Section 9.1.4.2

Enzymes

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CONTENTS

This text updates section 9.1.4.2 of Chapter 9, Principles Related to Specific Groups of Substances, of Environmental Health Criteria 240 (EHC 240), which was originally published in 2009. It was developed through an expert meeting of a working group established to consider the evaluation of enzyme preparations used in the manufacture of foods, held in December 2018. The text was available for public comment in December 2019, and the final version was discussed and approved at the eighty-ninth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), held in June 2020.

For abbreviations used in the text, the reader may refer to the list of abbreviations at the front of this section. Definitions of select terms may be found in the glossary in Annex 1 of EHC 240 (http://www.inchem.org/documents/ehc/ehc/ehc240_annex1.pdf).

List of abbreviations

ADI	acceptable daily intake
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
CAS	Chemical Abstracts Service
DNA	deoxyribonucleic acid
EC/IUBMB	Enzyme Commission/International Union of Biochemistry and Molecular Biology
EHC 240	Environmental Health Criteria 240
FAO	Food and Agriculture Organization of the United Nations
FASTA	FAST-All
GMP	Good Manufacturing Practice
IgE	immunoglobulin E
JECFA	Joint FAO/WHO Expert Committee on Food Additives
MOE	margin of exposure
NOAEL	no-observed-adverse-effect level
rDNA	recombinant deoxyribonucleic acid
RNA	ribonucleic acid
SCF	Scientific Committee on Food
SDS PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TOS	total organic solids
USA	United States of America
WHO	World Health Organization

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9.1.4.2 *Enzymes*

(a) Introduction

The history of enzyme use in food applications – especially in the making of bread, cheese, wine and beer, where enzymes are part of the manufacturing or maturation processes – is long and well known. Enzymes used in the food industry are produced from animal tissues, plants and microorganisms. However, most commercial enzymes are produced from microorganisms that are enhanced through natural selection, classical strain improvement techniques (e.g. mutagenesis and selection), recombinant DNA technologies and gene editing. Microbial enzymes are typically produced by controlled fermentation followed by removal of the production microorganism and purification and concentration of the enzyme. Final standardization with stabilizers, preservatives, carriers, diluents and other approved food-grade additives and ingredients is carried out after the purification and concentration steps. Enzyme preparations, depending on the application, may be produced as a liquid, semi-liquid or dried product. Enzyme preparations may contain either one major active enzyme that catalyses a specific reaction or two or more active enzymes that catalyse different reactions during food processing.[1]

Enzyme preparations often contain organic constituents of the production organism and compounds carried over from the manufacturing process – for example, the residues of the fermentation broth. In 2006, the Joint FAO/WHO Expert Committee on Food Additives (JECFA), at its sixty-seventh meeting, elaborated principles and procedures for the safety assessment of enzyme preparations for use in food, whereby an enzyme preparation must comply with the *General specifications and considerations for enzyme preparations*[2] *used in food processing* (FAO, 2006; FAO/WHO, 2007a). This document addressed certain aspects of the evaluation of the safety of all enzyme preparations, including the

[1] In this section, "enzyme" refers to the enzyme and its amino acid sequence; "enzyme concentrate" refers to the enzyme concentrate used in the toxicity studies; and "enzyme preparation" refers to the enzyme preparation formulated for commercial use.

[2] Note that "enzymes" rather than "enzyme preparations" was used in the title in FAO (2006).

safety of the production organism, the enzyme components, side activities and the manufacturing process, as well as the consideration of dietary exposure.

Some of the specific safety concerns related to enzyme preparations as well as an updated classification system for enzymes used in food are outlined in the following subsections.

(b) Potential for enzymes to cause allergic reactions

Food allergies. Food allergies are adverse immunological reactions to an otherwise harmless food, such as a protein. The severity of food allergies in susceptible individuals (atopy) can range from mild to severe and, in some cases, can be life-threatening. The most common type of food allergy is mediated by allergen-specific immunoglobulin E (IgE) antibodies. Allergens are almost always proteins (e.g. Ara h2 in peanuts, papain in papaya, lactoperoxidase in cow's milk), but not all food proteins are allergens. As there is no single test that can accurately predict whether a microbially synthesized enzyme will immunologically cross-react with an established allergen, a weight-of-evidence approach should be used (FAO/WHO, 2001). One approach that has routinely been used by JECFA is to compare the amino acid sequence of an enzyme against known linear IgE-binding epitopes in allergenic proteins using in silico methods and appropriate protein databases (e.g. AllergenOnline of the Food Allergy Research and Resource Program, University of Nebraska, Lincoln, Nebraska, USA: http://www.allergenonline.org). The possibility of immunological cross-reactivity between the expressed enzyme and a known allergen is considered when there is:

- at least 35% identity in the amino acid sequence of the expressed protein (i.e. without the leader sequence, if any), using a sliding window of 80 amino acids and a suitable gap penalty (for algorithms such as FAST-All [FASTA], Basic Local Alignment Search Tool [BLAST], or equivalent; Codex Alimentarius Commission, 2003, 2009); and/or
- identification of eight contiguous amino acids common to the expressed enzyme and a known allergen (FAO/WHO, 2016).

Amino acid sequence information is not available for most enzymes – either derived from animals or plants or produced by microorganisms – that are traditionally accepted constituents of

foods. Thus, the absence of allergenicity in humans is reasoned to have been demonstrated by the presence of these enzymes in widely consumed foods for a long period of time.

Allergenic food proteins and resistance to proteolysis. The susceptibility of a dietary protein to proteolytic degradation by digestive enzymes, such as gastric pepsin, could potentially provide information on its immunological safety for human consumption. Whereas most dietary proteins are readily hydrolysed to peptides and amino acids in the gastrointestinal tract, there is evidence that many potent food allergens are resistant to proteolysis (Schmidt et al., 1995; FAO/WHO, 2001; Bannon, 2004; Moreno et al., 2005). In vitro pepsinolysis assays (Thomas et al., 2004) have been proposed as an additional piece of information as part of a weight-of-evidence approach for evaluating the safety of newly expressed proteins (Codex Alimentarius Commission, 2009). A pepsinolysis assay that is based on simulated gastric fluid and frequently used in the preclinical testing of pharmaceuticals has been described by the United States Pharmacopeia (2000). The simulated gastric fluid assay is often used to allow comparisons between different newly expressed proteins under experimental conditions (Astwood, Leach & Fuchs, 1996). To date, however, such pepsin resistance data for enzymes have rarely been submitted to JECFA for consideration within a weight-of-evidence approach. This may be because there are studies – albeit not using the same conditions (pH, purity and activity of pepsin, and pepsin-to-substrate protein ratio) – showing that the correlation with allergenic potential is not absolute and that proteins that are resistant to pepsinolysis might not be allergenic under physiological conditions of dietary exposure; in contrast, labile proteins (e.g. β-casein) or peptides formed during proteolysis may be allergenic (Vieths et al., 1999; Yagami et al., 2000; Wal, 2001; Fu, Abbott & Hatzos, 2002; Bøgh & Madsen, 2015). Consequently, data on resistance to pepsinolysis from in vitro tests are currently not considered to be strong evidence for the absence of the intrinsic allergenicity of a protein, but still may have some utility as part of a weight-of-evidence approach.

Occupational hazards: respiratory allergies, skin and eye irritation. A known safety risk linked to industrial enzyme use is respiratory allergy (Quirce et al., 1992; Green & Beezhold, 2011). For most proteases, there is also some potential for skin and eye irritation (Vanhanen, 2001; Anderson, Long & Dotson, 2017).

(c) Safety concerns pertaining to enzyme preparations produced by genetically modified microorganisms

The *General specifications and considerations for enzyme preparations used in food processing* (FAO, 2006; FAO/WHO, 2007a) provides recommendations on the safety assessment of the genetic material inserted into the genome of the production microorganism. Two new considerations that were introduced in the most recent revision of the specifications (which were first elaborated by JECFA at its twenty-sixth meeting with several revisions proposed at subsequent meetings) read as follows:

> For enzyme preparations from recombinant-DNA microorganisms, the following should also be considered:
>
> 1. The genetic material introduced into and remaining in the production microorganism should be characterized and evaluated for function and safety, including evidence that it does not contain genes encoding known virulence factors, protein toxins, and enzymes involved in the synthesis of mycotoxins or other toxic or undesirable substances.
> 2. Recombinant-DNA production microorganisms might contain genes encoding proteins that inactivate clinically useful antibiotics. Enzyme preparations derived from such microorganisms should contain neither antibiotic inactivating proteins at concentrations that would interfere with antibiotic treatment nor transformable DNA that could potentially contribute to the spread of antibiotic resistance. [FAO/WHO, 2007a:87–88]

It must be pointed out that papers identified through extensive literature searches on the safety of enzymes from microbial sources support the general assumption that industrial enzyme preparations from non-pathogenic organisms are safe (Olempska-Beer et al., 2006). Most engineered enzymes exhibit no greater amino acid sequence variability than already exists for many isozymes in the diet (Préstamo & Manzano, 1993). Also, there is no evidence to suggest that changes in amino acid sequence made through protein engineering – to confer benefits such as tolerance to heat or pH or to simply increase yield – will result in an otherwise safe enzyme being rendered toxic. That said, comparing the amino acid sequence of an enzyme with the sequence of known toxic or allergenic proteins using in silico methods is one way to exclude the possibility that the enzyme may be toxic or allergenic or have some other adverse physiological effect.

(d) Toxicological assessments of enzyme preparations

Toxicological considerations. As noted above, enzyme preparations contain either one major active enzyme that catalyses a specific reaction or two or more active enzymes that catalyse different reactions during food processing. Each enzyme in the preparation must comply with the established identity and purity specifications.

Although food enzyme preparations are considered unlikely to cause any acute toxicity, genotoxicity or repeated-dose oral toxicity, the fermentation products of microorganisms remaining from the manufacturing process are of interest due to the potential presence of secondary metabolites that may induce toxicity when ingested (e.g. aflatoxins, fumonisins and ochratoxins) (OECD, 2018). The enzyme concentrate, containing both fermentation products and the food enzyme of interest, has traditionally been used in genotoxicity tests and in repeated-dose rodent feeding studies submitted to JECFA.

The Scientific Committee on Food (SCF, 1992) elaborated the points of potential toxicological concern, noting that:

1. Different strains belonging to the same species can behave differently. For many microorganisms it is known that some of the strains in one species are harmless, while others belonging to the same species are toxic.
2. For some fungal genera, especially *Penicillium* and *Aspergillus*, there have been many misidentifications of fungal isolates. As a consequence of this, there is a risk of misclassification of fungal strains. For example in some cases it has been difficult to distinguish *A. oryzae* from *A. flavus* which has the ability to produce aflatoxin. As long as there is a risk of misidentification of microbial isolates, it is very important that the microorganism used is correctly identified and, in case of doubt, the identity should be verified by an independent, recognized laboratory.
3. The ability of a microorganism to produce toxins depends – qualitatively and quantitatively – on environmental factors such as the composition of fermentation media, pH, temperature and fermentation period. Therefore there is a risk that a microorganism which does not produce toxins under some conditions will turn out to be toxin-producing under other conditions.
4. The continuous selection processes applied to source microorganisms in order to maximize and optimize enzyme production may result in spontaneous mutations which give rise to the possibility of changing a non-toxic strain to a toxic strain.

5. There is a considerable potential to apply new techniques of genetic modification in the production of food enzymes. Along with the introduction of desirable traits, there is also the potential for introducing toxin production and therefore there is a need explicitly to characterize and evaluate the genetic construct as to host, vector and insert. [SCF, 1992:14–15]

As a result of these safety concerns, the following basic toxicological testing requirements were provided (SCF, 1992):

9.1 For enzymes derived from edible parts of animals or plants no toxicological tests are normally required. Where parts which are not generally considered as a normal part of the diet are used, some toxicological testing may be required unless other satisfactory documentation for safety in use is provided.

9.2 For enzyme preparations derived from microorganisms the following tests are normally required:

(a) 90-day oral toxicity test in a rodent species;
(b) Two short-term tests:
    1. a test for gene-mutations in bacteria,
    2. a test for chromosomal aberrations (preferably in vitro).

The toxicological tests shall, where possible, be performed on a batch from the final purified fermentation product, before addition of carriers, diluents, etc. [SCF, 1992:19]

Exemptions from the basic toxicological requirements. The exemptions from performing toxicological bioassays in the safety assessments of enzymes, as described in the original SCF (1992) guidelines, are as follows:

From a toxicological point of view it is important to perform a toxicological testing procedure on each specific enzyme preparation produced from a microbiological source.

10.1 If, however, one enzyme 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 decided on a case-by-case basis.

10.2 If the microorganism used in the production

– has a long history of safety in food use, and

> – belongs to a **species** about which it has been documented that no toxins are produced, and
> – the actual **strain** used is of well documented origin,
>
> the acceptance of an enzyme preparation from this organism without specific toxicological testing may be justified. In this case a correct and confirmed identification of the organism is of extra importance. [SCF, 1992:20]

To date, very few exemptions from toxicological testing have been considered in safety assessments of enzymes by JECFA. This may be because of the uncertainty regarding compliance with the requirements of accurately identifying the microbial strain and assessing the ability of the microorganism to produce toxins. However, these requirements can more easily be met using current technologies such as analytical molecular biology techniques – for example, full genome sequencing, gene probing or RNA sequencing technologies to minimize misidentification (Yu et al., 2011) and chemometrics (Inui et al., 2012) to identify and quantify secondary metabolites in complex natural product mixtures that may result from microbial fermentation.

If the sponsor does not conduct toxicity testing, then the sponsor is obligated to provide other information to attest to the enzyme's safety. The full battery of toxicity tests may be waived for enzymes from a specific (new) strain if the manufacturing process does not differ significantly from that used for other enzymes from the same strain, a related strain or a lineage of related strains, provided other evidence is presented to support the safety of the enzyme preparation of interest (e.g. chemical assessment for known toxins, whole genome sequencing and assessment for possible toxin production).

(e) Dietary exposure and margin of exposure

Dietary exposure is calculated on the basis of the total organic solids (TOS) content in the final (commercial) enzyme preparation and is usually expressed in milligrams or micrograms of TOS per kilogram of body weight per day. TOS encompasses the enzyme component and other organic material originating from the production organism and the manufacturing process, while excluding intentionally added formulation ingredients. JECFA considers the estimated dietary exposure to an enzyme preparation based on the proposed uses and use levels in food and relates it to the no-observed-adverse-effect level (NOAEL) in its hazard assessment in order to determine a margin of exposure (MOE).

(f) Classification of enzymes

To aid in the decision-making process, in 2018, JECFA reassessed the requirements for testing the toxicity of enzyme preparations used in food and updated the classes as follows (FAO/WHO, 2019):

- *Class I: Enzymes obtained from sources that are considered safe for consumption and for which toxicological evaluations are NOT normally required*

  This class, which also includes immobilized enzymes from these sources, can be further categorized into:

  – ***Type i:*** Enzymes obtained from edible tissues of plants or animals commonly used as foods

    These enzymes are regarded as foods; consequently, their safety is considered acceptable, provided that satisfactory chemical and microbiological specifications can be established (e.g. papain, rennet). Uses and use levels should be considered.

  – ***Type ii:*** Enzymes produced by microorganisms that are traditionally accepted as constituents of foods or are normally used in the preparation of foods

    These enzymes are regarded as foods; consequently, their safety is considered acceptable, provided that satisfactory chemical and microbiological specifications can be established (e.g. *Saccharomyces* spp.). Enzymes produced by microorganisms modified by genetic engineering are not considered to be Class I Type ii, but fall into either Class I Type iii or Class II. Uses and use levels should be considered.

– ***Type iii:*** Enzymes produced by a Safe Food Enzyme Production Strain[1] or a Presumed Safe Progeny Strain[2]

  For food enzyme preparations in this group, a detailed chemical and microbiological (genomic) narrative confirming that the enzyme is produced by an organism that meets the definition of a Safe Food Enzyme Production Strain or a Presumed Safe Progeny Strain that has undergone appropriate toxicological testing (i.e. repeated-dose toxicity and genotoxicity testing) is required. Appropriate toxicological testing includes existing studies conducted on enzymes from other closely related strains derived from the same parental organism. This could be demonstrated with published or unpublished genomic sequence data of the genetically modified microorganism to exclude the possibility of the presence of genes for the production of toxic secondary metabolites. Safety assessments for these food enzymes should also include appropriate information or other experimental data to determine their potential to cause an allergic reaction when ingested.

  On completion of appropriate toxicological testing of the fermentation product from a production microorganism, this guidance anticipates that it should be possible to conclude that the microorganism can be classified as a source that is considered safe for human consumption. Such a declaration was made for *A. oryzae* at the sixty-eighth meeting of JECFA (FAO/WHO, 2007b). As of 2020, JECFA has

[1] A "Safe Food Enzyme Production Strain" is a non-pathogenic, non-toxigenic microbial strain with a demonstrated history of safe use in the production of food enzymes. Evidence supporting this history of safe use includes knowledge of taxonomy, genetic background, toxicological testing, other aspects related to the safety of the strain and commercial food use.

[2] A "Presumed Safe Progeny Strain" is developed from a Safe Food Enzyme Production Strain or from the parent of that Safe Food Enzyme Production Strain. The progeny strain is developed through specific well-characterized modifications to its genome; the modifications must be thoroughly documented, must not encode any harmful substances and must not result in adverse effects. This concept also applies to multiple generations of progeny. Evidence supporting their safety includes knowledge of taxonomy, genetic background and toxicological testing (including read-across of toxicological studies).

evaluated over 80 food enzyme preparations from a variety of microorganisms and has never recorded a positive result in any toxicity study, suggesting either that toxins were not present or that toxins were present at levels that were below the limit of detection of the bioassays. These data suggest that there are many strains of microorganisms that JECFA has previously reviewed (e.g. *Bacillus subtilis*, *B. licheniformis*, *Aspergillus niger* and *A. oryzae*) that are considered to be safe sources of food enzymes. Therefore, provided the genetic modification of the production organism, as the result of the use of either recombinant DNA or chemical mutagenesis, was well characterized, additional toxicological testing would not be required. However, as already described in the JECFA guidance (FAO, 2006; FAO/WHO, 2007a), information on other aspects of enzyme production would still be required (see Appendix in section 9.1.4.2(h) below). An acceptable daily intake (ADI) may be established.

- *Class II: Enzymes derived from sources that are NOT considered or presumed safe for consumption*

  For all enzymes that do not fall under any of the Class I subcategories listed above, chemical and microbiological specifications must be established. Similarly, enzymes from organisms that have not been previously reviewed by JECFA, although they may subsequently be considered Class I Type iii, require the submission of relevant microbiological, toxicological and chemical data. Each enzyme will be evaluated, and an ADI may be established.

  For enzymes produced by strains of microorganisms not previously evaluated by JECFA, information is required about the taxonomy, genetic background and other aspects related to the safety of the strain, and commercial use in foods (if any). Enzyme preparations produced by such microorganisms should not contain either antibiotic inactivating proteins at concentrations that would interfere with antibiotic treatment or transformable DNA that could potentially contribute to the spread of antibiotic resistance.

The absence of microorganism-derived secondary metabolites of toxicological significance in the enzyme concentrate also needs to be confirmed. This can be achieved by submitting the results of two genotoxicity (mutagenicity and clastogenicity) assays on the enzyme, as well as a short-term oral toxicity study. As an alternative to genotoxicity testing for the presence of undesirable secondary metabolites in the fermentation products, a detailed chemical characterization of the enzyme concentrate, including confirmation of the absence of toxicologically significant levels of toxic secondary metabolites (e.g. mycotoxins that are known to be generated by strains of the production microorganism or by species related to the production microorganism), can be performed using high-performance liquid chromatography or mass spectrometry. Such characterization must also be supported by detailed knowledge of the genomic sequence of the genetically modified microorganism to exclude the possible presence of genes capable of producing toxic secondary metabolites. Additional characterization of the enzyme protein would also be required, such as the inclusion of bioinformatics analyses to confirm the absence of any potential allergenic epitopes or significant amino acid sequence homology to known toxins.

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(h) Appendix: Information required for the safety assessment of enzyme preparations for use in foods

No.	Class(es)[a]	Information required	Details/rationale
Enzyme classification and description of active components of enzyme preparation			
1.	All	Name of enzyme(s)	e.g. Triacylglycerol lipase
2.	All	Systematic name(s) and number(s)	EC/IUBMB number; CAS number (where appropriate)
3.	All	Molecular weight(s)	As determined by SDS PAGE, gel filtration chromatography, etc.
4.	All	Amino acid sequence(s)	Predicted and determined primary amino acid sequence
5.	All	Catalytic activity	All reactions catalysed, including any secondary activities, conditions under which catalysis occurs (e.g. pH, temperature)
6.	All	Historical use(s) in food-based applications	Evidence of commercial food use, including from the parent strain or other strains in the lineage (e.g. as a processing aid in the manufacture of bakery products, pasta and noodles, in egg yolk and in oil degumming)
7.	All	Use levels in food(s)	Express each use as TOS in mg/kg food, substrate or raw material – specify

No.	Class(es)[a]	Information required	Details/rationale
8.	All	Fate in final food(s)	Is the enzyme active, inactive or removed? How is the enzyme inactivated/removed?
9.	All	Existing safety evaluations	Include any existing health-based guidance values (e.g. ADI)
Details about the production organism			
10.	All	Identity of the production organism	Identify genus, species, strain
11.	I(iii), II	Host/recipient organism	Identify genus, species
12.	I(iii), II	Donor of genetic material	e.g. Identify origins of genetic material by genus, species (if native or modified)
13.	I(iii), II	Details of genetic modification:	
		i. To host genome	History of development of host strain (e.g. deletion of gene clusters that encode for aflatoxins, modifications that make host extracellular protease deficient or make it non-sporulating, etc.), identification of genes removed/ added

No.	Class(es)[a]	Information required	Details/rationale
		ii. Addition of rDNA (gene of interest from another microorganism) to host microorganism through mobile genetic elements	Donor of genetic material, details on how the genetic element was designed and the identity of genes on the element, stability information, copy numbers, whether it integrates or does not integrate into the host genome, etc. Evidence that genetic material does not contain genes coding for virulence factors, protein toxins or any enzymes that may be involved in the synthesis of mycotoxins
14.	I(iii), II	Genetic modification techniques	Site-directed mutagenesis, chemical mutagenesis, rDNA technology, etc.
15.	I(iii), II	Description of intended and nonspecific effects resulting from genetic modification and any changes carried out to prevent unwanted side reactions/products	e.g. An intended effect may be increased yield; a nonspecific effect may be activation of toxin production Rectification measures may include genetic modifications, specific fermentation conditions, etc.
16.	All	Deposit information (if applicable)	e.g. ATCC number

No.	Class(es)[a]	Information required	Details/rationale
Production of enzyme concentrate and preparation			
17.	All	Detailed manufacturing process	For enzymes in Class I(i) and Class I(ii), and Class II enzymes obtained from plants and animals, manufacturing details are required. For enzymes in Class I(iii) and Class II produced by microorganisms, include details describing controlled fermentation inputs and conditions, the steps taken to retain genetic modifications, and further processing, purification and concentration steps. Indicate how production strains are maintained under conditions that ensure the absence of genetic drift, and, when used in the production of enzyme preparations, indicate the methods and conditions that are applied to ensure consistency and reproducibility from batch to batch. Such conditions must ensure the absence of toxin production by the organism and prevent the introduction of microorganisms that could be the source of toxic or other undesirable substances.

No.	Class(es)[a]	Information required	Details/rationale
18.	All	Formulation ingredients	Identify the carriers, diluents, excipients, supports and other additives and ingredients (including processing aids) used in the production, stabilization and application of enzyme preparations; must be acceptable for food use In order to distinguish the proportion of the enzyme preparation arising from the source material as opposed to that contributed by diluents and other additives and ingredients, individual specifications require a statement of percentage TOS, which is defined as follows: % TOS = 100 − (A + W + D) where A = % ash, W = % water and D = % diluents and/or other additives and ingredients.
Specifications and data required for enzyme concentrates and preparations			
19.	All	Description	Physical form of the enzyme preparation – liquid, semiliquid or dried product
20.	All	Purity	Impurities, including elemental and microbiological impurities Analytical test methods, validation data, representative batch data (minimum of five batches) are required

No.	Class(es)[a]	Information required	Details/rationale
21.	All	Enzyme characterization	Enzyme activity (including method of assay, activity unit definition), molecular weight determination for the enzyme and other specific identification techniques. A universally usable test method to define enzyme activity present in the preparation should be submitted. Analytical test methods, validation data, representative batch data (minimum of five batches) are required.
22.	All	Analysis of at least five non-consecutive batches of the **enzyme concentrate** (for enzymes in Class II, at least one of which should have been used for toxicological testing)	e.g. TOS, enzyme activity, protein concentration, impurities, absence of antibiotic inactivating proteins, etc.
23.	All	Composition of at least five non-consecutive batches of the **product(s) of commerce** (enzyme preparation)	e.g. Stabilizers, pH adjustment agents, carriers, diluents, preservatives, etc.
24.	I(iii), II	Information on carryover of allergens from the fermentation media to the enzyme concentrate	Identification of major food allergens in media components and in the enzyme concentrate
25.	I(iii), II	Evidence for absence of rDNA and production organisms in the enzyme concentrate or the enzyme commercial product	This requirement applies only to enzymes produced with those production organisms that express DNA sequences of concern, e.g. antibiotic-resistant markers.

No.	Class(es)[a]	Information required	Details/rationale
Assessment of potential allergenicity of the enzyme			
26.	I(iii), II	Comparison of the amino acid sequence of the enzyme with known allergens	In silico comparison of primary amino acid structure with allergen databases to confirm the absence of sequence homology with known allergenic proteins: i. Sequence homology (35% of a sliding window of 80 amino acids) ii. Sequence identity in contiguous stretches of 8 amino acids within the enzyme sequence All the information resulting from the sequence homology comparison between an expressed enzyme and known allergens should be reported. If any of the identity scores equals or exceeds 35%, this is considered to indicate significant homology and needs to be scientifically considered in the context of a safety assessment for enzymes in food.
27.	I(iii), II	Proteolysis resistance/digestibility of the enzyme	e.g. Simulated gastric fluid studies, etc.
Toxicology			
28.	II	Results of toxicological testing of the enzyme concentrate	It is necessary to conduct toxicological studies in order to assess whether an ADI needs to be established: (a) 90-day oral toxicity test in a rodent species;

No.	Class(es)[a]	Information required	Details/rationale
			(b) Two short-term genotoxicity tests (mutagenicity and clastogenicity) 1. A test for gene mutations in bacteria 2. An in vitro micronucleus test
29.	I(iii), II	Bioinformatic analysis of the amino acid sequence for potential matches with known toxins	Explanation of the analysis and interpretation should be provided.
Dietary exposure assessment			
30.	II	Estimate of dietary exposure to the enzyme preparation calculated on the basis of the TOS. Separate dietary exposure situations may need to be considered, depending on whether they are for: (a) enzyme preparations added directly to food and not removed; (b) enzyme preparations added to food but removed from the final product according to GMP; or (c) immobilized enzyme preparations that are in contact with food only during processing.	Express the dietary exposure as mg TOS/kg body weight per day; provide an explanation of the methodology used to derive the estimated dietary exposure.

No.	Class(es)[a]	Information required	Details/rationale
31.		Additional information and comments	Additional items considered helpful in the safety assessment.

ADI: acceptable daily intake; ATCC: American Type Culture Collection; CAS: Chemical Abstracts Service; DNA: deoxyribonucleic acid; EC/IUBMB: Enzyme Commission/International Union of Biochemistry and Molecular Biology; GMP: Good Manufacturing Practice; rDNA: recombinant deoxyribonucleic acid; SDS PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TOS: total organic solids

[a] Class I: Enzymes obtained from sources that are considered safe for consumption and for which toxicological evaluations are NOT normally required.

Type i: Enzymes obtained from edible tissues of plants or animals commonly used as foods: I(i).
Type ii: Enzymes produced by microorganisms that are traditionally accepted as constituents of foods or are normally used in the preparation of foods: I(ii).
Type iii: Enzymes produced by a Safe Food Enzyme Production Strain or a Presumed Safe Progeny Strain: I(iii).

Class II: Enzymes derived from sources that are NOT considered safe for consumption and are not in any of the subcategories listed above.