation” (as industry does it today already). Regarding skin irritation, test methods according to the OECD guideline 404 should be performed. For other toxic properties the following tests should be carried out:

**Acute toxicity:** It is assumed, that in some cases (certain production strains) an acute oral toxicity may be necessary due to possible toxin contaminations. This can only be decided on a case by case basis.

**Subacute toxicity:** It is assumed, that a repeated dose oral toxicity testing (e. g. with OECD 407) on rodents gives a basic indication about the subacute toxicity of the enzyme concentrate (e. g. information necessary to calculate a margin of safety). Since so far industrially produced enzymes are considered to be non-toxic, the testing is focussing on by-products and contaminants. Although, in case of alteration in enzymes structure or extremozymes, toxicity might also have to be re-tested. Re-testing requirements should generally be clearly linked to significant and „substantial” alterations in enzyme structure, production organism and production process. To a certain extent industry should carry out re-testing in the course of process surveillance (see: process surveillance by the company).

Therefore it is proposed, that a repeated dose oral toxicity testing is not mandatory (i. e. additional parameter) in reporting, but a lack of testing should give reason to verification. This is also valid for mutagenicity testing (i. e. in vitro bacterial and non bacterial mutagenicity tests). In vivo mutagenicity testing should not be considered to be essential unless the results of in vitro testing give an indication for a mutagenic potential. This approach might also help to avoid an excess of animal testing.

### **Ecotoxic properties**

**Ready biodegradability:** Though enzymes in general are ready biodegradable, the biodegradability of enzymes with increased stability (e. g. temperature) should be proved. The test on ready biodegradability should be performed only in case the enzyme has an „unusual” stability. This should be decided on a case-by-case basis by the authority. Decision criteria for „unusual stability” have to be set.

**Acute toxicity on aquatic organisms:** should solely be requested in case the enzyme is not ready biodegradable. For acute toxicity testing, the most sensitive species should be selected.

### **11.4.5 Quality assurance in manufacturing and safety testing**

Quality assurance in industrial manufacturing of enzymes should be guaranteed by complying with Good Manufacturing Practice (GMP) as it is presently done in case of food enzymes. According to industry „GMP in the microbial food enzyme industry ensures that enzyme preparations are produced, packed and handled in a hygienic way. All operations are designed to avoid contamination, formation of undesirable by-products, deterioration and handling errors. The principles of GMP include systems of quality control and quality assurance, employee qualifications, maintenance standards for equipment, control of raw materials and product stability. The key issues in GMP are the microbiological control of the microorganism selected for enzyme production, the control and monitoring systems ensuring pure culture and optimum enzyme productivity conditions during fermentation and control of the hygienic conditions throughout recovery and finishing of the enzyme preparations” (AMFEP 1997).

Quality assurance and validity in safety testing and characterisation of enzymes might be ensured either by complying with OECD/EC testing guidelines as presently done in EU chemical legislation. For tests or measuring methods which are not standardised Good Laboratory Practice (GLP) might be a valuable instrument for quality assurance and reliability. Tests according to OECD/EC or GLP can be carried out either by the manufacturer or by an independent institution. This also corresponds with a position paper from AMFEP/FEFANA (1999) which also recommends the use of GLP in case of feed enzymes.



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## 13 ANNEX

### 13.1 Tables and Figures

Table 52: Enzymes evolved from DNA-shuffling. Source: Reid (2000), modified.

System	Improvement	Size	Cycles	Mutations	Comments	References
TEM-1 $\beta$ -lactamase	Enzyme activity 32 000-fold	1 kb	3 + 2 <sup>a</sup>	6 aa	Selection. MIC 0.02 $\mu\text{g ml}^{-1}$ to 640 $\mu\text{g ml}^{-1}$ . In comparison, three cycles of mutagenic PCR and selection = 16-fold	Stemmer, 1994
Benzyl esterase	Antibiotic deprotection 150-fold	1.5 kb	2	8 aa	Screen. Four rounds of PCR mutagenesis and screening for improved variants prior to shuffling	Moore & Arnold, 1996
$\beta$ -Galactosidase	Fucosidase activity 66-fold. Substrate specificity 1000-fold	4 kb	7	6 aa 13 bp	Screen. 10 000 colonies per round, best 20–40 chosen per cycle. 66-fold fucosidase activity; 2–3-fold increase expression plus 20-fold increase activity	Zhang et al., 1997
Alkyl transferase	DNA repair 10-fold	0.5 kb	6	7 aa	Selection. Suicide enzyme: limited potential for improvement	F. C. Christians, G. Davies and W. P. C. Stemmer, unpublished results Liu et al., 1997
tRNA synthetase $\beta$ -Lactamase family	Charging of engineered tRNA 100-fold Enzyme activity 270–540-fold	2 kb 1 kb	7 1	nd Chimerics	Selection Selection. 500-fold jump in fitness in one round of shuffling and screening 5000 colonies	Cramer et al., 1998
Thymidine kinase (TK) genes from HSV types 1 and 2	Sensitize E. coli to 32-fold less AZT than HSV-1 TK and 16 000-fold less than HSV-2 TK	0.4 kb	4	Chimerics	Screen. 10 000 colonies per round. Kinetic measurements showed that chimeric enzymes acquired reduced $K_m$ for AZT and decreased specificity for thymidine	Christians et al., 1999
Human interferon- $\alpha$ (HU-IFN- $\alpha$ ) gene	Antiviral activity 285 000-fold	0.6 kb	2	Chimerics	Antiviral activities determined by cytopathic effect (CPE) reduction	Chang et al., 1999
Subtilisin	Activity at 23°C, thermostability, solvent stability, pH dependence increased	1.5 kb	1	Chimerics	Five conditions: measuring activity at pH 5.5, 7.5, and 10. Thermostability measured as residual activity at pH 10 after incubation at 70°C for 5 min. Function in organic solvent assayed as activity in 35% DMF at pH 7.5. All activities compared to Saimase	Ness et al., 1999
Catechol 2,3-dioxygenase	High stability at high temperature 13- to 26-fold	1 kb	2	Chimerics	Thermal stability tested at 50°C. Clones created by restriction enzyme digests	Kikuchi et al., 1999

Table 53: Hybrid enzymes. Source: NIXON et al. (1998)

Enzyme	Desired conversion	Enzyme assayed (substrate or cofactor)	Kinetic parameters			Change in $k_{cat}/K_m$ (ratio of activities of hybrid and wild type) <sup>j</sup>	Refs
			$K_m$ ( $\mu$ M)	$k_{cat}$ ( $s^{-1}$ )	$k_{cat}/K_m$ ( $M^{-1}s^{-1}$ )		
Trypsin <sup>a</sup>	Convert to chymotrypsin	Trypsin	650	30	$4.6 \times 10^4$	26	12
		Chymotrypsin	50	33	$6.0 \times 10^5$		
		Hybrid	27	33	$1.2 \times 10^6$		
Chymotrypsin <sup>b</sup>	Convert to trypsin	Chymotrypsin	110	7.2	$6.9 \times 10^4$	0.19	13
		Trypsin	1.3	$1.1 \times 10^4$	$8.4 \times 10^9$		
		Hybrid	1100	75	$1.3 \times 10^4$		
Glutathione reductase <sup>c</sup>	Convert cofactor specificity from NADPH to NADH	Glutathione reductase (NADPH)	22	267	$1.2 \times 10^7$	$4.2 \times 10^{-3}$	14
		Hybrid (NADPH)	220	11	$5.0 \times 10^4$		
		Glutathione reductase (NADH)	2000	11.3	$5.7 \times 10^3$		
		Hybrid (NADH)	86	35	$4.1 \times 10^5$		
Lactate dehydrogenase <sup>d</sup>	Convert substrate specificity from pyruvate to oxaloacetate	Lactate dehydrogenase (oxaloacetate)	1500	6	$4.0 \times 10^3$	1050	15
		Hybrid (oxaloacetate)	60	250	$4.2 \times 10^6$		
		Lactate dehydrogenase (pyruvate)	60	250	$4.2 \times 10^6$		
		Hybrid (pyruvate)	1800	0.9	$5.0 \times 10^2$		
Subtilisin BPN <sup>e</sup>	Convert substrate specificity to that of <i>Bacillus licheniformis</i> subtilisin	Subtilisin BPN <sup>e</sup>	$1.4 \times 10^5$	50	360	7.2	16
		Subtilisin from <i>B. licheniformis</i>	$2.0 \times 10^5$	510	2500		
		Hybrid	$9.4 \times 10^4$	250	2600		
Subtilisin BPN <sup>f</sup>	Convert substrate specificity from hydrophobic sequences to dibasic or tribasic sequences	Subtilisin BPN <sup>f</sup> (hydrophobic)	110	29	$2.6 \times 10^5$	$7.3 \times 10^{-3}$ $3.1 \times 10^{-5}$ 220	17,18
		Kexilisins hybrid (hydrophobic)	1800	3.4	$1.9 \times 10^3$		
		Furilisins hybrid (hydrophobic)	ND <sup>h</sup>	ND <sup>h</sup>	8.0 <sup>h</sup>		
		Subtilisin BPN <sup>f</sup> (dibasic)	1700	2.8	$1.7 \times 10^3$		
		Kexilisins hybrid (dibasic)	41	15	$3.7 \times 10^5$		
		Subtilisin BPN <sup>f</sup> (tribasic)	ND <sup>i</sup>	ND <sup>i</sup>	ND <sup>i</sup>		
		Furilisins hybrid (tribasic)	29	9.8	$3.4 \times 10^5$		
fH <sub>4</sub> F-hydrolase <sup>g</sup>	Construct GAR transformylase by domain recruitment	fH <sub>4</sub> F-hydrolase (fDDF)	7	$2.6 \times 10^{-2}$	$3.7 \times 10^4$	>400 <sup>i</sup>	19
		GAR transformylase (fDDF/GAR)	17/19	16	$9.4 \times 10^5$ / $8.4 \times 10^5$		
		Hybrid (fDDF/GAR)	35/16	$4 \times 10^{-4}$	11/25		

<sup>a</sup>Succinyl-AAPF-thiobenzyl ester as substrate; pH 6.5, 37°C

<sup>b</sup>Succinyl-AAPR-amino-4-methylcoumarin as substrate, pH 8.0, 37°C

<sup>c</sup>Oxaloacetate as substrate; pH 4.7 (wild type), pH 5.4 (mutant), 30°C

<sup>d</sup>pH 8.6, 25°C

<sup>e</sup>Subtilisin BPN<sup>f</sup>, subtilisin from *Bacillus amyloliquefaciens*; succinyl-AAPF-p-nitroanilide as substrate; pH 6.0, 25°C

<sup>f</sup>Kexilisins, hybrid subtilisin BPN<sup>f</sup> designed to cleave dibasic sequences; furilisins, hybrid subtilisin BPN<sup>f</sup> designed to cleave tribasic sequences; hydrophobic substrate, succinyl-AAPF-p-nitroanilide; dibasic substrate, succinyl-AAKR-p-nitroanilide; tribasic substrate, succinyl-KAKR-p-nitroanilide pH 8.2, 25°C

<sup>g</sup>fH<sub>4</sub>F, N<sup>10</sup>-formyltetrahydrofolate; GAR, glycineamide ribonucleotide; fDDF, formyl-dideazafolate; pH 7.5, 30°C

<sup>h</sup>Unable to saturate the enzyme; apparent  $k_{cat}/K_m$  calculated from rates at low substrate concentrations assuming  $v = (k_{cat}/K_m)[E][S]$ , where [E] and [S] are the concentrations of the enzyme and substrate, respectively.

<sup>i</sup>Cleavage of substrate so poor that assaying was impossible owing to predominant cleavage between succinyl-KAK and R-p-nitroanilide

<sup>j</sup>Change with regards to fH<sub>4</sub>F-hydrolase cannot be determined, because fH<sub>4</sub>F-hydrolase has no measurable GAR-transformylase activity. Based on the detection limit, the ratio of the  $k_{cat}$ s is >400. Fold change with regards to GAR transformylase is  $3.0 \times 10^{-5}$ .

Abbreviations: AAPF, AAPR, four-amino-acid linkers; ND, not determined.

Table 54: List of commercial enzyme presently manufactured in the EU. Source: AMFEP Oct. 2001

Principal enzymatic activity	Host organism/ Production organism	Donor organism	IUB	CAS	EINECS	Food	Feed	Technical
Acetolactate decarboxylase (alpha)	Bacillus amyloliquefaciens or subtilis	Bacillus sp.	4.1.1.5	9025-02-9		Bevr		
Aminoacylase	Aspergillus melleus	none	3.5.1.14	9012-37-7	232-732-3	Diet		
Aminopeptidase	Aspergillus niger	none	3.4.11.x@	9031-94-1	232-874-6	Ches		
Aminopeptidase	Aspergillus oryzae	none	3.4.11.x@	9031-94-1	232-874-6	Bevr Ches Soup Spic		Misc
Aminopeptidase	Lactococcus lactis	none	3.4.11.x@	9031-94-1	232-874-6	Ches Milk		
Aminopeptidase	Rhizopus oryzae	none	3.4.11.x@	9031-94-1	232-874-6	Ches Fish Meat		
Aminopeptidase	Trichoderma reesei or longibrachiatum	Aspergillus sp.	3.4.11.x@	9031-94-1	232-874-6	Ches Egg Meat Milk Spic		Misc
AMP deaminase	Aspergillus melleus	none	3.5.4.6	9025-10-9		Soup		
Amylase (alpha)	Aspergillus niger	none	3.2.1.1	9000-90-2	232-565-6	Bake Bevr Frut Stch		
Amylase (alpha)	Aspergillus oryzae	none	3.2.1.1	9000-90-2	232-565-6	Bake Bevr Stch	Feed	Ldry Misc Pulp Text
Amylase (alpha)	Bacillus amyloliquefaciens or subtilis	Bacillus sp.	3.2.1.1	9000-90-2	232-565-6	Bake Bevr Stch	Feed	
Amylase (alpha)	Bacillus amyloliquefaciens or subtilis	Thermoactinomyces sp.	3.2.1.1	9000-90-2	232-565-6	Bake	Feed	
Amylase (alpha)	Bacillus amyloliquefaciens or subtilis	none	3.2.1.1	9000-90-2	232-565-6	Bake Bevr Stch	Feed	Ldry Misc Pulp Text
Amylase (alpha)	Bacillus licheniformis	Bacillus sp.	3.2.1.1	9000-90-2	232-565-6	Bevr Stch Sugr		Dish Ldry Misc Pulp Text
Amylase (alpha)	Bacillus licheniformis	none	3.2.1.1	9000-90-2	232-565-6	Bake Bevr Stch		Text
Amylase (alpha)	Bacillus stearothermophilus	none	3.2.1.1	9000-90-2	232-565-6	Bevr Stch		
Amylase (alpha)	Microbacterium imperiale	none	3.2.1.1	9000-90-2	232-565-6	Bake Conf Sugr		
Arabinanase	Aspergillus niger	none	3.2.1.99	37325-54-5	253-463-8	Bevr, Choc	Feed	
Arabinofuranosidase	Aspergillus niger	Aspergillus sp.	3.2.1.55	9067-74-7	232-957-7	Bevr		
Arabinofuranosidase	Aspergillus niger	none	3.2.1.55	9067-74-7	232-957-7	Bake Bevr Choc		
Catalase	Aspergillus niger	Aspergillus sp.	1.11.1.6	9001-05-2	232-577-1	Bake Bev Ches Egg Fats Stch Sugr		Wast
Catalase	Aspergillus niger	none	1.11.1.6	9001-05-2	232-577-1	Ches Milk		Misc
Catalase	Micrococcus luteus or lysodeikticus	none	1.11.1.6	9001-05-2	232-577-1			Text
Catalase	Scytalidium thermophilum	none	1.11.1.6	9001-05-2	232-577-1			Text
Cellulase	Aspergillus niger	none	3.2.1.4	9012-54-8	232-734-4	Bevr Frut Stch		
Cellulase	Aspergillus oryzae	Myceliophthora sp.	3.2.1.4	9012-54-8	232-734-4			Text
Cellulase	Aspergillus oryzae	Thielavia sp.	3.2.1.4	9012-54-8	232-734-4			Text
Cellulase	Aspergillus oryzae	Humicola sp.	3.2.1.4	9012-54-8	232-734-4			Ldry Pulp Text
Cellulase	Bacillus amyloliquefaciens or subtilis	none	3.2.1.4	9012-54-8	232-734-4			Ldry Text
Cellulase	Humicola insolens	none	3.2.1.4	9012-54-8	232-734-4			Ldry Text
Cellulase	Penicillium funiculosum	none	3.2.1.4	9012-54-8	232-734-4	Stch	Feed	Text
Cellulase	Penicillium or Talaromyces emersonii	none	3.2.1.4	9012-54-8	232-734-4	Bevr Stch	Feed	
Cellulase	Streptomyces lividans	none	3.2.1.4	9012-54-8	232-734-7			Text

Principal enzymatic activity	Host organism/ Production organism	Donor organism	IUB	CAS	EINECS	Food	Feed	Technical
Cellulase	Trichoderma reesei or longibrachiatum	Trichoderma sp.	3.2.1.4	9012-54-8	232-734-4		Feed	Text
Cellulase	Trichoderma reesei or longibrachiatum	none	3.2.1.4	9012-54-8	232-734-4	Bake Bevr Choc Diet Fats Frut Stch	Feed	Misc Pulp Text
Cellulase	Trichoderma viride	none	3.2.1.4	9012-54-8	232-734-4	Bevr		
Chymosin	Aspergillus niger var. awamori	Calf stomach	3.4.23.4	9001-98-3	232-645-0	Ches		
Chymosin	Kluyveromyces lactis	Calf stomach	3.4.23.4	9001-98-3	232-645-0	Ches		
Cyclodextrin glucanotransferase	Bacillus licheniformis	Thermoanaerobacter sp.	2.4.1.19	9030-09-5		Stch		
Cyclodextrin glucanotransferase	Bacillus macerans	none	2.4.1.19	9030-09-5		Sugr		
Dextranase	Chaetomium erraticum	none	3.2.1.11	9025-70-1	232-803-9	Sugr		
Dextranase	Penicillium lilacinum	none	3.2.1.11	9025-70-1	232-803-9	Stch		
Esterase	Rhizomucor miehei	none	3.1.1.1	9016-18-6	232-773-7	Ches		
Galactosidase (alpha)	Aspergillus niger	none	3.2.1.22	9025-35-8	232-792-0	Diet		
Galactosidase (alpha)	Aspergillus oryzae	Aspergillus sp.	3.2.1.22	9025-35-8	232-792-0		Feed	
Galactosidase (alpha)	Saccharomyces cerevisiae	Guar plant	3.2.1.22	9025-35-8	232-792-0		Feed	
Glucanase (beta)	Aspergillus aculeatus	none	3.2.1.6	62213-14-3	263-462-4	Bevr Stch		
Glucanase (beta)	Aspergillus niger	none	3.2.1.6	62213-14-3	263-462-4	Bake Bevr Stch	Feed	
Glucanase (beta)	Bacillus amyloliquefaciens or subtilis	Bacillus sp.	3.2.1.6	62213-14-3	263-462-4	Bevr	Feed	
Glucanase (beta)	Bacillus amyloliquefaciens or subtilis	none	3.2.1.6	62213-14-3	263-462-4	Bevr	Feed	Pulp
Glucanase (beta)	Disporotrichum dimorphosporum	none	3.2.1.6	62213-14-3	263-462-4	Bevr		
Glucanase (beta)	Humicola insolens	none	3.2.1.6	62213-14-3	263-462-4	Bevr Stch	Feed	
Glucanase (beta)	Penicillium funiculosum	none	3.2.1.6	62213-14-3	263-462-4	Bevr Stch	Feed	
Glucanase (beta)	Penicillium or Talaromyces emersonii	none	3.2.1.6	62213-14-3	263-462-4	Bevr Stch	Feed	
Glucanase (beta)	Pseudomonas paucimobilis	none	3.2.1.6	62213-14-3	263-462-4	Soup		
Glucanase (beta)	Trichoderma reesei or longibrachiatum	none	3.2.1.6	62213-14-3	263-462-4	Bake Bevr Diet Stch	Feed	Text
Glucanase (beta)	Trichoderma reesei or longibrachiatum	Trichoderma sp.	3.2.1.6	62213-14-3	263-462-4	Stch	Feed	Text
Glucoamylase or Amyloglucosidase	Aspergillus niger	Aspergillus sp.	3.2.1.3	9032-08-0	232-877-2	Bevr Frut Stch		
Glucoamylase or Amyloglucosidase	Aspergillus niger	none	3.2.1.3	9032-08-0	232-877-2	Bake Bevr Conf Frut Stch		Misc Text
Glucoamylase or Amyloglucosidase	Rhizopus delemar	none	3.2.1.3	9032-08-0	232-877-2	Bake		
Glucoamylase or Amyloglucosidase	Rhizopus niveus	none	3.2.1.3	9032-08-0	232-877-2	Bevr Stch		
Glucoamylase or Amyloglucosidase	Rhizopus oryzae	none	3.2.1.3	9032-08-0	232-877-2	Bake Bevr Stch		
Glucose isomerase	Actinoplanes missouriensis	none	5.3.1.5	9055-00-9	232-944-6	Stch		
Glucose isomerase	Streptomyces lividans	Actinoplanes sp.	5.3.1.5	9055-00-9	232-944-6	Stch		
Glucose isomerase	Streptomyces murinus	none	5.3.1.5	9055-00-9	232-944-6	Stch		

Principal enzymatic activity	Host organism/ Production organism	Donor organism	IUB	CAS	EINECS	Food	Feed	Technical
Glucose isomerase	<i>Streptomyces olivochromogenes</i>	none	5.3.1.5	9055-00-9	232-944-6	Stch		
Glucose isomerase	<i>Streptomyces rubiginosus</i>	<i>Streptomyces</i> sp.	5.3.1.5	9055-00-9	232-944-6	Stch		
Glucose oxidase	<i>Aspergillus niger</i>	<i>Aspergillus</i> sp.	1.1.3.4	9001-37-0	232-601-0	Bake Egg		
Glucose oxidase	<i>Aspergillus niger</i>	none	1.1.3.4	9001-37-0	232-601-0	Bake Bevr Egg		Misc
Glucose oxidase	<i>Penicillium chrysogenum</i>	none	1.1.3.4	9001-37-0	232-601-0	Bake Bevr Egg		
Glucosidase (alpha)	<i>Aspergillus niger</i>	none	3.2.1.20	9001-42-7	232-604-7	Bevr Stch		
Glucosidase (beta)	<i>Aspergillus niger</i>	none	3.2.1.21	9001-22-3	232-589-7	Bevr Stch	Feed	Misc Text
Glucosidase (exo-1,3-beta)	<i>Trichoderma harzianum</i>	none	3.2.1.58	9073-49-8	232-968-7	Bevr		
Glucosyltransferase	<i>Aspergillus foetidus</i>	none	2.4.1.24	9030-12-0		Stch		Text
Glutaminase	<i>Bacillus subtilis</i>	none	3.5.1.2	9001-47-2		Soup		
Hemicellulase	<i>Aspergillus foetidus</i>	none	*	9025-56-3	232-799-9	Bake Stch	Feed	Misc
Hemicellulase	<i>Aspergillus niger</i>	none	*	9025-56-3	232-799-9	Bake Frut	Feed	Misc
Hemicellulase	<i>Bacillus amyloliquefaciens</i> or <i>subtilis</i>	none	*	9025-56-3	232-799-9	Bake		
Hemicellulase	<i>Bacillus amyloliquefaciens</i> or <i>subtilis</i>	<i>Bacillus</i> sp.	*	9025-56-3	232-799-9	Bake Stch	Feed	
Inulase	<i>Aspergillus niger</i>	none	3.2.1.7	9025-67-6	232-802-3	Stch		
Invertase or Fructofuranosidase (beta)	<i>Saccharomyces cerevisiae</i>	none	3.2.1.26	9001-57-4	232-615-7	Bevr Conf Sugr		
Laccase	<i>Aspergillus oryzae</i>	<i>Myceliophthora</i> sp.	1.10.3.2	80498-15-3				Text
Laccase	<i>Aspergillus oryzae</i>	<i>Polyporus</i> sp.	1.10.3.2	80498-15-3				Text
Lactase or Galactosidase (beta)	<i>Aspergillus oryzae</i>	none	3.2.1.23	9031-11-2	232-864-1	Ches Diet Ice Milk		
Lactase or Galactosidase (beta)	<i>Kluyveromyces lactis</i>	none	3.2.1.23	9031-11-2	232-864-1	Ice Milk		
Lactase or Galactosidase (beta)	<i>Aspergillus oryzae</i>	none	3.2.1.23	9031-11-2	232-864-1	Ches Diet Ice Milk		
Lactase or Galactosidase (beta)	<i>Kluyveromyces lactis</i>	<i>Klyveromyces</i> sp.	3.2.1.23	9031-11-2	232-864-1	Ice Milk		
Lipase, monoacylglycerol	<i>Penicillium camembertii</i>	none	3.1.1.23	9040-75-9		Ches Fats		
Lipase, triacylglycerol	<i>Aspergillus niger</i>	none	3.1.1.3	9001-62-1	232-619-9	Bake Ches Fats Milk Spic		
Lipase, triacylglycerol	<i>Aspergillus oryzae</i>	<i>Rhizomucor</i> sp.	3.1.1.3	9001-62-1	232-619-9	Ches Fats Spic		Misc
Lipase, triacylglycerol	<i>Aspergillus oryzae</i>	<i>Thermomyces</i> sp.	3.1.1.3	9001-62-1	232-619-9	Bake Fats		Dish Ldry Lthr Pulp
Lipase, triacylglycerol	<i>Aspergillus oryzae</i>	<i>Fusarium</i> sp.	3.1.1.3	9001-62-1	232-619-9	Bake Fats		
Lipase, triacylglycerol	<i>Aspergillus oryzae</i>	<i>Candida</i> sp.	3.1.1.3	9001-62-1	232-619-9	Fats		Misc Text
Lipase, triacylglycerol	<i>Candida lipolytica</i>	none	3.1.1.3	9001-62-1	232-619-9	Bake Ches Fats Spic		
Lipase, triacylglycerol	<i>Candida rugosa</i>	none	3.1.1.3	9001-62-1	232-619-9	Ches Fats		
Lipase, triacylglycerol	<i>Mucor javanicus</i>	none	3.1.1.3	9001-62-1	232-619-9	Ches Fats		
Lipase, triacylglycerol	<i>Penicillium roqueforti</i>	none	3.1.1.3	9001-62-1	232-619-9	Ches Fats		
Lipase, triacylglycerol	<i>Pseudomonas alcaligenes</i>	<i>Pseudomonas</i> sp.	3.1.1.3	9001-62-1	232-619-9			Ldry
Lipase, triacylglycerol	<i>Rhizomucor miehei</i>	none	3.1.1.3	9001-62-1	232-619-9	Ches		
Lipase, triacylglycerol	<i>Rhizopus delemar</i>	none	3.1.1.3	9001-62-1	232-619-9	Ches Fats		
Lipase, triacylglycerol	<i>Rhizopus niveus</i>	none	3.1.1.3	9001-62-1	232-619-9	Ches Fats		
Lipase, triacylglycerol	<i>Rhizopus oryzae</i> or <i>arrhizus</i>	none	3.1.1.3	9001-62-1	232-619-9	Bake Ches Fats		Misc
Lipoxygenase	<i>Penicillium chrysogenum</i>	none	1.13.11.12	9029-60-1	232-853-1	Bake		

Principal enzymatic activity	Host organism/ Production organism	Donor organism	IUB	CAS	EINECS	Food	Feed	Technical
Maltogenic amylase	Bacillus amyloliquefaciens or subtilis	Bacillus sp.	3.2.1.133	160611-47-2		Bake Stch		
Mannanase (endo-1,4-beta)	Aspergillus niger	none	3.2.1.78	37288-54-3	253-446-5	Bake Bevr Stch		
Mannanase (endo-1,4-beta)	Penicillium funiculosum	none	3.2.1.78	37288-54-3	253-446-5		Feed	
Mannanase (endo-1,4-beta)	Trichoderma reesei or longibrachiatum	Trichoderma sp.	3.2.1.78	37288-54-3	253-446-5		Feed	
Pectate lyase	Bacillus licheniformis	Bacillus sp.	4.2.2.2	9015-75-2	232-766-9			Text
Pectin lyase	Aspergillus niger	none	4.2.2.10	9033-35-6	232-894-5	Bevr Choc Frut	Feed	
Pectin lyase	Aspergillus niger var. awamori	Aspergillus sp.	4.2.2.10	9033-35-6	232-894-5	Bevr Choc Frut	Feed	Text
Pectin lyase	Trichoderma reesei or longibrachiatum	Aspergillus sp.	4.2.2.10	9033-35-6	232-894-5	Bevr Choc Frut	Feed	Text
Pectin methylesterase or Pectinesterase	Aspergillus niger	Aspergillus sp.	3.1.1.11	9025-98-3	232-807-0	Bevr Choc Frut	Feed	
Pectin methylesterase or Pectinesterase	Aspergillus oryzae	Aspergillus sp.	3.1.1.11	9025-98-3	232-807-0	Bevr Frut		Misc
Pectin methylesterase or Pectinesterase	Aspergillus niger	none	3.1.1.11	9025-98-3	232-807-0	Bevr Choc Frut	Feed	
Pectin methylesterase or Pectinesterase	Trichoderma reesei or longibrachiatum	Aspergillus sp.	3.1.1.11	9025-98-3	232-807-0	Bevr Frut	Feed	Text
Penicillin amidase	Alcaligenes faecalis	Alcaligenes sp.	3.5.1.11	9014-06-6	232-753-8			Misc
Pentosanase	Humicola insolens	none	*	9068-42-2		Bake		
Pentosanase	Trichoderma reesei or longibrachiatum	none	*	9068-42-2		Bake Bevr Stch	Feed	
Phosphatase	Aspergillus niger	none	3.1.3.2	9001-77-8	232-630-9	Bevr		
Phosphodiesterase	Leptographium procerum	none	3.1.4.1	9025-82-5	232-806-5	Spic		
Phosphodiesterase	Penicillium citrinum	none	3.1.4.1	9025-82-5	232-806-5	Soup		
Phospholipase A	Trichoderma reesei or longibrachiatum	Aspergillus sp.	3.1.1.4	9001-84-7	232-637-7	Bake Fats	Feed	Text
Phospholipase B	Aspergillus niger	none	3.1.1.5	9001-85-8		Stch		
Phospholipase B	Aspergillus niger var. awamori	none	3.1.1.5	9001-85-8		Bake Stch	Feed	
Phospholipase B	Trichoderma reesei or longibrachiatum	Aspergillus sp.	3.1.1.5	9001-85-8		Bake Stch	Feed	
Phytase	Aspergillus niger	Aspergillus sp.	3.1.3.8	9001-77-8	232-630-9		Feed	
Phytase	Aspergillus niger	none	3.1.3.8	9001-77-8	232-630-9	Stch		
Phytase	Aspergillus oryzae	Peniophora sp.	3.1.3.8	9001-77-8	232-630-9		Feed	
Phytase	Trichoderma reesei or longibrachiatum	Aspergillus sp.	3.1.3.8	9001-77-8	232-630-9		Feed	
Polygalacturonase or Pectinase	Aspergillus aculeatus	none	3.2.1.15	9032-75-1	232-885-6	Bevr Frut		
Polygalacturonase or Pectinase	Aspergillus niger	none	3.2.1.15	9032-75-1	232-885-6	Bevr Choc Frut	Feed	Misc
Polygalacturonase or Pectinase	Aspergillus pulverulentus	none	3.2.1.15	9032-75-1	232-885-6	Frut		



Principal enzymatic activity	Host organism/ Production organism	Donor organism	IUB	CAS	EINECS	Food	Feed	Technical
Polygalacturonase or Pectinase	Penicillium funiculosum	none	3.2.1.15	9032-75-1	232-885-6	Bevr		
Polygalacturonase or Pectinase	Trichoderma reesei or longibrachiatum	Aspergillus sp.	3.2.1.15	9032-75-1	232-885-6	Bevr Choc Frut	Feed	Text
Protease (incl. milkclotting enzymes)	Ananas comosus	none	3.4.2x.xx#	9001-92-7	232-642-4	Diet Fish Meat Spic		Misc
Protease (incl. milkclotting enzymes)	Bacillus halodurans or eltnus	none	3.4.2x.xx#	9001-92-7	232-642-4			Lydr Lthr Misc
Protease (incl. milkclotting enzymes)	Calf stomach	none	3.4.2x.xx#	9001-92-7	232-642-4	Ches		
Protease (incl. milkclotting enzymes)	Carida papaya	none	3.4.2x.xx#	9001-92-7	232-642-4	Bake Bevr Diet Fish Meat Spic		Misc Lthr
Protease (incl. milkclotting enzymes)	Cryphonectria or Endothia parasitica	Cryphonectria sp.	3.4.2x.xx#	9001-92-7	232-642-4	ches		
Protease (incl. milkclotting enzymes)	Ficus glabrata	none	3.4.2x.xx#	9001-92-7	232-642-4	Ches Diet Fish Meat Spic		Misc
Protease (incl. milkclotting enzymes)	Aspergillus melleus	none	3.4.2x.xx#	9001-92-7	232-642-4	Ches Fish Meat		
Protease (incl. milkclotting enzymes)	Aspergillus niger	none	3.4.2x.xx#	9001-92-7	232-642-4	Bake Bevr Fish Frut Meat Stch	Feed	
Protease (incl. milkclotting enzymes)	Aspergillus oryzae	none	3.4.2x.xx#	9001-92-7	232-642-4	Bake Bevr Ches Fish Frut Meat Milk Soup Spic Stch	Feed	Misc
Protease (incl. milkclotting enzymes)	Aspergillus oryzae	Aspergillus sp.	3.4.2x.xx#	9001-92-7	232-642-4			Lthr
Protease (incl. milkclotting enzymes)	Aspergillus oryzae	Rhizomucor sp.	3.4.2x.xx#	9001-92-7	232-642-4	Ches		
Protease (incl. milkclotting enzymes)	Aspergillus sojae	none	3.4.2x.xx#	9001-92-7	232-642-4	Fish Meat Milk Spic Stch	Feed	Misc
Protease (incl. milkclotting enzymes)	Bacillus alcalophilus	Bacillus sp.	3.4.2x.xx#	9001-92-7	232-642-4			Ldry
Protease (incl. milkclotting enzymes)	Bacillus amyloliquefaciens or subtilis	Bacillus sp.	3.4.2x.xx#	9001-92-7	232-642-4	Bake Bevr Ches Fish Meat Milk Stch	Feed	
Protease (incl. milkclotting enzymes)	Bacillus amyloliquefaciens or subtilis	none	3.4.2x.xx#	9001-92-7	232-642-4	Bake Bevr Ches Fish Meat Soup Spic Stch	Feed	Lthr Misc Pulp
Protease (incl. milkclotting enzymes)	Bacillus halodurans or lentus	Bacillus sp.	3.4.2x.xx#	9001-92-7	232-642-4			Dish Ldry Lthr
Protease (incl. milkclotting enzymes)	Bacillus licheniformis	Bacillus sp.	3.4.2x.xx#	9001-92-7	232-642-4	Fish Meat		Dish Ldry
Protease (incl. milkclotting enzymes)	Bacillus licheniformis	none	3.4.2x.xx#	9001-92-7	232-642-4	Ches Fish Meat Soup Spic	Feed	Dish Ldry Lthr Misc Text
Protease (incl. milkclotting enzymes)	Bacillus stearothermophilus	none	3.4.2x.xx#	9001-92-7	232-642-4	Fish Meat Soup		
Protease (incl. milkclotting enzymes)	Cryphonectria or Endothia parasitica	none	3.4.2x.xx#	9001-92-7	232-642-4	Ches		
Protease (incl. milkclotting enzymes)	Penicillium citrinum	none	3.4.2x.xx#	9001-92-7	232-642-4	Soup		

Principal enzymatic activity	Host organism/ Production organism	Donor organism	IUB	CAS	EINECS	Food	Feed	Technical
Protease (incl. milkclotting enzymes)	Rhizomucor miehei	none	3.4.2x.xx#	9001-92-7	232-642-4	Ches		Lthr
Protease (incl. milkclotting enzymes)	Rhizopus niveus	none	3.4.2x.xx#	9001-92-7	232-642-4	Meat		
Protease (incl. milkclotting enzymes)	Streptomyces fradiae	none	3.4.2x.xx#	9001-92-7	232-642-4		Feed	Misc
Pullulanase	Trichoderma reesei or longibrachiatum	Hormoconis sp.	3.2.1.41	9075-68-7	232-983-9	Bake	Feed	
Pullulanase	Bacillus acidopullulyticus	none	3.2.1.41	9075-68-7	232-983-9	Bevr Stch		
Pullulanase	Bacillus circulans	none	3.2.1.41	9075-68-7	232-983-9	Bevr Stch Sugr		
Pullulanase	Bacillus licheniformis	Bacillus sp.	3.2.1.41	9075-68-7	232-983-9	Stch		
Pullulanase	Bacillus subtilis	Bacillus sp.	3.2.1.41	9075-68-7	232-983-9	Bevr Stch		
Pullulanase	Klebsiella planticola	Klebsiella sp.	3.2.1.41	9075-68-7	232-983-9	Bevr Stch		
Pullulanase	Klebsiella planticola	none	3.2.1.41	9075-68-7	232-983-9	Bevr Stch		
Rhamnosidase (alpha-L)	Penicillium decumbens	none	3.2.1.40	37288-35-0		Bevr Frut		
Tannase	Aspergillus niger	none	3.1.1.20	9025-17-2	232-804-4	Choc Frut		
Transglutaminase	Streptovorticillium mobaraense	none	2.3.2.13	80146-85-6		Bake Ches Conf Diet Fish Ice Meat Milk Stch	Feed	
Xaa-Pro-dipeptidyl-aminopeptidase	Lactococcus lactis	none	3.4.14.5	9031-96-3	232-875-1	Ches Diet Egg Fish Meat Milk Spic		
Xylanase	Aspergillus foetidus	none	3.2.1.8	9025-57-4	232-800-2	Bake Stch	Feed	Misc
Xylanase	Aspergillus niger	none	3.2.1.8	9025-57-4	232-800-2	Bake	Feed	
Xylanase	Aspergillus niger	Aspergillus sp.	3.2.1.8	9025-57-4	232-800-2	Bake Bevr	Feed	
Xylanase	Aspergillus niger var. awamori	none	3.2.1.8	9025-57-4	232-800-2	Bake		
Xylanase	Aspergillus niger var. awamori	Aspergillus sp.	3.2.1.8	9025-57-4	232-800-2	Bake		
Xylanase	Aspergillus oryzae	Aspergillus sp.	3.2.1.8	9025-57-4	232-800-2	Stch		
Xylanase	Aspergillus oryzae	Thermomyces sp.	3.2.1.8	9025-57-4	232-800-2	Bake	Feed	
Xylanase	Bacillus amyloliquefaciens or subtilis	none	3.2.1.8	9025-57-4	232-800-2	Bake Bevr Stch		
Xylanase	Bacillus amyloliquefaciens or subtilis	Bacillus sp.	3.2.1.8	9025-57-4	232-800-2	Bake Bevr Stch	Feed	Pulp Text
Xylanase	Bacillus licheniformis	Bacillus sp.	3.2.1.8	9025-57-4	232-800-2	Stch		Pulp
Xylanase	Disporotrichum dimorphosporum	none	3.2.1.8	9025-57-4	232-800-2	Bevr		
Xylanase	Humicola insolens	none	3.2.1.8	9025-57-4	232-800-2		Feed	
Xylanase	Penicillium funiculosum	none	3.2.1.8	9025-57-4	232-800-2	Bevr Stch		
Xylanase	Trichoderma reesei or longibrachiatum	none	3.2.1.8	9025-57-4	232-800-2	Bake Bevr Stch	Feed	Pul
Xylanase	Trichoderma reesei or longibrachiatum	Trichoderma sp.	3.2.1.8	9025-57-4	232-800-2	Bevr Stch	Feed	Pulp
Xylanase	Trichoderma reesei or longibrachiatum	Actinomadura sp.	3.2.1.8	9025-57-4	232-800-2			
Xylanase	Trichoderma viride	none	3.2.1.8	9025-57-4	232-800-2	Bake Stch		Misc

\* Those enzymes which do not have an IUB number are enzyme complexes, where the listed activity is the result of the sum of many single active enzyme proteins. @ There is no general IUB number for aminopeptidases. But all these enzymes fall under the 3.4.11.x category. # There is no general IUB number for proteases. But all these enzymes fall under the 3.4.2x category. Bake = Bakery, Bevr = Beverages (soft drinks, beer, wine), Ches = Cheese, Choc = Cocoa, chocolate, coffee and tea, Conf = Confectionary, Diet =

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*Dietary food, Dish = Dishwashing powder, Egg = Egg, Fats = Fats and oils, Fish = Fish, Frut = Fruit and vegetables, Ice = Edible ice, Ldry = Laundry, Lthr = Leather, Meat = Meat, Milk = Milk, Misc = Miscellaneous, Pulp = Pulp and paper, Sald = Salads, Soup = Soups and broths, Spic = Spices and flavours, Stch = Cereal and starch, Sugr = Sugar and honey, Text = Textile, Wast = Wastewater treatment.*

Table 55: Production organisms of enzymes presently marketed in the EU. Source: Derived from Table 54, AMFEP Oct. 2001

	Host organism/ Production organism	Food	Feed	Technical
	<b>Actinoplanes</b> missouriensis	Stch		
	<b>Alcaligenes</b> faecalis			Misc
	<b>Ananas</b> comosus	Diet Fish Meat Spic		Misc
Aspergillus	<b>Aspergillus</b> aculeatus	Bevr Stch Frut		
	<b>Aspergillus</b> foetidus	Stch		Text
	<b>Aspergillus</b> foetidus	Bake Stch	Feed	Misc
	<b>Aspergillus</b> melleus	Ches Diet Fish Meat Soup		
	<b>Aspergillus</b> niger	Ches		
	<b>Aspergillus</b> niger	Bake Bevr Ches Choc Conf Diet Egg Fats Fish Frut Milk Meat Stch Sugr	Feed	Misc Text Wast
	<b>Aspergillus</b> niger var. awamori	Bake Bevr Ches Choc Frut	Feed	Text
	<b>Aspergillus</b> oryzae	Bake Bevr Ches Diet Fats Fish Frut Ice Meat Milk Soup Spic Stch	Feed	Dish Ldry Misc Pulp Text
	<b>Aspergillus</b> pulverulentus	Frut		
	<b>Aspergillus</b> sojae	Fish Meat Milk Spic Stch	Feed	Misc
	<b>Aspergillus</b> niger	Bevr Choc Frut	Feed	
Bacillus	<b>Bacillus</b> acidopullulyticus	Bevr Stch		
	<b>Bacillus</b> alcalophilus			Ldry
	<b>Bacillus</b> amyloliquefaciens or subtilis	Bake Bevr Ches Fish Meat Milk Stch	Feed	Ldry Misc Pulp Text
	<b>Bacillus</b> circulans	Bevr Stch Sugr		
	<b>Bacillus</b> halodurans or lentus			Dish Ldry Lthr Misc
	<b>Bacillus</b> licheniformis	Bake Bevr Ches Fish Meat Soup Spic Stch	Feed	Dish Ldry Lthr Misc Pulp Text
	<b>Bacillus</b> macerans	Sugr		
	<b>Bacillus</b> stearothermophilus	Bevr Fish Meat Soup Stch		
	<b>Bacillus</b> subtilis	Bevr Soup Stch		
	<b>Calf</b> stomach	Ches		
Candida	<b>Candida</b> lipolytica	Bake Ches Fats Spic		
	<b>Candida</b> rugosa	Ches Fats		
	<b>Carida</b> papaya	Bake Bevr Diet Fish Meat Spic		Misc Lthr
	<b>Chaetomium</b> erraticum	Sugr		
	<b>Cryphonectria</b> or Endothia parasitica	Ches		
	<b>Disporotrichum</b> dimorphosporum	Bevr		
	<b>Ficus</b> glabrata	Ches Diet Fish Meat Spic		Misc
	<b>Humicola</b> insolens	Bake Bevr Stch	Feed	Ldry Text
	<b>Klebsiella</b> planticola	Bevr Stch		
	<b>Kluyveromyces</b> lactis	Ches Ice Milk		
	<b>Lactococcus</b> lactis	Ches Diet Egg Fish Meat Milk Spic		
	<b>Leptographium</b> procerum	Spic		
	<b>Microbacterium</b> imperiale	Bake Conf Sugr		
	<b>Micrococcus</b> luteus or lysodeikticus			Text
	<b>Mucor</b> javanicus	Ches Fats		
	<b>Penicillium</b> camembertii	Ches Fats		

	Host organism/ Production organism	Food	Feed	Technical
	Penicillium chrysogenum	Bake Bevr Egg		
	Penicillium citrinum	Soup		
	Penicillium decumbens	Bevr Frut		
	Penicillium funiculosum	Bevr Stch	Feed	Text
	Penicillium or Talaromyces emersonii	Bevr Stch	Feed	
	Penicillium lilacinum	Stch		
	Penicillium roqueforti	Ches Fats		
Pseudomonas	Pseudomonas alcaligenes			Ldry
	Pseudomonas paucimobilis	Soup		
Rhizopus	Rhizomucor miehei	Ches		Lthr
	Rhizopus delemar	Bake		
	Rhizopus delemar	Ches Fats		
	Rhizopus niveus	Bevr Ches Fats Meat Stch		
	Rhizopus oryzae	Bake Bevr Ches Fish Meat Stch		
	Rhizopus oryzae or arrhizus	Bake Ches Fats		Misc
	<b>Saccharomyces cerevisiae</b>	Bevr Conf Sugr	Feed	
Streptomyces	<b>Scytalidium thermophilum</b>			Text
	Streptomyces fradiae		Feed	Misc
	Streptomyces lividans	Stch		Text
	Streptomyces murinus	Stch		
	Streptomyces olivochromogenes	Stch		
	Streptomyces rubiginosus	Stch		
	<b>Streptoverticillium mobaraense</b>	Bake Ches Conf Diet Fish Ice Meat Milk Stch	Feed	
Trichoderma	Trichoderma harzianum	Bevr		
	Trichoderma reesei or longibrachiatum	Bake Bevr Ches Choc Egg Frut Diet Fats Frut Meat Milk Spic Stch	Feed	Misc Pulp Text
	Trichoderma viride	Bake Bevr Stch		Misc

Bake = Bakery, Bevr = Beverages (soft drinks, beer, wine), Ches = Cheese, Choc = Cocoa, chocolate, coffee and tea, Conf = Confectionary, Diet = Dietary food, Dish = Dish-washing powder, Egg = Egg, Fats = Fats and oils, Fish = Fish, Frut = Fruit and vegetables, Ice = Edible ice, Ldry = Laundry, Lthr = Leather, Meat = Meat, Milk = Milk, Misc = Miscellaneous, Pulp = Pulp and paper, Sald = Salads, Soup = Soups and broths, Spic = Spices and flavours, Stch = Cereal and starch, Sugr = Sugar and honey, Text = Textile, Wast = Wastewater treatment.

Table 56: List of Enzymes marketed in the EU and produced by GMM. Source: Derived from Table 54, AMFEP Oct. 2001

Principal enzymatic activity	Host organism/ Production organism	Donor organism	IUB	CAS	EINECS	Food	Feed	Technical
Acetolactate decarboxylase (alpha)	Bacillus amyloliquefaciens or subtilis	Bacillus sp.	4.1.1.5	9025-02-9		Bevr		
Aminopeptidase	Trichoderma reesei or longibrachiatum	Aspergillus sp.	3.4.11.x@	9031-94-1	232-874-6	Ches Egg Meat Milk Spic		Misc
Amylase (alpha)	Bacillus amyloliquefaciens or subtilis	Bacillus sp.	3.2.1.1	9000-90-2	232-565-6	Bake Bevr Stch	Feed	
Amylase (alpha)	Bacillus amyloliquefaciens or subtilis	Thermoactinomyces sp.	3.2.1.1	9000-90-2	232-565-6	Bake	Feed	
Amylase (alpha)	Bacillus licheniformis	Bacillus sp.	3.2.1.1	9000-90-2	232-565-6	Bevr Stch Sugr		Dish Ldry Misc Pulp Text
Arabinofuranosidase	Aspergillus niger	Aspergillus sp.	3.2.1.55	9067-74-7	232-957-7	Bevr		
Catalase	Aspergillus niger	Aspergillus sp.	1.11.1.6	9001-05-2	232-577-1	Bake Bev Ches Egg Fats Stch Sugr		Wast
Cellulase	Aspergillus oryzae	Humicola sp.	3.2.1.4	9012-54-8	232-734-4			Ldry Pulp Text
Cellulase	Aspergillus oryzae	Myceliophthora sp.	3.2.1.4	9012-54-8	232-734-4			Text
Cellulase	Aspergillus oryzae	Thielavia sp.	3.2.1.4	9012-54-8	232-734-4			Text
Cellulase	Trichoderma reesei or longibrachiatum	Trichoderma sp.	3.2.1.4	9012-54-8	232-734-4		Feed	Text
Chymosin	Aspergillus niger var. awamori	Calf stomach	3.4.23.4	9001-98-3	232-645-0	Ches		
Chymosin	Kluyveromyces lactis	Calf stomach	3.4.23.4	9001-98-3	232-645-0	Ches		
Cyclodextrin glucanotransferase	Bacillus licheniformis	Thermoanaerobacter sp.	2.4.1.19	9030-09-5		Stch		
Galactosidase (alpha)	Aspergillus oryzae	Aspergillus sp.	3.2.1.22	9025-35-8	232-792-0		Feed	
Galactosidase (alpha)	Saccharomyces cerevisiae	Guar plant	3.2.1.22	9025-35-8	232-792-0		Feed	
Glucanase (beta)	Bacillus amyloliquefaciens or subtilis	Bacillus sp.	3.2.1.6	62213-14-3	263-462-4	Bevr	Feed	
Glucanase (beta)	Trichoderma reesei or longibrachiatum	Trichoderma sp.	3.2.1.6	62213-14-3	263-462-4	Stch	Feed	Text
Glucoamylase or Amyloglucosidase	Aspergillus niger	Aspergillus sp.	3.2.1.3	9032-08-0	232-877-2	Bevr Frut Stch		
Glucose isomerase	Streptomyces lividans	Actinoplanes sp.	5.3.1.5	9055-00-9	232-944-6	Stch		
Glucose isomerase	Streptomyces rubiginosus	Streptomyces sp.	5.3.1.5	9055-00-9	232-944-6	Stch		
Glucose oxidase	Aspergillus niger	Aspergillus sp.	1.1.3.4	9001-37-0	232-601-0	Bake Egg		
Hemicellulase	Bacillus amyloliquefaciens or subtilis	Bacillus sp.	*	9025-56-3	232-799-9	Bake Stch	Feed	
Laccase	Aspergillus oryzae	Myceliophthora sp.	1.10.3.2	80498-15-3				Text
Laccase	Aspergillus oryzae	Polyporus sp.	1.10.3.2	80498-15-3				Text
Lipase, triacylglycerol	Aspergillus oryzae	Candida sp.	3.1.1.3	9001-62-1	232-619-9	Fats		Misc Text
Lipase, triacylglycerol	Aspergillus oryzae	Rhizomucor sp.	3.1.1.3	9001-62-1	232-619-9	Ches Fats Spic		Misc
Lipase, triacylglycerol	Aspergillus oryzae	Thermomyces sp.	3.1.1.3	9001-62-1	232-619-9	Bake Fats		Dish Ldry Lthr Pulp
Lipase, triacylglycerol	Pseudomonas alcaligenes	Pseudomonas sp.	3.1.1.3	9001-62-1	232-619-9			Ldry
Maltogenic amylase	Bacillus amyloliquefaciens	Bacillus sp.	3.2.1.133	160611-47-2		Bake Stch		

Principal enzymatic activity	Host organism/ Production organism	Donor organism	IUB	CAS	EINECS	Food	Feed	Technical
Mannanase (endo-1,4-beta)	or subtilis Trichoderma reesei or longibrachiatum	Trichoderma sp.	3.2.1.78	37288-54-3	253-446-5		Feed	
Pectate lyase	Bacillus licheniformis	Bacillus sp.	4.2.2.2	9015-75-2	232-766-9			Text
Pectin lyase	Aspergillus niger var. awamori	Aspergillus sp.	4.2.2.10	9033-35-6	232-894-5	Bevr Choc Frut	Feed	Text
Pectin lyase	Trichoderma reesei or longibrachiatum	Aspergillus sp.	4.2.2.10	9033-35-6	232-894-5	Bevr Choc Frut	Feed	Text
Pectin methylesterase or Pectinesterase	Aspergillus niger	Aspergillus sp.	3.1.1.11	9025-98-3	232-807-0	Bevr Choc Frut	Feed	
Pectin methylesterase or Pectinesterase	Aspergillus oryzae	Aspergillus sp.	3.1.1.11	9025-98-3	232-807-0	Bevr Frut		Misc
Pectin methylesterase or Pectinesterase	Trichoderma reesei or longibrachiatum	Aspergillus sp.	3.1.1.11	9025-98-3	232-807-0	Bevr Frut	Feed	Text
Penicillin amidase	Alcaligenes faecalis	Alcaligenes sp.	3.5.1.11	9014-06-6	232-753-8			Misc
Phospholipase A	Trichoderma reesei or longibrachiatum	Aspergillus sp.	3.1.1.4	9001-84-7	232-637-7	Bake Fats	Feed	Text
Phospholipase B	Trichoderma reesei or longibrachiatum	Aspergillus sp.	3.1.1.5	9001-85-8		Bake Stch	Feed	
Phytase	Aspergillus niger	Aspergillus sp.	3.1.3.8	9001-77-8	232-630-9		Feed	
Phytase	Trichoderma reesei or longibrachiatum	Aspergillus sp.	3.1.3.8	9001-77-8	232-630-9		Feed	
Polygalacturonase or Pectinase	Trichoderma reesei or longibrachiatum	Aspergillus sp.	3.2.1.15	9032-75-1	232-885-6	Bevr Choc Frut	Feed	Text
Protease (incl. milkclotting enzymes)	Aspergillus oryzae	Aspergillus sp.	3.4.2x.xx#	9001-92-7	232-642-4			Lthr
Protease (incl. milkclotting enzymes)	Aspergillus oryzae	Rhizomucor sp.	3.4.2x.xx#	9001-92-7	232-642-4	Ches		
Protease (incl. milkclotting enzymes)	Bacillus alcalophilus	Bacillus sp.	3.4.2x.xx#	9001-92-7	232-642-4			Ldry
Protease (incl. milkclotting enzymes)	Bacillus amyloliquefaciens or subtilis	Bacillus sp.	3.4.2x.xx#	9001-92-7	232-642-4	Bake Bevr Ches Fish Meat Milk Stch	Feed	
Protease (incl. milkclotting enzymes)	Bacillus halodurans or lentus	Bacillus sp.	3.4.2x.xx#	9001-92-7	232-642-4			Dish Ldry Lthr
Protease (incl. milkclotting enzymes)	Bacillus licheniformis	Bacillus sp.	3.4.2x.xx#	9001-92-7	232-642-4	Fish Meat		Dish Ldry
Pullulanase	Bacillus licheniformis	Bacillus sp.	3.2.1.41	9075-68-7	232-983-9	Stch		
Pullulanase	Bacillus subtilis	Bacillus sp.	3.2.1.41	9075-68-7	232-983-9	Bevr Stch		
Pullulanase	Klebsiella planticola	Klebsiella sp.	3.2.1.41	9075-68-7	232-983-9	Bevr Stch		
Xylanase	Aspergillus niger	Aspergillus sp.	3.2.1.8	9025-57-4	232-800-2	Bake Bevr	Feed	
Xylanase	Aspergillus niger var. awamori	Aspergillus sp.	3.2.1.8	9025-57-4	232-800-2	Bake		
Xylanase	Aspergillus oryzae	Aspergillus sp.	3.2.1.8	9025-57-4	232-800-2	Stch		
Xylanase	Aspergillus oryzae	Thermomyces sp.	3.2.1.8	9025-57-4	232-800-2	Bake	Feed	
Xylanase	Bacillus amyloliquefaciens or subtilis	Bacillus sp.	3.2.1.8	9025-57-4	232-800-2	Bake Bevr Stch	Feed	Pulp Text

Principal enzymatic activity	Host organism/ Production organism	Donor organism	IUB	CAS	EINECS	Food	Feed	Technical
Xylanase	Bacillus licheniformis	Bacillus sp.	3.2.1.8	9025-57-4	232-800-2	Stch		Pulp
Xylanase	Trichoderma reesei or longibrachiatum	Trichoderma sp.	3.2.1.8	9025-57-4	232-800-2	Bevr Stch	Feed	Pulp
Lactase or Galactosidase (beta)	Kluyveromyces lactis	Kluyveromyces sp.	3.2.1.23	9031-11-2	232-864-1	Ice Milk		
Lipase, triacylglycerol	Aspergillus oryzae	Fusarium sp.	3.1.1.3	9001-62-1	232-619-9	Bake Fats		
Phytase	Aspergillus oryzae	Peniophora sp.	3.1.3.8	9001-77-8	232-630-9		Feed	
Protease (incl. milkclotting enzymes)	Cryphonectria or Endothia parasitica	Cryphonectria sp.	3.4.2x.xx#	9001-92-7	232-642-4	ches		
Pullulanase	Trichoderma reesei or longibrachiatum	Hormoconis sp.	3.2.1.41	9075-68-7	232-983-9	Bake	Feed	
Xylanase	Trichoderma reesei or longibrachiatum	Actinomadura sp.	3.2.1.8	9025-57-4	232-800-2			

\* Those enzymes which do not have an IUB number are enzyme complexes, where the listed activity is the result of the sum of many single active enzyme proteins. @ There is no general IUB number for aminopeptidases. But all these enzymes fall under the 3.4.11.x category. # There is no general IUB number for proteases. But all these enzymes fall under the 3.4.2x category. Bake = Bakery, Bevr = Beverages (soft drinks, beer, wine), Ches = Cheese, Choc = Cocoa, chocolate, coffee and tea, Conf = Confectionary, Diet = Dietary food, Dish = Dishwashing powder, Egg = Egg, Fats = Fats and oils, Fish = Fish, Frut = Fruit and vegetables, Ice = Edible ice, Ldry = Laundry, Lthr = Leather, Meat = Meat, Milk = Milk, Misc = Miscellaneous, Pulp = Pulp and paper, Sald = Salads, Soup = Soups and broths, Spic = Spices and flavours, Stch = Cereal and starch, Sugr = Sugar and honey, Text = Textile, Wast = Wastewater treatment.



Table 57: Enzymes listed in EINECS

	<b>EINECS-Name</b>	<b>EINECS-No</b>	<b>CAS-No</b>
1	Coccarboxylase	205-836-1	154-87-0
2	Esterase, acetyl choline	232-559-3	9000-81-1
3	Amylase, bacterial	232-560-9	9000-85-5
4	Aminotransferase, alanine	232-561-4	9000-86-6
5	Oxidase, d-amino acid	232-563-5	9000-88-8
6	Oxidase, l-amino acid	232-564-0	9000-89-9
7	Amylase, alpha-	232-565-6	9000-90-2
8	Amylase, beta-	232-566-1	9000-91-3
9	Amylase	232-567-7	9000-92-4
10	Apyrase	232-569-8	9000-95-7
11	Arginase	232-570-3	9000-96-8
12	Aminotransferase, aspartate	232-571-9	9000-97-9
13	Carbohydrase	232-575-0	9001-02-9
14	Dehydratase, carbonate	232-576-6	9001-03-0
15	Catalase	232-577-1	9001-05-2
16	Chitinase	232-578-7	9001-06-3
17	Esterase, choline	232-579-2	9001-08-5
18	Collagenase	232-582-9	9001-12-1
19	Coagulase	232-583-4	9001-13-2
20	Kinase (phosphorylating), creatine	232-585-5	9001-15-4
21	Oxidase, cytochrome	232-586-0	9001-16-5
22	Dehydrogenase, lipoamide	232-587-6	9001-18-7
23	Amylase, alpha-, Aspergillus oryzae	232-588-1	9001-19-8
24	Glucosidase, beta-	232-589-7	9001-22-3
25	Galactosidase	232-600-5	9001-34-7
26	Oxidase, glucose	232-601-0	9001-37-0
27	Dehydrogenase, glucose 6-phosphate	232-602-6	9001-40-5
28	Isomerase, glucose phosphate	232-603-1	9001-41-6
29	Glucosidase, alpha-	232-604-7	9001-42-7
30	Glucosidase, 1,3-alpha-	232-605-2	9001-43-8
31	Glucuronidase, beta-	232-606-8	9001-45-0
32	Dehydrogenase, glutamate	232-607-3	9001-46-1
33	Reductase, glutathione	232-608-9	9001-48-3
34	Dehydrogenase, glyceraldehyde phosphate	232-609-4	9001-50-7
35	Kinase (phosphorylating), hexo-	232-611-5	9001-51-8
36	Phosphatase, fructose di-	232-612-0	9001-52-9
37	Oxidase, diamine	232-613-6	9001-53-0
38	Hyaluronidase	232-614-1	9001-54-1
39	Fructofuranosidase, beta-	232-615-7	9001-57-4

	<b>EINECS-Name</b>	<b>EINECS-No</b>	<b>CAS-No</b>
40	Kinase (phosphorylating), pyruvate	232-616-2	9001-59-6
41	Dehydrogenase, lactate	232-617-8	9001-60-9
42	Aminopeptidase, cytosol	232-618-3	9001-61-0
43	Lipase, triacylglycerol	232-619-9	9001-62-1
44	Dehydrogenase, malate	232-622-5	9001-64-3
45	Oxidase, monoamine	232-623-0	9001-66-5
46	Neuraminidase	232-624-6	9001-67-6
47	Carbamoyltransferase, ornithine	232-625-1	9001-69-8
48	Proteinase, pancreatic	232-626-7	9001-72-3
49	Penicillinase	232-628-8	9001-74-5
50	Phosphatase, acid	232-630-9	9001-77-8
51	Phosphatase, alkaline	232-631-4	9001-78-9
52	Kinase (phosphorylating), phosphofructo-	232-633-5	9001-80-3
53	Phosphomutase, glucose	232-634-0	9001-81-4
54	Dehydrogenase, phosphogluconate	232-635-6	9001-82-5
55	Kinase (phosphorylating), phosphoglycerate	232-636-1	9001-83-6
56	Phospholipase A2	232-637-7	9001-84-7
57	Phospholipase C	232-638-2	9001-86-9
58	Phospholipase D	232-639-8	9001-87-0
59	Proteinase	232-642-4	9001-92-7
60	Oxidase, pyruvate	232-644-5	9001-96-1
61	Nuclease, ribo-	232-646-6	9001-99-4
62	Kinase (enzyme-activating), strepto-	232-647-1	9002-01-1
63	Decarboxylase, tyrosine	232-652-9	9002-09-9
64	Oxygenase, monophenol mono-	232-653-4	9002-10-2
65	Oxidase, urate	232-655-5	9002-12-4
66	Urease	232-656-0	9002-13-5
67	Oxidase, xanthine	232-657-6	9002-17-9
68	Nuclease, deoxyribo-	232-667-0	9003-98-9
69	Peroxidase	232-668-6	9003-99-0
70	Lipase, lipoprotein	232-669-1	9004-02-8
71	Elastase	232-670-7	9004-06-2
72	Methyltransferase, catechol	232-727-6	9012-25-3
73	Cephalosporinase	232-728-1	9012-26-4
74	Synthetase, acetyl coenzyme A	232-729-7	9012-31-1
75	Acetylglucosaminidase, beta-	232-730-2	9012-33-3
76	Aminoacylase	232-732-3	9012-37-7
77	Glucosidase, amylo-1,6-	232-733-9	9012-47-9
78	Cellulase	232-734-4	9012-54-8
79	Amidase	232-736-5	9012-56-0

	<b>EINECS-Name</b>	<b>EINECS-No</b>	<b>CAS-No</b>
80	Phosphorylase b	232-737-0	9012-69-5
81	Lyase, citrate	232-740-7	9012-83-3
82	Nucleotidyltransferase, deoxyribonucleate	232-741-2	9012-90-2
83	Amylase, fungal	232-742-8	9013-01-8
84	Kinase (phosphorylating), adenylate	232-743-3	9013-02-9
85	Phosphatase	232-744-9	9013-05-2
86	Carboxykinase, phosphopyruvate (pyrophosphate)	232-745-4	9013-12-1
87	Synthetase, acyl coenzyme A	232-747-5	9013-18-7
88	Nuclease, micrococcal	232-748-0	9013-53-0
89	Peroxidase, glutathione	232-749-6	9013-66-5
90	Luciferase	232-751-7	9014-00-0
91	Amidase, penicillin	232-753-8	9014-06-6
92	Hydratase, phosphopyruvate	232-754-3	9014-08-8
93	Nucleotidyltransferase, polyribonucleotide	232-755-9	9014-12-4
94	Nucleotidyltransferase, ribonucleate	232-756-4	9014-24-8
95	Synthetase, succinyl coenzyme A (guanosine diphosphate-forming)	232-758-5	9014-36-2
96	Transaldolase	232-759-0	9014-46-4
97	Peptidase, entero-	232-761-1	9014-74-8
98	Asparaginase	232-765-3	9015-68-3
99	Lyase, pectate	232-766-9	9015-75-2
100	Glucanase	232-767-4	9015-78-5
101	Dehydrogenase, beta-hydroxy steroid	232-769-5	9015-81-0
102	Synthetase, polydeoxyribonucleotide	232-770-0	9015-85-4
103	Sulfatase, aryl-	232-772-1	9016-17-5
104	Esterase, carboxyl	232-773-7	9016-18-6
105	Isomerase, triose phosphate	232-774-2	9023-78-3
106	Isomerase, mannose phosphate	232-775-8	9023-88-5
107	Protopectinase	232-776-3	9023-92-1
108	Lyase, chondroitin ABC	232-777-9	9024-13-9
109	Ammonia-lyase, phenylalanine	232-778-4	9024-28-6
110	Lyase, mandelonitrile	232-780-5	9024-43-5
111	Aldolase, fructose diphosphate	232-781-0	9024-52-6
112	Decarboxylase, glutamate	232-782-6	9024-58-2
113	Decarboxylase, lysine	232-783-1	9024-76-4
114	Pyrophosphatase, inorganic	232-784-7	9024-82-2
115	Decarboxylase, oxalate	232-785-2	9024-97-9
116	Creatininase	232-786-8	9025-13-2
117	Amidase, aryl acyl-	232-787-3	9025-18-7
118	Carboxypeptidase B	232-788-9	9025-24-5

	<b>EINECS-Name</b>	<b>EINECS-No</b>	<b>CAS-No</b>
119	Carboxypeptidase, glycine	232-789-4	9025-25-6
120	Dipeptidase, proline	232-791-5	9025-32-5
121	Galactosidase, alpha-	232-792-0	9025-35-8
122	Keratinase, Streptomyces	232-793-6	9025-41-6
123	Mannosidase, alpha-	232-794-1	9025-42-7
124	Proteinase, Aspergillus acid	232-796-2	9025-49-4
125	Xylosidase, exo-1,4-beta-	232-797-8	9025-53-0
126	Xylanase, endo-1,3-	232-798-3	9025-55-2
127	Hemicellulase	232-799-9	9025-56-3
128	Xylanase, endo-1,4-	232-800-2	9025-57-4
129	Nuclease, deoxyribo-, II	232-801-8	9025-64-3
130	Inulinase	232-802-3	9025-67-6
131	Dextranase	232-803-9	9025-70-1
132	Tannase	232-804-4	9025-71-2
133	Phosphodiesterase	232-806-5	9025-82-5
134	Esterase, pectin	232-807-0	9025-98-3
135	Esterase, cholesterol	232-808-6	9026-00-0
136	Nuclease, guanyloribo-	232-809-1	9026-12-4
137	Uridyltransferase, hexose 1-phosphate	232-810-7	9026-21-5
138	Uridyltransferase, glucose 1-phosphate	232-811-2	9026-22-6
139	Kinase (phosphorylating), nucleoside mono-phosphate	232-812-8	9026-50-0
140	Kinase (phosphorylating), nucleoside di-phosphate	232-813-3	9026-51-1
141	Kinase (phosphorylating), guanylate	232-814-9	9026-59-9
142	Kinase (phosphorylating), choline	232-815-4	9026-67-9
143	Deaminase, adenosine	232-817-5	9026-93-1
144	Kinase (phosphorylating), acetate	232-818-0	9027-42-3
145	Nucleotidyltransferase, terminal deoxyribo-	232-819-6	9027-67-2
146	Nucleotidase, 5'-	232-820-1	9027-73-0
147	Synthase, citrate	232-821-7	9027-96-7
148	Dehydrogenase, alcohol (nicotinamide adenine dinucleotide phosphate)	232-823-8	9028-12-0
149	Dehydrogenase, glycerol	232-824-3	9028-14-2
150	Dehydrogenase, l-iditol	232-825-9	9028-21-1
151	Dehydrogenase, uridine diphosphoglucose	232-826-4	9028-26-6
152	Reductase, glyoxylate	232-828-5	9028-32-4
153	Dehydrogenase, d-lactate	232-829-0	9028-36-8
154	Dehydrogenase, 3-hydroxybutyrate	232-830-6	9028-38-0
155	Dehydrogenase, 3-hydroxyacyl coenzyme A	232-831-1	9028-40-4
156	Dehydrogenase, 20beta-hydroxy steroid	232-832-7	9028-42-6

	<b>EINECS-Name</b>	<b>EINECS-No</b>	<b>CAS-No</b>
157	Dehydrogenase, malate (decarboxylating)	232-833-2	9028-46-0
158	Dehydrogenase, malate (oxalacetate-decarboxylating) (nicotinamide adenine dinucleotide phosphate)	232-834-8	9028-47-1
159	Dehydrogenase, isocitrate (nicotinamide adenine dinucleotide phosphate)	232-835-3	9028-48-2
160	Dehydrogenase, glucose	232-836-9	9028-53-9
161	Dehydrogenase, galactose	232-837-4	9028-54-0
162	Dehydrogenase, 3alpha-hydroxy steroid	232-839-5	9028-56-2
163	Oxidase, choline	232-840-0	9028-67-5
164	Oxygenase, lactate 2-mono-	232-841-6	9028-72-2
165	Oxidase, cholesterol	232-842-1	9028-76-6
166	Oxidase, galactose	232-843-7	9028-79-9
167	Dehydrogenase, formate	232-844-2	9028-85-7
168	Dehydrogenase, aldehyde	232-845-8	9028-86-8
169	Dehydrogenase, aldehyde (nicotinamide adenine dinucleotide (phosphate))	232-846-3	9028-88-0
170	Dehydrogenase, alanine	232-847-9	9029-06-5
171	Dehydrogenase, glutamate (nicotinamide adenine dinucleotide (phosphate))	232-848-4	9029-12-3
172	Oxidase, sarcosine	232-850-5	9029-22-5
173	Oxidase, thiol	232-851-0	9029-39-4
174	Oxidase, ascorbate	232-852-6	9029-44-1
175	Oxygenase, lip-	232-853-1	9029-60-1
176	Acetyltransferase, carnitine	232-854-7	9029-90-7
177	Acetyltransferase, phosphate	232-855-2	9029-91-8
178	Phosphorylase, maltose	232-856-8	9030-19-7
179	Phosphorylase, purine nucleoside	232-857-3	9030-21-1
180	Phosphoribosyltransferase, orotate	232-858-9	9030-25-5
181	Aminotransferase, glutamine-keto acid	232-859-4	9030-44-8
182	Kinase (phosphorylating), glucono-	232-861-5	9030-55-1
183	Kinase (phosphorylating), glycerol	232-862-0	9030-66-4
184	Dehydrogenase, 15-hydroxyprostaglandin	232-863-6	9030-87-9
185	Galactosidase, beta-	232-864-1	9031-11-2
186	Linamarinase	232-865-7	9031-18-9
187	Peroxidase, iodide	232-866-2	9031-28-1
188	Isomerase, steroid DELTA-	232-867-8	9031-36-1
189	Sphingomyelinase C	232-869-9	9031-54-3
190	Dehydrogenase, alcohol	232-870-4	9031-72-5
191	Epimerase, aldose 1-	232-872-5	9031-76-9
192	Oxidase, oxalate	232-873-0	9031-79-2
193	Aminopeptidase	232-874-6	9031-94-1

	<b>EINECS-Name</b>	<b>EINECS-No</b>	<b>CAS-No</b>
194	Peptidase	232-875-1	9031-96-3
195	Deaminase, formiminotetrahydrofolate cyclo-	232-876-7	9032-05-7
196	Amylase, gluco-	232-877-2	9032-08-0
197	Phosphorylase a	232-878-8	9032-10-4
198	Peroxidase, nicotinamide adenine dinucleotide	232-879-3	9032-24-0
199	Phosphomutase, glycerate (diphosphoglycerate cofactor)	232-880-9	9032-62-6
200	Adenylyltransferase, nicotinamide mononucleotide	232-883-5	9032-70-6
201	Esterase, aryl	232-884-0	9032-73-9
202	Polygalacturonase	232-885-6	9032-75-1
203	Formiminotransferase, glutamate	232-886-1	9032-83-1
204	Hydratase, fumarate	232-887-7	9032-88-6
205	Glycosidase	232-888-2	9032-92-2
206	Glucosidase	232-889-8	9033-06-1
207	Glycosyltransferase	232-890-3	9033-07-2
208	Lyase, lactoylglutathione	232-891-9	9033-12-9
209	Deaminase, guanine	232-892-4	9033-16-3
210	Lyase, pectin	232-894-5	9033-35-6
211	Phosphorylase	232-904-8	9035-74-9
212	Dehydrogenase	232-907-4	9035-82-9
213	Proteinase, Streptomyces griseus	232-909-5	9036-06-0
214	Nuclease, exodeoxyribo-, III	232-912-1	9037-44-9
215	Fucosidase, alpha-I-	232-914-2	9037-65-4
216	Aminotransferase, aminobutyrate	232-915-8	9037-67-6
217	Methyltransferase, noradrenaline N-	232-916-3	9037-68-7
218	Kinase (enzyme-activating), uro-	232-917-9	9039-53-6
219	Proteinase, Bothrops atrox serine	232-918-4	9039-61-6
220	Phosphodiesterase, cyclic 3',5'-nucleotide	232-920-5	9040-59-9
221	Glucanase, 1,3-beta-	232-927-3	9044-93-3
222	Polygalacturonase, exo-	232-929-4	9045-35-6
223	Glutamyltransferase, gamma-	232-931-5	9046-27-9
224	Oxidase, glycerol phosphate	232-932-0	9046-28-0
225	Proteinase, Agkistrodon serine	232-933-6	9046-56-4
226	Carboxypeptidase, serine	232-934-1	9046-67-7
227	Aminopeptidase, microsomal	232-942-5	9054-63-1
228	Dismutase, superoxide	232-943-0	9054-89-1
229	Isomerase, glucose	232-944-6	9055-00-9
230	Amylase, iso-	232-956-1	9067-73-6
231	Arabinofuranosidase, alpha-I-	232-957-7	9067-74-7

	<b>EINECS-Name</b>	<b>EINECS-No</b>	<b>CAS-No</b>
232	Carboxylase, phosphopyruvate (phosphate)	232-958-2	9067-77-0
233	Deaminase	232-959-8	9067-84-9
234	Dehydrogenase, 2-hydroxybutyrate	232-960-3	9067-92-9
235	Dehydrogenase, malate (pig heart)	232-961-9	9067-93-0
236	Naringinase	232-962-4	9068-31-9
237	Nucleotidyltransferase, deoxyribonucleate, RNA-dependent	232-964-5	9068-38-6
238	Phosphodiesterase II	232-965-0	9068-54-6
239	Proteinase, microbial neutral	232-966-6	9068-59-1
240	Arabinogalactanase	232-967-1	9073-41-0
241	Glucosidase, exo-1,3-beta-	232-968-7	9073-49-8
242	Kinase (phosphorylating), pyruvate (rabbit muscle)	232-969-2	9073-59-0
243	Lactamase, beta-	232-970-8	9073-60-3
244	Oxidase, alcohol	232-971-3	9073-63-6
245	Proteinase, Bacillus alkaline	232-972-9	9073-77-2
246	Proteinase, Mucor aspartic	232-975-5	9073-79-4
247	Carboxykinase, phosphopyruvate (adenosine triphosphate)	232-976-0	9073-94-3
248	Proteinase, Aspergillus alkaline	232-977-6	9074-07-1
249	Hydrolase, gamma-glutamyl	232-978-1	9074-87-7
250	Glucanase, beta-	232-979-7	9074-98-0
251	Glucanase, endo-beta-	232-980-2	9074-99-1
252	Aminopeptidase, pyroglutamate	232-981-8	9075-21-2
253	Dehydrogenase, glycerol phosphate	232-982-3	9075-65-4
254	Pullulanase	232-983-9	9075-68-7
255	Dehydrogenase, lactate (cytochrome)	232-986-5	9078-32-4
256	Dehydrogenase, reduced nicotinamide adenine dinucleotide	232-988-6	9079-67-8
257	Proteinase, Aspergillus oryzae neutral	232-990-7	9080-55-1
258	Proteinase, Bacillus subtilis metallo-	232-991-2	9080-56-2
259	Proteinase, oryzin	232-992-8	9081-30-5
260	Carboxypeptidase A	234-297-5	11075-17-5
261	Proteinase, Bacillus subtilis, sutilain	235-390-3	12211-28-8
262	Glucanase, 1,3-alpha-	253-393-8	37205-50-8
263	Nuclease, ribo- (purine-specific)	253-394-3	37205-57-5
264	Proteinase, Endothia aspartic	253-395-9	37205-60-0
265	Kinase (phosphorylating), polynucleotide 5'-hydroxyl	253-400-4	37211-65-7
266	Hesperidinase	253-402-5	37213-47-1
267	Dehydrogenase, sarcosine	253-414-0	37228-65-2
268	Glucanase, 1,6-beta-	253-415-6	37228-69-6

	<b>EINECS-Name</b>	<b>EINECS-No</b>	<b>CAS-No</b>
269	Dehydrogenase, alcohol (nicotinamide adenine dinucleotide (phosphate))	253-428-7	37250-10-5
270	Dehydrogenase, succinate semialdehyde (nicotinamide adenine dinucleotide (phosphate))	253-429-2	37250-88-7
271	Lyase, hyaluronate	253-430-8	37259-53-3
272	Proteinase, serine	253-431-3	37259-58-8
273	Nuclease, ribo- (non-base specific)	253-438-1	37278-25-4
274	Xylanase	253-439-7	37278-89-0
275	Nuclease, single-stranded nucleate endo-	253-443-9	37288-25-8
276	Glucosidase, exo-1,4-beta-	253-444-4	37288-52-1
277	Mannanase, endo-1,4-beta-	253-446-5	37288-54-3
278	Carrageenanase, kappa-	253-447-0	37288-59-8
279	Proteinase, Myxobacter alpha-lytic	253-448-6	37288-76-9
280	Deiminase, creatinine	253-449-1	37289-15-9
281	Proteinase, Serratia extracellular	253-457-5	37312-62-2
282	Arabinanase	253-463-8	37325-54-5
283	Hyaluronoglucosaminidase	253-464-3	37326-33-3
284	Cellobiohydrolase, exo-	253-465-9	37329-65-0
285	Creatinase	253-471-1	37340-58-2
286	Hydrolase, creatinine cyclo-	253-472-7	37340-59-3
287	Nuclease, streptococcal deoxyribo-	253-473-2	37340-82-2
288	Diaphorase	253-474-8	37340-89-9
289	Proteinase, Aspergillus parasiticus neutral	254-418-5	39335-09-6
290	Galactanase	254-421-1	39346-28-6
291	Proteinase, Aspergillus niger neutral	254-422-7	39346-39-9
292	Dehydratase, carbonate (human erythrocyte B)	254-441-0	39386-01-1
293	Elastase (pig pancreas)	254-453-6	39445-21-1
294	Proteinase, Tritirachium album serine	254-457-8	39450-01-6
295	Helicase	255-913-9	42613-29-6
296	Proteinase, Bacillus polymyxa neutral	255-914-4	42613-33-2
297	Lyase, methionine	255-916-5	42616-25-1
298	Galactomannanase	256-778-9	50812-17-4
299	Carrageenanase, lambda-	256-860-4	50936-37-3
300	Proteinase, microbial acid	256-862-5	50936-52-2
301	Proteinase, microbial alkaline	256-863-0	50936-53-3
302	Proteinase, Bacillus subtilis	257-520-8	51931-23-8
303	Nuclease, bacteriophage T4 endodeoxyribo-, V	257-759-8	52227-85-7
304	Galactosidase, endo-beta-	258-130-0	52720-51-1
305	Xylosidase, alpha-	258-495-6	53362-86-0



	<b>EINECS-Name</b>	<b>EINECS-No</b>	<b>CAS-No</b>
306	Xylosidase, beta-	258-496-1	53362-87-1
307	Dehydratase, carbonate (human erythrocyte C)	258-634-0	53570-56-2
308	Pancrelipase	258-659-7	53608-75-6
309	Glucanase, alpha-	258-785-2	53801-47-1
310	Lipase cofactor	259-490-1	55126-92-6
311	Proteinase, Staphylococcus aureus serine	259-611-8	55354-32-0
312	Reductase, riboflavin mononucleotide (reduced nicotinamide adenine dinucleotide phosphate)	260-292-2	56626-29-0
313	Mannanase	262-402-4	60748-69-8
314	Luciferase (firefly luciferin)	263-359-4	61970-00-1
315	Glucanase, endo-1,3(4)-beta-	263-462-4	62213-14-3
316	Peroxidase (horseradish protein moiety reduced)	263-659-5	62628-26-6
317	Reductase, riboflavin (reduced nicotinamide adenine dinucleotide (phosphate))	264-772-2	64295-83-6
318	Proteinase, Bacillus subtilis serine	266-557-9	67051-83-6
319	Prothrombinase	276-433-6	72162-96-0
320	Proteinase, Myxobacter AL-1, II	276-716-4	72561-05-8
321	Proteinase, Bacillus neutral	278-547-1	76774-43-1
322	Proteinase, Aspergillus neutral	278-588-5	77000-13-6
323	Proteinase, aspartic	278-856-1	78169-47-8
324	Proteinase, Bacillus licheniformis serine	279-361-3	79986-26-8
325	Nuclease, restriction endodeoxyribo-, BglI	279-474-8	80449-04-3
326	Nuclease, restriction endodeoxyribo-, EcoRI	279-487-9	80498-17-5
327	Nuclease, restriction endodeoxyribo-, AluI	279-719-9	81295-04-7
328	Nuclease, restriction endodeoxyribo-, BamHI	279-720-4	81295-09-2
329	Nuclease, restriction endodeoxyribo-, BglII	279-722-5	81295-12-7
330	Nuclease, restriction endodeoxyribo-, EcoRII	279-723-0	81295-15-0
331	Nuclease, restriction endodeoxyribo-, HaeII	279-724-6	81295-17-2
332	Nuclease, restriction endodeoxyribo-, HaeIII	279-725-1	81295-18-3
333	Nuclease, restriction endodeoxyribo-, HhaI	279-726-7	81295-20-7
334	Nuclease, restriction endodeoxyribo-, HindII	279-727-2	81295-21-8
335	Nuclease, restriction endodeoxyribo-, HindIII	279-728-8	81295-22-9
336	Nuclease, restriction endodeoxyribo-, HinfI	279-729-3	81295-23-0
337	Nuclease, restriction endodeoxyribo-, HpaI	279-730-9	81295-24-1
338	Nuclease, restriction endodeoxyribo-, HpaII	279-731-4	81295-25-2
339	Nuclease, restriction endodeoxyribo-, KpnI	279-733-5	81295-27-4
340	Nuclease, restriction endodeoxyribo-, PstI	279-734-0	81295-32-1
341	Nuclease, restriction endodeoxyribo-, PvuI	279-735-6	81295-33-2
342	Nuclease, restriction endodeoxyribo-, PvuII	279-736-1	81295-34-3

	<b>EINECS-Name</b>	<b>EINECS-No</b>	<b>CAS-No</b>
343	Nuclease, restriction endodeoxyribo-, Sall	279-737-7	81295-38-7
344	Nuclease, restriction endodeoxyribo-, TaqI	279-738-2	81295-40-1
345	Nuclease, restriction endodeoxyribo-, XbaI	279-739-8	81295-42-3
346	Nuclease, restriction endodeoxyribo-, HincII	279-830-2	81811-55-4
347	Nuclease, restriction endodeoxyribo-, MspI	279-831-8	81811-56-5
348	Proteinase, submandibular gland serine, A	279-893-6	82047-85-6
349	Nuclease, restriction endodeoxyribo-, SmaI	279-944-2	82391-42-2
350	Nuclease, restriction endodeoxyribo-, ClaI	280-504-7	83589-01-9
351	Kinase (phosphorylating), protein, cAMP-dependent	283-495-8	84650-14-6
352	Enzymes, com., Driselase	286-055-3	85186-71-6
353	Nuclease, restriction endodeoxyribo-, SphI	286-569-8	85270-15-1
354	Dehydrogenase, glucose 6-phosphate, 5H-dibenz[b,f]azepine-5-carboxamide conjugate	291-170-7	90342-33-9
355	Dehydrogenase, glucose 6-phosphate, 5-ethylidihydro-5-phenyl-4,6(1H,5H)-pyrimidinedione conjugate	291-171-2	90342-34-0
356	Dehydrogenase, glucose 6-phosphate, 5-ethyl-5-phenyl-2,4,6(1H,3H,5H)-pyrimidinetrione conjugate §95979-76-3	291-172-8	90342-35-1
357	Chymopapain	232-580-8	9001-09-6
358	Ficin	232-599-1	9001-33-6
359	Papain	232-627-2	9001-73-4
360	Plasmin	232-640-3	9001-90-5
361	Rennin	232-645-0	9001-98-3
362	Thrombin	232-648-7	9002-04-4
363	Trypsin	232-650-8	9002-07-7
364	Chymotrypsin	232-671-2	9004-07-3
365	Subtilisin	232-752-2	9014-01-1
366	Clostripain	232-822-2	9028-00-6
367	Thermolysin	232-973-4	9073-78-3
368	Pancreatin	232-468-9	8049-47-6

Table 58: Enzymes listed in Annex I of Directive 67/548/EEC

Substance Name	Index No	EEC No	CAS No	Annex I Classification	Comment
delta-glucosidase	647-001-00-8	232-589-7	9001-22-3	R42	ESR <sup>1</sup>
cellulase	647-002-00-3	232-734-4	9012-54-8	R42	ESR, HPVC <sup>2</sup>
exo-cellobiohydrolase	647-003-00-9	253-465-9	37329-65-0	R42	ESR
cellulases, with the exception of those specified elsewhere in this Annex	647-004-00-4	#	#	R42	
alpha-amylase	647-015-00-4	232-565-6	9000-90-2	R42	ESR, LPVC <sup>3</sup>
amylases, with the exception of those specified elsewhere in the Annex	647-016-00-X	#	#	R42	
ficin	647-006-00-5	232-599-1	9001-33-6	Xi; R36/37/38 R42	ESR
papain	647-007-00-0	232-627-2	9001-73-4	Xi; R36/37/38 R42	ESR, LPVC
pepsin A	647-008-00-6	232-629-3	9001-75-6	Xi; R36/37/38 R42	ESR
rennin	647-009-00-1	232-645-0	9001-98-3	Xi; R36/37/38 R42	ESR
trypsin	647-010-00-7	232-650-8	9002-07-7	Xi; R36/37/38 R42	ESR
chymotrypsin	647-011-00-2	232-671-2	9004-07-3	Xi; R36/37/38 R42	ESR
proteinase, microbial neutral	647-013-00-3	232-966-6	9068-59-1	Xi; R36/37/38 R42	ESR
proteases, with the exception of those specified elsewhere in the Annex	647-014-00-9	#	#	Xi; R36/37/38 R42	

Substance Name	Index No	EEC No	CAS No	Annex I Classification	Comment
nex					
bromelain, juice	647-005-00-X	232-572-4	9001-00-7	Xi; R36/37/38 R42	ESR
subtilisin	647-012-00-8	232-752-2	9014-01-1	Xi; R37/38-41 R42	ESR, LPVC

<sup>1</sup> ESR: defined as existing substance according to the Existing Substances Regulation (793/93/EEC); <sup>2</sup> high production volume chemical; <sup>3</sup> low production volume chemical

*R-phrases relevant for classification of enzymes: R42: May cause sensitisation by inhalation. R36/37/38: Irritating to the eyes, respiratory system and skin.*

*R37/38-41: Irritating to the respiratory system and skin. Risk of serious damage to eyes.*

## 13.1.1 Annex

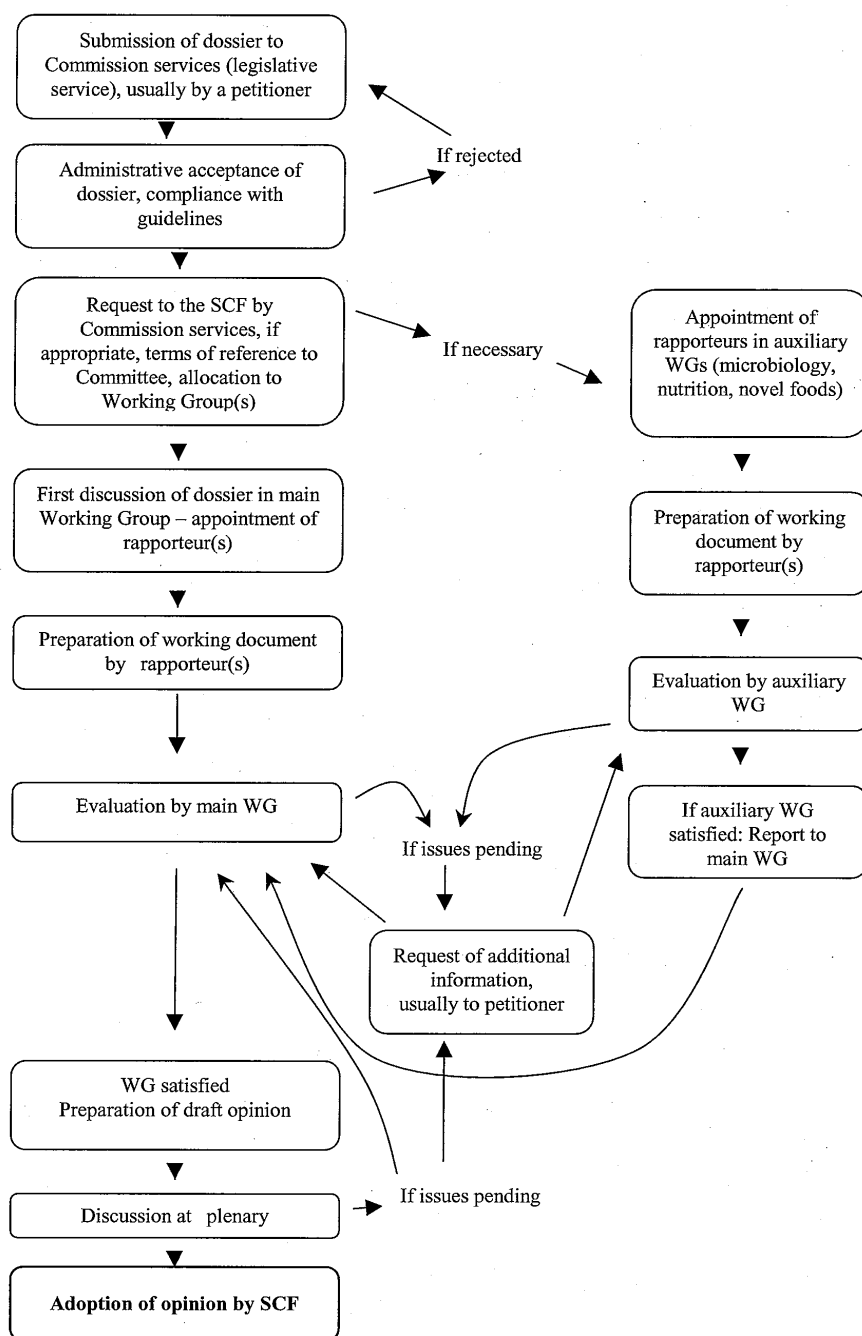


Figure 10: Flowchart of the process of evaluation of the SCF of a dossier on a food additive. Source: Scientific Committee on Food: Guidance on submissions for additive evaluations by the Scientific Committee on Food (opinion expressed on 11 July 2001). WG = working group.

Table 59: SCF Guidelines for food enzymes: Information to be supplied for evaluation by the SCF and conditions of use for an enzyme preparation to be used in foodstuff. Conditions and requirements regarded as relevant are displayed. The structure is according to the guidelines.

	Conditions of Use	Data Requirements	Method
<b>Administrative data</b>			
	N. a.	<ul style="list-style-type: none"> <li>– Name of applicant</li> <li>– Manufacturer of enzyme</li> <li>– Person responsible for the dossier</li> </ul>	N. a.
<b>Technical data</b>			
<b>1. Active components</b>			
1.1. Principal enzyme activities	N. s.	<ul style="list-style-type: none"> <li>– Systematic names</li> <li>– EC numbers</li> </ul>	N. s.
1.2. Enzyme activity	N. s.	Units/Weight or Units/Volume or (Quantity of enzyme preparation to be added to a given quantity of food)	N. s.
1.3. subsidiary enzymatic activities (side activities)	N. s.	List of subsidiary enzymatic activities (side activities) „whether they perform a useful function or not” (page 15)	N. s.
<b>2. Source materials</b>			
2.1. In case of animal sources	Source must comply with meat inspection requirements	Identification of animal and part of the animal	N. a.
2.2. In case of plant sources	N. s.	Identification of plant and part of the plant	N. a.
2.3. In case of microbial sources <sup>b</sup> (including GMM)	Must be discrete stable strains and well characterised (to enable to be assigned unique identities as the sources of the enzyme preparation)	N. s.; for GMM see 2.4 in this table	N. a.
2.4. In case of GMM			
– Host organism	N. s.	Information about host organism	N. a.

	Conditions of Use	Data Requirements	Method
– Vector	Must be free of harmful sequences, non-conjugative, and non-mobilizable	<ul style="list-style-type: none"> <li>– Identity and biology</li> <li>– Size</li> <li>– Restriction map</li> <li>– Possible full DNA sequence</li> <li>– Genes found on the vector</li> <li>– Marker genes</li> </ul>	N. s.
– Characterisation of introduced DNA	N. s.	<ul style="list-style-type: none"> <li>– DNA sequence(s) introduced</li> <li>– Number of inserted genes</li> <li>– Type of regulation (promoter activity)</li> <li>– Gene product(s)</li> <li>– Origin and pedigree of the genetic construct</li> </ul>	N. s.
– Donor organism		Identification of the donor organism	
<b>3. Manufacturing process</b>			
3.1. Method of manufacture	N. s.	In case of MO: <ul style="list-style-type: none"> <li>– Information on fermentation media</li> <li>– Information on fermentation conditions</li> </ul>	N. a.
3.2. Purification procedure	N. s.	Information on purification procedure	N. a.
<b>4. Carriers and other additives and ingredients</b>			
4.1. Carriers, diluents etc.	Must either be substances acceptable for the relevant food use or which are insoluble in food and removed before consumption	Information on carriers, diluents, excipients, supports and other additives and ingredients (including processing aids)	N. a.
4.2. In case of immobilised enzymes:	Carriers, immobilisation agents should be acceptable for the relevant use	Testing of potential leakage of immobilisation agents	N. s.
4.3. TOS (Total Organic Solids)	N. s.	TOS	TOS; see section 3.4
<b>5. Usage</b>			

	Conditions of Use	Data Requirements	Method
	N. s.	Technological function of the enzyme	N. a.
	N. s.	Types of foodstuff in which the enzyme is intended to be used	N. a.
	N. s.	Maximum amount of enzyme preparation to be used in each foodstuff	N. a.
<b>6. Stability and fate in food</b>			
	N. s.	Amount of enzyme preparation in the final food	N. a.
	N. s.	Main reaction products and possible reaction products not considered normal constituents of the diet, formed during production and storage	N. a.
	N. s.	Possible effects on nutrients	N. a.
<b>General requirements and specifications</b>			
<b>7. Hygiene</b>			
	Manufacturing of enzymes should be done according to GMP  Stock cultures of MO used as source should be periodically tested for their purity	N. s.	N. s.
	Addition of the enzyme preparation must not cause any increase in the total microbial counts in the food stuff	N. s.	N. s.
<b>8. Contaminants</b>			
8.1. Heavy metals	Preparations should not contain toxicologically significant amount of heavy metals such as lead, cadmium, arsenic, and mercury	Level of heavy metals	N. s.
8.2. Microbial contaminants	<ul style="list-style-type: none"> <li>– No pathogenic micro-organisms should be detectable</li> <li>– Coliforms not more than 30 per gram as determined</li> <li>– Total viable count not more than</li> </ul>	N. s.	Tests according to * FDA Bacteriological Analytical Manual, Sixth Edition 1984 ** Microbiology - General guidance for enumeration of Coliforms. Colony count techniques



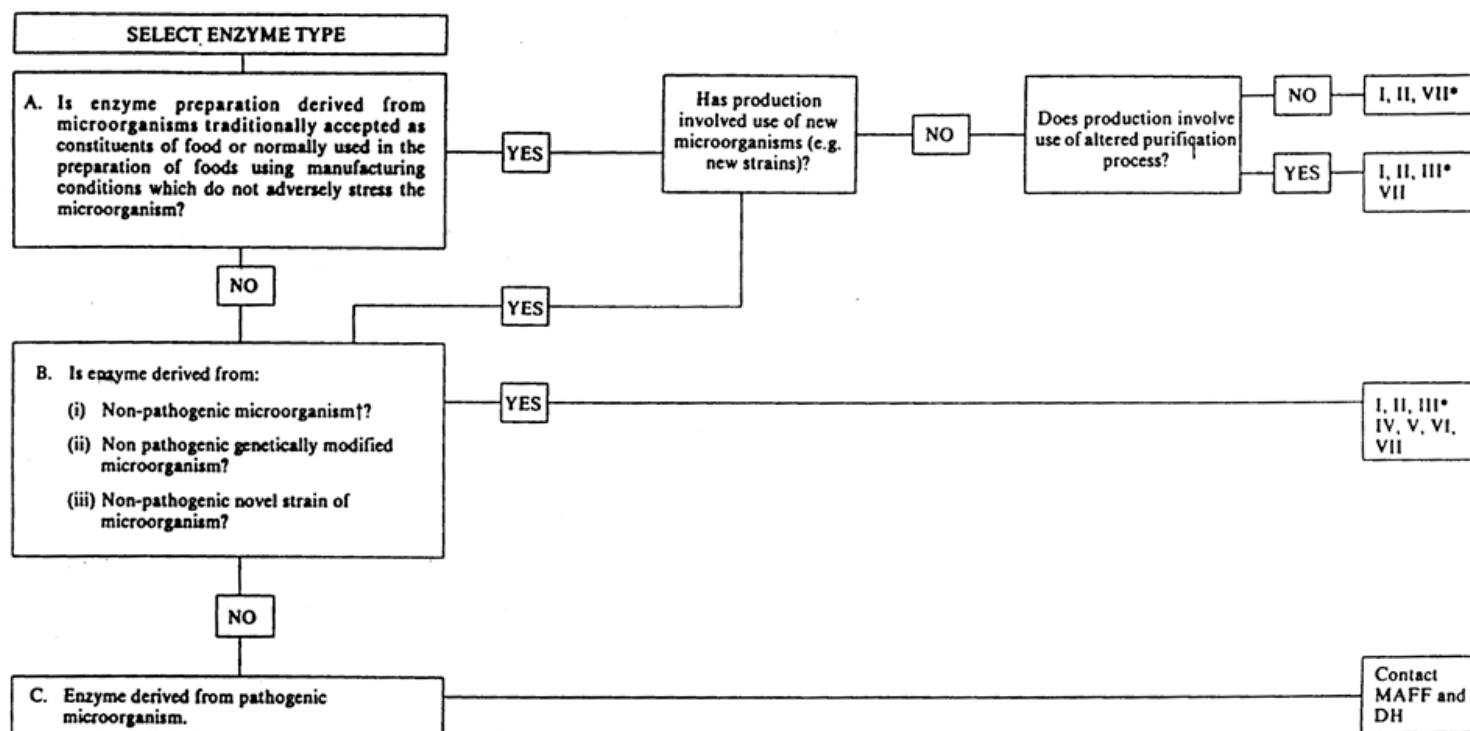
	Conditions of Use	Data Requirements	Method
	$10^2$ - $10^4$ per gram		at 30°C. ISO International Standard Ref. No. ISO 4833, First Edition 1978
8.3. Viable cells from the production strain	Viable cells from the production strain should not be present in the final product	N. s.	N. s.
8.4. Antibiotic activity in Enzyme preparation	Enzyme preparation may not contain any antibiotic activity	N. s.	FAO Food and Nutrition Paper No 49. JECFA, 35 <sup>th</sup> Session 1990 Specifications for identity and purity of certain food additives
8.5. Toxins in enzyme preparation	Enzyme preparation may not contain detectable amount of toxins	When a given source is known to be able to produce toxins the absence of those toxins shall be shown	N. s.
<b>Documentation for safety in use</b>			
<b>9. Basic toxicological requirements</b>			
9.1. Enzymes derived from edible parts of animals and plants	N. s.	No toxicological tests are required	N. a.
9.2. Parts are not considered as normal part of the diet	N. s.	Some toxicological testing may be required („unless other satisfactory documentation for safety in use is provided”)	N. a.
9.3. Enzyme preparations from MO	N. s.	Test should be performed <ul style="list-style-type: none"> <li>– on a batch from the final purified product, before addition of carriers, diluents etc.</li> <li>– in accordance to EC/OECD guidelines</li> </ul>	<ul style="list-style-type: none"> <li>– 90-day oral toxicity test in rodent species</li> <li>– test for gene mutations in bacteria</li> <li>– test for chromosomal aberrations (preferably in vitro)</li> </ul>
<b>10. Exemptions from the basic toxicological requirements</b>			
10.1.	If other enzyme preparations from the particular strain have thoroughly been tested before and if the manufacturing process does not significantly differ	Full testing battery <u>may</u> be waived	N. a.
10.2.	If the production MO <ul style="list-style-type: none"> <li>– Has a long history of safety in</li> </ul>	Acceptance of enzyme preparation without specific toxicological testing <u>may</u> be justified	N. a.

	Conditions of Use	Data Requirements	Method
	food use, and – Belongs to a species where no toxins are produced, and – The particular strain is of well documented origin		
10.3.	If a production organism of an already approved enzyme preparation is replaced by a mutant strain	A modified, less comprehensive test procedure <u>may</u> be possible	N. a.
10.4. In case of immobilised enzymes:	If the immobilisation techniques and the enzyme preparation have already been approved on the basis of adequate toxicity testing and if it is ensured that the leakage of components of the immobilisation system is within acceptable limits	The combinatorial product <u>may</u> not be subjected to any additional testing	N. a.
10.5. In case of non-toxin producing GMO:	If high purity and specificity of the enzyme product could be demonstrated	Full toxicity testing <u>may</u> not be needed	N. a.

<sup>a</sup> Modifications of the standard test protocols may be necessary due to e. g. the enzymatic activities of certain enzyme preparations and will be accepted if they are supported by arguments. <sup>b</sup> „Microbial sources can be native strains or variants of microorganisms, or be derived from native strains or variants by the process of selective serial culture or genetic modification” (p. 15, 2.3). N. s. ... Not specified; N. a. . Not applicable. Source: SCE (1992)

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*Figure: 11: Decision Tree of the COT for assessment of microbial enzymes used in food. Source: BATTERSHILL (1993)*



**Key to data requirements:**

- I = Identity, use and specification
- II = Quality assurance data
- III = Purification data
- IV = Resistance data (genetically modified organisms)
- V = Toxicology data
- VI = Intake data
- VII = Additional data for immobilized enzyme preparations. Contact MAFF and DH

**NOTE:** Some data may already be available for enzyme preparations previously considered acceptable by the COT and placed in Group B.

\* Some data may be omitted if reasoned justification can be provided.

† Non-pathogenic: Manufacturers should evaluate the pathogenicity of the production strain (see Para 7(a)) of the guidelines.

Table 60: Enzymes evaluated by JECFA for use in food

	Substance Name	Production organism	Donor organism
1.	alpha-Acetolactate Decarboxylase	Bacillus brevis	Bacillus subtilis
2.	alpha-Amylase and Glucoamylase .	Aspergillus oryzae, var	N.a.
3.	alpha-Amylase	Aspergillus oryzae, var.	N.a.
4.	alpha-Amylase	Bacillus megaterium	Bacillus subtilis
5.	alpha-Amylase	Bacillus stearothermophilus	N.a.
6.	alpha-Amylase	Bacillus stearothermophilus	Bacillus subtilis
7.	alpha-Amylase	Bacillus subtilis	N.a.
8.	Amyloglucosidase	Aspergillus niger, var.	N.a.
9.	Avian Pepsin	N.sp.	N.a.
10.	beta-Glucanase	Aspergillus niger, var.	N.a.
11.	beta-Glucanase	Trichoderma harzianum	N.a.
12.	Bromelain	N.sp.	N.a.
13.	Carbohydrase	Aspergillus awamori, var.	N.a.
14.	Carbohydrase	Aspergillus niger, var.	N.a.
15.	Carbohydrase	Bacillus licheniformis	N.a.
16.	Carbohydrase	Rhizopus oryzae, var.	N.a.
17.	Carbohydrase	Saccharomyces species	N.a.
18.	Catalase	Bovine Liver	N.a.
19.	Catalase	Micrococcus lysodeicticus	N.a.
20.	Cellulase	Penicillium funiculosum	N.a.
21.	Cellulase	Trichoderma longibrachiatum	N.a.
22.	Chymosin A	Escherichia coli K-12 containing the Prochymosin A Gene	N.sp.
23.	Chymosin B	Aspergillus niger var. awamori containing the Prochymosin B Gene	N.sp.
24.	Chymosin B	Kluyveromyces lactis containing the Prochymosin B Gene	N.sp.
25.	Ficin	n.sp.	N.a.
26.	Glucose Isomerase	Actinoplanes missouriensis	N.a.
27.	Glucose Isomerase	Bacillus coagulans	N.a.
28.	Glucose Isomerase	Streptomyces olivaceus	N.a.
29.	Glucose Isomerase	Streptomyces olivochromogen	N.a.
30.	Glucose Isomerase	Streptomyces rubiginosus	N.a.
31.	Glucose Isomerase	Streptomyces violaceoniger	N.a.
32.	Glucose Oxidase and Catalase	Aspergillus niger, var.	N.a.
33.	Hemicellulase	Aspergillus niger, var.	N.a.

	<b>Substance Name</b>	<b>Production organism</b>	<b>Donor organism</b>
34.	Lipase	Animal	N.a.
35.	Lipase	<i>Aspergillus oryzae</i> , var.	N.a.
36.	Malt Carbohydrase	N.sp.	N.a.
37.	Maltogenic Amylase	<i>Bacillus stearothermophilus</i>	<i>Bacillus subtilis</i>
38.	Mixed Microbial Carbohydrase and Protease	<i>Bacillus subtilis</i> , var.	N.a.
39.	Papain	N.sp.	N.a.
40.	Pectinase	<i>Aspergillus niger</i> , var.	N.a.
41.	Pepsin	Hog Stomach	N.a.
42.	Protease	<i>Aspergillus oryzae</i> , var.	N.a.
43.	Protease	<i>Streptomyces fradiae</i>	N.a.
44.	Pullulanase	<i>Klebsiella aerogenes</i>	N.a.
45.	Rennet	N.sp.	N.a.
46.	Rennet Bovine	N.sp.	N.a.
47.	Rennet	<i>Bacillus cereus</i>	N.a.
48.	Rennet	<i>Endothia parasitica</i>	N.a.
49.	Rennet	<i>Rhizomucor</i> species	N.a.
50.	Trypsin	N.sp.	N.a.

Source: JECFA: Compendium of food additives specifications. N.a. ... Not applicable; N.sp. ... Not specified.

Table 61: Compilation of enzymes listed as food additives, or permitted enzymes, or affirmed as GRAS

Status	Regulation	Enzyme/source organism/application
FA	§ 173.110	Amyloglucosidase derived from <i>Rhizopus niveus</i> for use in degrading gelatinised starch into constituent sugars.
FA	§ 173.120	Carbohydrase and cellulase derived from <i>Aspergillus niger</i> for use in clam and shrimp processing.
FA	§ 173.130	Carbohydrase derived from <i>Rhizopus oryzae</i> for use in the production of dextrose from starch.
FA	§ 173.135	Catalase derived from <i>Micrococcus lysodeikticus</i> for use in the manufacture of cheese.
FA	§ 173.140	Esterase-lipase derived from <i>Mucor miehei</i> var. Cooney et Emerson as a flavour enhancer in cheeses, fats and oils, and milk products.
FA	§ 173.145	-galactosidase derived from <i>Mortierella vinaceae</i> var. raffinoseutilizer for use in the production of sucrose from sugar beets
FA	§ 173.150	Milk-clotting enzymes, microbial for use in the production of cheese (Milk-clotting enzymes are derived from <i>Endothia parasitica</i> <i>Bacillus cereus</i> , <i>Mucor pusillus</i> Lindt and <i>Mucor miehei</i> Cooney et Emerson and <i>Aspergillus oryzae</i> modified to contain the gene for aspartic proteinase from <i>Rhizomucor miehei</i> var Cooney et Emerson).
P	§ 137.105	Flour may contain -amylase obtained from the fungus <i>Aspergillus oryzae</i> .
GRAS	§ 184.1012	Alpha-amylase enzyme preparation from <i>Bacillus stearothermophilus</i> used to hydrolyse edible starch to produce maltodextrin and nutritive carbohydrate sweeteners.
GRAS	§184.1024	Bromelain derived from pineapples, <i>Ananas comosus</i> and <i>Ananas bracteatus</i> used to hydrolyse proteins and polypeptides.
GRAS	§184.1027	Mixed carbohydrase and protease enzyme product derived from <i>Bacillus licheniformis</i> for use in hydrolysing proteins and carbohydrates in the preparation of alcoholic beverages, candy, nutritive sweeteners and protein hydrolysates.
GRAS	§ 184.1034	Catalase from bovine liver used to decompose hydrogen peroxide
GRAS	§ 184.1316	Ficin (peptide hydrolase) from the genus <i>Ficus</i> to hydrolyse proteins and polypeptides.
GRAS	§ 184.1372	Insoluble glucose isomerase enzyme preparations are derived from recognised species of precisely classified, non-pathogenic, and non-toxicogenic microorganisms, including <i>Streptomyces rubiginosus</i> , <i>Actinoplanes missouriensis</i> , <i>Streptomyces olivaceus</i> , <i>Streptomyces olivochromogenes</i> and <i>Bacillus coagulans</i> grown in a pure culture fermentation that produces no antibiotic.
GRAS	§ 184.1387	Lactase enzyme preparation from <i>Candida pseudotropicalis</i> for use in hydrolysing lactose to glucose and galactose.
GRAS	§ 184.1388	Lactase enzyme preparation from <i>Kluyveromyces lactis</i> (previously called <i>Saccharomyces lactis</i> ) for use in hydrolysing lactose in milk.
GRAS	§ 184.1415	Animal lipase (triacylglycerol hydrolase) derived from the edible fore-stomach of calves, kids or lambs used to hydrolyse fatty acid glycerides.
GRAS	§ 184.1420	Lipase enzyme preparation from <i>Rhizopus niveus</i> used in the inter-esterification of fats and oils.

Status	Regulation	Enzyme/source organism/application
GRAS	§ 184.1443	Malt (-amylase and -amylase) from barley to hydrolyse starch.
GRAS	§ 184.1583	Pancreatin (peptide hydrolase) from porcine or bovine pancreatic tissue used to hydrolyse proteins or polypeptides.
GRAS	§ 184.1585	Papain derived from papaya, <i>Carica papaya</i> L. .
GRAS	§ 184.1595	Pepsin (peptide hydrolase) from hog stomach used to hydrolyse proteins.
GRAS	§ 184.1685	Rennet (animal derived) and chymosin preparation from <i>Escherichia coli</i> K-12, <i>Kluyveromyces marxianus</i> var. <i>lactis</i> or <i>Aspergillus niger</i> var. <i>awamori</i> to coagulate milk in cheeses and other dairy products.
GRAS	§ 184.1914	Trypsin (peptide hydrolase) from porcine or bovine pancreas used to hydrolyze proteins.
GRAS	§ 184.1924	Urease enzyme preparation from <i>Lactobacillus fermentum</i> for use in the production of wine.
GRAS	§ 184.1985	Aminopeptidase enzyme preparation from <i>Lactococcus lactis</i> used as an optional ingredient for flavour development in the manufacture of cheddar cheese.
GRAS*		Carbohydrase, cellulase, glucose oxidase-catalase, pectinase, and lipase from <i>Aspergillus niger</i>
GRAS*		Carbohydrase and protease from <i>Aspergillus oryzae</i>
GRAS*		Carbohydrase and protease from <i>Bacillus subtilis</i>
GRAS*		Invertase from edible baker's yeast or brewer's yeast ( <i>Saccharomyces cerevisiae</i> )

*FA... enzymes listed as food additives; P... permitted enzymes, GRAS... affirmed as GRAS by FDA for specified or unspecified food uses and listed in 21 CFR Part 184; GRAS\* ... recognised as GRAS in opinion letters issued in the 1960ies (FDA has not affirmed as GRAS all food ingredients that it may consider GRAS. Therefore, this does not represent a complete list of all enzymes that FDA may view as GRAS for some uses). Source: FDA (2001)*



Table 62: Comparison of information requirements for enzymes in different guidelines and regulations

	SCF <sup>a</sup>	FDA/GRAS <sup>b</sup>	JECFA <sup>c</sup>	AMFEP <sup>d</sup>	France <sup>e</sup>
<b>Technical data</b>					
<b>Active components</b>					
<b>Principal enzyme activities</b>	<ul style="list-style-type: none"> <li>– Systematic names</li> <li>– EC numbers</li> </ul>	<ul style="list-style-type: none"> <li>– Systematic names</li> <li>– EC numbers</li> <li>– CAS Registry Number</li> <li>– Common and/or trade names</li> </ul>	<ul style="list-style-type: none"> <li>– Systematic names</li> <li>– EC number</li> </ul>	<ul style="list-style-type: none"> <li>– Systematic names</li> <li>– EC number</li> </ul>	N. i.
<b>Enzyme Activity</b>	<ul style="list-style-type: none"> <li>– Units/Weight or Units/Volume or (Quantity of enzyme preparation to be added to a given quantity of food)</li> </ul>	<ul style="list-style-type: none"> <li>– Units</li> <li>– Enzymatic function</li> <li>– Mode of action</li> <li>– Substrate specificity</li> <li>– Molecular weight<sup>g</sup></li> <li>– Isoelectric point<sup>g</sup></li> <li>– Kinetic properties<sup>g</sup></li> <li>– Specific activity<sup>g</sup></li> <li>– Temperature<sup>h</sup></li> <li>– pH<sup>h</sup></li> <li>– Inorganic ions<sup>h</sup></li> </ul>	<ul style="list-style-type: none"> <li>– Units/Weight</li> <li>– Reaction catalysed</li> <li>– Detailed description of suitable methods for each evaluated enzyme is provided</li> </ul>	<ul style="list-style-type: none"> <li>– International units or manufacturer units</li> </ul>	N. i.
<b>Subsidiary enzymatic activities</b>	List of subsidiary enzymatic activities „whether they perform a useful function or not” (page 15)	Other major enzymatic activities	Secondary enzyme activities	N.sp.	N. i.

	SCF <sup>a</sup>	FDA/GRAS <sup>b</sup>	JECFA <sup>c</sup>	AMFEP <sup>d</sup>	France <sup>e</sup>
<b>recombi- nant en- zymes from GMM:</b>	N.sp.	<ul style="list-style-type: none"> <li>– Description of permanent or transient structural modifications (by chemical <u>or</u> genetic engineering)</li> <li><i>Comparative analysis<sup>i</sup>:</i> <ul style="list-style-type: none"> <li>– <i>Enzymatic activity</i></li> <li>– <i>Kinetic parameters</i></li> <li>– <i>Amino acid composition</i></li> <li>– <i>Amino sugar composition</i></li> <li>– <i>Amino acid sequence (full or partial)</i></li> <li>– <i>Molecular weight</i></li> <li>– <i>Isoelectric point</i></li> <li>– <i>Gel-migration</i></li> <li>– <i>Chromatographic (or similar) properties</i></li> </ul> </li> </ul>	<ul style="list-style-type: none"> <li>– molecular weight;</li> <li>– isoelectric point;</li> <li>– substrate specificity;</li> <li>– reaction kinetics;</li> <li>– activity as a function of pH and temperature;</li> <li>– amino acid composition;</li> <li>– amino acid sequence;</li> <li>– a peptide map; and</li> <li>– DNA base sequence coding for the enzyme.</li> </ul>	N.sp.	N. i.
<b>Source materials</b>					
<b>animal sources</b>	Identification of animal and part of the animal  Source must comply with meat inspection requirements	Should be characterised; should comply with the General requirements for enzyme preparations set forth in the Food and Chemicals Codex 3rd edition, 1981	Sources must comply with meat-inspection requirements and be handled in accordance with good hygienic practice	N.sp.	N. i.
<b>plant sources</b>	Identification of plant and part of the plant	Should be identified	No residues harmful to health must be left in the processed finished food under normal conditions of use	N.sp.	N. i.
<b>microbial sources (includ-</b>	Must be discrete stable strains and well characterised (to enable to be assigned unique identities as the	Should be taxonomically and genetically identified	Must be discrete and stable strains or variants which are sufficiently well characterised to enable them	Taxonomy of the MO should be precisely	N.sp.

	SCF <sup>a</sup>	FDA/GRAS <sup>b</sup>	JECFA <sup>c</sup>	AMFEP <sup>d</sup>	France <sup>e</sup>
<b>ing GMM</b> ) <sup>94</sup>	sources of the enzyme preparation)		to be assigned unique identities as the sources of the enzyme preparations which are the subject of individual specifications	defined.	
<b>GMM</b>	Information about host organism	<p>All procedures and steps involved in the construction of the production organism should be thoroughly described</p> <ul style="list-style-type: none"> <li>– Genetic stability</li> <li>– Growth properties</li> </ul>	<p>Host microorganisms should be taxonomically and genetically characterised</p> <p>Identification at the level of genus and species is usually adequate for those that have been determined to be safe and suitable<sup>95</sup></p> <p>Documentation that the host microorganism is non-pathogenic and non-toxicogenic should be provided</p> <p>Production microorganism should be characterised with respect to the introduced DNA, its genetic stability, and its growth properties</p>	Host organism should be described	Introduction of DNA-sequences should only be done if they are strictly needed to maintain and express the genes of interest
<b>Vector</b>	<p>Must be free of harmful sequences, non-conjugative, and non-mobilizable</p> <ul style="list-style-type: none"> <li>– Identity and biology</li> <li>– Size</li> <li>– Restriction map</li> <li>– Possible full DNA sequence</li> <li>– Genes found on the vector</li> <li>– Marker genes</li> </ul>	Should be genetically identified and characterised	<p>characterised and a description of its construction provided</p> <p>genetic material does not contain genes coding for virulence factors, protein toxins, or enzymes that may be involved in the synthesis of mycotoxins or any other toxic or undesirable substances</p> <p>As with the citation of strains, the citation of specific expression plasmids is generally unnecessary and may impose unnecessary re-</p>	should be described	Should avoid heterologous markers which present asserted risks (i. e. genes for resistance to antibiotics)

<sup>94</sup> Including wild-type strains, „variants” ? or strains resulting from selection procedures or genetic modification (page 25).

<sup>95</sup> According to 53<sup>rd</sup> JECFA meeting, identification at the strain level may impose unnecessary constraints on the development of production organisms. The committee further concluded that in the case of a non-pathogenic, non-toxicogenic strain that belongs to a species that includes pathogenic and toxicogenic strains (e. g. *Escherichia coli*), there should be a requirement in the monograph that the strain be non-pathogenic and non-toxicogenic. Citation of a suitable strain number may be included by way of example.

	SCF <sup>a</sup>	FDA/GRAS <sup>b</sup>	JECFA <sup>c</sup>	AMFEP <sup>d</sup>	France <sup>e</sup>
			strictions on the development of enzyme preparations.		
<b>introduced DNA</b>	<ul style="list-style-type: none"> <li>– DNA sequence(s) introduced</li> <li>– Number of inserted genes</li> <li>– Type of regulation (promoter activity)</li> <li>– Gene product(s)</li> <li>– Origin and pedigree of the genetic construct</li> </ul>	Introduced DNA	N.sp.	should be described	<p>Nucleotide sequence of the whole construction introduced</p> <p>Precise knowledge of the gene products of the inserted genes</p>
<b>Donor organism</b>	Identification of the donor organism	Should be taxonomically and genetically identified and characterised	The source of the DNA encoding the enzyme of interest should be identified where possible.	should be described	N. i.
<b>Additives and other ingredients</b>					
<b>Enzyme preparation</b>	TOS	<p>Content of proteins and nucleic acids, carbohydrates, fats, total solids, and ash measured for at least five representative batches</p> <p>Percentage of enzyme protein in enzyme preparation</p> <p>TOS</p>	TOS	The typical composition of a commercial enzyme preparation, such as content of protein, carbohydrates, fat, ash, water and diluents should be given.	N. i.
<b>Additives, Processing Aids</b>	<p>Information on carriers, diluents, excipients, supports and other additives and ingredients (including processing aids)</p> <p>Must either be substances acceptable for the relevant food use or which are insoluble in food and removed before consumption</p>	Should be identified	Must be substances that are acceptable for the relevant food uses of the enzyme preparations concerned, or substances which are insoluble in food and removed from the food material after processing	Identity and quality of stabilisers, standardizers, preservatives, and formulating agents should be given.	N. i.

	SCF <sup>a</sup>	FDA/GRAS <sup>b</sup>	JECFA <sup>c</sup>	AMFEP <sup>d</sup>	France <sup>e</sup>
<b>immobi- lised en- zymes:</b>	Testing of potential leakage of immobilisation agents  Carriers, immobilisation agents should be acceptable for the relevant use	All components should be listed along with their percentages on a dry-solids basis;  Immobilisation agent should be characterised with respect to composition, purity, chemical and physical properties;  method of immobilisation should be described	Leakage of carriers, immobilisation agents and active enzymes must be kept within acceptable limits as specified in the individual specifications.	N.sp.	
<b>Manufacturing process</b>					
<b>Method of manufacture</b>	In case of MO:  – information on fermentation media  – information on fermentation conditions	Should be thoroughly described  In case of GMM:  – Fermentation process  – Components of fermentation medium should be identified	N.sp.	N.sp.	N. i.
<b>Purification procedure</b>	Information on purification procedure	Should be thoroughly described  In case of GMM:  Isolation of the enzyme including all physical and chemical treatment should be described	N.sp.	N.sp.	
<b>Usage</b>					
	Technological function of the enzyme	N.sp.	N.sp.	N.sp.	N. i.
	Types of foodstuff in which the enzyme is intended to be used	N.sp.	N.sp.	N.sp.	
	Maximum amount of enzyme preparation to be used in each foodstuff	N.sp.	N.sp.	N.sp.	
<b>Stability and fate in food</b>					
	Amount of enzyme preparation in the final food	N.sp.	N.sp.	N.sp.	N. i.
	Main reaction products and possible reaction products not considered normal constituents of the diet, formed during production and	N.sp.	N.sp.	N.sp.	N. i.

	SCF <sup>a</sup>	FDA/GRAS <sup>b</sup>	JECFA <sup>c</sup>	AMFEP <sup>d</sup>	France <sup>e</sup>
	storage				
	Possible effects on nutrients	N.sp.	N.sp.	N.sp.	N. i.
<b>Safety data</b>					
<b>Hygiene</b>					
	Manufacturing of enzymes should be done according to GMP Stock cultures of MO used as source should be periodically tested for their purity	N.sp.	N.sp.	N.sp.	N. i.
	Addition of the enzyme preparation must not cause any increase in the total microbial counts in the food stuff	N.sp.	N.sp.	N.sp.	N. i.
<b>Contaminants in Enzyme preparation</b>					
	N.sp.	Impurities specific to the source of the enzyme or the manufacturing process should also be identified and measured (at least five batches should be analysed)	N.sp.	N.sp.	N. i.
<b>Heavy metals</b>	Level of heavy metals Preparations should not contain toxicologically significant amount of heavy metals such as lead, cadmium, arsenic, and mercury	Food Chemicals Codes specification for enzymes	Arsenic: not more than 3 mg/kg ( <i>rev.<sup>f</sup>: threshold eliminated</i> ) Lead: not more 10 mg/kg ( <i>rev.<sup>f</sup>: 5 mg/kg</i> ) Heavy metals: not more than 40 mg/kg ( <i>rev.<sup>f</sup>: threshold eliminated</i> ) Detailed description of methods is provided	correspond to recommendations given by JECFA: Arsenic: not more than 3 mg/kg Lead: not more 10 mg/kg Heavy metals: not more than 40 mg/kg  Methods: JECFA	Cadmium: not more 0.5 mg/kg Mercury: not more 0.5 mg/kg Arsenic: not more 3 mg/kg Lead: not more 10 mg/kg
<b>Microbial contami-</b>	No pathogenic micro-organisms should be detectable*	Food Chemicals Codes specification for enzymes	Use of enzyme preparation should not increase the total microbial	correspond to recommendations given by	Total viable mesophilic aerobic count:

	SCF <sup>a</sup>	FDA/GRAS <sup>b</sup>	JECFA <sup>c</sup>	AMFEP <sup>d</sup>	France <sup>e</sup>
<b>nants</b>	<p>Coliforms not more than 30 per gram as determined<sup>††</sup></p> <p>Total viable count not more than 10<sup>2</sup> - 10<sup>4</sup> per gram</p> <p>Tests according to</p> <p>* FDA Bacteriological Analytical Manual, Sixth Edition 1984</p> <p>** Microbiology - General guidance for enumeration of Coliforms. Colony count techniques at 30°C. ISO International Standard Ref. No. ISO 4833, First Edition 1978</p>		<p>count in treated food ...</p> <p>Total viable mesophilic aerobic count: no more than 50,000/g (<i>rev. f: threshold eliminated</i>)</p> <p>Coliforms: not more than 30/g</p> <p>Salmonella: absence in 25 g</p> <p>Escherichia coli: absence in 25 g</p> <p>Methods: ISO 4833, FDA Bacteriological Analytical Manual</p>	<p>JECFA:</p> <p>Total viable mesophilic aerobic count: no more than 50,000/g</p> <p>Coliforms: not more than 30/g</p> <p>Salmonella: absence in 25 g</p> <p>Escherichia coli: absence in 25 g</p> <p>Methods: JECFA</p>	<p>no more than 50,000/g</p> <p>Coliforms: not more than 30/g</p> <p>Salmonella: absence in 25 g</p> <p>Anaerobic sulfito-reducing count: no more than 30/g</p> <p>Staphylococcus aureus: absence in 1 g</p>
<b>Production strain</b>	Viable cells from the production strain should not be present in the final product	Food Chemicals Codes specification for enzymes	Not specifically mentioned	N.sp.	N. i.
<b>Antibiotic activity</b>	<p>Enzyme preparation may not contain any antibiotic activity</p> <p>FAO Food and Nutrition Paper No 49. JECFA, 35<sup>th</sup> Session 1990 Specifications for identity and purity of certain food additives</p>	In case of MO: should not be present:	<p>Antibiotic activity of enzymes from MO should be absent</p> <p>Detailed description of a suitable method is provided</p> <p>In case of GMM: If the production microorganism is capable of producing proteins that inactivate clinically useful antibiotics, documentation should be provided that the finished enzyme preparation contains neither antibiotic-inactivating proteins at concentrations that would interfere with antibiotic treatment nor DNA that is capable of transforming microorganisms, which potentially could lead to the spread of antibiotic re-</p>	Antibiotic activity of enzymes from MO should be negative by test	Enzyme preparation may not contain any antibiotic activity

	SCF <sup>a</sup>	FDA/GRAS <sup>b</sup>	JECFA <sup>c</sup>	AMFEP <sup>d</sup>	France <sup>e</sup>
			sistance.		
<b>Toxins</b>	When a given source is known to be able to produce toxins the absence of those toxins shall be shown  Enzyme preparation may not contain detectable amount of toxins	In case of MO: should not be present	Enzyme preparation derived from fungal sources shall not contain detectable amounts of (rev.: those mycotoxins, that are known to be produced by strains of the species used in the production of the enzyme preparation or related species) aflatoxin B1, ochratoxin A, sterigmatocystin, T-2 toxin or zearalenone  Method: PATTERSON & ROBERTS (1979)	Enzyme preparation derived from fungal sources shall not contain detectable amounts of mycotoxins by test	Enzyme preparation may not contain detectable toxins, special attention is given to mycotoxins
<b>DNA</b>	N.sp.	N.sp.	N.sp.	N.sp.	In case of GMM: prove of the absence of active DNA in the enzyme preparation (indicated by threshold of the detection method)
<b>Toxicological requirements</b>					
<b>Source: edible parts of animals and plants</b>	No toxicological tests are required	According to FDA 1997 <sup>j</sup>	Specified in: Principles for the safety assessment of food additives and contaminants in food. WHO Environmental Health Criteria, No. 70, Geneva, World Health Organization, 1987	N.sp.	N. i.
<b>Source: not considered as normal part of the diet</b>	Some toxicological testing may be required („unless other satisfactory documentation for safety in use is provided”)	According to FDA 1997 <sup>j</sup>	Enzyme preparations are grouped into classes I to V depending on their production organism  Normally, acute and short-term toxigenicity tests are required. In case of MO they do not normally occur in food, also long term test have to be provided. In case of fungi or bacteria from genus that might produce (myco)toxins specific test have to be carried out. In case of testing of genetic toxicity, teratology and mutagenicity are also required	N.sp.	N. i.



	SCF <sup>a</sup>	FDA/GRAS <sup>b</sup>	JECFA <sup>c</sup>	AMFEP <sup>d</sup>	France <sup>e</sup>
<b>Source: MO</b>	<p>Test should be performed on a batch from the final purified product, before addition of carriers, diluents etc.</p> <p>in accordance to EC/OECD guidelines<sup>96</sup></p> <p>90-day oral toxicity test in rodent species</p> <p>test for gene mutations in bacteria</p> <p>test for chromosomal aberrations (preferably in vitro)</p>	According to FDA 1997 <sup>j</sup>		„[...] extensive animal feeding trials of the enzyme preparation. Evidence of non-pathogenicity of the production strain should be provided either by literature or by actual experiments such as biological tests of the strain in rodents.“	
<b>Source: GMM</b>	N.sp.	According to FDA 1997 <sup>j</sup> ; Transformable DNA coding for toxins and/or proteins that inactivate therapeutic antibiotics should not be present	<p>All enzyme preparations should be evaluated for their potential to elicit allergic reactions. As a general rule, if the food is known to cause an allergic reaction in humans, its use as a source of DNA encoding the enzyme of interest should be avoided. In exceptional cases, where there is a demonstrated need to use an allergenic source of DNA, documentation should be provided indicating that the enzyme is not associated with the allergic reaction. The most common allergenic foods on a world-wide basis are fish, crustacea, peanuts, tree nuts, soybeans, milk, eggs, and wheat</p> <p><i>Rev.<sup>f</sup>: „In case of GMM the need for an evaluation for allergenic potential of the gene products en-</i></p>	N.sp.	

<sup>96</sup> Modifications of the standard test protocols may be necessary due to e. g. the enzymatic activities of certain enzyme preparations and will be accepted if they are supported by arguments.

	SCF <sup>a</sup>	FDA/GRAS <sup>b</sup>	JECFA <sup>c</sup>	AMFEP <sup>d</sup>	France <sup>e</sup>
			<i>coded should be assessed. “ No need to further assess the allergic potential in case the DNA sequence is comparable to that coding for an enzyme with a long history of safe use in food” (page 19).</i>		
<b>Exemptions from the toxicological requirements</b>					
	If other enzyme preparations from the particular strain have thoroughly been tested before and if the manufacturing process does not significantly differ  Full testing battery <i>may</i> be waived	N. i.	N.sp.	N.sp.	N. i.
	If the production MO – Has a long history of safety in food use, and – Belongs to a species where no toxins are produced, and – The particular strain is of well documented origin  Acceptance of enzyme preparation without specific toxicological testing <i>may</i> be justified	N. i.	N.sp.	N.sp.	N. i.
	If a production organism of an already approved enzyme preparation is replaced by a mutant strain  A modified, less comprehensive test procedure may be possible	N. i.	N.sp.	N.sp.	N. i.
	In case of immobilised enzymes: if the immobilisation techniques and the enzyme preparation have already been approved on the basis of adequate toxicity testing and if it is ensured that the leakage of components of the immobilisation system is within acceptable limits	N. i.	N.sp.	Where no leakage of either carrier / immobilisation agent or enzyme can be demonstrated in validated	N. i.

	SCF <sup>a</sup>	FDA/GRAS <sup>b</sup>	JECFA <sup>c</sup>	AMFEP <sup>d</sup>	France <sup>e</sup>
	The combinatorial product <i>may</i> not be subjected to any additional testing			assays, toxicological tests are not necessary.	
	In case of non-toxin producing GMO: If high purity and specificity of the enzyme product could be demonstrated  Full toxicity testing <i>may</i> not be needed	N. i.	N.sp.	N.sp.	N. i.

<sup>a</sup> (SCF, 1992); <sup>b</sup> FDA 1993; <sup>c</sup> Prepared at the 35<sup>th</sup> JECFA (1989) and published in FNP 49 (1990) and in FNP 52 (1992), superseding general specifications prepared at the 25<sup>th</sup> JECFA (1981) and published in FNP 19 (1981) and in FNP 31/2 (1984). Amended at the 55<sup>th</sup> JECFA (1999) and partially published in FNP 52 Add 7 (1999). For Enzymes from GMM: Prepared at the 53<sup>rd</sup> JECFA (1998) and published in FNP 52 Add 6 (1998), superseding Appendix prepared at the 37<sup>th</sup> JECFA (1990) and published FNP 52 (1992). Amended at the 55<sup>th</sup> JECFA (1999) and partially published in FNP 52 Add 7 (1999); <sup>d</sup> (AMFEP, 1992); <sup>e</sup> SCOOP Task 7.4 preliminary results; <sup>f</sup> Amendments decided at the 57<sup>th</sup> JECFA (2001); <sup>g</sup> If available or necessary to support the enzyme identity; <sup>h</sup> If such characteristics are relevant for the intended use of the enzyme preparation; <sup>i</sup> If an enzyme normally produced in one organism is expressed in another organism through molecular cloning, especially in case of gene transfer to genetically distant organisms; <sup>j</sup> The extent of toxicological testing of food additives is depending on the on the assignment of a „concern level”, on structural features and on an estimation of exposure. Minimum testing requirements are recommended for each concern level as well as each structural and exposure group (FDA, 1997). However, these requirements are included in the investigations of this section. N.sp. ... Not specified; N. i.... Not investigated.

Table 63 : Commercial names of approved enzyme products in the EU

Nr.	Commercial name	Animal category	R / Doc. (until)
E 1600	Natuphos (1)	piglets	1353/00 (no time limit)
		pigs for fatt.	1353/00 (no time limit)
		sows	1353/00 (no time limit)
		chicken for fatt.	1353/00 (no time limit)
		laying hens	1353/00 (no time limit)
1	Natuphos (1)	turkey	2200/01 (14.12.03)
2	Phytase Novo (1)	piglets	2200/01 (30.06.04)
		pigs for fatt.	2200/01 (30.06.04)
		chicken for fatt.	2200/01 (30.06.04)
		laying hens	2200/01 (30.06.04)
3	Alpha Gal (2)	chicken for fatt.	2200/01 (30.06.04)
4	Energex (3)	piglets	2200/01 (30.06.04)
		chicken for fatt.	2200/01 (01.04.04)
5	Biofeed Wheat (4)	chicken for fatt.	2200/01 (30.06.04)
		turkey for fatt.	2200/01 (30.06.04)
		piglets	2200/01 (30.06.04)
6	Biofeed Plus (3+4)	chicken for fatt.	2200/01 (30.06.04)
		piglets	2200/01 (30.06.04)
		pigs for fatt.	2200/01 (30.06.04)
7	Grindazym GP (3+4)	chicken for fatt.	2200/01 (01.04.04)
	coated and liquid: 1.4.2004 solid: 30.9.2004	turkey for fatt.	2200/01 (01.04.04)
		laying hens	2200/01 (01.04.04)
		piglets	2200/01 (01.04.04)
8	Grindazym GV (3+4)	chicken for fatt.	2200/01 (01.04.04)
	coated and liquid: 1.4.2004 solid: 30.9.2004	piglets	2200/01 (01.04.04)
		laying hens	2200/01 (01.04.04)
9	Lyxasan (4)	chicken for fatt.	2200/01 (30.06.04)
		laying hens	2200/01 (30.06.04)
		turkey for fatt.	2200/01 (30.06.04)
10	Vevozyme (5)	piglets	2200/01 (30.06.04)
		pigs for fatt.	2200/01 (30.06.04)
		sows	2200/01 (30.06.04)
11	Roxazyme G2 liquid (3'+4)	chicken for fatt.	2200/01 (30.06.04)
	Broiler: liquid.. granulat:	turkey for fatt.	2200/01 (31.05.05)
12	Roxazyme G (3'+4)	chicken for fatt.	2200/01 (30.06.04)
	(3'= 2 different glucanases)	laying hens	2200/01 (30.06.04)
		turkey for fatt.	2200/01 (30.06.04)
13	Natugrain/Barlican (3+4)	chicken for fatt.	2200/01 (30.06.04)
		laying hens	2200/01 (01.04.04)
		turkey for fatt.	2200/01 (01.04.04)

Nr.	Commercial name	Animal category	R / Doc. (until)
14	Allzyme PT (4)	chicken for fatt.	2200/01 (30.06.04)
15	Allzyme BG (3)	chicken for fatt.	2200/01 (30.06.04)
16	Hostazym C (3)	chicken for fatt.	2200/01 (30.06.04)
	liquid: 30.06.04	laying hens	2200/01 (30.06.04)
	solid: 17.07.04	piglets	2200/01 (30.06.04)
		pigs for fatt.	2200/01 (30.06.04)
17	Hostazym X (4)	chicken for fatt.	2200/01 (30.06.04)
	liquid: 30.06.04	laying hens	2200/01 (30.06.04)
	solid: 17.07.04	piglets	2200/01 (30.06.04)
		pigs for fatt.	2200/01 (30.06.04)
		turkey for fatt.	2200/01 (17.07.04)
18 (3)	Rovabio $\beta$ -Gluc. AN P/LC	chicken for fatt.	2200/01 (30.06.04)
19	Feedlyve AGL (3)	chicken for fatt.	2200/01 (30.06.04)
20	Rovabio Xyl. TR P/LC (4)	chicken for fatt.	2200/01 (30.06.04)
21	Feedlyve AXC (4)	chicken for fatt.	2200/01 (30.06.04)
22	Safizym G (3)	chicken for fatt.	2200/01 (30.06.04)
23	Safizym X (4)	chicken for fatt.	2200/01 (30.06.04)
		turkey for fatt.	2200/01 (28.02.05)
		laying hens	2200/01 (28.02.05)
24	Quatrazyme (3+4)	chicken for fatt.	2200/01 (30.06.04)
25	Endofeed (3+4)	chicken for fatt.	2200/01 (30.06.04)
		laying hens	2200/01 (30.06.04)
26	Econase Barley (3)	chicken for fatt.	2200/01 (30.06.04)
		piglets	2200/01 (30.06.04)
27	Econase Wheat (3+4)	chicken for fatt.	2200/01 (30.06.04)
		piglets	2200/01 (28.02.05)
28	Finase (1)	piglets	2200/01 (30.06.04)
		pigs for fatt.	2200/01 (30.06.04)
		chicken for fatt.	2200/01 (28.02.05)
29	Rovabio $\beta$ -Gluc. GEP (3)	chicken for fatt.	2200/01 (30.06.04)
30 (3+4)	Rovabio $\beta$ -Gluc. PF P/LC	chicken for fatt.	2200/01 (30.06.04)
		laying hens	2200/01 (28.02.05)
		pigs for fatt.	2200/01 (28.02.05)
		turkey for fatt.	2200/01 (28.02.05)
31	Volible P/L (4)	chicken for fatt.	2200/01 (30.06.04)
		laying hens	2200/01 (30.06.04)
32	Avizyme/Porzyme Gluc. (3)	chicken for fatt.	2200/01 (30.06.04)
		piglets	2200/01 (30.06.04)
		pigs for fatt.	2200/01 (30.06.04)
33	Avizyme/Porzyme Xyl. (4)	chicken for fatt.	2200/01 (30.06.04)

Nr.	Commercial name	Animal category	R / Doc. (until)
		laying hens	2200/01 (30.06.04)
		piglets	2200/01 (30.06.04)
		pigs for fatt.	2200/01 (30.06.04)
34	Amylofeed (3+4+5)	piglets	2200/01 (26.07.04)
35	Avizyme 2100 (3+4)	laying hens	2200/01 (26.07.04)
36	Avizyme SX (3+4)	chicken for fatt.	2200/01 (26.07.04)
		laying hens	2200/01 (26.07.04)
37	Avizyme 1300 (4+7)	chicken for fatt.	2200/01 (26.07.04)
		turkey	2200/01 (26.07.04)
38	Porzyme 8300 (4+7)	piglets	2200/01 (26.07.04)
39	Porzyme 9100 (3+4)	pigs for fatt.	2200/01 (26.07.04)
40	Avizyme 1100 (3+4+7)	chicken for fatt.	2200/01 (26.07.04)
41	Avizyme 1200 (3+4+7)	chicken for fatt.	2200/01 (26.07.04)
		laying hens	2200/01 (26.07.04)
42	Porzyme 9305 (4)	piglets	2200/01 (26.07.04)
		pigs for fatt.	2200/01 (17.07.04)
43	Porzyme TP 100 G (3+4+5)	piglets	2200/01 (06.01.04)
44	Porzyme 8100 (3+4+5)	piglets	2200/01 (06.01.04)
45	Porzyme SP (3+4+5)	piglets	2200/01 (06.01.04)
46	Porzyme SF 100 (3+4+6)	pigs for fatt.	2200/01 (06.01.04)
47	Porzyme TP 100 (3+4+5+6)	piglets	2200/01 (06.01.04)
48	Biofeed Alpha (3+5)	chicken for fatt.	2200/01 (01.04.04)
		turkey for fatt.	2200/01 (01.04.04)
49	Avizyme TX (3+4+5+6+8)	chicken for fatt.	2200/01 (17.07.04)
		laying hens	2200/01 (17.07.04)
50	Biofeed phytase (1)	chicken for fatt.	2200/01 (17.07.04)
		laying hens	2200/01 (17.07.04)
		turkey for fatt.	2200/01 (17.07.04)
		piglets	2200/01 (17.07.04)
		pigs for fatt.	2200/01 (17.07.04)
51	Belfeed (4)	chicken for fatt.	2200/01 (17.07.04)
		piglets	2200/01 (31.05.05)
52	Kemzyme liqid (3+3+5)	chicken for fatt.	2200/01 (17.07.04)
53	Kemzyme W Dry (3+3+4+5+8)	piglets	2200/01 (23.11.04)
		chicken for fatt.	2200/01 (23.11.04)
54	Kemzyme W Liquid (3+3+4+5)	chicken for fatt.	2200/01 (23.11.04)
		turkey for fatt.	2013/01 (13.10.05)
55	Kemzyme Dry (3+3+5+8)	piglets	2200/01 (23.11.04)
		pigs for fatt.	2200/01 (23.11.04)

Nr.	Commercial name	Animal category	R / Doc. (until)
		chicken for fatt.	2200/01 (23.11.04)
		laying hens	2200/01 (23.11.04)
56	Kemzyme B Dry (3+3+5+8)	chicken for fatt.	2200/01 (23.11.04)
57	Kemzyme HF Dry (3+3+5+8)	chicken for fatt.	2200/01 (23.11.04)
58	Kemzyme PS Dry (3+3+5+8)	piglets	2200/01 (23.11.04)
59	Avizyme 1500 (3+4+5+6+7)	chicken for fatt.	2200/01 (28.02.05)
60	Avizyme 1210 (3+4)	chicken for fatt.	2200/01 (28.02.05)
61	Wheatzyme (3+4)	chicken for fatt.	2200/01 (28.02.05)

## ENZYMES:

- 1--- 3-phytase
- 2--- alpha galactosidase
- 3--- glucanase
- 4--- xylanase
- 5--- alpha amylase
- 6--- polygalacturonase
- 7--- subtilisin
- .8--- bacillolysine

### 13.1.2 AMFEP list of toxicity testing on enzymes

Principal enzymatic activity	Host organism (production organism)	Donor organism	Toxicology (in brackets: number of companies)							
			Acute	Sub-acute	Subchron	Chron	Mut. bact	Mut. mam	Other	JECFA -ev.
Acetolactate decarboxylase (alpha)	Bacillus amyloliquefaciens or subtilis	Bacillus sp.			1		1	1		yes
Aminoacylase	Aspergillus melleus	none	1		1	1			H,I	
Aminopeptidase	Aspergillus niger	none		1	1		1	1		
Aminopeptidase	Aspergillus oryzae	none			1		1	1		
Aminopeptidase	Lactococcus lactis	none		1	1					
Aminopeptidase	Rhizopus oryzae	none			1		1	1		
Aminopeptidase	Trichoderma reesei or longibrachiatum	Aspergillus sp.								
AMP deaminase	Aspergillus melleus	none	1		2(1)		1	1	H,I	
Amylase (alpha)	Aspergillus niger	none			1					yes
Amylase (alpha)	Aspergillus oryzae	none	2(2)	1	2(2)		3(3)	2(2)		yes
Amylase (alpha)	Bacillus amyloliquefaciens or subtilis	Bacillus sp.	2(2)	2(2)	2(2)		3(2)	2(2)	D,E,F,G	?
Amylase (alpha)	Bacillus amyloliquefaciens or subtilis	Thermoactinomyces sp.								
Amylase (alpha)	Bacillus amyloliquefaciens or subtilis	none			1		1	1	D,E,F	yes
Amylase (alpha)	Bacillus licheniformis	Bacillus sp.	6(2)	1	5(3)		4(3)	3(3)	A,D,F,I,G	
Amylase (alpha)	Bacillus licheniformis	none			1		1	1		yes
Amylase (alpha)	Bacillus stearothermophilus	none	1	1	1		1	1	E,F	yes
Amylase (alpha)	Microbacterium imperiale	none	1	1	1		1	1		
Arabinanase	Aspergillus niger	none								
Arabinofuranosidase	Aspergillus niger	Aspergillus sp.		2 (1)			1	1		
Arabinofuranosidase	Aspergillus niger	none			1		1	1		
Catalase	Aspergillus niger	Aspergillus sp.	1				1		F	
Catalase	Aspergillus niger	none	1		1		2(2)	1		
Catalase	Micrococcus luteus or lysodeikticus	none						1	D,E,F	
Catalase	Scytalidium thermophilum	none		1			1			
Cellulase	Aspergillus niger	none	1		2(1)	1	1	1	H,I	yes



Principal enzymatic activity	Host organism (production organism)		Toxicology (in brackets: number of companies)							
			Acute	Sub-acute	Subchron	Chron	Mut. bact	Mut. mam	Other	JECFA -ev.
Cellulase	<i>Aspergillus oryzae</i>	<i>Humicola</i> sp.	1				1		D,E,F	
Cellulase	<i>Aspergillus oryzae</i>	<i>Myceliophthora</i> sp.							I	
Cellulase	<i>Aspergillus oryzae</i>	<i>Thielavia</i> sp.							E,F	
Cellulase	<i>Bacillus amyloliquefaciens</i> or <i>subtilis</i>	none								
Cellulase	<i>Humicola insolens</i>	none			1		1	1	D,E,F	
Cellulase	<i>Penicillium</i> or <i>Talromyces emersonii</i>	none	1	1	1		1	1	E ,F	
Cellulase	<i>Penicillium funiculosum</i>	none	1	1	1		1	1	E,F	
Cellulase	<i>Streptomyces lividans</i> <i>Trichoderma reesei</i> or <i>longibrachiatum</i>	none								
Cellulase	<i>Streptomyces lividans</i> <i>Trichoderma reesei</i> or <i>longibrachiatum</i>	<i>Trichoderma</i> sp.								
Cellulase	<i>Streptomyces lividans</i> <i>Trichoderma reesei</i> or <i>longibrachiatum</i>	none	2(1)	2(2)	5(5)		4(4)	4(4)	A,D,E,F,G	yes
Cellulase	<i>Trichoderma viride</i>	none	1		1	1			H,I	
Chymosin	<i>Aspergillus niger</i> var. <i>awamori</i>	Calf stomach			1		1	1	A,E,F,G	yes
Chymosin	<i>Kluyveromyces lactis</i>	Calf stomach	1	3(1)	2(1)		3(1)	2(1)	A,C	yes
Cyclodextrin glucanotransferase	<i>Bacillus licheniformis</i>	<i>Thermoanaerobacter</i> sp.			1		1	1		
Cyclodextrin glucanotransferase	<i>Bacillus macerans</i>	none			1					
Dextranase	<i>Chaetomium erraticum</i>	none	1				1			
Dextranase	<i>Penicillium lilacinum</i>	none		1			1		E,F	
Esterase	<i>Rhizomucor miehei</i>	none		2(1)	1	1	1	1	B	
Galactosidase (alpha)	<i>Aspergillus niger</i>	none			1		1	1		
Galactosidase (alpha)	<i>Aspergillus oryzae</i>	<i>Aspergillus</i> sp.			1		1	1	D,E,F	
Galactosidase (alpha)	<i>Saccharomyces cerevisiae</i>	Guar plant			1		1	1		
Glucanase (beta)	<i>Aspergillus aculeatus</i>	none			1		1	1		
Glucanase (beta)	<i>Aspergillus niger</i>	none	2(2)	1	3(3)		4(3)	3(2)	A,D,E,F,G	yes
Glucanase (beta)	<i>Bacillus amyloliquefaciens</i> or <i>subtilis</i>	<i>Bacillus</i> sp.	1	1	1		2(2)	1		
Glucanase (beta)	<i>Bacillus amyloliquefaciens</i> or <i>subtilis</i>	none		2(1)	3(2)		3(2)	1		

Principal enzymatic activity	Host organism (production organism)		Toxicology (in brackets: number of companies)							
			Acute	Sub-acute	Subchron	Chron	Mut. bact	Mut. mam	Other	JECFA -ev.
Glucanase (beta)	Disporotrichum dimorphosporum	none	1	1	2(1)		3(1)	4(1)	A	
Glucanase (beta)	Humicola insolens	none					1	1		
Glucanase (beta)	Penicillium or Talaromyces emersonii	none	2(2)	4(2)	6(2)		4(2)	3(2)	E,F	
Glucanase (beta)	Penicillium funiculosum	none	1	1	1		1	1	E,F	
Glucanase (beta)	Pseudomonas paucirnobilis	none	1		1		1		H,I	
Glucanase (beta)	Trichoderma reesei or longibrachiatum	Trichoderma sp.			1			1	D,E,F	
Glucanase (beta)	Trichoderma reesei or longibrachiatum	none	3(2)	2(1)	4(3)		4(3)	3(3)	A,D,E,F,G	yes
Glucoamylase or Amyloglucosidase	Aspergillus niger	Aspergillus sp.		1				1		
Glucoamylase or Amyloglucosidase	Aspergillus niger	none	2(2)		4(4)	1	1	1	H, I	yes
Glucoamylase or Amyloglucosidase	Rhizopus delermar	none								
Glucoamylase or Amyloglucosidase	Rhizopus niveus	none	1		1	1			H, I	
Glucoamylase or Amyloglucosidase	Rhizopus oryzae	none								
Glucose isomerase	Actinoplanes missouriensis	none	1	1	1		1		H,I,J	yes
Glucose isomerase	Streptomyces lividans	Actinoplanes sp.	1	1	1			1		
Glucose isomerase	Streptomyces murinus	none			1		1			
Glucose isomerase	Streptomyces olivochromogenes	none								yes
Glucose isomerase	Streptomyces rubiginosus	Streptomyces sp.			1					
Glucose oxidase	Aspergillus niger	Aspergillus sp.			1		1	1	A	
Glucose oxidase	Aspergillus niger	none	4(2)	3(2)	3(3)		4(4)	4(3)		yes
Glucose oxidase	Penicillium chrysogenum	none								
Glucosidase (alpha)	Aspergillus niger	none	1				1	1		
Glucosidase (beta)	Aspergillus niger	none	1	3(2)			2(2)	2(2)		
Glucosidase (exo-1 .3-beta)	Trichoderma harzianum	none			1		1	1		
Glucosyltransferase	Aspergillus foetidus	none								

Principal enzymatic activity	Host organism (production organism)		Toxicology (in brackets: number of companies)							
			Acute	Sub-acute	Subchron	Chron	Mut. bact	Mut. mam	Other	JECFA -ev.
Glutaminase	Bacillus subtilis	none								
Hemicellulase	Aspergillus foetidus	none								
Hemicellulase	Aspergillus niger	none	6(3)	1	5(3)	1	8(3)	9(3)	D,E,F,G,H,I	yes
Hemicellulase	Bacillus amyloliquefaciens or subtilis	Bacillus sp.	2(2)	2(2)	3(3)		3(3)	3(3)	D,E,F	
Hemicellulase	Bacillus amyloliquefaciens or subtilis	none								
Inulase	Aspergillus niger	none			1		1	1		
Invertase or Fructofuranosidase (beta)	Saccharomyces cerevisiae	none								
Lactase	Aspergillus oryzae	Myceliophthora sp.					1	1		
Lactase	Aspergillus oryzae	Polyporus sp.			1		1	1		
Lactase or Galactosidase (beta)	Aspergillus oryzae	Aspergillus sp.						1		
Lactase or Galactosidase (beta)	Aspergillus oryzae	none	1		1	1			B	
Lactase or Galactosidase (beta)	Kluyveromyces lactis	Kluyveromyces sp.		1				1		
Lactase or Galactosidase (beta)	Kluyveromyces lactis	none	1	1	1		1			
Lipase, monoacylglycerol	Penicillium camembertii	none	1		1		1	1		
Lipase, triacylglycerol	Aspergillus niger	none	1		1	1	1	1	B,H,I	
Lipase, triacylglycerol	Aspergillus oryzae	Candida sp.		1			1	1		
Lipase, triacylglycerol	Aspergillus oryzae	Fusarium sp.		1	1		1	1		
Lipase, triacylglycerol	Aspergillus oryzae	Rhizomucor sp.			1		1	1		
Lipase, triacylglycerol	Aspergillus oryzae	Thermomyces sp.			1		1	1		
Lipase, triacylglycerol	Candida lipolytica	none								
Lipase, triacylglycerol	Candida rugosa	none	1		1		1	1		
Lipase, triacylglycerol	Mucor javanicus	none	1		1	1	1		B,H,I,J	
Lipase, triacylglycerol	Penicillium roqueforti	none	1		1		1	1		
Lipase, triacylglycerol	Pseudomonas alcaligenes	Pseudomonas sp.								
Lipase, triacylglycerol	Rhizomucor miehei	none		2(1)	1	1	1	1	B	

Principal enzymatic activity	Host organism (production organism)		Toxicology (in brackets: number of companies)							
			Acute	Sub-acute	Subchron	Chron	Mut. bact	Mut. mam	Other	JECFA -ev.
Lipase, triacylglycerol	Rhizopus deleamar	none								
Lipase, triacylglycerol	Rhizopus niveus	none	1		1	1	1		H,I	
Lipase, triacylglycerol	Rhizopus oryzae or arrhizus	none	2(2)	2(2)	5(2)	1	3(3)	1	A,B	
Lipoxygenase	Penicillium chrysogenum	none								
Maltogenic amylase	Bacillus amyloliquefaciens or subtilis	Bacillus Sp.			1		1	1		yes
Mannanase (endo-1 ,4-beta)	Aspergillus niger	none								
Mannanase (endo-1 ,4-beta)	Penicillium funiculosum	none	1	1	1		1	1	E,F	
Mannanase (endo-1 ,4-beta)	Trichoderma reesei or longibrachiatum	Trichoderma sp.								
Pectate lyase	Bacillus licheniformis	Bacillus sp.	1				1		D,E,F	
Pectin lyase	Aspergillus niger var. awamori	Aspergillus sp.		1	1		2(1)	1		
Pectin lyase	Aspergillus niger	none	1		1		2(1)	2(1)	E	yes
Pectin lyase	Trichoderma reesei or longibrachiatum	Aspergillus sp.								
Pectin methylesterase or Pectinesterase	Aspergillus niger	Aspergillus sp.		1			1	1		
Pectin methylesterase or Pectinesterase	Aspergillus niger	none	1	1	2(2)		3(2)	3(2)	E	yes
Pectin methylesterase or Pectinesterase	Aspergillus oryzae	Aspergillus sp.			1		1	1		
Pectin methylesterase or Pectinesterase	Trichoderma reesei er longibrachiatum	Aspergillus sp.	3(1)							
Penicillin amidase	Alcaligenes faecalis	Alcaligenes sp.								
Pentosanase	Humicola insolens	none			1		1	1		
Pentosanase	Trichoderma reesei or longibrachiatum	none			1		1	1		
Phosphatase	Aspergillus niger	none								
Phosphodiesterase	Leptographium procerum	none		2(1)			1	1		
Phosphodiesterase	Penicillium citrinum	none	1		2(1)		1	1		
Phospholipase A	Trichoderma reesei er longibrachiatum	Aspergillus sp.								

Principal enzymatic activity	Host organism (production organism)		Toxicology (in brackets: number of companies)							
			Acute	Sub-acute	Subchron	Chron	Mut. bact	Mut. mam	Other	JECFA -ev.
Phospholipase B	<i>Aspergillus niger</i>	none	1	1	1		1	1	E,F	
Phospholipase B	<i>Aspergillus niger</i> var. <i>awamori</i>	none								
Phospholipase B	<i>Trichoderma reesei</i> or <i>longibrachiatum</i>	<i>Aspergillus</i> sp.								
Phytase	<i>Aspergillus niger</i>	<i>Aspergillus</i> sp.	2(1)	4(1)	3(1)		4(1)	3(1)	A,D,E,F	
Phytase	<i>Aspergillus niger</i>	none	1		1	1	1	1		
Phytase	<i>Aspergillus oryzae</i>	<i>Peniophore</i> sp.		1			1	1	D,E,F	
Phytase	<i>Trichoderma reesei</i> or <i>longibrachiatum</i>	<i>Aspergillus</i> sp.			1		1	1	D,E,F	
Polygalacturonase or Pectinase	<i>Aspergillus aculeatus</i>	none			1		1	1		
Polygalacturonase or Pectinase	<i>Aspergillus niger</i>	none	2(2)	5(1)	8(5)	1	8(5)	8(5)	E,H,I	yes
Polygalacturonase or Pectinase	<i>Aspergillus pulverulentus</i>	none	1	1	1	1				
Polygalacturonase or Pectinase	<i>Penicillium funiculosum</i>	none	1	1	1		1	1	E,F	
Polygalacturonase or Pectinase	<i>Trichoderma reesei</i> or <i>longibrachiatum</i>	<i>Aspergillus</i> sp.	3(1)							
Protease (incl. milkclotting enzymes)	<i>Aspergillus melleus</i>	none	1			1		1	B,H,I	
Protease (incl. milkclotting enzymes)	<i>Aspergillus niger</i>	none			1		1			
Protease (incl. milkclotting enzymes)	<i>Aspergillus oryzae</i>	<i>Aspergillus</i> sp.			1		1	1	D,E,F	
Protease (incl. milkclotting enzymes)	<i>Aspergillus oryzae</i>	<i>Rhizomucor</i> sp.		2(1)	2(1)		1	2(1)	B	
Protease (incl. milkclotting enzymes)	<i>Aspergillus oryzae</i>	none	1		2(2)		2(2)	2(2)	H,I	yes
Protease (incl. milkclotting enzymes)	<i>Aspergillus sojae</i>	none								
Protease (incl. milkclotting enzymes)	<i>Bacillus alcalophilus</i>	<i>Bacillus</i> sp.	1	1	1		1	1	D,E,F,G	
Protease (incl. milkclotting enzymes)	<i>Bacillus amyloliquefaciens</i> or <i>subtilis</i>	<i>Bacillus</i> sp.	2(2)	2(2)	1		2(2)	2(2)		

Principal enzymatic activity	Host organism (production organism)		Toxicology (in brackets: number of companies)							
			Acute	Sub-acute	Subchron	Chron	Mut. bact	Mut. mam	Other	JECFA -ev.
Protease (incl. milkclotting enzymes)	Bacillus amyloliquefaciens or subtilis	none	1	1	2(2)		2(2)	2(2)		yes
Protease (incl. milkclotting enzymes)	Bacillus halodurans or lentus	Bacillus sp.	1				1	1		
Protease (incl. milkclotting enzymes)	Bacillus halodurans or lentus	none			1		1	1		
Protease (incl. milkclotting enzymes)	Bacillus licheniformis	Bacillus sp.	3(1)				2(1)	1	E, G	
Protease (incl. milkclotting enzymes)	Bacillus licheniformis	none	2(1)	3(1)	1		1	1	D,E,F,G	
Protease (incl. milkclotting enzymes)	Bacillus stearothermophilus	none	1		1		1	1		
Protease (incl. milkclotting enzymes)	Cryphonectria or Endothia parasitica	Cryphonectria sp.	1	1			1	1		
Protease (incl. milkclotting enzymes)	Cryphonectria or Endothia parasitica	none	1		2(1)					yes
Protease (incl. milkclotting enzymes)	Penicillium citrinum	none	1		2(1)		1	1		
Protease (incl. milkclotting enzymes)	Rhizomucor miehei	none	5(1)	6(1)	1	2(1)	7(2)	3(1)	ABDEFGH	yes
Protease (incl. milkclotting enzymes)	Rhizopus niveus	none	1		1	1	1		HI	
Protease (incl. milkclotting enzymes)	Streptomyces fradiae	none	1			1			HJ	yes
Pullulanase	Bacillus acidopullulyticus	none			1		1	1		
Pullulanase	Bacillus circulans	none			1		1			
Pullulanase	Bacillus licheniformis	Bacillus sp.		1	2(2)		2(2)	2(2)	A,D,E,F	
Pullulanase	Bacillus subtilis	Bacillus sp.								
Pullulanase	Klebsiella planticola	Klebsiella sp.	1	1	1		1	1		
Pullulanase	Klebsiella planticola	none								
Pullulanase	Trichoderma reesei or longibrachiatum	Hormoconis sp.								
Rhamnosidase (alpha-L)	Penicillium decumbens	none					1			

Principal enzymatic activity	Host organism (production organism)	Donor organism	Toxicology (in brackets: number of companies)							
			Acute	Sub-acute	Subchron	Chron	Mut. bact	Mut. mam	Other	JECFA -ev.
Tannase	Aspergillus niger	none								
Transglutaminase	Streptovercillium mobaraense	none	1	1	1	1	1	1		
Xaa-Pro-dipeptidyl-aminopeptidase	Lactococcus lactis	none								
Xylanase	Aspergillus foetidus	none			1		1	1		
Xylanase	Aspergillus niger	Aspergillus sp.	2(1)	1	1		2(1)	1	D E F G	
Xylanase	Aspergillus niger	none	3(1)		2(1)		3(1)	5	DE,F,G	
Xylanase	Aspergillus niger var. awamori	Aspergillus sp.			1		1	1		
Xylanase	Aspergillus niger var. awamori	none			1		1	1		
Xylanase	Aspergillus oryzae	Aspergillus sp.			1		1	1		
Xylanase	Aspergillus oryzae	Thermomyces sp.			1		1	1	DEF	
Xylanase	Bacillus amyloliquefaciens or subtilis	Bacillus sp.	1	2(1)	2(2)		2(2)	2(2)	DEF	
Xylanase	Bacillus amyloliquefaciens or subtilis	none								
Xylanase	Bacillus licheniformis	Bacillus sp.	1				1		E,F	
Xylanase	Disporotrichum dimorphosporum	none		1	1		1	1	A	
Xylanase	Humicola insolens	none			1		1	1	D,E,F	
Xylanase	Penicillium funiculosum	none	1	1	1		1	1	E,F	
Xylanase	Trichoderma reesei or longibrachiatum	Actinomadura sp.								
Xylanase	Trichoderma reesei or longibrachiatum	Trichoderma sp.			1		1	1	A,D,E,F	
Xylanase	Trichoderma reesei or longibrachiatum	none	2(2)	3(3)	5(5)		5(5)	4(4)	ADEFG	

\* Those enzymes which do not have an IUS number are enzyme complexes, where the listed activity is the result of the sum of many single active enzymes prot LEGEND~

@ There is a general IUB number for aminopeptidases. But all these enzymes fall under the 3.4.11 x category.

# There is no general IUB number for proteases. But all these enzymes fall under the 3.4.2x category

#### TOX-STUDIES:

Acute: studies with a single oral dose given

Subacute: studies with daily oral doses given (more than 1 day, usually 28 days)

Subchron: studies with daily oral doses given (more than 28 days, usually 91 days)

Chron: studies with daily oral doses given (more than 91 days)

Mut.bact.: in vitro mutation studies using bacterial cells

Mut.mam.: in vitro mutation studies using mammalian cells

#### OTHER STUDIES:

A) in vivo genotoxicity

- B) Reproduktion (incl. teratogenicity)
- C) in vitro cytotoxicity
- D) acute inhalation
- E) acute dermal irritation
- F) acute eye irritation
- G) skin sensitisation
- H) acute interperitoneal
- I) acute subcutaneous
- J) acute intravenous

REMARKS:

- a) as carbohydrase
  - b) part of studies as „carbohydrase“
  - c) part of studies as „carbohydrase mixed with protease“
  - d) with xylanase
  - e) part of studies as „pectinases“
  - f) part of studies as „hemicellulase“
  - g) with  $\beta$ -glucanase
  - 1) includes studies performed on mice
  - 2) includes studies performed on ducklings
  - 3) includes studies performed on rabbits
  - 4) includes studies performed on dogs
  - 5) includes relay-studies with cheese
  - 6) includes studies performed with guinea pigs
  - 7) includes studies performed with cats
  - 8) includes studies performed with monkeys
  - 9) includes relay-study with High Fructose Corn Syrup
- studies performed on equivalent product from classical production organism



Table 64: Selected protein and enzyme databases

Name; Internet Address; Statistics	Description
<b>BRENDA</b> <a href="http://brenda.bc.uni-koeln.de">http://brenda.bc.uni-koeln.de</a> 12,530 entries (indexed 16-Feb-2001)	The development of BRENDA, the enzyme database was started in 1987 at the GBF in Braunschweig the book „Enzyme Handbook“ representing the printed version of this data bank. The enzymes in the data base are classified according to the Enzyme Commission list of enzymes and later supplements. According to E.C. numbers some 3,400 „different“ enzymes are covered. Frequently very different enzymes are included under the same E.C. number. Meanwhile all classified enzymes are covered (so far ca. 35,000 literature references are implemented).
<b>Enzyme</b> <a href="http://www.expasy.ch/enzyme">http://www.expasy.ch/enzyme</a> 3,751 entries (Release 26.0, May 2000, and updates up to 05-May-2001)	The ENZYME database is a repository of information relative to the nomenclature of enzymes. It is primarily based on the recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB), and it contains data for each type of characterised enzyme for which an EC (Enzyme Commission) number has been provided.
<b>The Swiss-Prot Data Bank</b> <a href="http://www.expasy.ch/sprot/">http://www.expasy.ch/sprot/</a> 95,674 protein entries (SWISS-PROT Release 39.18 of 04-May-2001; presumably 34,529 enzymes due to „ase“)	The Swiss-Prot Data Bank is a protein sequence database maintained collaboratively by the University of Geneva and the European Institute of Bioinformatics (EBI). A high level of annotations (such as the description of the function of a protein, its domains structure, post-translational modifications, variants, etc.), a minimal level of redundancy, and a high level of integration with other databases is strived. The SWISS-PROT protein sequence data bank consists of sequence entries.
<b>The Brookhaven Protein Data Bank</b> <a href="http://www.rcsb.org/pdb/index.html">http://www.rcsb.org/pdb/index.html</a> 7,231 enzymes (search performed 15-May-2001)	The Protein Data Bank (PDB) is the single international repository for the processing and distribution of 3-D macromolecular structure data primarily determined experimentally. The Protein Data Bank is operated by the Research Collaboratory for Structural Bioinformatics (RCSB) under a contract to the U.S. National Science Foundation and is supported by other funds. Out of a total of 15,299 entries for „Proteins, Peptides, and Viruses“ (15-Jan-2002 update), there are 12,881 structures determined by X-ray Diffraction and other; 2,105 by NMR and 313 by theoretical modelling.
<b>EMP, The Enzymology Database</b> <a href="http://www.empproject.com/">http://www.empproject.com/</a> Statistics: See description	The Enzymes and Metabolic Pathways database, EMP covers all aspects of enzymology and metabolism and represents the whole factual content of original journal publications. (EMP contains about 30,000 records compiled from about 15,000 original experimental journal publications). The database is being created in Pushchino (Moscow Region, Russia) by a team of scientists, bibliographers and programmers. A metabolic part of EMP constitutes a separate database, EMP Pathways (earlier known as MPW). It has over 3,000 metabolic diagrams.
<b>Protherm</b> <a href="http://www.rtc.riken.go.jp/jouhou/Protherm/protherm.html">http://www.rtc.riken.go.jp/jouhou/Protherm/protherm.html</a> Statistics: Total number of entries: 8816; Number of unique proteins: 426 (122 enzymes found by „ase“); Number of Protein Names with mutant: 147 (39 enzymes found by „ase“)	The thermodynamic database for proteins and mutants (ProTherm) contains numerical data of several thermodynamic parameters, namely, Gibbs free energy, enthalpy, heat capacity, transition temperature etc. for wild type and mutant proteins, that are important for understanding the structure and stability of proteins upon mutations. It also contains information about secondary structure, accessibility of wild type residues, experimental conditions (pH, temperature etc.), measurements and methods used for each data, and activity information ( $K_M$ and $K_{cat}$ etc.).

Table 65: Survey on the information covered by the protein and enzyme databases described in Table 64

	Information/Parameter	BRENDA	Enzyme	Swiss-Prot	PDB	EMP	Pro-therm
	<b>Nomenclature</b>						
N1	EC Number	X	X	X			X
N2	Recommended Name	X					
N3	Systematic Name	X					
N4	Enzyme Names	X	Official name	name	name	name	name
N5	Synonyms	X		X			
N6	CAS Registry Number	X					
N7	Reaction	X	x	function	Biological function	X	
N8	Reaction Type	X				Metabolism	
N9	Organism	X					
	<b>Enzyme-Ligand Interactions</b>						
E1	Ligands	X	X			Enzyme regulation	
E2	Substrates and Products	X					
E3	Substrates	X					
E4	Products	X					
E5	Natural Substrates	X					
E6	Cofactors	X					
E7	Metals/Ions	X					
E8	Inhibitors	X					
E9	Activating Compounds	X					
	<b>Functional Parameters</b>						
F1	K <sub>M</sub> Value	X				X	X
F2	Turnover Number (k <sub>cat</sub> )	X					X
F3	Specific Activity	X					
F4	pH Optimum	X					
F5	pH Range	X					
F6	Temperature Optimum	X					
F7	Temperature Range	X					
	Thermodynamics					X	X <sup>97</sup>
	<b>Organism related Information</b>						
O1	Source Tissue	X					
O2	Localisation	X					
	Enzyme Structure						

<sup>97</sup> Thermodynamic features covering: method used to measure thermodynamic parameters (fluorescence spectroscopy, circular dichroism, differential scanning calorimetry); experimental method of denaturation; pH, dG, concentration of denaturant, ddG (dG(mutant) - dG(wild)), dH, reversibility, number of transition states.

	Information/Parameter	BRENDA	Enzyme	Swiss-Prot	PDB	EMP	Pro-therm
	Number of amino acids			X	X		X
S1	Sequence	X					
S2	PDB	X					
S3	Molecular Weight	X		X		X	X
S4	Subunits	X		X	X	X	
	Domains and Sites			X			
	Secondary Structure			X			X
S5	Crystallization	X			X		
S6	Posttranslational Modification	X		X		X	
	<b>Molecular Properties</b>						
M1	pH Stability	X					
M2	Temperature Stability	X					
M3	General Stability	X					
M4	Organic Solvent Stability	X					
M5	Oxidation Stability	X					
M6	Storage Stability	X					
M7	Purification	X				X	
M8	Cloned	X					
M9	Engineering	X					
M10	Renatured	X					
M11	Application	X					
	<b>Bibliography/Disease</b>						
B1	References	X	X	X	X	X	X
B2	Disease	X		X			
	Immunochemistry					X	

EMP... The Enzymology Database; PDB... Protein Data Bank.

Table 66: Material Safety Data Sheets (MSDS) for enzyme preparation Xx

<b>1. Identification of the Preparation and the Company</b>	
Commercial product name:	Xx
Description:	Granulated protease enzyme preparation
Appearance:	Off-white granulate
Responsible company:	xy
<b>2. Information on Ingredients</b>	
Chemical characterisation of active component:	Enzyme protein
Synonyms:	Protease (Subtilisin)
IUB number:	3.4.21.62

CAS number:	9014-01-1
EINECS number:	232-752-2
Hazardous ingredients:	Protease enzyme protein (1-10%)
Classification of preparation:	Xn (harmful), R-42 , R-36/38
Exposure limit:	0.00006 mg/m3 (as 100% pure protease)
<b>3. Hazards Identification</b>	
Granulated enzymes are coated to prevent formation of enzyme dust. However, inappropriate handling may release dust. For appropriate handling see section 6 and 7. Inhalation of enzyme dust or aerosols resulting from inappropriate handling may induce sensitisation and may cause allergic reactions in sensitised individuals. Prolonged skin contact may cause minor irritation. The preparation is irritating to eyes.	
<b>4. First Aid Measures</b>	
Skin contact:	Wash skin with plenty of water.
Eye contact:	Rinse eyes with plenty of water for at least 15 minutes and see an eye specialist.
Ingestion:	Rinse mouth and throat thoroughly with water. Drink water.
Inhalation:	Remove from exposure. If symptoms of irritation or sensitisation occur (shortness of breath, wheezing or laboured coughing), call a doctor.
<b>5. Fire-fighting Measures</b>	
Protection against fire and explosions:	No special requirements
Suitable fire extinguishing media:	Water, foam
Non-suitable media:	None
Special exposure hazards:	None
<b>6. Accidental Release Measures</b>	
Spilled preparation should be removed immediately. Avoid formation of dust. Take up by mechanical means, preferably by a vacuum cleaner equipped with a high efficiency filter. Flush remainder carefully with plenty of water. Avoid splashing and high-pressure washing (avoid formation of aerosols). Ensure sufficient ventilation. Wash contaminated clothing.	
<b>7. Handling and Storage</b>	
Avoid formation of dust. Avoid splashing and high-pressure washing. Ensure good ventilation of the room when handling this preparation. Store container in a cool and dry place.	
<b>8. Exposure Controls/Personal Protection</b>	
Recommended personal protective equipment:	
Respiratory protection:	Respirator with P3 filter
Hand protection:	Impermeable gloves
Eye protection:	Protective glasses or eye shield
Clothing:	Wear suitable protective clothing
<b>9. Physical and Chemical Properties</b>	
Appearance:	Off-white granulate.
Odour:	Slight fermentation odour
pH, boiling point, melting point, flash point, ignition temperature, vapour pressure, density and solubility are not relevant to safety. For further information see the Product Specification and Product Sheet for this preparation.	
<b>10. Stability and Reactivity</b>	
This material is stable under normal conditions of use.	
Conditions to avoid:	None
Materials to avoid:	None
Hazardous decomposition products:	None
<b>11. Toxicological Information</b>	

Inhalation of aerosols or dust resulting from inappropriate handling may induce sensitization and may cause allergic reactions in sensitized individuals. Prolonged skin contact may cause minor irritation. The preparation is irritating to eyes. Oral rat LD-50 > 2g/kg b.w. classifies the preparation as „non-toxic“.	
<b>12. Ecological Information</b>	
LC-50(fish) > 100 mg/l, EC-50(daphnia) > 100 mg/l and IC-50(algae) > 100 mg/l, which classifies the preparation as „non-dangerous“ to the environment.	
The preparation is biodegradable.	
<b>13. Disposal Considerations</b>	
No special disposal method required, except as in accordance with current local authority regulations.	
<b>14. Transport Information</b>	
UN No:	Not applicable
Sea:	Not applicable
Road/Rail:	Not applicable
Air:	Not applicable
<b>15. Regulatory Information</b>	
The preparation is a dangerous preparation according to EU regulations.	
Labelling:	
Xn	(harmful)
R-42	May cause sensitization by inhalation
R-36/38	Irritating to eyes and skin
S-22	Do not breathe dust.
S-24	Avoid contact with skin
S-26	In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
S-36/37/39	Wear suitable protective clothing, gloves and eye/face protection.
<b>16. Other Information</b>	
As of the date of issue the information contained in this Enzyme Material Safety Data Sheet is believed to be true and correct. However, the accuracy or completeness of this information and any recommendations or suggestions are made without warranty or guarantee. Since the conditions of use are beyond the control of our company, it is the responsibility of the user to determine the conditions for safe use of this preparation. The information in this data sheet does not represent analytical specifications, for which please refer to our Product Specification.	

## 13.2 Roster of Questions to AMFEP

### 13.2.1 Questions to AMFEP supplementing the AMFEP list of enzymes (Roster 1)

#### Governing Remarks

The questions listed below refer to the AMFEP list of enzymes and are intended to extend this list with additional information.<sup>98</sup> Consequently each question should be answered for each individual enzyme stated in the AMFEP list, e.g. alpha-Amylase from *Aspergillus niger*. If one particular enzyme entry in the AMFEP list comprises at least two *structurally different* enzymes<sup>99</sup>, *questions 2 to 22* should be answered *separately* for each enzyme variant. Please note that the requested information is specifically demanded by the Environment DG of the European Commission. Any data provided from the industry and marked as confidential will only be disclosed to the principal. However, if you skip any questions, please, *state the particular reasons*, e.g. „no data available” or „confidential”.

Furthermore, it would be favourable if the *AMAFE enzymes* could be considered in the AMFEP list. Consequently, the AMFEP list will have to be updated and the questions will also apply to these enzymes.

#### Roster of Questions

- 1) Please state the number of enzyme variants differing in amino acid structure for each enzyme entry in the AMFEP list as described above.
- 2) Please enclose the corresponding product sheet (technical sheet) and the Material Safety Data Sheet for each enzyme.
- 3) Please specify the catalytic properties of this particular enzyme.
  - a) the main activity (range of substrate specificity)
  - b) side activities (range of substrate specificity)
- 4) Please give data on kinetic parameters  $K_m$  and  $V_{max}$  of this particular enzyme.
- 5) Is there any data on the enzyme structure available?
  - a) Amino acid sequence (Yes/No)
  - b) 2-dimensional structure (Yes/No)
  - c) 3-dimensional structure (Yes/No)
  - d) Quaternary structure (Yes/No)
  - e) Active centre (Yes/No)
  - f) Posttranslational modification (Yes/No)
  - g) If data are already published, please give the full citation
  - h) If the protein (amino acid sequence) is already registered in a protein or enzyme database (PIR, PDB, BRENDA, EXPASY etc.), please specify the registration number.

<sup>98</sup> Already given in the AMFEP list of enzymes: Type of enzyme (depending on the enzyme activity according to IUB), IUB number and number of the corresponding EINECS entry, source of isolation/production (microorganisms, cell culture, animal or plant tissue), use of the enzyme.

<sup>99</sup> „Structurally different” means enzymes of different amino acid sequence e.g. isoenzymes or enzyme variants produced by protein engineering.

- 6) Please specify if the particular enzyme is marketed as a multi-enzyme preparation or as a single-enzyme preparation.<sup>100</sup>
- 7) In case the enzyme is also or exclusively marketed as a multi-enzyme preparation, please give data on the accompanying enzymes in each of these multi-enzyme preparations.
- 8) Please specify if the particular enzyme is marketed as an enzyme preparation that contains more than one enzyme of technical importance (Yes/No/Not Investigated).<sup>101</sup>
- 9) In case the answer to question 8) is yes, please specify the accompanying enzymes.
- 10) Please specify if this enzyme is also or exclusively marketed/used in an immobilised form.
- 11) Please specify if this enzyme is manufactured to different grades of purity (according to their area of application).
- 12) Please specify the annual tonnage (round figure) of the particular enzyme manufactured / marketed in the EU.
- 13) Please specify the year the particular enzyme was launched on the EU market.
- 14) Please describe in brief the way the enzyme is purified.
- 15) Please specify the enzyme assay(s) routinely used to detect the enzyme activity and give the definition of Units used. If the assay has already been published, please give the full citation. If either a modified version of the published assay is used or the assay is not described in a publicly accessible document, please enclose a brief description of this enzyme assay(s).
- 16) Please specify any methods in use for *specifically* and routinely detecting this particular enzyme (e.g. immunological methods). Please describe any limitations concerning the specificity of the method in use, e.g. certain enzyme variants (due to protein engineering) might not be distinguishable.
- 17) Please specify any post-translational modification of the enzyme (peptide processing, glycosylation, phosphorylation, peptide bound cleavage etc.). If published data are available, please give full citation of the corresponding paper(s).

**In case there are any differences in post-translational modification of the enzyme between the donor organism and the host organism or in case this is not known, please, answer to question 18).**

- 18) Please, specify in which properties the wild type enzyme *significantly* differs from the enzyme which is commercially marketed.<sup>102</sup> (Possible answers: Yes/No/Not investigated)
  - a) Isoelectric point
  - b) pH optimum
  - c) pH range
  - d) Temperature optimum
  - e) Temperature range

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<sup>100</sup> Multi-enzyme preparation is understood as a mixture of at least two different enzymes which have been manufactured separately.

<sup>101</sup> Different to questions 6) and 7) this question aims at enzyme preparation resulting from a single source and from one fermentation process and containing *more than one enzyme of technical importance*.

<sup>102</sup> In this regard „significantly“ is not understood in a regulatory sense, but rather in a scientific sense.

- f) Substrate spectrum (main activity)
- g) Substrate spectrum (side activity)
- h) Product spectrum
- i) Kinetic parameters ( $V_{\max}$ ,  $K_m$ )
- j) Co-factors
- k) Stability (pH, temperature, oxidation, storage etc.)
- l) Immunological properties
- m) Ecotoxicological properties
- n) Toxicological properties
- o) Other.....

Comments .....

**In case the enzyme is structurally modified by protein engineering, please answer to questions 19) to 21).**

- 19) Please give the exact number of amino acids which are changed/deleted or introduced.
- 20) Please specify (enclose) more details on these structural modifications. If the data have already been published, please give the full citation.
- 21) Please specify which properties of the enzyme are significantly affected<sup>103</sup> by protein engineering (Yes/No/Not investigated)
  - a) Isoelectric point
  - b) pH optimum
  - c) pH range
  - d) Temperature optimum
  - e) Temperature range
  - f) Substrate spectrum (main activity)
  - g) Substrate spectrum (side activity)
  - h) Product spectrum
  - i) Kinetic parameters ( $V_{\max}$ ,  $K_m$ )
  - j) Co-factors
  - k) Stability (pH, temperature, oxidation, storage etc.)
  - l) Immunological properties
  - m) Ecotoxicological properties
  - n) Toxicological properties
  - o) Other.....

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<sup>103</sup> See footnote 102.



Comments .....

22) Please specify any post-fermentation processing of the enzyme which could/will lead to changes in at least one of the properties described in question 21) a - o.

### 13.2.2 Questions to AMFEP not related to individual enzymes (Roster 2)

#### Governing Remarks

These questions should rather be answered on a general basis than on the basis of individual enzymes. However, it is assumed that the answers will differ significantly between the enzyme products for different areas of application, thereby reducing the significance of the data obtained. It will thus be most favourable to define certain categories such as food enzymes, feed enzymes, detergent enzymes, enzymes for personal care, enzymes for medical purposes etc. and give answers separately for each category.

The purpose of the questions listed in the following is to get more precise information on the type and the range of both additives and impurities in enzyme products. Likewise, we would like to get information on physico-chemical properties investigated and on the testing procedures for health and environmental properties applied in the safety assessment of enzyme preparations..

#### Roster of Questions

- 1) Please state the approximate range of active enzyme<sup>104</sup> in the enzyme isolate (column A) and also the approximate range of enzyme isolate in the final enzyme preparation (column B) for each category of enzymes.<sup>105</sup>

Category of enzymes	A Active enzyme/ Enzyme isolate [Range (percentage)]	B Enzyme isolate/ En- zyme preparation [Range (percentage)]
Technical enzymes <sup>106</sup>		
Food enzymes		
Feed enzymes		
Enzymes for personal care products <sup>107</sup>		
Enzymes for therapeutic applications <sup>108</sup>		
Enzymes for analytical/diagnostic application		
other		

- 2) Please specify the impurities in the enzyme isolate for each enzyme category.
  - a) Type of impurities:
  - b) Approximate range of impurities

<sup>104</sup> The enzyme of technical importance.

<sup>105</sup> The enzyme isolate is understood as the purified enzyme from the fermentation broth containing the pure enzyme and impurities originating from the fermentation or purification process. The enzyme preparation is understood as the commercial enzyme product marketed in the EU containing the enzyme isolate and any additives needed, e.g. stabilisers.

<sup>106</sup> E. g. detergent enzymes, enzymes for the pulp and paper industry, for fuel production, leather and textile industries, enzymes used in oil drilling.

<sup>107</sup> E. g. cosmetics, denture cleansers, toothpaste, contact lens cleansers.

<sup>108</sup> E. g. digestive enzymes.

Category of enzymes	Type of impurities	Range (percentage)
Technical enzymes		
Food enzymes		
Feed enzymes		
Enzymes for personal care products		
Enzymes for therapeutic applications		
Enzymes for analytical/diagnostic application		
other		

3) Please specify the additives in the final enzyme product for each enzyme category.

- a) Name and purpose of additive
- b) Approximate range of additives

Category of enzymes	Type of additive	Purpose	Range (percentage)
Technical enzymes			
Food enzymes			
Feed enzymes			
Enzymes for personal care products			
Enzymes for therapeutic applications			
Enzymes for analytical/diagnostic application			
Other			

4) Please, specify the testing of physico-chemical, health and environmental properties for each enzyme category for enzymes marketed within the EU. If different tests are applied (e. g depending on the enzyme manufacturer) to comply with particular regulations, please, specify each test applied.

Category of enzymes	Type of physico-chemical, health or environmental effect / method ( e. g. OECD, EEC, others) /	Testing is performed voluntarily (V) or required by regulation(R)	Test substance enzyme isolate <sup>110</sup> (EI) and / or enzyme prepara-
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	GLP principles applied or not applied <sup>109</sup>		tion <sup>14</sup> (EP) and / or others (please specify) (O)
Technical enzymes			
Food enzymes			
Feed enzymes			
Enzymes for personal care products			
Enzymes for therapeutic applications			
Enzymes for analytical/diagnostic application			
Other (please specify)			

- 5) Please enclose reports or documents on (potential) human health and environmental problems caused by enzymes which are not published or might not be accessible for the project team.

<sup>109</sup> E.g. eye irritation / according to OECD test 405 / performed according to the principles of Good Laboratory Practice (GLP).

<sup>110</sup> The enzyme isolate is understood as the purified enzyme from the fermentation broth containing the pure enzyme and impurities originating from the fermentation or purification process. The enzyme preparation is understood as the commercial enzyme product marketed in the EU containing the enzyme isolate and also any additives needed, e.g. stabilisers.

### 13.2.3 Roster of Questions concerning Enzyme Regulation (Roster 3)

The questions below mainly refer to regulatory issues of either enzymes in general or enzymes for specific applications. Questions 2 to 5 do not need to be answered systematically and in their entirety, e.g. for each country where enzyme preparations are marketed. Their intention is rather to ask about enzyme regulation that is considered as being relevant.

#### *Regulation of enzymes in Directive 67/548/EEC*

- 1) Certain preparations are exempted from regulation according to Article 1 (2) of Directive 92/32/EEC. If any enzyme categories are exempted from this Directive, please name the category of enzymes and the reason why they are exempted.

#### *Regulation of enzymes*

- 2) Which countries inside and outside the EU have a *national* regulation for enzymes (covering enzymes in general, not enzymes for a particular application e.g. feed enzymes)? Please, give the name of a contact person for each country (name, department/authority, e-mail, phone number/fax) for further inquiries.

#### *Enzymes in personal care products*

- 3) Which countries *inside and outside* the EU have a national regulation or notification system for enzymes in personal care products (e. g. cosmetics, denture cleansers, toothpaste, contact lens cleansers)? Please, give the name of a contact person for each country (name, department/authority, e-mail, phone number/fax) for further inquiries.

#### *Food enzymes*

- 4) Which countries *outside* the EU have a national regulation or notification system for food enzymes? Please, give the name of a contact person for each country (name, department/authority, e-mail, phone number/fax) for further inquiries.

#### *Feed enzymes*

- 5) Which countries *inside and outside* the EU have a national regulation or notification system for feed enzymes? Please, give the name of a contact person for each country (name, department/authority, e-mail, phone number/fax) for further inquiries.

#### *Enzymes for analytical/diagnostic/therapeutic application*

- 6) Enzymes which are produced in very small amounts and/or which are used either in laboratory research work (analytical purposes, DNA- or RNA-modifying enzymes) or in medicine (for therapeutic or diagnostic purposes) are not the focal point of our work. Since these enzymes should also be considered in this study, however, we would like to ask you for information on the following issues:
  - a) Does AMFEP also represent major manufacturers of enzymes for analytical, scientific, diagnostic or therapeutic application?
  - b) Are these enzymes already included in the AMFEP list?

- 
- 7) If these enzyme manufacturers are not represented in AMFEP (as it presumably is in the case of analytical enzymes for molecular biology), please specify a contact person (name, department, e-mail, phone number/fax) for further inquiries.
- 8) What kind of regulation within the EU do you have to consider for the marketing of
- i) analytical/diagnostic/scientific enzymes
  - ii) enzymes in personal care products (please specify a contact person for AMFEP and - if possible - in the European Commission (name, department, e-mail, phone number/fax) for further inquiries)
  - iii) therapeutic enzymes<sup>111</sup> (please specify a contact person for AMFEP and - if possible - in the European Commission (name, department, e-mail, phone number/fax) for further inquiries)

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<sup>111</sup> E.g. digestive aids.

### 13.3 AMFEP Position on Roster of Questions

#### **Amfep Position on Roster of Questions (2002.02.27)**

##### **Introduction**

Amfep has expressed its interest cooperating with the Commission and its Austrian Task Force on the work aiming at a future scheme for enzymes within the scope of EU environmental and chemicals legislation.

A number of documents and data have already been provided by Amfep, in particular the updated List of Commercial Enzymes. Furthermore, meetings have been held with the Task Force and DG ENV.

However, as already explained in previous communications, the very comprehensive Roster of Questions was considered as going much too far as compared to the study objectives and the ability of companies.

Following discussions with the Task Force, DG ENV and within our association, Amfep, however, decided to provide this consolidated, joint response to the Roster. The earlier draft response to section 2 has been edited and included in this document.

Our response comprises answers to some questions but also explanations why data are not available and positions on why requested data are not considered critical or relevant for the purpose of a study aiming at regulation.

Questions related to regulation and notification of enzymes is still being discussed within Amfep and with the Commission regarding food and feed enzymes. Therefore, the earlier draft response to questions on enzyme regulation has not been included in the document.

We would like to come back to these issues in a coming meeting with the Task Force and DG ENV.

##### **Applying strict chemical identity criteria would neither be logical nor operational for enzymes.**

From a safety point of view, this is relevant only for conventional, small molecular weight substances.

The 3-D structure of the majority of enzymes has not been determined and the data are of no relevance with respect to identification, classification and risk assessment of enzyme preparations.

Per entry of the Amfep List of Commercial Enzymes each company may market more than one product varying in terms of trade name, formulation and structure (amino acid sequence variants).

Amfep does not have an overview of these product variations, which in part are subject to proprietary company information.

Concerning structural variation, Amfep is of the opinion that from a safety and regulatory point of view no distinction should be made between variants occurring in nature and such produced by modern biotechnology.

In any case the company intending to market such enzyme will make a safety assessment. In doing so, necessary testing will be considered based on the similarity with known enzymes subject to more extensive testing and/or a history of safe use.

The regulatory status of the variant should be determined based on the catalytic activity, systematic classification (IUB) and inventory compliance (EINECS).

Enzyme families within a class occur naturally and specific enzymes marketed have been selected and further optimized by classical or modern biotechnology techniques.

In order to maintain the overall 3-dimensional structure determining the specific catalytic activity associated with one class or family, it is essential that certain regions of the amino acid sequence are conserved. This is the case for naturally occurring as well as protein engineered variants. Almost super imposable 3-D structures may result from amino acid sequences showing only around 50% homology (e.g. Siezen, R.J., 1997). Generally, such enzymes are characterized by partial or full immunochemical identity.

In the case of enzymes preparations falling under the Amfep scope (i.e. food, feed and technical applications), this variation is not important from a safety point of view. The only known safety risk linked to all enzymes is occupational allergenicity and for certain proteases the minor risk of skin/eye irritation. Although one type of enzyme might be able to induce allergy sooner than another type, the risk of allergenicity is valid for all types of enzymes. Therefore, in this context there is no need to analyse the structure of the enzyme since all enzyme preparations are labelled with the risk phrase 'May cause sensitisation by inhalation'.

The natural occurring structural variations of enzymes (between species and/or within one species) are only partially known, because their amino acid sequences and 3-D structures have not been analysed. In some cases, e.g. if genetic modification of the production organism was involved, the amino acid sequence of a specific enzyme has been determined. But even for these enzymes, it is generally not known in how many other variations they occur in other species or even within the same species. To illustrate how many variations can naturally occur within one organism, the complete DNA sequencing of *A. niger* revealed that one and the same enzyme can occur in 60 variations.

Detergent enzyme producers among Amfep have prepared a document to the German health authority BgVV [attached]. This document addresses the relationship between general enzyme properties, enzyme variants and allergenicity as well as other toxicological endpoints.

**Data Sheets are not prepared for a specific enzyme, but for each formulated enzyme preparation.**

**All key information is given in the Amfep list.**

Technical Data Sheets and Material Safety Data Sheets are not prepared for a specific enzyme, but for each formulated enzyme preparation. As a result, there are hundreds more Data Sheets than there are enzymes on the Amfep list. The Technical Data Sheet contains information that is relevant for the users and their application. They do not contain data on the safety of the enzyme preparation. The variations in the Material Safety Data Sheets of different enzyme preparations are only minor (e.g. other brand name, other formulation) and therefore do not give much relevant information for a specific enzyme preparation. The information in the Material Safety Data Sheets regarding the enzyme activity, CAS and IUB number is already given in the Amfep list. In [Enclosure], some examples of several Material Safety Data Sheets for various formulations and from various Amfep members are given. In [Enclosure], some examples of Technical Data Sheets are given.

The main enzyme activities are mentioned in the Amfep list. Most enzymes are named after the substrate they act upon. Information about the catalytic properties, including substrate specificity, can be found in the IUB „Enzyme Nomenclature” <http://www.chem.qmw.ac.uk/iubmb/enzyme/>. The methods to measure the enzymatic activity make use of the substrate specificity of the enzyme.

In principle, an enzyme preparation may contain minor amounts of all enzyme activities (several hundreds) the production organism makes. In general, only those side activities are measured which may support or affect the enzyme preparation in its specific application. The enzyme activities listed in the Amfep list as main activities can be found as side activities in other enzyme preparations.

K<sub>m</sub> and V<sub>max</sub> values are not determined for the majority of enzymes in the Amfep List since they are not considered important with respect to identification and not relevant for classification and risk assessment of enzyme preparations.

Determination of K<sub>m</sub> and V<sub>max</sub> may be useful for research purposes (mechanistic studies etc.). This requires high enzyme purification and data are related to the specific method of analysis used (i.e. the substrate, pH, temperature, etc). K<sub>m</sub> and V<sub>max</sub> values can only be determined on non-polymeric and well-defined substrates (having a unique, molecular structure).

However, for the performance analysis of commercial enzyme preparations, substrates are used that are relevant for the specific application in question.

**Mixtures of enzymes (blends) are based on declared enzymes from the Amfep list.**

The enzyme industry feels free to market any mixture (called „blends” by the enzyme industry) of two or more different enzyme concentrates, as long as the individual enzyme concentrates are suitable and approved for the application in question. The types of blends on the market are subject to frequent changes, depending on customer demand.



**Individual enzyme concentrates may contain non-declared side-activities.**

From a safety point of view, the types of accompanying enzymes are irrelevant.

From a regulatory perspective, the declared enzyme activities should be considered.

In general, an enzyme preparation is standardised on one specific enzyme activity, also called „main enzyme activity“. This is regarded as the enzyme of technical importance. In some cases, however, the „main enzyme activity“ does not refer to a single enzyme but to multiple enzymes needed to hydrolyse complex substrates. An example of such a complex substrate is hemicellulose. Various enzymes are needed to break down this substrate. This enzyme-complex is sold under one name: Hemicellulase.

The number and type of accompanying enzymes may vary, depending on the production organism and the production process used. A production organism can be triggered to produce one or more enzymes in higher amounts, depending on the raw materials that are offered to the production organism. In the case of hemicellulase, the enzyme preparation may contain a selection of the following enzymes: ( $\beta$ ) glucanases, galactanases, mannanases, xylanases (endo-1, 3- $\beta$ -xylanase, endo-1, 4- $\beta$ -xylanase, exo-1, 3- $\beta$ -xylosidase, exo-1, 4- $\beta$ -xylosidase), arabinanases and arabinosidases (arabinofuranosidases).

There are merely a couple of enzyme preparations marketed in immobilised form, primarily for use in the food industry. Immobilisation should be seen as a specific type of formulation. With respect to identification and classification of the enzyme, the type of formulation is irrelevant although immobilisation effectively reduces the risk of sensitisation. All immobilisation agents used should be safe and suitable for the application and in compliance with relevant legislation.

In principle, the fermentation broth is concentrated to a certain enzyme activity (enzyme concentrate). The enzyme concentrate is subsequently diluted (i.e. standardised) to various strengths and thus purities (even within the same area of application), depending on the market demand.

**The Commission can provide existing information about tonnages.**

In 1998, Amfep member companies reported independently to the Joint Research Centre (JRC) according to the Existing Chemicals Regulation (93/793).

JRC should be able to provide the total tonnage levels for the enzymes passing 10 t/y\*, which we assume comprises 5-10 enzyme types and many of the Amfep products. In accordance with the Existing Chemicals Regulation reporting was done in relatively broad intervals thus covering the present tonnages on the market.

Other enzyme types on the Amfep List are marketed at lower or much lower tonnage levels but it would be a very resource demanding exercise for member companies to compile such additional information. We appreciate that for testing and notification purposes a number of lower volume triggers is in place for chemicals and may be useful also for enzymes in the future. However, we do not find the requested, additional reporting of lower volumes for a large number of enzymes necessary and justified for this purpose.

\*) Amfep recommended reporting on enzyme concentrate basis. 10 t/y corresponds to approx. 0.5-5 t/y on enzyme protein basis.

Due to circumstances as for example acquisitions and mergers, the exact date of introduction of particular enzyme preparations is hard to trace back. Furthermore, clarification of this question is needed in case a similar product from a competitor was on the market earlier. In our opinion, the data are of no relevance with respect to identification, classification and risk assessment of enzyme preparations.

**General downstream processing methods to prepare enzyme concentrates are adequately described in the report on detergent enzymes made in 1998 by Armin Spoek (section 5.2).**

**Published methods (JECFA, FCC) measuring enzyme activities are enclosed**

Each manufacturer feels free to develop its own enzyme assay, depending on the application for which the enzyme preparation is sold. Thus, for one and the same enzyme various methods may exist. If needed, the individual enzyme producers can submit all the assays used, however, the relevance should be explained.

Routinely, only the methods as described above are used. In case more specific and sensitive methods are required, e.g. for air monitoring of occupational exposure, immunological assays (ELISA) can be used. Protein engineered variants may not be distinguishable if they show immunochemical identity or partial identity. However, In terms of allergenic potential, such protein engineered variants should not be considered as separate entities either.

#### **Post-translational modification is only an issue for protein pharmaceuticals.**

Glycosylation a.o. post-translational modifications are not considered essential to the identity, classification and safety characteristics of enzymes. Enzymes expressed in GMO's may differ slightly in glycosylation from the wild type, however, our experience is that with respect to catalytical and sensitising properties, no significant effects have been observed. Naturally occurring enzymes within one class show such variation as well. We only consider such variation relevant for injectable protein pharmaceuticals.

#### **Compared to classical selection and mutation, protein engineering enables optimal improvement of enzyme properties. However, in terms of enzyme structure it does not go beyond natural variation.**

So far only a few enzyme entries on the Amfep list comprise variants modified by protein engineering. As explained in Question 1, similar variations also occur in nature or after classical mutation/selection. With respect to identification, classification and risk assessment of enzyme preparations, such variants can be considered substantially equivalent.

In most cases exact information on changes will be confidential information. Furthermore, the characterization data requested will usually not be available. Protein engineering aims at improving the enzyme by changing some of the properties listed but selection of mutants is based on application relevant screening. Some enzyme chemical characterisation may be part of a research programme but data should not be required for regulatory purposes.

As far as we are aware, post-fermentation processing of the enzyme molecule is very unusual outside the pharmaceutical area and probably not employed for any of the enzymes currently on the Amfep List.

#### **Enzyme preparations vary considerably in terms of purity and formulation (additives) among companies and depending on application.**

Amfep cannot give a comprehensive overview since some of this information is proprietary and not subject to labelling requirements. However, the following is an attempt to provide a general answer and probably suffices according to the purpose of the questions.

Most enzyme applications do not require high enzyme concentration of the concentrate or the preparation. We prefer the term „enzyme concentrate” instead of „enzyme isolate”, because for the most enzymes on the Amfep list this more accurately describes what happens during Downstream Processing.

In some cases limited purity and concentration is desirable in terms of formulation of the preparation, improving stability/performance, facilitating homogeneous blending etc.

However, the increasing use of GMO production strains and new recovery techniques has generally increased purity and enzyme concentration.

We do not find the categorisation into the various application areas (technical, food, feed, personal care) necessary, as the variations in terms of purity or concentration can be found for all enzyme preparations, independent of the application. Moreover, production strain characteristics, fermentation conditions and key recovery steps are quite similar for enzyme production within all categories and largely determine the composition.

With regard to enzyme for therapeutic applications or analytical/diagnostic application: these are enzymes not covered by Amfep.

### Terminology

Table 1 describes the terminology commonly used for enzymes.

**Table 1: Terminology on Enzymes**

Nomenclature	Description
Enzyme	A pure protein with a specified activity, on basis of which the enzyme is identified. The activity has an IUB and a CAS number
Enzyme concentrate	An enzyme-containing mixture in its most concentrated form as it occurs during commercial production, thus before formulation. Enzyme concentrates are not commercially available as such, but some may be used directly by the producer as a processing aid for food or to synthesize certain chemicals (i.e. „captive use“)
Enzyme formulation	An enzyme concentrate, diluted to a standardised activity, stabilized and commercially available on the market
Enzyme blend	A formulation of an intended mixture of enzyme concentrates originating from different sources
Enzyme preparation	Enzyme formulation or enzyme blend
Declared enzyme	The enzyme component(s) in the preparation of significance in terms of application and regulation

‘Enzyme’ may also be referred to as ‘active enzyme’ or ‘active enzyme protein’.

‘Enzyme concentrate’ may also be referred to as ‘enzyme isolate’.

### Purity

The „amount“ of active enzyme is measured on basis of its enzymatic activity (expressed in certain Units/g of enzyme concentrate) and not on basis of its weight. Recalculation from enzymatic activity into pure enzyme weight requires knowledge of the so-called „specific activity“ of an enzyme (i.e. Units/g pure enzyme). Due to the extensive effort needed to obtain a pure enzyme and the fact that pure enzymes are often unstable, reliable data about the specific activities are often unavailable. Moreover, some enzyme activities – which are (as usual) named after their substrate – do not refer to a single enzyme, but to enzyme complexes. This is for instance the case with the enzyme (complex) hemicellulase: several different enzymes are needed to hydrolyse the substrate hemicellulose. For such complexes it is also not possible to determine their „purity“.

Only if the „active enzyme“ present in a concentrate reaches a purity of 80% or higher (w/w on basis of Dry Matter), it becomes possible to determine the purity fairly accurate even if the specific activity is unknown. This can be done by measuring the protein content in the concentrate in combination with the relative amount of the enzyme protein in a SDS-PAGE profile. For less pure products, this method does not lead to accurate figures.

Based on the limited data that are presently available, it can be stated that the percentage of „active enzyme“ in enzyme concentrates may range between 2% and 70% (w/w on basis of Dry Matter).

Enzyme concentrates are usually diluted to obtain enzyme preparations of various strengths, depending on the market demand. The dilution factor, obviously, depends on the amount of „active enzyme“ present in the enzyme concentrate. For the amounts of diluents used, please see below.

### By-products

Enzyme concentrates will contain substances derived from the micro-organism and the fermentation medium. These are proteins, peptides, amino acids, carbohydrates, minerals and other minor components. The exact type and level may vary, depending on natural variations (e.g. seasonal) of the raw materials used for the fermentation. The range of by-products is complementary to the range of the „active enzyme“ (see above).

The relative amounts of these components vary considerably within and between categories of enzyme preparations. However, in terms of function and toxicology we do not consider such variation significant. Our experience from numerous studies is, that the main non-enzymic constituents: proteins, peptides, amino acids and carbohydrates originating from the fermentation as substrate residues or produced by the organisms have no toxicological or ecotoxicological potential (e.g. Pariza and Johnson, 2001).

Enzymes used for Food and Feed comply with FAO/WHO (JECFA) purity specifications (\*) comprising limits for heavy metals and contaminating microorganisms and absence of mycotoxins, antibiotics, and the production strain.

\*) Specifications for Enzyme Preparations used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives in Compendium of Food Additive Specifications, Vol. 1, Annex 1, (FAO 1992), as supplemented in FAO Food and Nutrition Paper (FNP) 52, Add.6, (1998), and FNP 52, add. 7 (1999)

**In order to secure safe handling, stability, suitable mixing etc. and functionality in the various applications, most enzyme preparations are formulated in a variety of liquid and granular forms.**

Some enzyme preparations are immobilised.

The choice of formulation ingredients (additives) is not specific for a given category, but certain substances used for e.g. technical enzymes may not comply with food, feed and cosmetics regulations, specifications etc. Some applications may require special substances and technologies, e.g. in the case of immobilised enzymes or enteric coating of digestive aid preparations.

Product formulation also comprises company specific and proprietary information, so Amfep can only outline typical types of additives and functionalities.

The concentrations indicated in Table 2 below are typical, approximate ranges/levels.

**Table 2: Formulation additives used in enzyme preparations.**

Type of formulation	Type of additive (typical examples)	Purpose	Range (percentage)
Liquid/slurry	Sucrose, sorbitol, glycerol, propylene glycol	Stabiliser	20-50%
Liquid/slurry	Ethoxylated fatty alcohols (technical products only)	Stabiliser	20-40%
Liquid/slurry	Sodium chloride	Stabiliser	10-20%
Liquid/slurry	Sodium benzoate, potassium benzoate, BIT (isothiazoline derivative, technical products only)	Preservative	0.1-0.5%
Granular	Inorganic salts, e.g. sodium chloride, sulphate, calcium carbonate	stabilising, granulation,	50-75%
Granular	Cellulose, dextrin, sucrose	stabilising, granulation, coating	10-75%

Type of formulation	Type of additive (typical examples)	Purpose	Range (percentage)
Granular	Kaolin, titanium dioxide (Technical products only)	(de)colouring	0-20%
Granular	Polyethylene glycol (all PEG variants, Technical products only)	stabilising, granulation, coating	0-10%
Granular	Vegetable oil (e.g. hydrogenated palm oil)	stabilising, coating	5-15%

**Testing of enzyme concentrates are done for companies own safety clearance and for regulatory approvals.**

Test methods: Generally OECD/EEC methods and GLP standards are always complied with when performing phys/chem, tox and ecotox tests as required by regulation.

Test substance: Generally, the enzyme concentrate is used as test substance in order to cover the safety of both enzyme and other components derived from the fermentation. In a few cases, toxicological or ecotoxicological properties of formulation components (additives) are taken into account with regard to classification and labelling.

Technical enzymes: The classification and labelling of enzymes comprised by the 16 types on Annex I to Directive 67/548/EEC have been fixed by the Commission and testing for regulatory purposes (self-classification) is therefore not required. All 16 types of enzymes are within three classification categories: subtilisins, non-subtilisin proteases and non-proteases. However, ad hoc testing may be done due to producers' need for risk assessment, MSDS information, customer requirements etc. For a completely new enzyme (i.e. not on EINECS): enzyme relevant tox/ecotox testing as described for the notification of new substances (Directive 92/32/EEC).

See also the attached document on safety assessment of detergent enzymes.

Food enzymes: For evaluation according to SCF guidelines (Report of the Scientific Committee for Food, 27<sup>th</sup> series, Guidelines for the presentation of data on food enzymes, Opinion expressed on 11 April 1991), a 90 days subchronic feeding study in rodents, a bacterial mutagenicity (Ames) test and an in vitro chromosome aberration test is required.

Only France and Denmark have implemented this as a regulatory requirement (pre-market approval).

Feed enzymes: For approval of enzymes as animal feed additives, comprehensive dossiers including specified toxicity testing is required according to Dir. 94/40/EC, sect. IV.

Briefly, the tests comprise: Tolerance test in target species, acute toxicity in rat and one other animal species, acute inhalation toxicity, skin/eye irritation, Ames and in vitro chromosome aberration mutagenicity tests, a 90 days subchronic toxicity test in one rodent species. Allergenic potential (by inhalation) is acknowledged for all enzymes (no testing required).

Enzymes for personal care products: Ingredients for personal care products, e.g. cosmetics, must be covered by safety documentation records, but specified testing and pre-market approval is not required. The specific application, human exposure level etc will determine the amount of testing needed for risk assessment.

**After more than three decades of industrial uses of enzyme preparations, it is well known that the only critical toxicological endpoint is their ability to cause inhalation sensitisation (Type I hypersensitivity).**

Predictive (to humans) inhalation sensitisation assays have never been established although several studies have been carried out in various animal species (guinea pigs, mice, rabbits, and monkeys) using many different routes of exposure since the early 70's.

The existing data collectively support the lack of adverse human health or environmental effects associated with industrial enzymes used throughout the last three decades. Documentation of relevant adverse effects from consumer exposures is non-existent. In occupational settings, improved formula-

tions and manufacturing methods along with stricter safety measures have almost annulled workers' adverse risks. Isolated cases of allergy alleged from occupational exposure are reported but those incidences happened only from production sites not meeting current enzyme industry engineering and monitoring standards.

### Relevant publications

Cullinan P., Harris JM. et al: An outbreak of asthma in a modern detergent industry. *The Lancet*, Vol. 356(9245), pp. 1899-1900, Dec. 2000.

Schweigert M.K. et al.: Occupational asthma and allergy associated with the use of enzymes in the detergent industry – A review of the epidemiology, toxicology, and methods of prevention. *Clin Exp Allergy*, Vol. 30, pp. 1511-1518, 2000.

Vanhanen M, Tuomi T. et al: Risk of enzyme allergy in the detergent industry. *Occup Environ Med*, Vol. 57, pp. 121-1215, 2000.

Sorensen, T.B. et al.: Consumer Allergy Risk from Enzyme Residues in Food, Amfep, Aug. 1998.

### Other references

Siezen, R.J. and Leunissen, J.A.M. *Protein Science*, Vol. 6 (3) pp. 501-523 (1997)

Position of detergent enzyme producers concerning the adequacy of toxicological data for the safety assessment of detergent enzymes, Amfep detergent enzyme producers, 2. May 2001 (TEC/01/11)

Pariza, M.W. and Johnson, E.A. *Regulatory Toxicology and Pharmacology* **33**:173-186 (2001)

### Conclusion

We see no point in trying to make a very comprehensive assessment of all enzyme variants marketed by Amfep for the purpose of considering a future, general enzyme regulation and principles for notification of 'new' enzymes. The environmental benefits of introducing new and better enzymes in e.g. detergents and by substitution of undesirable chemicals in industrial processes and products should not be limited by a pre-market approval scheme for variants based on chemical identity, i.e. amino acid sequence identity or close similarity.

## Overview of general methods of analysis for enzymes

Mentioned in the JECFA Compendium: [http://apps3.fao.org/jecfa/additive\\_specs/foodad-q.jsp](http://apps3.fao.org/jecfa/additive_specs/foodad-q.jsp)

### Substance Name

alpha-Acetolactate Decarboxylase from *Bacillus brevis* expressed in *Bacillus subtilis*

Amyloglucosidase from *Aspergillus niger*, var.

Avian Pepsin

Carbohydrazase from *Aspergillus niger*, var.

Carbohydrazase from *Saccharomyces species*

Cellulase from *Penicillium funiculosum*

Hemicellulase from *Aspergillus niger*, var.

Lipase from Animal

Lipase from *Aspergillus oryzae*, var.

Malt Carbohydrazase

Maltogenic Amylase from *Bacillus stearothermophilus* expressed in *Bacillus subtilis*

Pectinase from *Aspergillus niger*, var.

Pepsin from Hog Stomach

Trypsin

(n.b. for carbohydrazase (invertase) from *Saccharomyces* sp. only a reference is given)

Mentioned in the JECFA Guide to Specifications, Food and Nutrition Paper 5, Rev. 2 (1991):

Alpha-amylase, bacterial

Alpha-amylase, fungal

Alpha-amylase, malt

Catalase

Cellulase

Glucoamylase

Beta-Glucanase

Glucose Isomerase

Glucose Oxidase

Hemicellulase

Milk Clotting activity

Protease, Viscometer

Proteolytic activity, bacterial

Proteolytic activity, fungal (HUT)

Proteolytic activity, fungal (SAP)

Proteolytic activity, plant

Pullulanase

Mentioned in the Food Chemical Codex, 4th edition:

Acid phosphatase from *Aspergillus niger*

Alpha-Amylase from *Aspergillus niger*, *Aspergillus oryzae*, *Rhizopus oryzae*, barley malt

Alpha-Amylase from *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus stearothermophilus*

Catalase from *Aspergillus niger*, *Micrococcus lysodeikticus*, bovine liver

Cellulase

Chymotrypsin from porcine or bovine pancreas

Diastase from barley malt and other enzyme preparations

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Alpha-Galactosidase from *Aspergillus niger*  
Beta-Glucanase from *Aspergillus niger* and *Bacillus subtilis*  
Glucoamylase from *Aspergillus niger*, *Aspergillus oryzae*, *Rhizopus oryzae*  
Glucose isomerase  
Glucose oxidase from *Aspergillus niger*  
Beta-D-Glucosidase from *Aspergillus niger*, *Trichoderma longibrachiatum*  
Hemicellulase from *Aspergillus niger*  
Invertase from *Saccharomyces* sp (*Kluyveromyces*)  
Lactase (neutral) from *Kluyveromyces marxianus* var. *lactis*, *Saccharomyces* sp.  
Lactase (acid) from *Aspergillus oryzae*  
Lipase from microbial sources and pancreatic tissues  
Lipase/esterase from forestomach sources  
Maltogenic amylase from *Bacillus subtilis* containing a *Bacillus stearothermophilus* gene  
Milk-clotting activity from animal or microbial sources  
Pancreatin  
Pepsin from porcine or other animal sources  
Phospholipase A2 from porcine pancreas  
Phytase  
Plant proteolytic activity (papain, ficin and bromelain)  
Proteolytic activity, bacterial (PC) from *Bacillus subtilis*, *Bacillus licheniformis*  
Proteolytic activity, fungal (HUT) from *Aspergillus oryzae*, *Aspergillus niger*  
Proteolytic activity, fungal (SAP) from *Aspergillus oryzae*, *Aspergillus niger*  
Pullulanase from *Bacillus acidopullulyticus*  
Trypsin from porcine or bovine pancreas



## 13.4 Position of detergent enzyme producers concerning the adequacy of toxicological data for the safety assessment of detergent enzymes

### POSITION OF DETERGENT ENZYME PRODUCERS CONCERNING THE ADEQUACY OF TOXICOLOGICAL DATA FOR THE SAFETY ASSESSMENT OF DETERGENT ENZYMES

This position paper is submitted in response to comments and concerns raised by officials from BgVV (Federal Institute for Consumer Health Protection and Veterinary Medicine) and UBA (German Environmental Protection Agency) to representatives of three detergent enzyme producers (Genencor International, Henkel KGaA, and Novozymes A/S) during a meeting held in Berlin, Germany on November 30, 2000. Responses to UBA's subsequent letter dated December 13, 2000 relative to environmental risk assessment are also included in this document.

BgVV, during the review of toxicological information submitted under the *Voluntary Commitment: Toxicological Evaluation of Enzymes in Laundry and Cleaning Products*, noticed differences among companies relative to study format, study procedures, toxicological endpoints measured, and types of toxicology studies submitted. The detergent enzyme producers have then been requested to discuss the adequacy of the submission to assist German authorities in their assessment of the safety of detergent enzymes.

This paper summarizes the detergent enzyme producers' position relative to the toxicology testing requirement and the overall safety of detergent enzymes.

#### I. GENERAL POSITION

##### 1. Data submission format:

In the spirit of the *Voluntary Commitment* signed between detergent enzyme producers and German authorities, the industry has agreed to inform authorities on the safety of detergent enzymes by the submission of summarized toxicology information. Since the submission is voluntary and emphasis is placed on safety, the studies are not submitted following any pre-approved format. Notwithstanding the differences in format used by the industry, the information submitted does fulfill the purpose of the *Voluntary Commitment*, i.e. to convey safety information to the authorities. For future submission, a format could be agreed upon between the enzyme producers and the authorities.

##### 2. Study Procedures

Toxicology studies conducted in support of enzyme safety were generated years apart with many of the assays conducted even prior to the issuance of official guidelines such as those of the OECD and the US.EPA. Consequently, the procedures, the range of dose levels, the number of animals per group, etc. are expected to vary among submissions for the same type of study. For more recent submissions, the procedures are more closely related due to the availability of testing guidelines. However, procedural differences still remain, albeit minor ones, due to the lack of harmonization among regulatory agencies. Regardless of the procedures used, all results are scientifically valid and support the conclusions of the studies.

##### 3. Testing Requirement

The detergent enzyme industry consented on the *Voluntary Commitment* in 1997 due to the absence of an official German Regulation concerning the requirement of toxicology studies for detergent enzymes. The industry, in an effort to generate safety data, has voluntarily sponsored and/or conducted toxicology studies on its own. The industry's approach is to do appropriate toxicology testing, including *in-vitro* and animal testing, to ensure the safety of our products for our employees, our customers, the public and the environment. The industry also endorses the position of (a) reducing the number of animals tested, (b) not conducting unnecessary testing with animals, and (c) eliminating discomfort to the animals. It is up to each enzyme producer to determine the type of toxicology data needed to substantiate the safety of each producer's product as long as safety is not compromised. Therefore, it is expected that the number of toxicology studies generated for each class of enzyme may differ among companies

but never at the expense of safety. Notwithstanding the quantitative difference, the detergent enzyme industry affirms that the safety of its enzymes is never compromised.

#### 4. Overall Position

The detergent enzyme producers conclude that although differences in toxicology study format, procedures, endpoints, types of studies, and number of studies may exist among submissions, the safety of the detergent enzymes has never been compromised. The existing data collectively support the lack of adverse effects associated with detergent enzymes used throughout the last three decades. Documentation of relevant adverse effects from consumer exposures is non-existent. In occupational settings, improved formulations and manufacturing methods along with stricter safety measures have almost annulled workers' adverse risks. Isolated cases of allergy alleged from occupational exposure are reported (Cullinan et al., 2000; Vanhanen et al., 2000) but those incidences happened only from production sites not meeting the standards of the current detergent enzyme industry.

## II. POSITION ON SPECIFIC TOXICOLOGICAL ENDPOINTS

### 1. Acute Toxicity

#### 1.1 Oral LD<sub>50</sub> study

Extensive data on the acute oral toxicity of detergent enzyme have been generated to satisfy regulatory requirements, worker safety issues, and detergent manufacturers' necessities. The acute oral LD<sub>50</sub> assays were primarily conducted in rats with the highest dose in some studies reaching 10 g/kg body weight. Many of those studies were conducted prior to official guidelines, hence the variation in dose range. Nevertheless, the results substantiate the lack of toxicity associated with accidental ingestion of detergent enzymes. The following information is taken from data submitted under the *Voluntary Commitment*.

Proteases:	oral LD <sub>50</sub> > 1.5 g/kg (limit test)
	oral LD <sub>50</sub> > 9.0 g/kg (highest dose tested)
Amylases:	oral LD <sub>50</sub> > 2.0 g/kg (limit test)
	oral LD <sub>50</sub> > 10 g/kg (highest dose tested)
Cellulases:	oral LD <sub>50</sub> > 2.0 g/kg (limit test)
	oral LD <sub>50</sub> > 10 g/kg (highest dose tested)
Lipases:	oral LD <sub>50</sub> s > 2.0 g/kg (limit test)
	oral LD <sub>50</sub> > 5 g/kg (highest dose tested)

#### Industry current position on acute oral LD<sub>50</sub> testing:

- The industry feels that acute oral LD<sub>50</sub> assays are no longer necessary for safety assessment of the types of enzymes currently in the market since the existing data have adequately documented the low degree of oral toxicity. Furthermore, conducting acute oral LD<sub>50</sub> study with the „neat” enzyme preparation is not scientifically sound since the concentration of enzymes used in consumer products are toxicologically irrelevant. Consumer potential exposure to enzymes is thus negligible. Potential occupational exposure to a large amount of enzymes is unrealistic due to stringent work practices and adherence to the voluntary Occupational Exposure Guidelines (OEGs) at or below the established ACGIH threshold limit value.
- The industry believes that there is no demand for acute oral LD<sub>50</sub> testing for new enzymes that differ from currently used by minor substitutions (i.e., amino acid changes). There is no reason to expect that a minor substitution could lead to significant enhancement of toxicity. Existing data from the original enzyme suffice to cover for the substituted ones.
- The industry considers that oral toxicity study is only needed for new enzymes that differ completely from the types currently used. If a study has to be generated, the limit test approach is preferred.

- The industry confirms that acute oral LD<sub>50</sub> assays should not be considered as essential for safety assessment due to humane reasons. In-vitro evaluation might, however, be performed.

## 1.2 Inhalation LC<sub>50</sub> study

In the past, inhalation toxicity data were generated for some enzyme preparations and the LC<sub>50</sub> values generally support the low degree of toxicity of detergent enzymes by this route of exposure.

### Industry current position on acute inhalation LC<sub>50</sub> testing:

- The industry believes that conducting acute inhalation LC<sub>50</sub> testing would serve no purpose to the assessment of detergent enzyme safety. An acute inhalation study is designed primarily to test for lethality, which is not the endpoint of interest for detergent enzymes.
- The industry realizes that the endpoint of concern, respiratory allergenicity, cannot be evaluated in the acute inhalation LC<sub>50</sub> assay in which animals are exposed continuously for 4 hours. The dose levels of enzyme preparations needed to be generated in an acute inhalation assay are relatively high (5 mg/L) or reaching the maximal achievable atmospheric concentration in order to produce lethality and/or serious toxic effects. This may represent a procedural error since high concentrations of enzyme preparations (dust, aerosol, mist, etc.) in the inhalation chamber may result in coughing and /or congestion of the respiratory tract restricting the usefulness of this type of assay. Furthermore, the maximally generated atmospheric concentration is irrelevant to both occupational and consumer exposure scenarios.
- The industry attests that respiratory irritation due to enzyme preparation is a very rare phenomenon and would not occur at the low concentrations of enzymes found in detergents. Risk to consumers is non-existent.
- The industry has taken measures to minimize occupational exposure. Subtilisin preparations, depending on atmospheric concentration, may be irritating to the respiratory tract, but due to the risk of sensitization, these enzyme preparations are now specifically formulated to avoid exposure by inhalation. Worker safety is further assured through current proper work practices, engineering controls, and use of personal protective equipments.
- The industry considers that acute inhalation LC<sub>50</sub> assays should not even be planned for some classes of enzymes such as cellulase and amylase in light of the absence of corresponding substrates in the respiratory tract and due to the lack of toxicological findings in previous studies.

## 1.3 Dermal LD<sub>50</sub> study

Dermal LD<sub>50</sub> study in either rabbit or rat is not supported by the detergent enzyme industry and no study has been submitted under the *Voluntary Commitment*.

### Industry current position on acute dermal LD50 testing:

The industry considers this type of study as not necessary to assess the safety of detergent enzymes. Enzymes, due to their relatively large molecular weight, are not expected to be absorbed through the skin and, therefore, it can be safely assumed that detergent enzymes do not exert any acute dermal toxicity.

## 2. Skin and Eye irritation

The purpose of conducting skin and eye irritation assays is to assess and quantify the degree of irritation produced by an enzyme preparation for both worker safety, consumer protection, and appropriate MSDS (Material Safety Data Sheet) classification and labeling.

### 2.1 Skin irritation

The skin irritation assay is primarily conducted with rabbits and studies have been generated and submitted for every class of enzymes. From the data submitted under the *Voluntary Commitment*, it can be concluded that the majority of the enzymes is categorized as non-irritating to the skin.

#### Current industry position on skin irritation testing:

Generally, the enzymes can be classified as non- to slight-irritating products. The industry construes that:

- For proteases, the industry has agreed to the EU-labelling of R38 for Subtilisins products. Therefore, skin irritation assay is not necessary.
- Previous assays conducted with cellulases and lipases demonstrate the lack of irritation associated with exposure to these enzymes. Using the weight of evidence approach, further skin irritation testing for these two enzymes is pointless.
- In case of a new enzyme preparation the need for a dermal irritation assay should be assessed on a case-by-case basis depending on the type of enzymes and their respective concentration in the preparations.
- An *in-vitro* evaluation may be considered before eventually progressing into *in-vivo* testing.
- Potential irritating properties are not a problem concerning consumers since the concentrations of enzymes in end products are toxicologically insignificant.

## 2.2 Eye Irritation

An eye irritation study is primarily conducted in rabbits. Although different procedures were used (low volume, OECD, DOT, etc.), the purpose remains the same, i.e. to assess the irritating property of enzymes when instilled onto the eyes. Eye irritation assays were conducted and submitted under the *Voluntary Commitment* and, except for proteases, enzymes are non-irritating to the eyes. Proteases, due to its proteolytic activities, might be irritating to the mucous membrane of the eyes.

#### Current industry position on eye irritation testing:

The industry confirms that:

- Adequate information has been generated to address the irritating property of amylases, cellulases and lipases. These enzymes are non-irritating to the eyes. Any additional eye irritation assay on these three classes of enzymes would be superfluous.
- *In-vivo* eye irritation study for proteases should not be requested due to humane reasons. Proteases are known to produce irritation. Therefore, any additional study would be considered as a waste of animals. *In-vitro* assay should be used to assess the irritating property of new enzymes.
- Although the industry has agreed to the EU-labelling of R36 (min) for Subtilisin products, results from the AMFEP project on in-vitro alternatives indicate that the current EU irritation classification of subtilisins should be re-evaluated.

## 3. Sensitization

### 3.1 Skin sensitization

Skin sensitization assays were conducted in the past in guinea pigs. Controversial and non-reproducible results were obtained even for the same class of enzyme. Consequently, the validity of the skin sensitization assay for enzymes has been questioned by some regulatory agencies.

#### Current industry position on skin sensitization testing:

Skin sensitization tests are not considered as useful for the safety assessment of detergent enzymes since:

- Detergent enzymes are proteins with molecular weight in the range of 20,000 to 100,000, which makes their absorption through the skin unlikely.
- Enzymes are not skin sensitizers since to behave as a skin sensitizer, enzymes must first penetrate the outer layer of the skin, the stratum corneum, an improbable event for proteins with large molecular weight.
- Enzymes are not classified as skin sensitizer in the context of the Dangerous Substances Directive 67/548/EEC.
- There is no animal model that has been developed or validated for assessing proteins as contact skin sensitizers; a fact recognized by the US. EPA (US. EPA Subdivision M).
- There has not been a case of allergic contact dermatitis (Type IV hypersensitivity) in both workers and consumers since the introduction of detergent enzymes in the mid 60's despite repeated and frequent exposures.
- A few cases of contact dermatitis had occurred in occupational setting in response to irritating enzyme preparations (e.g. proteases), but this is a non-immunologic phenomenon (also known as irritant contact dermatitis) unrelated to allergic contact hypersensitivity, which is a cell mediated delayed type hypersensitivity.
- Contact urticaria has been reported in occupational setting but this is also a non-immunologic event or antibody mediated type I hypersensitivity; Contact urticaria (also known as protein contact dermatitis) is unrelated to allergic contact hypersensitivity, which is a cell mediated delayed type hypersensitivity.
- In detailed investigations of individuals with possible adverse reactions to enzymes, follow-up skin patch tests are negative.

### 3.2 Inhalation sensitization

After more than three decades of industrial uses of enzyme preparations, it is well known that the only critical toxicological endpoint is their ability to cause inhalation sensitization of the Type I hypersensitivity. Predictive (to humans) inhalation sensitization assays have never been established although several studies have been carried out in various animal species (guinea pigs, mice, rabbits, and monkeys) using many different routes of exposure since the early 70's.

#### Current industry position on inhalation sensitization testing:

- The enzyme producers do not recommend animal studies for this purpose.
- A few members of the detergent producers may use the guinea pig (intratracheal or subcutaneous administration) and/or the mouse (intranasal administration) model as an internal tool. The information collected from those studies together with other factors (e.g. detergent matrix characteristic, types and concentration of enzymes in the detergent product, worker sensitization frequency, etc.) are used to establish the Occupational Exposure Guidelines (OEGs) relevant to the handling of enzyme in that respective producer.
- In these animal model assays, a benchmark enzyme (Alcalase, a Subtilisin type enzyme) is always used and the results are compared to those of the benchmark enzyme.
- The enzyme producers recognize that enzymes, like any other foreign proteins (e.g. pollen, animal hair, various foods, etc.) are potential inhalation sensitizers. However, for regulatory or any other purpose it is irrelevant to require inhalation sensitization assays due to their lack of predictability of the human situation and workers are well protected by current occupational exposures based on the established ACGIH (American Conference of Governmental Industrial Hygienists) threshold limit values (TLV) of 60 ng/m<sup>3</sup> for the benchmark enzyme Subtilisin.
- Finally, requirement for inhalation sensitization assay is unwarranted since all enzyme preparations will produce positive responses in these animal model assays. The enzyme producers,

on the other hand, trusts that development of new sensitization assays in suitable animal and/or in in-vitro models might become a valuable futuristic tool in the development and screening of low or non allergenic enzymes (e.g. using of epitope mapping and various forms of surface modifications, etc.)

- The industry assures that worker safety has been achieved through re-formulation of enzymes to keep the dust/aerosol levels low, through proper work practices, engineering controls, and eventually through the use of personal protective equipment.

#### 4. Genotoxicity

Genotoxicity studies are currently conducted to satisfy regulatory requirements or customer's needs. The most commonly used assays are:

- a. Bacterial point mutation using 4 strains of *Salmonella typhimurium* and 1 strain of *Escherichia coli*.
- b. Chromosomal aberrations test in mammalian cells *in vitro* (usually human lymphocytes).

##### Current industry position on mutagenicity testing:

The enzyme producers believe that additional genotoxicity assays for enzyme preparations are no longer warranted in light of:

- Availability of extensive mutagenicity data base on enzyme preparations
- Documented and recognized lack of genotoxic effects with enzyme preparations in both bacterial and mammalian systems.
- Substantial amounts of genotoxicity data are available from regulatory agencies in support of enzymes used in food and feed.
- Mutagenicity assays should only be required when the enzyme type and/or the production organism is new or not well defined.

#### 5. Repeated dose toxicity

The purpose of conducting a repeated dose toxicity study is to assess the cumulative systemic toxic effects of a chemical, to establish a safe dose level and to identify potential target organs. Repeated dose studies (14-day feeding, 28-day feeding; 90-day feeding) have been generated for food and feed enzymes and for detergent enzymes only to satisfy some regulatory requirements. Those studies collectively demonstrate the lack of systemic toxicity associated with repeated exposure to enzyme preparations.

##### Current industry position on repeated dose toxicity testing:

The enzyme producers confirm that assaying the toxicity of detergent enzymes after repeated exposure is unessential since:

- By ingestion, detergent enzymes are expected not to produce systemic toxicity due to their susceptibility to digestion. This is due to the fact that enzymes are proteins, which are susceptible to destruction by the digestive enzymes. Therefore, it is expected that enzymes will not accumulate in the body and no adverse effects would be produced, albeit subtle ones.
- By dermal application, enzymes are not expected to be absorbed in any relevant amount due to their large molecular weight. The only effects expected would be localized skin reaction at the application site for some enzyme preparations. Therefore, it is expected that enzymes will not accumulate in the body and no adverse effects would be produced, albeit subtle ones.
- Repeated inhalation exposure, these assays should generally be precluded due to the enzymes known sensitizing properties after inhalation exposure. Further, the current practice of enzyme

granule coating and strict occupational safety surveillance (personal protective equipment, engineering control, etc.) has proven effective as a precaution against inhalation allergies.

## 6. Reproductive toxicity

### Current industry position on reproductive toxicity testing:

In order for a chemical to affect the reproductive system, the chemical must be absorbed into the systemic circulation and be delivered to the respective target organs or must be able to affect the endocrine system. Reproductive toxicity assays for enzyme preparations should not be conducted since:

- As proteins, enzymes are readily biodegraded in the gastrointestinal tract resulting in negligible bioavailability.
- Due to their large molecular weight, enzymes do not readily penetrate the skin or mucous membranes to attain consequential concentration in the systemic circulation.
- Enzymes are not structurally related to any known endocrine disrupter.

## 7. Carcinogenicity

### Current industry position on carcinogenicity testing:

Conduction of carcinogenicity assay should not be considered for enzyme preparations in light of:

- There is absolutely no indication in the public literature that detergent enzymes possess carcinogenic properties – neither by genotoxic nor epigenetic mechanisms.
- It has been well demonstrated that the systemic bioavailability for enzymes is expected to be extremely low and toxicologically insignificant.
- The existing genotoxicity database on enzyme preparations support the lack of genotoxic effects.

## 8. Toxicokinetics

### Current industry position on toxicokinetic assays:

Toxicokinetic studies are relevant for materials that are absorbed into the body either via the skin, mucous membranes or gastrointestinal tract. Since enzymes are practically excluded from those paths due to their molecular weight and particle size, investigating the kinetics of enzymes is not scientifically valid.

## 9. Immunotoxicity

### Current industry position on immunotoxicity assays:

Besides inhalation sensitization (Type I hypersensitivity) that has been associated with enzymes, no other immunologic effects have been ascribed to enzyme preparations. Further, there are no obvious or suspected mechanisms of action regarding the adversity of enzymes on the immune system.

## III. POSITION ON CHANGE IN PRODUCTION PROCESS AND THEIR CONSEQUENCES

### **Changes in Production Process**

Today, new detergent enzymes typically are wild type or variant enzymes from well-characterised GMO production strains. Enzyme producers usually conduct internal risk assessments to assess the safety of the enzymes prior to marketing. The changes in production process could include:

- Optimization: Changes in production process would normally be understood as optimization of the production strain, fermentation, recovery, or formulation of an existing enzyme preparation. Examples of optimized strains are new transformants and yield mutants. A new submission and/or notification is/are not warranted for those inconsequential changes in detergent enzymes.
- New GMO strain constructions: All new GMO strain constructions for enzyme production are subject to national and local authorities' approval based on the EU 'contained use' Directive. These approvals require use of safe (GILSP/Class I) organisms and comprise occupational and environmental risk assessments. Such strains are subject to notification to authorities.
- New GMO host strains may involve a new genus/species or an existing strain improved in various ways: e.g. by removal of antibiotic resistance markers, removal of genes coding for inherent enzymatic activities or undesirable metabolites such as certain mycotoxins, by insertion of multiple enzyme gene copies or efficient promoters for expression of the target enzyme. Such strains are subject to internal risk assessments and notification to authorities.

Conditions for new notifications to UBA according to AMFEP/IKW commitment:

**Enzyme producers realize that under certain conditions, notification to German authorities is necessary. Those conditions are:**

- New enzyme molecules belonging to the same IUB class as existing enzymes (on EINECS or ELINCS) do not require notification for detergent application in the EU. However, UBA will be notified according to the commitment with AMFEP/IKW.
- In case of new enzymes (not on EINECS or ELINCS) these will be notified as new substances in the EU with the required data (EU Base Set) before marketing. UBA will be notified according to the commitment with AMFEP/IKW

#### IV. DETERGENT ENZYME PRODUCERS' POSITION ON TOXICOLOGICAL NEEDS

The detergent enzyme producers recognize that some toxicology data, either *in-vivo* or *in-vitro*, must be generated to assess the safety of enzymes in detergent products and to adequately prevent harm to its employees as well as customers and consumers. In light of the extensive toxicology data base on enzymes and their safe uses, the enzyme producers trust that toxicology requirements for detergent enzymes are minimal and should be required only under certain conditions. Enzymes from existing safe strain lines, substantially equivalent to enzymes previously tested, e.g. new Subtilisin proteases, are generally subject to analytical product characterization and limited in vitro screening comparing with known enzymes as basis for the risk assessment.

**Conditions that do not warrant additional toxicological assays:**

- Additional data are not required for labeling of existing enzymes: the EU Commission has fixed three sets of classification and labeling for 16 types of enzymes, comprising Subtilisins, other proteases than Subtilisins and non-proteases. AMFEP has agreed to use these classifications also for enzyme types not yet listed on Annex I of Directive 67/548.
- Acceptance of pre-classification as inhalation sensitizer: There is no need for inhalation sensitization assay since all enzymes currently on the market are classified Xn, R 42 – „Harmful, Risk of Sensitisation by Inhalation”.
- Compliance with EU regulations on MSDS: No or very limited testing is required.
- Concentration limit rules apply for classification of enzyme preparations (Directive 88/379).
- Volume production: The volume of each individual detergent enzyme is lower than the threshold limit of 1000 tons/year established by Regulation 793/93, which requires data on systemic toxicity and ecotoxicity for existing enzymes exceeding that threshold level.



**Conditions that might require some toxicological assays:**

Detergent enzyme producers within AMFEP have established internal guidelines for safety testing and risk assessment for introduction of enzymes based on modified production strains or modified production processes/formulation.

- For new enzymes, i.e. not on EINECS or ELINCS, toxicological information needed to be generated correlates with the production volume. For production volumes above 1 ton/year (enzyme protein or TOS), extensive data must be generated according to EU Base Set.
- Enzymes listed on EINECS but not previously marketed are generally subject to analytical product characterization and limited *in vitro/in vivo* testing in order to investigate the acute toxicity and ecotoxicity as basis for the risk assessment

**Types of toxicological information to be generated:**

The actual tests performed are decided on a case-by-case basis and may comprise: Analytical-chemical characterization

- Protein and immunochemistry (e.g., SDS-PAGE, CIE)
- *In vitro/ in vivo* toxicology (acute oral and skin/eye irritation)
- Ecotoxicity (Biodegradability, fish, Daphnia, algae)
- Specific detergent manufacturers' needs (e.g. allergenicity test) or non-EU authority requirements (e.g. Ames test for import to Japan) are taken into account.

**V. DETERGENT ENZYME PRODUCERS' POSITION ON FUTURE NOTIFICATION FORMAT**

The detergent enzyme industry is of the opinion that notifications to UBA according to the current Voluntary Commitment should be in the form of summaries and remain flexible in terms of the amount of studies performed, data and other information submitted. However, in order to harmonize the notification we propose that the 'IUCLID' summary format is used to report all toxicology studies, including ecotoxicity, to the extent possible.

The following data should be provided in the notifications:

- Trade name of enzyme
- Proposal for UBA number code
- Name (IUB) of enzyme, synonyms
- IUB/CAS/EINECS numbers
- Donor and host names of microorganisms (genus and species)
- Indication if enzyme is a GMO and/or a protein engineered variant

Based on the notifications, UBA, BgVV or other relevant German authorities may request, as needed, further information from each detergent enzyme producer. The requested information could extend from data explanation, types of studies performed, additional data, more extensive summaries or study reports or to other available information.

Companies should inform UBA (at least on an annual basis) if notified enzymes are no longer marketed in Germany.

**VI. Position of AMFEP concerning ecotoxicology endpoints****1. Biodegradation**

Numerous studies have demonstrated that enzymes from all major classes (subtilisins, amylases, cellulases, lipases etc.) are readily biodegradable. This is expected considering their globular protein structure.

Tests for ready biodegradability are usually performed according to OECD 301. Different versions (A-F) are available, based upon removal of dissolved organic carbon (DOC), CO<sub>2</sub> evolution or O<sub>2</sub> uptake at  $22 \pm 2$  °C over 28 days. Test substances achieving 60% (70% for DOC methods) degradation within 10 days of reaching 10% degradation are regarded as readily biodegradable (the 10-day window criterion).

Commercial enzyme preparations are based on fermentation concentrates, which, in addition to protein, may contain small quantities of nutrient residues, carbohydrates, salts and lipids. If such concentrates are used as test material, a normal exponential biodegradation profile may not be observed and the guideline required 10-day window criterion of 60% or 70% might occasionally not be reached. However, biodegradation will pass 60% (and usually 85%) in 28 days.

Ready biodegradability tests with enzymes from GMO's including PE-variants have not shown any different characteristics compared to naturally occurring or wild type GMO enzymes. This would also be expected, since these techniques do not change the general globular protein structure.

On this basis, the detergent enzyme producers conclude that enzymes, as a group of substances, are readily biodegradable. Therefore, testing of enzyme variants and currently used enzymes from new microbial sources will therefore normally not be performed.

New classes of enzymes will be tested, if warranted.

## 2. Bioaccumulation

As would be expected from the globular protein nature of enzymes, their high water solubility and ready biodegradability characteristics, there are no indications or reports to suggest that enzymes or their intermediate degradation products (peptides and amino acids) can lead to bioaccumulation.

On this basis, the detergent enzyme producers conclude that bioaccumulation assay should only be required if warranted.

## 3. Acute toxicity to aquatic organisms

Considering detergent enzymes general biodegradability characteristics, their potential acute toxicity effects on aquatic organisms are not critical. Three aquatic assays are usually conducted with the following test organisms, fish (OECD 203), Daphnia (OECD 202) and algae (OECD 201). The results obtained from these aquatic studies are

Fish: 96h LC<sub>50</sub> (median lethal concentration) in mg/L

Daphnia: 48h EC<sub>50</sub> (median effect concentration) in mg/L

Algae: 72h IC<sub>50</sub> (median growth inhibition concentration) in mg/L

For environmental classification, the outcome from biodegradability assays as well as data on median concentrations are evaluated together. According to EU classification, for readily biodegradable substances, the harm to the environment is of concern only when the aquatic median concentration in one or more aquatic organisms is below 1 mg/L. For the majority of enzyme concentrates, the aquatic median concentrations are in the ranges of 100 to 1000 mg/L suggesting that detergent enzymes would not do harm to the environment.

Effective aquatic median concentration at less than 100 mg/L may occur with proteolytic enzymes, such as Subtilisins due to their particular enzymatic function. However, it should be noted that a substantial inactivation of enzyme activity takes place during and following wash before discharge to waste water streams.

In conclusion, the acute toxicity to aquatic organisms is generally very low for enzymes and even in the case of concentrated Subtilisins, the combination of readily biodegradability and slight to moderate aquatic toxicity means that detergent enzymes are not harmful to the environment.

Acute toxicity tests with enzymes from GMO's including PE-variants have not shown any different characteristics compared to naturally occurring or wild type GMO enzymes. This would also be expected, since these techniques are not used to change the general catalytic function of the enzymes, i.e. they will still belong to the same class.

Testing of enzyme variants and from new microbial sources will therefore normally not be performed but generally limited to new classes of enzymes.

## VII. CONCLUSIONS FROM DETERGENT ENZYME PRODUCERS

- The existing toxicology database is adequate to support the safety of existing detergent enzymes.
- Although differences were noted among submissions, they are insignificant and do not compromise the safety of the detergent enzymes.
- For future submission, the IUCLID format is proposed.
- Toxicological information for new submissions should not be restricted to in-vivo testing but should be extended to in-vitro assays.
- Ecotoxicity testing is performed, if warranted.

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