

## Safety Regulations of Food Enzymes

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### Summary

The majority of industrial enzymes available at present is used in food industry. Safety regulations of food enzymes differ among countries, including fundamental aspects, whether a pre-market approval is needed and on the level of details, *e.g.* what particular information manufacturers have to provide in the course of safety evaluation. Occupational safety concerns focus on allergenic properties as it is well established that enzymes are potent inhalative sensitizers and can cause allergic reactions including asthma. Otherwise toxic substances including bacterial toxins and mycotoxins might also be present in enzyme isolates and might thus constitute a safety risk to consumers. Safety evaluation procedures seem to be appropriate as no incidents have been reported so far, resulting in suggestion for reduced test packages. Safety precaution and monitoring measures established by industry have also reduced but not entirely eradicated occupational risks. Challenges to regulators and industry arise from unresolved issues, *e.g.* whether enzymes might be contact sensitizers, and from the lack of harmonisation of both legislation and safety evaluation. In the EU, most food enzymes are not covered by food safety regulations neither on Community nor on national level. On top of this the availability of enzymes with new and unusual properties raises questions of safety. In the EU there seems to be a chance that these challenges will be tackled in the course of establishing a harmonised legislation on food enzymes.

*Key words:* food enzymes, enzyme legislation, risk assessment, safety evaluation, food safety

### Introduction

Industrial enzymes are a growing business worldwide worth about two billion US\$ – with food enzymes capturing about half of it. While both the number of available food enzymes and their annual turnover have been steadily increasing for many years so has the number and kind of applications. As of 2001 the major industry association AMFEP (Association of Manufacturers and Formulators of Enzyme Products) lists about 160 enzymes manufactured for use in food industry, at

least 36 of which were produced from genetically modified microorganisms (1). Most enzymes are applied in the beverage industry, bakery industry, in the production of dairy products and in the processing of starch.

In many cultural contexts various applications of enzyme producing microbes in food processing have been practiced for centuries or even thousands of years. The application of purified enzymes of microbial origin is a rather recent invention that dates back to the first half of the 20th century. Improvements in bioprocessing and

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*Abbreviations:* ADI: acceptable daily intakes, AMFEP: Association of Manufacturers and Formulators of Enzyme Products, CFR: Code of Federal Regulations, COT: UK Committee on the Toxicity of Chemicals in Foods, Consumer Products and the Environment, DNA: deoxyribonucleic acid, EFSA: European Food Safety Authority, FDA: US Food and Drug Administration, FDC: Food, Drug and Cosmetic Act, GMM: genetically modified microorganism, GMO: genetically modified organism, GRAS: generally recognised as safe, JECFA: Joint FAO/WHO Expert Committee on Food Additives, SCF: Scientific Committee on Food

strain development and especially the advent of genetic engineering have greatly increased the availability of enzymes and tailoring of enzyme properties in order to meet the sometimes very distinct requirements of the technical environments where they are used. As enzymes are often used to replace steps in food processing encompassing harsh chemical or physical conditions (temperature, pressure, chemicals), they are frequently perceived to be in line with both sustainable industrial production and careful processing of food in order to maintain nutritionally important ingredients such as vitamins, *etc.*

The rise of food enzymes as substances applied in food processing has also captured the interests of regulators in most industrialised countries. Newly marketed food enzymes might need a prior market authorisation by the US Food and Drug Administration (FDA) and – if they are considered food additives – also by the European Food Safety Authority (EFSA). In addition, a small number of EU Member States do have national legislations on food enzymes in place. These legislations mainly serve to ensure the safety of enzyme preparations for the final consumers and sometimes include specifications for purity and activity. In certain cases occupational health issues that might arise from manufacturing and handling enzymes are also part of the regulatory overview.

Risks of allergic reactions in workers handling enzymes are not specific to enzymes and have been discussed at great length in the scientific literature, within industry themselves and among stakeholders concerned, and even made it to the general press. In fact, the occurrence of severe allergic responses in workers of detergent enzyme manufacturers and allegedly also in consumers of washing powders almost led to the collapse of the enzyme industry between the late 1960s and early 1970s (reviewed in 2). Safety concerns for consumers largely focus on toxic by-products that might be inadvertently present in the enzyme preparation and might thereby be transferred to foodstuff.

Given the restricted scope of the EU food legislation the former aspect has mainly been an issue in the context of EU chemical legislation. Under chemical legislation occupational hazards have to be considered for both notification of new substances and for classification and labelling purposes as well.

Apart from the problems with enzyme dust 25 years ago, enzymes have neither been a focal issue for regulators nor for consumer groups or general public. In the beginning of the 1990s this started to change when consumer and environmental groups were alarmed by the increasing use of genetically modified microorganisms (GMM) for enzyme production. This eventually led to reviews of safety concerns and regulatory aspects, especially in Germany, Switzerland and Austria (reviewed in 2). Both environmental and health risks were discussed along with the benefits. However, enzymes from GMM were soon superseded by other topical issues, especially the cultivation and use for food processing of genetically modified crops. Beyond safety aspects in terms of labelling and tracking of genetically modified food as well as for organic agriculture, food enzymes have continued to be an issue – at least for regulators and food industry.

In the »back yards« of EU food authorities, however, awareness has been growing that enzymes in general and food enzymes in particular are not covered by appropriate regulations. Especially the exemption of almost all food enzymes from any kind of mandatory regulatory overview have been perceived as problematic for years. The accessibility of novel types of enzymes with unusual properties and from exotic sources such as deep sea and hot springs, and the use of certain techniques of genetic engineering added to this problem (3,4) and eventually led to a proposal for a harmonised EU legislation on food enzymes. The debates that will take place in the run-up to such legislation are likely to draw attention to food enzymes from several quarters beyond regulators and industry – especially from consumer and environmental organisations. Thus, safety and regulatory aspects of enzymes will not be discussed only in terms of compliance costs and of harmonisation to facilitate free-trade. Given the consumer awareness of food products and ingredients especially in the EU, it might again be discussed in terms of safety. Thus, it might be more than appropriate to review the status quo of food enzymes in terms of legislation and safety aspects – issues which might still be rather remote for University scientists but might be much closer to the business of enzyme manufacturing and food industry.

The first part of this review provides an overview on the occupational health and consumer safety issues associated with enzymes and enzyme production. The regulatory frameworks and the bodies responsible for safety evaluation and market authorisation in the EU and beyond are then described in the second part. The third part briefly summarises the genetic engineering techniques applied in enzyme manufacturing and a concluding part summarises and briefly discusses challenges to regulators and industry.

## Safety Concerns Associated with Enzymes

Safety concerns associated with industrial enzymes in general are possible allergenic, irritative and otherwise toxic properties. Allergenic and irritative risks are mainly issues of occupational health in the industrial production and application of enzymes, whereas risks of oral toxicity are especially relevant to consumers of food enzymes.

### *Allergenic and irritative properties*

In occupational contexts enzyme exposure includes mainly to dust or liquid aerosols that are set free while handling enzyme preparation in either manufacturing of the enzyme itself or using enzyme preparations in other industrial contexts. This is generally true for all enzymes regardless of the particular end-use.

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.

Immunologists differ between allergenic effects and sensitization. The latter means that individuals who are exposed to a possible antigen (here: the enzyme) for the first time may develop antigen-specific IgG and/or IgE antibodies. The formation of IgG indicates exposure, and IgE antibodies indicate allergic sensitization but not allergic disease. Thus, individuals with a positive skin prick test or specific IgE in their blood would not necessarily be considered ill. If sensitized individuals would, however, subsequently be exposed to the antigen, this might result in an allergic response. So, the question is whether enzymes could be antigens, whether there is evidence that enzymes can elicit allergic reactions and be sensitizers, and eventually, if they can act as irritants to the skin. Table 1 provides an overview of evidence on these kinds of health effects.

Many allergens, especially in food and pollen, are proteins. Whether a protein becomes an allergen depends on a set of factors including structural features of the protein. Several common allergens, *e.g.* from house dust mites, storage mites, ragweed, pollens, *Alternaria*, cat dander, and bee venoms, are furthermore proteins with enzymatic activity (5). And if common antigens are enzymes by nature, enzymes may correspondingly represent antigens. In fact, certain 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 the origin of allergy (6,7).

Many medical reports on allergenic and sensitizing properties of enzymes come from the detergent industry. In fact, it was the handling of enzyme containing washing powders in the second half of the 1960s that led to numerous reports of respiratory illnesses at the workplace. By the early 1970s for every 1000 workers an average of 400 were sensitised and 150 went on to develop respiratory symptoms (8–10). Similar reports were made in the enzyme manufacturing industry (*e.g.* 11 reviewed in 3). Since then the problem seems to have decreased, because the industry introduced quite extensive measures to diminish and monitor the exposure of workers, including encapsulation of enzymes, using immobilised preparations, avoiding direct contact, introduction of safe working practices and training of workers, replacement of older products by antigenically distinct proteases, and exclusion of potentially pre-disposed and especially sensitive workers from directly working with enzyme preparations (2).

The highest percentage of exposed people nowadays seems to be affected in the bakery industry and in the enzyme manufacturing industry, but sensitisation may also occur in the animal feed industry, resulting in concerns for meat consumers. 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 (ref. 12 in Table 2).

Table 2. Reported health effects of enzymes in the biotechnical industry (12)

Industrial sector/enzyme	Reported effects on	
	Skin	Airways
<i>Starch processing and baking</i>		
$\alpha$ -amylase	CA, CU	RA
amyloglucosidase	n.r.	RA
proteases	CA	RA
<i>Dairy industry</i>		
proteases	CA	RA
lipases	n.r.	RA
papain	CU	RA
trypsin	CU	RA
chymotrypsin	n.r.	RA
<i>Food industry</i>		
amylases	CA, CU	RA
cellulases	CA, CU	RA
hemicellulases	CA, CU	RA
<i>Protein industry</i>		
papain	CA	RA
pancreatic proteinase	n.r.	RA
<i>Animal feed industry</i>		
cellulases	CA, CU	RA
hemicellulases	CA, CU	RA
proteases	CA	RA
amylases	CA, CU	RA
phytase	CU	RA
<i>Pharmaceutical industry</i>		
chymotrypsin	n.r.	RA
trypsin	CU	RA

Key: CA enzyme causing contact allergy, CU enzyme causing contact urticaria, n.r. not reported, RA enzyme causing respiratory allergy

Table 1. Health impacts of enzymes in occupational settings reported in the scientific literature

Causative agent	Exposure	Health impact	Evidence
Enzyme dust, aerosols	Inhalation	Immediate type allergic reactions (type I) <i>e.g.</i> conjunctivitis, rhinitis, asthma	Good empirical evidence
	Skin contact	Immediate type allergic reactions (type I) <i>e.g.</i> allergic contact urticaria	Good empirical evidence but lack of differentiation from allergic contact dermatitis
		Delayed type allergic reaction (type IV) <i>e.g.</i> allergic contact dermatitis	Sensitization still contested
Enzyme (containing) powder or liquids		Skin irritation	Literature reports from the 1970s, recent reports missing
Food enzymes	Ingestion		Rare cases following respiratory sensitization

In accordance to what is said above, reports from the detergent, the baking and flour industry show that most exposed workers produce IgG antibodies (13,14) and several develop IgE antibodies (become sensitised) (15). In bread producing factories, exposure rates are high and enzymes have become a major cause of occupational disease. Recent reports have shown that of all the workers in the industry who were sensitized after the exposure, up to 36 % work in the enzyme manufacturing, up to 22 % in detergent industry, up to 30 % in the bakery industry, and 31 % in the pharmaceutical industry. Lower rates of sensitization were reported from the animal feed and the textile industry (16,17). These numbers are strikingly high despite the fact that industry – especially enzyme and detergent manufacturing industry, has done a lot to diminish worker exposure. However, only a minority of sensitised workers ever get ill. Whereas symptoms are usually mild, they are sometimes bothering enough and hinder individuals to continue working with those enzymes.

Overlooking the literature, there is sufficient evidence that enzymes can cause allergy, the main problems being allergies of the respiratory tract and, probably secondary, of the skin. In contrast, the question whether enzymes cause primary skin sensitisation or contact dermatitis directly is still controversially discussed: denied by some articles (18–21), but supported by other publications (12,22,23). Beyond occupational risks there is little evidence of any allergic risks to the final consumers of enzyme containing products. A rare exception might be workers who were previously respiratory sensitized with enzyme preparations and who might react to food enzymes – even if the food were heat-treated as in the case of bread (reviewed in 3).

Whether an enzyme in fact sensitises depends mostly on factors like the molecular structure and binding capacities to certain reactive epitopes. Presently, no predictive tools exist that would allow for sufficiently reliable prediction of allergenic or *de novo* sensitizing properties of a given enzyme – especially in case when a protein has not been part of the food chain before (24). Differences between different regulatory contexts in coping with these uncertainties seem to exist: the present allergenicity assessment of introduced proteins in genetically modified plants used for food purposes is largely restricted to indirect evidence such as homology comparisons to known allergens and *in vitro* digestibility tests simulating the gastrointestinal tract. In contrast, enzyme manufacturers that produce enzymes under the EU chemical legislation have acknowledged that – in the absence of any tests that could either predict or exclude allergenic properties – enzymes are generally considered as inhalative sensitizers and are handled and labelled accordingly (2).

Beyond possible allergenic properties enzymes are also supposed to be mild skin irritants in the workplace (18). The extent of damage will mostly depend on the time and intensity of exposure, the nature of the enzyme (e.g. proteases might attack skin proteins) 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. Since the concentration of enzymes in end products is very low, irri-

tating properties are not considered a problem for consumers. Validated animal models are available to routine tests for irritative properties of the substances on skin and eyes (25). A systematic adjustment of these methods for high molecular mass substances such as proteins is still missing.

### *Toxic properties*

Allergenic properties of enzymes are intrinsic to the structure of the enzyme protein (and possibly other proteins present in the enzyme preparation). Toxic concerns are, in contrast, rather focused on by-products and contaminants present in enzyme preparations. In the case of food enzymes, these concerns are especially relevant for obvious reasons. The range of the active enzyme in the final product from the fermentation processes, the enzyme concentrate, varies significantly between 2 and 70 % – hence, by-products amount up to 98 % (AMFEP, personal communication). These by-products are varying in terms of quantity and nature, depending on the production organisms and the particular conditions of the bioprocess. Toxic substances could result from contaminants during fermentation or from the production organisms themselves. It is well known that some production organisms or at least phylogenetically closely related strains (same genus or same species) can – under certain conditions – produce toxins (e.g. in the case of certain bacteria and numerous filamentous fungi).

A recently updated review of about 30 toxicity evaluations of food enzymes published in the scientific literature did not reveal any toxic effects neither of enzymes themselves nor of by-products. These evaluations include acute, subacute, subchronic toxicity, and *in vitro* genotoxicity (Spök, unpublished results). These published toxicity evaluations in fact represent only a small portion of toxicity evaluations conducted by the enzyme manufacturing industry. As of 2001 almost 800 toxicity tests conducted on more than 180 enzymes have been reported by AMFEP member companies alone. In addition to the endpoints mentioned above these studies included occasionally chronic toxicity, *in vivo* genotoxicity, reproduction (including teratogenicity), and *in vitro* cytotoxicity (AMFEP, personal communication). According to AMFEP these tests did not disclose toxic concerns.

In contrast to the overwhelming evidence about allergic properties of enzymes there seems to be no corresponding evidence of toxic effects of enzyme preparations neither in occupational environments nor in consumers. Generally concluding the absence of toxic risks in enzyme preparations might however be somehow premature. This may rather denote that present safety evaluation procedures have so far been sufficient to detect toxic contaminants before marketing of enzyme preparations.

Nevertheless, the possibility of toxic contaminants in enzyme preparations is still widely acknowledged. Thus, one of the most important factors in safety assurance of enzyme preparations is the toxic and pathogenic potential (26) and a reliable identification (e.g. including DNA fingerprinting) of the microorganisms used along with the quality assurance system to safeguard against drift in taxonomy, mutations or selection of strains with



unwanted abilities. Given the possibility that microorganisms belonging to the same species but different strains might differ in their ability to produce toxins, both quality assurance and identification have to be on the strain level (27).

Secondly, changes in bioprocessing conditions such as pH, temperature, purification process or media ingredients might affect the nature and quantity of by-products – including possible toxins. According to Rasmussen and Skovgaard (27) even microorganisms not known to be harmful in food applications, might under different conditions turn out to be toxin producers. Thus, there is also a safety reason to thoroughly describe and maintain the process conditions.

Furthermore, enzyme concentrates can be, and in fact are, subjected to animal testing for a variety of toxicological endpoints as described above. Besides, microbial toxins that might occur as contaminants can be identified and measured. The latter are chemical analytical tests that have to be conducted for each individual toxin, the former are intended to detect all (known and unknown) toxins that might be present. Beyond toxic substances microbes might contaminate enzyme preparations and even antibiotic substances might be present. Thus, total viable counts are usually measured in enzyme preparations and the presence of coliforms, *Salmonella* sp., *E. coli*, and pathogenic microorganisms is investigated (26).

What kind of toxicity relevant information should be required is still contested in the scientific literature. Pariza and Johnson (26) are advocating either acute or 14–91-day subchronic oral toxicity studies depending largely on the species of the host organism and consideration of the nature of toxins that could theoretically be present (e.g. mycotoxins or bacterial enterotoxins). Other authors, in contrast, are proposing 90-day studies only (27). The latter is also suggesting mutagenicity testing while the former is explicitly disregarding these kind of tests largely because they have only revealed either negative or false positive so far. These differences are also mirrored in the different guidelines for enzyme safety evaluation as discussed in the following section of this paper.

## Regulatory Contexts and Requirements for Safety Evaluations

In terms of food legislation enzymes have so far been distinguished into food additives and processing

aids. The main distinction between food additives and processing aids is that additives have a technological function in the final food, whereas processing aids do not (ref. 28 in Table 3). This distinction is important because in some jurisdictions a pre-market authorisation, including safety evaluation, is mandatory for additives only. The definitions underlying this distinction vary between jurisdictions. In Canada, USA and Japan for instance, all food enzymes are regulated as food additives. In Australia food enzymes are considered processing aids. Also, the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which has been conducting voluntary safety reviews on food enzymes since 1971, does not differentiate between these categories.

In the EU food legislation the situation is more complex. Most food enzymes are considered as processing aids. So far, only two enzymes, lysozyme and invertase, are considered as additives. Interestingly, this differentiation was not followed by the European Commission's own Scientific Committee, the Scientific Committee on Food (SCF), which was responsible for the evaluation of food 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« (29). A few enzymes are covered by the EU specialised legislation, e.g. on winemaking. The majority of about 160 food enzymes produced/ marketed in the EU are, however, considered as processing aids, the regulation of which is still governed by national legislation – if there is any legislation in place at all.

National regulations on enzymes used as processing aids differ substantially among the EU Member States. In France, Denmark, Poland and Hungary these enzymes are subjected to an authorisation procedure, in the United Kingdom a voluntary approval system is in place. In many other Member States no national regulation is in place. Furthermore, the range of enzyme products and enzyme applications permitted by national legislation varies among those Member States that have a regulation in place (4,30).

The following subsections provide a brief overview on the regulatory contexts of the EU and the USA and also touches on the JECFA – given their importance for worldwide standards.

### European Union

In the EU food additives are regulated by Council Directive 89/107/EEC (28, amended by Directive 94/

Table 3. Definitions of food additives and processing aids according to Directive 89/107/EEC (28)

Enzyme category	Definitions
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.« (Article 1.2)
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.« (Article 1.2)

34/EC) on the approximation of the laws of the Member States concerning food additives authorised for use in foodstuffs intended for human consumption and Directive 95/2/EC (31, amended by Directives 96/85/EC, 98/72/EC, 2001/5/EC, 2003/52/EC and 2003/114/EC) on food additives other than colours and sweeteners. 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, if they do not mislead the consumer, and if they present no hazard to the health of the consumer. All food additives have to fulfil purity criteria which are set out in detail in three Commission directives: Directive 96/77/EC (32, amended by Directive 96/86/EC and Directive 2000/63/EC) for additives other than colours and sweeteners.

Prior to their authorisation, food additives are evaluated for their safety by the Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) at the European Food Safety Authority (EFSA), which took over this responsibility from the SCF in 2003. Guidance on safety evaluation is provided for food additives in general (33) and for enzymes in particular (29). Requirements for toxicological data are dealt with in the SCF Guidance on submissions for food additive evaluations (33). This document includes some guidance for additives produced from GMOs and also refers to additional requirements for guidance documents on genetically modified food.

The 2001 guidance is, however, not intended to cover additives of complex nature such as proteins. Enzymes have thus been evaluated according to the SCF Guidelines for the presentation of data on food enzymes (29). These guidelines specify conditions of use, requirements for information, documentation and testing and occasionally refer to concrete methods of testing. The SCF guidelines focus on enzyme safety evaluation and are considered 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 mainly focus on toxicological requirements of enzyme preparations, on the safety of the source organism and on unintended reaction products in the food caused by enzymatic reactions in the final foodstuff. Exposure deals mainly with the quantity of enzymes consumed. Allergies or irritative effects are just briefly mentioned by stating that these effects are considered primarily as occupational problems. 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.

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 or in case of highly pure and specific enzyme preparations (which will become possible due to the use of non-toxin-producing GMMs as hosts). Whether the term »mutant strain« also include GMM is not specified. 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.

The main concern of the SCF regarding GMMs was the possibility of unintentionally introducing toxin production into the production organism. Furthermore, the potential for causing 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« (29).

Any evaluation is 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« (29). 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 resulted 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.

So far, five food enzymes have been evaluated by the SCF and one by the AFC (thrombin and fibrinogen from cattle and pigs; urease from *Lactobacillus fermentum*; papain from papaya; invertase from *Saccharomyces cerevisiae*; chymosin from *E. coli*, *Kluyveromyces lactis*, and *Aspergillus niger*; lactoperoxidase together with glucose oxidase), and two enzymes, lysozyme (E1105) and invertase (E1103), have been authorised as food additives in the EU. Additives authorised under Directive 95/2/EC are listed in the annexes to this Directive, which are regularly updated. The specific guidelines on enzymes of 1992 have not been updated so far but might nevertheless be replaced soon. In March 2005, the European Commission circulated a »Draft Working Paper for a Regulation of the European Parliament and of the Council on Food Enzymes« (34). If this draft regulation becomes law, it will establish for the first time a harmonised legislation on food enzymes, regardless of their former differentiation into additives and processing aids. However, it is yet too early to anticipate the changes the proposed regulation will bring about. What nevertheless seems to be clear is that a mandatory authorisation system for enzymes that will include a safety evaluation will be established.

Also recently, EFSA has circulated a »Draft Guidance Document for the Risk Assessment of GMMs and Their Derived Products Intended for Food and Feed Use« (35). This document provides primarily guidance for the risk assessment of GMMs and/or derived food and feed within the framework of Regulation 1829/2003. However, food additives in the meaning of Directive 89/107/

EEC produced from/with GMM are also covered. It seems quite likely that this guidance will be used in addition to or even instead of the SCF guidance of 1992 if enzymes from GMM have to be evaluated.

### United States

Enzymes used in food are regulated by the US Food and Drug Administration (FDA) under the Food, Drug and Cosmetic (FDC) Act. Since 1958 the FDA has perceived all enzymes as food additives and therefore subjected to restrictions pertaining to food additives. In general food enzymes may either be classified as (i) substances that are 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. 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.

So far, about 50 enzymes have either been 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 additive 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.

Detailed recommendations for enzymes are given in a guidance document (36). 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. The recommendations do not address other data needs, such as those pertaining to microbiological, toxicological and environmental considerations. The extent of toxicological testing of food additives depends on the assignment of a »concern level«, on structural features and on an estimation of exposure. Minimum testing requirements are recommended for each concerned level as well as each structural and exposure group (37). According to Zeman (30), North American enzyme manufacturers are using and FDA accepts decision tree for evaluating enzymes as proposed by Pariza and Johnson (26).

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« (38).

This approach is based on the concept of substantial equivalence (in earlier documents also referred to 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 (39,40). 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 microorganisms that had 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*, and *Aspergillus niger*) (41).

### FAO/WHO

About ten years before the SCF guidelines on enzyme safety evaluation were issued the Joint FAO/WHO Expert Committee on Food Additives (JECFA) had already been conducting safety evaluations of enzymes. The JECFA is an international expert scientific committee that 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.

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 (42). 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. 43). Right from the beginning the JECFA specification has 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 microorganisms.

In 1991 the »General Considerations and Specifications for Enzymes from Genetically Manipulated Microorganisms« were published to supplement the »General Specifications« (43). 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« (44) specified especially toxicological testing requirements.

In the revision of the »General Specifications« in 1989 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 (45). 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 (45).

The requirements to define the strain of the production organism were loosened in 1999 when it was concluded that citation of genus and species of host organisms is usually adequate for those that had been determined to be safe and suitable. The reason given for this amendment was that identification at the strain level might impose unnecessary constraints on the development of production microorganisms used to produce food-grade enzymes.

The guidelines including all amendments and supplements until 1999 were published in an updated version of the »Compendium of Food Additive Specifications« (Annex 1, originally published as *FAO Food and Nutrition Papers*, 52). In June 2001, the Committee revised their guidelines on enzyme preparations again (46,47). Revisions included threshold limits for heavy metals and contaminating microorganisms, testing requirements for mycotoxins, and the need for evaluation of the allergic potential. 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 on working papers which are themselves often based on proprietary unpublished reports. These reports are voluntarily submitted to the Committee by the manufacturers and in many cases they represent the only safety data available on these sub-

stances. All these studies are available to the Committee when it makes 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 (therefore and for reasons stated in the individual evaluations, the establishment of an ADI expressed in numerical form is not deemed necessary). The evaluation procedure by the JECFA is neither mandatory nor does an approval or rejection have any legal status; the results of the evaluations are nevertheless widely acknowledged in many states all over the world. So far, 68 enzyme preparations have been evaluated by the JECFA.

### *Industry perspective*

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 evaluations. Industry acknowledges the guidelines for safety evaluations issued by JECFA (48), SCF and the UK Committee on the Toxicity of Chemicals in Foods, Consumer Products and the Environment (COT) (49) and has come up with their own recommendations as well (48).

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 microorganisms grown on materials which do not contain components which might be hazardous to health.

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 microorganisms that can be recognized as safe for food production (50). AMFEP 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.

### *Comparison of safety requirements*

Although the requirements for safety evaluations in guidance documents and the scientific literature differ, the overall structure is fairly similar and includes:

- 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 (microorganisms, heavy metals, toxins) present in the final enzyme preparation.
- In the case of GMMs additional information is required on the host organism, the donor organism, the vector, or introduced DNA.



Enzymes are identified by their catalytic activity in all guidelines and provisions. In general, guidelines and provisions do not describe the requirements precisely. Thus, much is left to interpretation. Most detailed physicochemical information on the enzyme is required by the FDA (enzymatic function, mode of action, substrate specificity, molecular mass, 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 detailed information on the manufacturing and purification process. The JECFA guidelines also 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 mass, isoelectric point, gel migration, chromatographic properties).

SCF describes basic toxicological requirements, which 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. Particular requirements for toxicological testing primarily depend on the nature of the microbial source, e.g. if it is a microbe that also naturally occurs in food, if it is a GMM, etc. Unlike the contested proposals for toxicity testing in the scientific literature (see preceding section), toxicity endpoints suggested in guidance documents are fairly similar. SCF and JECFA ask for a 90-day subchronic toxicity test along

with mutagenicity tests on bacteria and an *in vitro* test for chromosomal aberration (see Table 4). AMFEP does not specify particular toxicity endpoints for food enzymes.

Potential allergenic properties are mentioned by the SCF only in the introduction, whereas no requirements are given in the guidelines themselves. Allergenic properties are mentioned by the JECFA only in case of GMM.

Other differences are:

- 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 not in general 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 takes into account long-term experience with certain enzymes. A GRAS status may be assigned to such enzymes.

If the recently drafted EFSA »Guidance Document for the Risk Assessment of Genetically Modified Microorganisms and Their Derived Products Intended for Food and Feed Use« (34) were applied to food enzymes from GMM as well, this would establish detailed information requirements for characterisation of the enzyme protein similar to those proposed by FDA including amino acid sequence and post-translational modifications.

Table 4. Toxicity endpoints proposed for food enzymes derived from microorganisms

Toxicological endpoints/References	27	26	29	34	48
Acute toxicity (oral)	n.r.	Y <sup>a</sup>	n.r.	n.r.	n.r. <sup>d</sup>
Repeated dose toxicity (on rodents)	90-day	14–91 day <sup>a</sup>	90-day	28-day	90-day <sup>d</sup>
Mutagenicity <i>in vitro</i> bacteriological test	Y	n.r. <sup>b</sup>	Y	c	Y <sup>d</sup>
Mutagenicity <i>in vitro</i> non-bacteriological test	Y	n.r. <sup>b</sup>	Y		Y <sup>d</sup>
Exemptions	n.sp.	possible	e	f	n.i.

Key: n.i. not investigated, n.r. not required, n.sp. not specified, Y required

<sup>a</sup>Either acute or repeated dose tests suggested on a case-by-case basis, depending largely on the species of the host organism and consideration of the nature of toxins that could theoretically be present (e.g. mycotoxin or bacterial enterotoxin). According to Pariza and Johnson (26) bacterial toxins are acute toxins and are produced by a few species only

<sup>b</sup>Relevant only in case of new enzyme types

<sup>c</sup>Depending on the outcome of 28-day study additional tests may be required

<sup>d</sup>Not explicitly required but generally accepted in recent enzyme evaluations

<sup>e</sup>If the production microorganism 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 may be justified. In case of non-toxin producing GMO: if high purity and specificity of the enzyme product could be demonstrated, full toxicity testing may not be needed

<sup>f</sup>If both the GMM and the protein have a history of safe consumption by humans and animals, specific toxicity testing might not be required

Toxicity relevant information would include homology comparisons to proteins known to cause adverse effects, information on stability of the protein under processing and storage conditions, and the expected treatment of the food and testing for resistance to proteolytic enzymes. Toxicity testing would include a 28-day oral toxicity study – additional studies would depend on the outcome of the 28-day study. Exemptions from toxicity testing might be justified if both the GMM and the protein have a history of safe consumption by humans. Compared to the other guidance documents, the EFSA Draft would put much more emphasis on allergenicity assessment, including homology comparison, digestibility testing and might even include *in vitro* tests with serum from allergic patients.

This guidance is, however, not laid out for industrial enzymes and certain enzyme specific information requirements, such as for additives in the enzyme preparation, hygiene (total viable counts, production strain), contaminants including thresholds (heavy metals, microbes, antibiotic activity in enzyme preparation, presence of mycotoxins) are not included. Hence, it is likely that for food enzymes additional guidance needs to be provided by EFSA.

### New Methods in Enzyme Production

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

Enzyme yield has been improved – among other factors – by the use of strong expression or multi-copy systems (51,52). Enzymes that were not accessible before, *e.g.* because it would not have been feasible to set-up a production process with their source organisms, can now be cloned into and produced from a well-known host organism. Thereby, enzymes from almost any source in nature become accessible exhibiting unusual properties such as extreme thermostability. This includes thermophilic and psychrophilic microbes and even non-culturable microbes (53,54).

Combinatorial approaches of rational protein design and directed evolution methods turn 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, and kinetic parameters have been successfully modified (*e.g.* 55,56). Even enzyme activities were switched (57). Protein shuffling and related techniques dramatically increase the variability of enzymes and might lead to enzymes not present in nature so far (*e.g.* 58,59). Apart from manufacturing enzymes from microorganisms plants are also investigated for the production of enzymes (60). Furthermore, enzymes can be chemically modified, *e.g.* by incorporation of cofactors, or chemical glycosylation (61).

Since the marketing of the first genetically modified industrial enzyme about 15 years ago and the subsequent development of these methods for modifying enzyme structure during the last 10 years, the number of available enzymes has dramatically increased and en-

zyme properties have been enhanced significantly. For instance, between 1993 and 1997 more than 130 fungal enzyme genes were cloned at Novozymes only (53).

### Challenges for Food Enzyme Regulation and Safety Evaluation

From the review above several challenges for both further development of food enzyme regulation and for safety evaluation can be identified. These challenges can be attributed to differences in both existing legislation and in requirements for safety evaluation, to prevalent scientific uncertainties and to the introduction of new production technologies, especially genetic engineering.

#### Regulatory aspects

Whether enzymes are categorised as processing aids or as additives and whether the definitions for both categories differ among countries is not important only to bureaucrats. In the EU, for instance, only food additives are covered by harmonised legislation and are required to undergo a pre-market approval procedure. This might not only be relevant for trade liberalisation but also in terms of consumer safety and perhaps of occupational safety as well. For, if no pre-market approval is mandatory – neither is safety evaluation. Consequently, safety evaluation of food enzymes used as processing aids would be a voluntary task depending on the particular manufacturer only. Although internal industry guidelines for safety of enzyme preparations and safe handling of enzymes exist, this might not be considered sufficient as both consumer awareness of food production methods and scepticism towards the food industrial system have been increasing considerably. In the EU, this picture might soon change though, if a new regulation on food enzymes along with a mandatory safety evaluation is actually established.

Another question is whether occupational health risks in manufacturing and handling of food enzymes are actually covered by harmonised EU legislation. In case of technical enzymes occupational health risks are dealt with under harmonised chemical legislation. If a new enzyme is marketed, the pre-market application will also include information on occupational health issues. Similarly, certain occupational health aspects have to be considered if applying for an authorisation of a new feed enzyme under harmonised EU legislation. In the absence of harmonised EU legislation for food enzymes that are used as processing aids only, they would thus be subjected to hazard classification and labelling according to chemical legislation. Whereas the manufacturing processes and associated risks of most industrial enzymes are similar it would have to be clarified whether this regime establishes different degrees of scrutiny of occupational risks. Given that about 90 enzymes presently manufactured by AMFEP member companies are solely used in food industry, this might be a relevant question to clarify (1).

#### Occupational and consumer safety

Industry has achieved a lot in reducing health risks for workers. However, there are still reports on occupa-

tional health problems. For instance, British authors reported an outbreak of asthma, at least equal in size to the numbers reported in the 60s, in a modern European factory which has used exclusively encapsulated enzymes (62,63). A survey revealed that enzyme sensitisation and work-related respiratory symptoms were positively correlated with airborne enzyme exposure. With bakers the number of workers that have to leave their job because of work-related problems is still increasing (64). This kind of reports keep the discussion ongoing and urge for a critical review and improvement of the safety precaution measures to be established.

Following contradicting evidence on skin sensitizing properties of enzymes, this calls upon further research.

With respect to enzymes from GMM it has to be mentioned that there are currently no reports on specific problems caused by these enzymes; they appear to have the same sensitising potential and are capable of sensitising exposed employees at the same rate as traditional enzymes (65). However, special attention might be appropriate. Changes in the amino acid sequence of enzymes, their structure, or properties such as thermostability might change allergenic properties. This is probably less important for occupational health as industry voluntarily labels and accordingly handles the enzymes as respiratory sensitizers. As enzymes might also be present in food it could nevertheless be important for consumers. So far, a possible allergenicity of food enzymes was a rather neglected issue in enzyme safety evaluations (30,46,47). A recent draft guidance document suggests a change in perception (34) – at least for enzymes from GMM. If this guidance document is to be used for enzyme evaluations the allergenic properties of new enzymes will have to be evaluated in light of their similarities to known enzymes, as well as food and environmental allergens. Such assessment procedures were originally introduced for foods derived from genetically modified crops (66). These procedures are increasingly contested for evaluating food from GMOs (reviewed in 24) but might nevertheless still be appropriate for food enzymes. At least well characterised and purified enzyme isolates would perhaps not allow for a high percentage of other proteins to be present.

As the use of enzymes expands into new areas there is also a risk of exposure of the general population, which differs from the current situation. And if enzymes are increasingly used 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.

Further research on understanding how enzymes act as allergens, linked with an understanding of how individuals become sensitised to enzymes is therefore important for the continued control of occupational and non-occupational disease caused by enzymes (67).

With respect to otherwise toxic properties of enzyme preparations, it seems that present safety evaluation approaches seem to work well to identify both critical amounts of toxins inadvertently present in the enzyme isolate and possible unknown toxic effects including the enzyme protein itself. Furthermore, it has to be acknowledged that the use of GMMs has the potential to im-

prove product safety by reducing the number of production organisms to a small set of well characterised strains that sometimes have safely been used in enzyme manufacturing and even in food processing for decades. This seems to be important for both occupational risks and for consumer safety.

The application of genetic engineering techniques resulted in increased variability and modified properties of enzymes such as thermo- and pH-stability. Furthermore, enzymes from exotic sources might be used in food for which there is no prior experience of exposure to humans. For this reasons it might be premature to generally relax the requirements for toxicity testing as demanded by Pariza and Johnson (26).

Furthermore, as shown in the sections above, there are differences in the requirements for safety evaluations of food enzymes among countries and scientific committees alike, e.g. toxicity endpoints. These differences are indicators of scientific uncertainties and point to a need for either clarification and/or scientific research. This would be helpful to clarify the relevance of endpoints and test methods contested in the scientific literature and in guidance documents for industry. Thereby, consistency in safety evaluation would be improved and unnecessary testing avoided.

The challenges mentioned above will most likely concern regulators and industry alike. The introduction of an EU harmonised legislation on food enzyme would offer an opportunity to reconsider regulatory approaches and scope and requirements for safety evaluations as well. Legislation and the implantation process will take place under critical observation and perhaps involvement of well-informed consumer and environmental groups. Scientists should also be aware of this process and might be interested and willing to contribute in order to arrive at a sound and reasonable regulatory framework. Ideally, further research will be conducted to clarify the open questions indicated.

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### References

1. AMFEP (Association of Manufacturers and Formulators of Enzyme Products): *AMFEP List of Commercial Enzymes* (2001).
2. A. Spök, W. Aberer, A. Boyer, G. Getzinger, P. Krajnik, B. Kränke, M. Preiss, B.M. Scherbler, J. Steindl, J. Suschek-Berger, F. Weinberger, *Enzyme in Wasch- und Reinigungsmitteln. Technikfolgenabschätzung und -bewertung unter besonderer Berücksichtigung der Anwendungen der Gentechnologie (Enzymes in detergents and cleansers. Tech-*



- nology assessment focussing on the application of genetic engineering methods), *Schriftenreihe des Bundesministeriums für Umwelt, Jugend und Familie, Band 29* (1998).
3. Federal Environment Agency/Inter-University Research Center for Technology Work, and Culture: *Collection of Information on Enzymes*, Final Report, Luxembourg: European Commission (2002) (<http://europa.eu.int/comm/environment/dansub/enzymerepcomplete.pdf>).
  4. SCOOP (Reports on Tasks for Scientific Cooperation): *Study of the Enzymes Used in Foodstuffs and Collation of Data on Their Safety*, Reports of experts participating in Task 7.4, Directorate General Health and Consumer Protection (2000) ([http://europa.eu.int/comm/food/fs/scoop/7.4.1\\_en.pdf](http://europa.eu.int/comm/food/fs/scoop/7.4.1_en.pdf)).
  5. M.H. Chabane, A. Abuaf, F. Leynadier, Pourquoi certains allergènes sont-ils des enzymes? (Why are some allergens enzymes?), *Ann. Biol. Clin.* 52 (1994) 425–431.
  6. C. Robinson, N.A. Kalsheker, N. Spinivasan, C.M. King, D.R. Garrod, P.J. Thompson, G.A. Stewart, On the potential significance of the enzymatic activity of mite allergens to immunogenicity: Clues to structure and function revealed by molecular characterization, *Clin. Exp. Allergy*, 27 (1997) 10–21.
  7. H.F. Kauffman, J.F.C. Tomee, M. van de Riet, A.J.B. Timmerman, P. Borger, Protease-dependent activation of epithelial cells by fungal allergens leads to morphologic changes and cytokine production, *J. Allergy Clin. Immunol.* 105 (2000) 1185–1193.
  8. D.C. Little, J. Dolovich, Respiratory disease in industry due to *Bacillus subtilis* enzyme preparations, *Can. Med. Assoc. J.* 108 (1973) 1120–1125.
  9. R.G. Slavin, C.R. Lewis, Sensitivity to enzyme additives in laundry detergent workers, *J. Allergy*, 48 (1971) 262–266.
  10. H. Weill, Z. Waggenspack, T. DeRouen, M. Ziskind, Respiratory reactions to *Bacillus subtilis* enzymes in detergents, *J. Occup. Med.* 15 (1973) 267–271.
  11. C.R. Johnsen, T.B. Sorensen, A. Ingemann-Larsen, A. Bertelsen-Secher, E. Andreasen, G.S. Kofoed, L. Fredslund-Nielsen, F. Gyntelberg, Allergy risk in an enzyme producing plant: A retrospective follow up study, *J. Occup. Environ. Med.* 54 (1997) 671–675.
  12. T. Tuomi: Biotechnical Industry Workers. In: *Handbook of Occupational Dermatology, Chapter 105*, L. Kanerva, P. Elsner, J.E. Wahlberg, H.I. Maibach (Eds.), Springer, Berlin, Germany (2000).
  13. Y. Matsumura, T. Niitsuma, H. Ito, A study of factors contributing to bakers' allergy symptoms, *Jpn. J. Allergol.* 43 (1994) 625–633.
  14. L. Moneo, E. Alday, M. Gonzalez-Munoz, J. Maqueda, G. Curiel, R. Lucena, Alpha-amylase hypersensitivity in non-exposed millers, *Occup. Med.* (London), 44 (1994) 91–94.
  15. M. Vanhanen, T. Tuomi, U. Tiikkainen, O. Tupasela, R. Voutilainen, H. Nordman, The risk of enzyme allergy in the detergent industry, *J. Occup. Environ. Med.* 57 (2000) 121–125.
  16. G. Doekes, N. Kamminga, L. Helwegen, D. Heederik, Occupational IgE sensitization to phytase, a phosphatase derived from *Aspergillus niger*, *J. Occup. Environ. Med.* 56 (1999) 454–459.
  17. M. Vanhanen, T. Tuomi, U. Tiikkainen, O. Tupasela, A. Tuomainen, R. Luukkonen, H. Nordman, Sensitisation to enzymes in the animal feed industry, *J. Occup. Environ. Med.* 58 (2001) 119–123.
  18. G. Peters, D.P. Mackenzie: Worker Safety: How to Establish Site Enzyme Capability. In: *Enzymes in Detergency*, J.H. Van Ee, O. Misset, E.J. Baas (Eds.), Marcel Dekker Inc., New York, USA (1997) pp. 327–440.
  19. C. Rodriguez, G. Calvin, C. Lally, J.M. LaChapelle, Skin effects associated with wearing fabrics washed with commercial laundry detergents, *J. Toxicol.-Cut. Ocular Toxicol.* 13 (1994) 39–45.
  20. R.M. Bolam, R. Hepworth, L.T. Bowerman, In-use evaluation of safety to skin of enzyme-containing washing products, *Br. Med. J.* 2 (1971) 499–501.
  21. E.A. Bannan, J.E. Griffith, T.L. Nusair, L.J. Sauers, Skin testing of laundered fabrics in the dermal safety assessment of enzyme-containing detergents, *J. Toxicol.-Cut. Ocular Toxicol.* 11 (1992) 327–339.
  22. P. Podmore, F.J. Storrs, Contact lens intolerance; Allergic conjunctivitis?, *Contact Dermatitis*, 20 (1989) 98–103.
  23. R.A. Herbst, H.I. Maibach, Contact dermatitis caused by allergy to ophthalmic drugs and contact lens solutions, *Contact Dermatitis*, 25 (1991) 305–312.
  24. A. Spök, H. Gaugitsch, S. Laffer, G. Pauli, H. Saito, H. Sampson, E. Sibanda, W. Thomas, M. van Hage-Hampsten, R. Valenta, Suggestions for the assessment of the allergenic potential of genetically modified organisms, *Int. Arch. Allergy Immunol.* 137 (2005) 167–180.
  25. OECD (Organisation for Economic Co-Operation and Development): *Guidelines for Testing of Chemicals, Acute Dermal Irritation/Corrosion (404) and Acute Eye Irritation/Corrosion (405)* (2001).
  26. M.W. Pariza, E.A. Johnson, Evaluating the safety of microbial enzyme preparations used in food processing: Update for a new century, *Regul. Toxicol. Pharmacol.* 33 (2001) 173–186.
  27. F. Rasmussen, N. Skovgaard: Microbiological and Toxicological Testing of Microbial Food Enzymes/Enzyme Products. In: *SCOOP (Reports on Tasks for Scientific Cooperation): Study of the Enzymes Used in Foodstuffs and Collation of Data on Their Safety*, Annexes, Reports of experts participating in Task 7.4 (2000).
  28. Council Directive 89/107/EEC of 21 December 1988 on the approximation of the laws of the Member States concerning food additives authorized for use in foodstuffs intended for human consumption, *Official Journal, L 040* (1989) 27–33.
  29. SCF (Scientific Committee on Food): Guidelines for the Presentation of Data on Food Enzymes. In: *Food, Science and Techniques: Reports of the Scientific Committee for Food* (1992) pp. 13–22.
  30. N.W. Zeman: Regulation of Enzymes Used in Foods: International Procedures for Safety Assessment. In: *Industrial Enzymes for Food Production. Past, Present and Future Perspectives*, Brasilia, Brazil (2002) ([www.anbio.org.br/palestras/palestra\\_nancy.ppt](http://www.anbio.org.br/palestras/palestra_nancy.ppt)).
  31. European Parliament and Council Directive No 95/2/EC of 20 February 1995 on food additives other than colours and sweeteners, *Official Journal, L 061* (1995) 0001–0040.
  32. Commission Directive 96/77/EC of 2 December 1996 laying down specific purity criteria on food additives other than colours and sweeteners, *Official Journal, L 339* (1996) 1–69.
  33. SCF (Scientific Committee on Food): *Guidance on Submissions for Safety Evaluation of Sources of Nutrients or of Other Ingredients Proposed for Use in the Manufacture of Foods*, SCF/CS/ADD/GEN/26/Final (2001) ([http://europa.eu.int/comm/food/fs/sc/scf/out98\\_en.pdf](http://europa.eu.int/comm/food/fs/sc/scf/out98_en.pdf)).
  34. EC (European Commission): *Draft Working Paper for a Regulation of the European Parliament and of the Council on Food Enzymes*, WGA/003/03/rev8, Brussels, Belgium (2005).
  35. EFSA (European Food Safety Authority): *Draft Guidance Document for the Risk Assessment of GMMs and Their Derived Products Intended for Food and Feed Use* (2005) ([http://www.efsa.eu.int/science/gmo/gmo\\_consultations/1035/gmo\\_consultation\\_guide\\_gmm2\\_en1.pdf](http://www.efsa.eu.int/science/gmo/gmo_consultations/1035/gmo_consultation_guide_gmm2_en1.pdf)).

36. FDA (Food and Drug Administration): *Enzyme Preparations: Chemistry Recommendations for Food Additive and GRAS Affirmation Petitions* (1993).
37. FDA (Food and Drug Administration): *Toxicological Testing of Food Additives* (1997).
38. FDA (Food and Drug Administration), Enzyme preparations from animal and plant sources; Affirmation of GRAS status as direct food ingredients, *Federal Register*, 60 (1995) 32904–32912.
39. OECD (Organization for Economic Cooperation and Development): *Safety Evaluation of Foods Derived by Modern Biotechnology – Concepts and Principles*, Paris, France (1993).
40. FAO/WHO (Food and Agricultural Organization/World Health Organization), *Biotechnology and food safety, Report of a Joint FAO/WHO Consultation*, Rome, Italy (1996).
41. FDA (Food and Drug Administration), Carbohydrase and protease enzyme preparations derived from *Bacillus subtilis* or *Bacillus amyloliquefaciens*; Affirmation of GRAS status as direct food ingredients, *Federal Register*, 64 (1999) 19887–19895.
42. FAO/WHO (Food and Agricultural Organization/World Health Organization), Specifications for identity and purity of carrier solvents, emulsifiers and stabilizers, enzyme preparations, flavouring agents, food colours, sweetening agents and other food additives, *FAO Food Nutr. Pap.* 19 (1981).
43. FAO/WHO (Food and Agricultural Organization/World Health Organization), Chemicals in foods: The role of the joint FAO/WHO expert committee on food additives (JECFA) in the area of new foods, foods derived from biotechnology, traditional foods and food components, *FAO/WHO Conference on Food Standards: Chemicals in Food and Food Trade*, Rome, Italy (1990).
44. WHO (World Health Organization), Principles for the safety assessment of food additives and contaminants in food, *WHO Environmental Health Criteria*, 70 (1987).
45. D. Atkins, C.A. Lawrie, S. Wearne, Future regulatory controls on food enzymes, *Bulletin of the IDF*, 269 (1992) 18–23.
46. JECFA (Joint FAO/WHO Expert Committee on Food Additives), *Report of the 57th Meeting*, Rome, Italy (2001).
47. JECFA (Joint FAO/WHO Expert Committee on Food Additives), *Summary and Conclusions of the 57th Meeting*, Rome, Italy (2001).
48. JECFA (Joint FAO/WHO Expert Committee on Food Additives): *Methods for Enzyme Preparations. General Specifications and Considerations for Enzyme Preparations Used in Food Processing*. In: *JECFA: Compendium of Food Additives Specifications and the Guide to Specifications* (2005) (<http://www.fao.org/es/esn/jecfa/database/cover.htm>).
49. AMFEP (Association of Manufacturers and Formulators of Enzyme Products): *Safety Aspects of Enzymes Produced by Genetically Modified Microorganisms*, AMFEP, Brussels, Belgium (1996).
50. AMFEP (Association of Manufacturers and Formulators of Enzyme Products): *Regulatory Aspects of Microbial Food Enzymes*, AMFEP, Brussels, Belgium (1992).
51. N. Kulkarni, A. Shendye, Molecular and biotechnological aspects of xylanases, *FEMS Microbiol. Rev.* 23 (1999) 411–456.
52. H. Ruttloff: *Industrielle Enzyme (Industrial Enzymes)*, Behr's, Hamburg, Germany (1994).
53. H. Dalbøge, L. Lange, Using molecular techniques to identify new microbial biocatalysts, *TIBTECH*, 16 (1998) 265–272.
54. D.C. Demirjian, F. Moris-Varas, C.S. Cassidy, Enzymes from extremophiles, *Curr. Opin. Chem. Biol.* 5 (2001) 144–151.
55. P.N. Bryan, Protein engineering of subtilisin, *Biochim. Biophys. Acta*, 1543 (2000) 203–222.
56. D.D.Y. Ryu, D.H. Nam, Recent progress in biomolecular engineering, *Biotechnol. Progr.* 16 (2000) 2–16.
57. M.M. Altamirano, J.M. Blackburn, C. Aguayo, A.R. Fersth, Directed evolution of new catalytic activity using the  $\alpha/\beta$ -barrel scaffold, *Nature*, 403 (2000) 617–622.
58. H.Y. Kim, D.H. Nahm, H.S. Park, D.C. Choi, Occupational asthma and IgE sensitization to cellulase in a textile industry worker, *Ann. Allergy Asthma Immunol.* 82 (1999) 174–178.
59. A. Singh, K. Hayashi, Construction of chimeric  $\beta$ -glucosidases with improved enzymatic properties, *J. Biol. Chem.* 270 (1995) 21928–21933.
60. J.A. Howard, E. Hood, Commercial production of proteases in plants. *United States patent No. 6087558* (2000).
61. G. DeSantis, J.B. Jones, Chemical modification of enzymes for enhanced functionality, *Curr. Opin. Biotechnol.* 10 (1999) 324–330.
62. P. Cullinan, J.M. Harris, A.J. Newman Taylor, A.M. Hole, M. Jones, F. Barnes, G. Jolliffe, An outbreak of asthma in a modern detergent factory, *Lancet*, 356 (2000) 1899–1900.
63. G.M. Liss, J.R. Kominsky, J.S. Gallagher, J. Melius, S.M. Brooks, I.L. Bernstein, Failure of enzyme encapsulation to prevent sensitization of workers in the dry bleach industry, *J. Allergy Clin. Immunol.* 73 (1984) 348–355.
64. M.J. Nieuwenhuijsen, D. Heederik, G. Doekes, K.M. Venables, A.J. Newman Taylor, Exposure-response relations of  $\alpha$ -amylase sensitization in British bakeries and flour mills, *J. Occup. Environ. Med.* 56 (1999) 197–201.
65. T. Dauvrin, G. Groot, K.H. Maurer, D. De Rijke, H. Ryssov-Nielsen, M. Simonsen, T.B. Sorensen: *Consumer Allergy Risk from Enzyme Residues in Food*, AMFEP (1998).
66. D.D. Metcalfe, J.D. Astwood, R. Townsend, H.A. Sampson, S.L. Taylor, R.L. Fuchs, Assessment of the allergenic potential of foods derived from genetically engineered crop plants, *Crit. Rev. Food Sci. Nutr.* 36 (1996) 165–186.
67. M.K. Schweigert, D.P. Mackenzie, K. Sarlo, 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*, 30 (2000) 1511–1518.