Food and Agriculture Organization of the United Nations

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JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES

Expert meeting of Working Group established to consider the evaluation of enzyme preparations used in the manufacture of foods

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FINAL REPORT

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Evaluation of enzyme preparations used in the manufacture of foods

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1. Background

The Working Group (WG) met to discuss recent advances in the understanding of the safety of enzyme preparations for use in food and the need to revise Chapter 9.1.4.2 of *Principles and methods for the risk assessment of chemicals in food; Environmental Health Criteria* 240[1], (EHC 240). The WG also discussed the possibility of reducing the extent of toxicological testing necessary for enzyme preparations derived from well characterized micro-organisms and how this should be reflected in Chapter 9.1.4.2. Since the publication of the JECFA guidance on the evaluation of enzymes for use in foods in 2006, there has been a steady increase in the number of enzymes from genetically engineered microbial sources. In the guidance, JECFA had previously acknowledged the possibility of toxin production and the generation of an allergenic protein, among other potential issues. This Working Group identified ways to approach these aspects in the evaluation process and proposed revisions to the guidance in EHC 240, Chapter 9.1.4.2 pertaining to enzyme preparations for use in food. The Working Group also recognised that these revisions would need to be reflected in the "*Combined Compendium of Food Additive Specifications. Volume 4 – Analytical methods, test procedures and laboratory solutions used by and referenced in the food additive specifications*."

At the time of the meeting, the Codex Committee on Food Additives (CCFA) *Priority list of substances proposed for evaluation by JECFA* contained 29 enzyme preparations produced by microorganisms (REP18/FA Appendix X[2]). To assist in addressing these requests, the Working Group considered the current safety evaluation process for enzyme preparations for use in foods and the state of the art of food enzyme production. To lead into the discussions, the Working Group considered a background document (*Annex 1*) presenting newer approaches in the consideration of enzyme safety since the last revision was made in 2006. The factors presented in Table 1 framed the deliberations of the Working Group.

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[1] EHC 240 (2009) Principles and methods for the risk assessment of chemicals in food; Environmental Health Criteria 240, Food and Agricultural Organization of the United Nations and the World Health Organization. Inchem.org/documents/ehc/ehc/ehc240_index.htm

[2] http://www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FMeetings%252FCX-711-50%252FReport%252FREP18_FAe.pdf

Table 1: Factors considered by the experts for the revision of the safety evaluation of enzyme preparations for use in foods

Factors	Elements
History of safe use of the production micro-organism	Genetic stability/instability of a given construct Toxigenicity and pathogenicity Safety data from production strains of the same lineage
Enzyme preparations from the same source organism	Strain performance/productivity Enzyme activity and TOS Whole genome sequence Identification of the specific modification
GMP production	Control of toxin production Carryover of residual components in the growth medium from manufacturing Enzyme purity in TOS
Presence or absence of production strain in final product	Carryover of secondary metabolites
Dietary exposure	Levels of use in food production and in the final food
In silico database comparisons	Prediction of similarity with known allergens Prediction of protein toxicity
Digestibility	Determination of *in vitro* digestibility to assess absence of allergenic protein fragments

These discussions led to the a proposal for revision of Chapter 9.1.4.2 in EHC 240, including revision to the classification of enzymes (Section 2, below); a list of Recommendations for adoption by JECFA, below (Section 3, below); the development of definitions for "Safe Food Enzyme Production Strain" and "Presumed Safe Progeny Strain" for use by JECFA (*Annex 2*); a checklist of information required for the safety evaluation of enzyme preparations for use in foods (*Annex 3*); and list of terms and definitions related to the safety evaluation of enzyme preparations for use in food (*Annex 4*).

2. Draft Changes to Chapter 9.1.4.2 EHC 240 proposed by the JECFA Working Group on Enzymes

The history of enzyme use in food applications is long and well known, especially in bread-, cheese-, wine-, and beer-making where enzymes are part of the processing or maturation processes. Enzymes used in the food industry are derived 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, 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. The formulated enzymes are referred to as enzyme preparations, which, depending upon the application, may be produced as a liquid, semi-liquid or dried product. Enzyme preparations may contain either one major active enzyme that catalyzes a specific reaction during food processing or two or more active enzymes that catalyze different reactions.

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 sixty-fifth JECFA Committee 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 Used in Food Processing* (FAO, 2006a; FAO, 2006b). The documents addressed certain aspects that apply to the safety evaluation of all enzyme preparations, including the safety evaluation of the production organism, the enzyme component, side activities, the manufacturing process and the consideration of dietary exposure.

Some of the specific safety concerns are:

1. Potential for the enzyme to cause an allergic reaction

1.1 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, lacto-peroxidase 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. Food Allergy Research and Resource Program, University of Nebraska; AllergenOnline (http://www.allergenonline.org)]. The possibility of immunological cross-reactivity between the expressed enzyme and a known allergen is considered when there is:

a. 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 FASTA or BLASTP (Codex Alimentarius, 2003), or equivalent);

b. identification of eight contiguous amino acids common to the expressed enzyme and a known allergen (JECFA, 2016).

1.2 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. While 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 newly expressed proteins (Codex Alimentarius, 2009). A pepsinolysis assay that is based on simulated gastric fluid (SGF) and usually used in pre-clinical testing of pharmaceuticals, has been described by the United States Pharmacopeia (2000). The SGF is often used to allow comparisons between different newly-expressed proteins under experimental conditions (Astwood *et al.*, 1999). However, to date, 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 pH, purity and activity of pepsin and pepsin-to-substrate protein ratio, which have shown that the correlation with allergenic potential is not absolute and that proteins which are resistant to pepsinolysis might not be allergenic under physiological conditions of dietary exposure whereas labile proteins (eg. β-casein) or peptides formed during proteolysis may be allergenic (Vieths *et al.*, 1999; Yagami *et al.*, 2000; Wal, 2001; Fu *et al.*, 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 intrinsic allergenicity of a protein, but still may have some utility as part of a Weight-of-Evidence approach.

1.3 Occupational hazards – respiratory allergies, skin and eye irritation

A known safety risk linked to industrial enzyme use is respiratory allergy and for most proteases there is also some potential for skin and eye irritation

(Vanhanen, 2001; Anderson *et al*., 2017). Enzymes present a risk of a respiratory allergy (e.g. Aspergillus-derived enzymes in bakers' asthma) and it is well described in the scientific literature (Quirce *et al.*, 1992; Green & Beezhold, 2001).

1. *Safety concerns pertaining to enzyme preparations derived from genetically modified microorganisms.*

The *General Specifications and Considerations for Enzyme Preparations Used in Food Processing* (2006) provides recommendations on the safety assessment of the genetic material inserted into the genome of the production microorganism. Two additional considerations that were introduced in the 2006 revision of the document state:

a. For enzyme preparations from recombinant-DNA-modified microorganisms 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.

b. Recombinant-DNA-modified 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.

It must be pointed out that extensive literature searches citing 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 and/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 against the sequence of known toxic proteins using *in silico* methods is one way to exclude the very remote possibility that the enzyme may be toxic or have some physiological effect.

2. Toxicological assessments of enzyme preparations

Enzyme preparations contain either one major active enzyme that catalyzes a specific reaction during food processing or two or more active enzymes that catalyze different reactions. Each enzyme preparation must comply with the identity and purity specifications, which are established for each enzyme preparation.

While food enzyme preparations are considered unlikely to cause any acute toxicity, genotoxicity, or repeat-dose oral toxicity, it is the fermentation product(s) of microorganisms from the manufacturing process that is/are of interest due to the potential presence of secondary metabolites that may induce toxicity when ingested (eg. aflatoxins, fumonisins and/or ochratoxins) (OECD, 2018). The fermentation product, which also includes the food enzyme of interest, has traditionally been used in genotoxicity tests and in repeat-dose rodent feeding studies submitted to JECFA.

The *General Specifications and Considerations for Enzyme Preparations Used in Food Processing* that was published by JECFA (2006) and the Scientific Committee on Food (SCF, 1992) elaborated the points of potential toxicological concern noting that:

a. 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 may produce toxins.

b. For some fungal genera, especially *Penicillium* and *Aspergillus*, there have been many misidentifications of fungal isolates. Consequently, 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*; the latter may produce aflatoxins. 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.

c. The ability of microorganisms to turn on genes that code for toxins can depend on fermentation conditions 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 produce toxins under other conditions.

d. 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-toxin-producing strain into a toxin-producing strain, providing its genetic predisposition is such that these mutations are sufficient to turn on the expression of toxin producing genes.

e. 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 or deleting genes for toxin production and, therefore, there is a need to explicitly characterize and evaluate the genetic construct in the host, vector and insert.

As a result of these safety concerns, the toxicological testing requirements are:

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

b. For enzyme preparations derived from microorganisms, the toxicological tests shall, where possible, be performed on batches of the final purified, concentrated fermentation product before addition of formulation ingredients (e.g. carriers, diluents, etc). The following tests are normally required:

i. 90-Day oral toxicity test in a rodent species;

ii. Two short-term tests:

1. A test for gene-mutations in bacteria,

2. A test for chromosomal aberrations (preferably *in vitro*).

3.1 Dietary exposure and Margin of Exposure (MoE)

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 TOS per kilogram body weight per day. TOS encompasses the enzyme component and other organic material derived from the production organism and the manufacturing process while excluding intentionally-added formulation ingredients. JECFA then considers the estimated dietary exposure to an enzyme preparation based on the proposed uses and use levels in food and relates it to the NOAEL in its hazard assessment in order to determine an MoE.

3.2 Exemptions from the basic toxicological requirements

The original SCF and JECFA guidelines, which described exemptions from performing toxicological bioassays in the safety assessments for enzymes, are:

a. 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. 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 such enzymes. This will be decided on a case-by-case basis.

b. If the microorganism used in the production has a long history of safety in food use, belongs to a species that has been well-documented, does not produce toxins, and the strain itself is of well documented origin, the acceptance of an enzyme preparation from such a microorganism with no specific toxicological testing may be justified. In this case, a correct and confirmed identification of the microorganism is of paramount importance.

Thus far, there are very few examples of these exemptions from toxicological testing being considered in a safety assessment 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 with current technologies such as analytical molecular biology techniques, for example, full genome sequencing, gene probing or RNA-Seq technologies, to minimize misidentification (Yu *et al*., 2011) and biochemometrics (Inui *et al*., 2012) to identify and quantify secondary metabolites in complex natural product mixtures that may result from microbial fermentation..

Classification of Enzymes

To aid in the decision-making process for whether toxicological studies are required, JECFA has grouped enzyme preparations for use in food into the following classes:

I. *Class I: Enzymes derived from sources which 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:

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

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

ii. Type ii: Enzymes derived from microorganisms that are traditionally accepted as constituents of foods or are normally used in the preparation of foods.

These products are regarded as foods and, consequently, their safety is considered acceptable, provided that satisfactory chemical and microbiological specifications can be established. (e.g. *Saccharomyces* sp.).

iii. Type iii: Enzymes derived from a Safe Food Enzyme Production Strain or a Presumed Safe Progeny Strain (for definitions see *Annex 2*).

For enzyme preparations in this group, a detailed chemical and microbiological narrative needs to be provided confirming that the source organism producing a food enzyme has undergone appropriate toxicological testing (i.e. repeat dose toxicity and genotoxicity testing) together with a thorough chemical characterization of the enzyme concentrate and genomic analysis. This could be demonstrated with published or unpublished genomic sequence data of the genetically modified micro-organism to exclude the possibility of secondary metabolite toxin genes. 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 micro-organism, the guidelines anticipate that it should be possible to conclude that the micro-organism can be classified as a source that is considered safe for human consumption. Such a declaration was made for *A. oryzae* at the 68th meeting of JECFA in 2008 (JECFA, 2008). Up until 2018, JECFA has evaluated over 80 food enzyme preparations from a variety of micro-organisms and has never recorded a positive result in any toxicity study, suggesting that either toxins were not present or were present at levels that were below the limit of detection of the bioassays. These data suggest that

there are many strains of microorganisms which JECFA has previously reviewed (e.g. *Bacillus subtilis, B. licheniformis, Aspergillus niger* and *A. oryzae)* that are considered to be sources of food enzymes that are safe for human consumption. Therefore, provided the genetic modification of the production organism, either as the result of the use of recombinant-DNA or chemical mutagenesis, was well characterized, additional toxicological testing would not be required. However, as already described in the 2006 JECFA guidelines, information on other aspects of enzyme production would be still required (see Annex 3).

II. Class II: *Enzymes derived from sources which are NOT considered safe for consumption*

For all enzymes that do not fall under any of the sub-categories listed above and which have not been previously reviewed by JECFA, chemical and microbiological specifications must be established. Each enzyme must be evaluated and an ADI must be established.

For enzymes derived from strains of micro-organisms not previously considered by JECFA, information is required about the taxonomy, genetic background, other aspects related to safety of the strain, and commercial use in foods (if any). Enzyme preparations derived from such micro-organisms 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.

The absence of micro-organism-derived secondary metabolites of toxicological importance 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 these enzymes, as well as a subchronic oral toxicity study. As an alternative to genotoxicity testing for secondary metabolites in fermentation products, a detailed chemical characterization of the extracts (e.g. confirmation that they do not contain toxicologically significant amounts of mycotoxins or other toxic secondary metabolite that are known to be synthesized by strains of the production microorganism species or

of species related to the production microorganism), can be performed using analytical tests like high-performance liquid chromatography and/or mass spectrometry. This must also be supported with detailed knowledge of the genomic sequence of any genetically modified microorganisms to exclude the possible presence of secondary metabolite toxin genes. Additional characterization of the enzyme protein would also be required, for instance including a bioinformatics analysis of the amino acid sequence to confirm the absence any potential allergenic epitopes or significant amino acid sequence homology to known toxins.

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3. List of Recommendations for adoption by JECFA

The Working Group recommends that JECFA considers:

1. The implementation [adoption] of:
    a. The definition for Safe Food Enzyme Production Strain and Presumed Safe Progeny Strain (*Annex 2*);
    b. Revisions to Chapter 9.1.4.2 of EHC 240 pertaining to enzymes, including a revision of the classification of enzymes and their definitions (Section 2);
    c. A checklist of data requirements (*Annex 3*) for the risk assessment of enzyme preparations in submissions for review by JECFA based on their assigned class in the proposed revisions to Chapter 9.1.4.2 of EHC 240 (Section 2);
    d. A list of terms and definitions as related to submissions of enzyme preparations for use in food (*Annex 4*).
2. Whether an allergenicity assessment should be conducted on enzyme preparations proposed for inclusion in all classes or only on proposals for Class I Type iii and Class II as presented in Annex 2 and Questions 26 and 27 in Annex 3.
3. Whether the association between the establishment of an ADI and the presence of the enzyme preparation in the final food can be considered unnecessary and reference to the ADI can be deleted from the text in EHC 240 Chapter 9.1.2.4, as shown in Annex 2.
4. Whether it is appropriate to combine the consideration of immobilized enzyme preparations that are in contact with foods only during processing with the consideration of enzyme preparations added to foods but removed from the final products (Annex 2).
5. Whether a separate online database should be established to present the combined toxicological and specification information for enzyme preparations for use in food

evaluated by JECFA as a means of providing a simplified presentation of the data to users (similar to the presentation currently used for flavourings).

6. Whether a separate JECFA identification number should be established to help further identify enzyme preparations with completed JECFA safety evaluations (similar to the JECFA numbering system used for flavourings).

7. Whether an enzyme-preparation-specific template for the submission of analytical methods including method performance characteristics (method validation data) and quality control data should be developed.

4. List of Abbreviations

ADI – Acceptable daily intake

ATCC – American Type Culture Collection

bw – body weight

CAS – Chemical Abstracts Service

CCFA - Codex Committee on Food Additives

EC – Enzyme Commission of the IUBMB

EHC 240 – Environmental Health Criteria 240 Principles and methods for the risk assessment of chemicals in food

GMM – Genetically Modified Microorganisms

GMP – Good Manufacturing Practice

IUBMB – International Union of Biochemistry and Molecular Biology

JECFA – Joint FAO/WHO Expert Committee on Food Additives

NOAEL – No Observed Adverse Effect Level

NOEL – No Observed Effect Level

OECD – Organisation for Economic Co-operation & Development

SGF – Simulated Gastric Fluid

SCF – Scientific Committee on Food (Advisory body to the European Commission)

TOS – Total Organic Solids

Annex 1. **Draft Report on Enzyme Assessment for JECFA Food Additives**

FAO JECFA Project to Review and Revise the Protocol for Enzyme Review

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1. Summary

A number of documents are available regarding the evaluation of enzyme preparations for safe use in the manufacture of food products. These documents have been focused on the submission of relevant information to bodies such as JECFA, EFSA and specific governments for approval for food use. The enzyme industry also has produced documents related to the requirements for submissions for safety evaluations. Most of the processes and procedures have been routed through the Technology committee of the Enzyme Technical Association (ETA). ETA also hosts the International Enzyme Coordination Group (IECG), whose members represent regionally located professional enzyme associations and advocacy groups (ABIAM, AMFEP, CERF, ETA and JEA). meetings were held in July and October 2018 with members of the ETA Executive Committee to understand processes for enzyme evaluation from an industry perspective and to discuss requirements for future enzyme safety evaluations. A literature search generated surprisingly only a few papers on the evaluation of food enzymes and included notifications of enzymes evaluated by various bodies. From this research a proposal is made to streamline the safety evaluation of enzymes used in food using experience gained in the JECFA safety evaluation of flavors and JECFA discussions on information required for Modified Starches.

2. Background (taken from JECFA reports [WHO 2016])

> "In 1987, the Committee outlined criteria for evaluating the safety of enzymes and proposed to categorize enzyme preparations into five main groups on the basis of their origin: (i) animal tissues, (ii) portions of edible plants, (iii) microorganisms traditionally accepted as constituents of food, microorganisms normally used in the preparation of foods, (iv) non-pathogenic microorganisms commonly found as contaminants of foods or (v) microorganisms that are less well known. At the same time, the Committee envisaged three cases for assessing the safety of enzymes in groups (iv) and (v) – those added directly to food and not removed, those added to food but removed, and immobilized enzyme preparations – and indicated guidelines appropriate for evaluations of safety in each case (IPCS, 1987)."
>
> "Enzymes produced by genetically modified microorganisms were not considered at this time. Subsequently, the Committee evaluated several enzymes in this category, including laccase from *Myceliophthora thermophila* expressed in *Aspergillus oryzae* and xylanase from *Thermomyces lanuginosus* expressed in *Fusarium venenatum*. The Committee evaluated the safety of these enzyme preparations on the basis of toxicological data files, both of which included a 90-day toxicity study in rats, and two *in vitro* genotoxicity tests - a test for reverse mutations in bacteria and a test for chromosomal aberrations in mammalian cells. The Committee allocated an ADI "not specified" to these enzyme preparations."

"The sixty-fifth Committee (FAO/WHO, 2006) evaluated an enzyme preparation of phospholipase A1 produced by the same host strain of *A. oryzae* that had been modified to produce other enzymes. It could not, however, assess the safety of this preparation by comparison with the information available on one of the other enzymes but acknowledged that alternatives to toxicity testing such as evidence that no unintended compounds were present in the enzyme preparation or a complete molecular characterization of the enzyme production strain were acceptable. The Committee concluded that guidelines should be drawn up for the safety assessment of enzymes produced by genetically modified microorganisms. These guidelines should include the essential information for various situations and details of molecular characterization of the producing microbial strain necessary to allow adequate assessment of the safety of the preparation."

"At the sixty-eighth meeting of JECFA (FAO/WHO, 2007), the Committee reviewed comments on these considerations submitted by the Enzyme Technical Association and the Association of Manufacturers and Formulators of Enzyme Products. The Committee also noted the ongoing international initiatives to elaborate guidelines for the safety evaluation of enzymes (including those from genetically modified microorganisms) and microorganisms intended for food applications. These documents were expected to be finalized shortly thereafter. The Committee recommended that the subject of guidelines for the safety evaluation of enzymes produced by genetically modified microorganisms be addressed at a future meeting."

"At the seventy-first meeting of JECFA (FAO/WHO, 2010), the Committee discussed the new regulation for enzymes enacted by the European Parliament and related guidance documents. The Committee decided to update the General Specifications and Considerations for Enzymes Used in Food Processing (FAO, 2006) to expand recommendations for microbiology and molecular biology information to be submitted in dossiers for enzymes from microorganisms (including those from genetically modified microorganisms) and to discuss toxicological and other safety studies for enzymes from all sources. The Committee recommended the establishment of a working group to update the current guidance document on enzymes for discussion at a future meeting."

3. Data sources used in the preparation of this report

Information was requested be email from a variety of national governments, trade organizations and experts in the field who had previously indicated interest in the topic. A number of positive responses were received from governments and technical experts referencing published literature, documents and websites. the Enzyme Trade Association represented the international consortium IECG including ABIAM, AMFEP,

CERF, ETA and JEA. Their support was augmented with two face-to face meetings and phone calls. In addition, a literature search and Google searches were performed.

Source materials not directly referenced elsewhere include:

i. Documents and processes referred to by Australia, Canada, China, Japan, USA
ii. IECG – representing ABIAM, AMFEP, CERF, ETA and JEA
iii. ETA - references from ETA website
iv. Literature search - (Enzymes AND food safety AND regulations) OR (Enzymes AND food safety AND evaluation), 1997-2018 – conducted Jan 2018;
v. JECFA specifications – see enzyme evaluations in recent WHO/FAO series
vi. EHC 240 (2009) Chapter 9.1.4.2
vii. GRAS Notifications
viii. EFSA reviews

4. Introduction

Enzymes are classified in the International Union of Biochemistry and Molecular Biology (IUBMB) system into six major classes (IUBMB, 1992) (EC 1-6): oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. Each class is subdivided into subclasses that are further subdivided. Each enzyme has an IUBMB record consisting of the Enzyme Commission (EC) number and nomenclature and a Chemical Abstract Service (CAS) number. In general, an enzyme name includes both the substrate(s) and the reaction type, though in many situations the enzyme is referred to by a shortened or trivial name, whereas the full name may be found in JECFA, EFSA and other listings of approved enzymes for food use.

Enzymes are found in all living organisms and provide a variety of catalytic functions necessary for cell viability. From a structural perspective, enzymes are composed of single or multiple proteins made up of amino acids that are common to all proteins found in food. Enzymes with the same catalytic activity have similar active site configurations on a structural backbone of amino acids. When ingested, enzymes from all sources, like most other proteins, are readily digested, absorbed and further metabolized. While it has been noted that many food allergens are proteins, it is clear that not all food proteins are allergens. Reviewing the amino acid sequence of an enzyme against known allergenic sequences has become a key requirement in determining the safety of enzymes.

The history of enzyme use in food applications is long and well known. Enzymes have been sourced from animal tissues, plants and microorganisms. In some cases, the microorganism is added to food during its preparation, e.g. bread-, cheese-, wine-, beer-making, allowing the enzymes present to work on the food as part of processing or maturation. More recently microorganisms have become a major source of

enzymes used as stand-alone technical treatments in the manufacture of food ingredients and products.

A large number of enzymes used by the food industry are sourced from both native and genetically engineered microorganisms (also termed genetically modified microorganisms [GMM]). Enzyme are produced by controlled fermentation, followed by removal of the production strain, purification and concentration and formulated using food-grade raw materials.

4.1 Enzymes from microorganisms

Enzymes with specific catalytic activities have been identified in a large number of microorganisms, but industrial scale enzyme production is limited to a small subset of well-characterized microorganisms. The selection of such microorganisms relies on the knowledge of their safety and ability to reliably produce enzymes under large-scale, controlled, fermentation conditions. As knowledge of the safety of, and experience with microorganisms has developed, a relatively small number of host strains have been subjected to further genetic modification. Genetic modifications may include:

- improvements to strain stability and performance in large scale
- incorporation of genetic material coding for enzymes from the same or from other well-characterized donor microorganisms
- deletion of genes capable of producing toxins or other undesirable metabolites
- improvement of the functionality of the desired enzyme (e.g. enhanced stability and performance in different environments such as pH, temperature)

In the development and characterization of native and recombinant strains, the inserted DNA and in some cases the complete genome of the organism has been sequenced. The large body of knowledge about specific host organisms and the safety of the inserted DNA contribute to the overall safety of the enzymes produced.

From a production perspective, the enzyme should be produced in large quantities by a well-characterized microorganism under fermentation conditions that do not stress the organism or lead to the production of toxic materials. In a system similar to Good Manufacturing Practice, enzymes from microorganism are produced according to Good Large Scale Fermentation Practices in line with guidelines published by OECD (OECD, 1992).

Since a limited number of well characterized microorganisms has been exploited by the food enzyme industry, their history of use and growth characteristics in culture have been well documented. The regular use has led to the development of the Safe Strain Lineage concept (SSL) by the food enzyme industry. Originally proposed by

Pariza & Foster (1983), SSL has been revisited and strengthened on a number of occasions since then (see figure 1). Supported by IECG and their international industry members, the decision tree approach allows food enzyme manufacturers to assess likely safety concerns and make appropriate decisions on the use of the host strain and its gene-modified counterparts. The decision tree approach addresses concerns regarding the production strain, the host strain, the inserted DNA as well as the safety of the enzyme and possible antibiotic resistance, presence of antibiotics and toxic metabolites.

EFSA has also developed Qualified Presumption of Safety (QPS) for microorganisms used in production of food enzymes and the US FDA has released enzyme guidance and a final rule on GRAS evaluations (REFS).

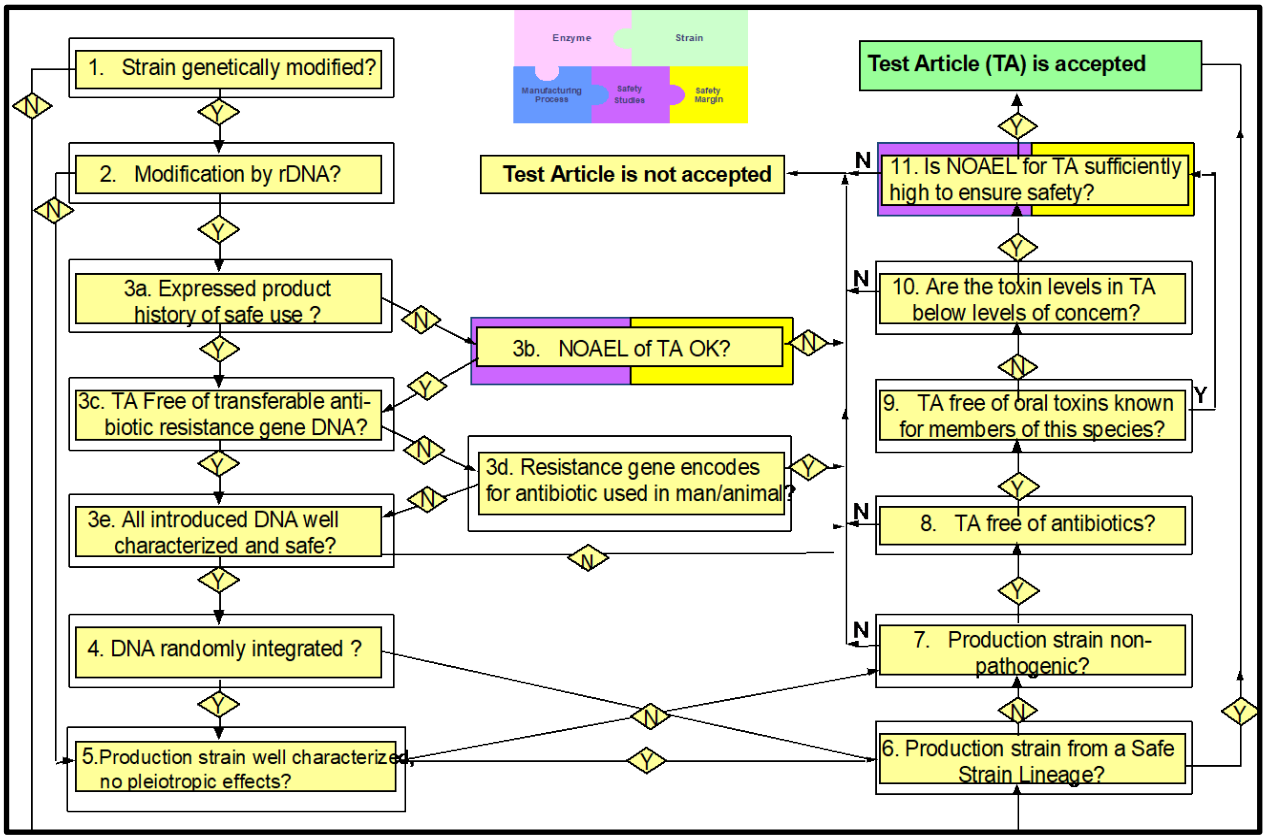

Fig.1. Decision tree proposed by Pariza & Johnson (2001) as provided; courtesy of ETA, July 2018.

4.2 Current enzyme safety evaluation

Enzyme preparations for food use have been grouped for safety evaluation according to the following criteria:

a) enzyme preparations added directly to food but not removed; e.g. enzymes used in bakery applications;

b) enzyme preparations added to food but removed from the final product according to Good Manufacturing Practice (GMP); e.g. enzyme preparations used the preparation of food ingredients from complex starting materials such as carbohydrases; or
c) immobilized enzyme preparations that are in contact with food only during processing; e.g. immobilized lipase and phospholipases.

These three categories reflect varying degrees of likelihood of the enzyme or other components of the enzyme preparation being present in the finished food. In terms of likelihood, case b) reflects only traces of enzyme and organism/medium components that might be left behind, and case c) covers the possibility of cross-linking reagents or support components (monomers) being leached during processing. The primary safety concern in each case is not the enzyme itself but the presence of potential toxins in the preparations. For immobilized enzymes it was potential genotoxins leaching from some cross-linking agents (e.g. ethylenimine from polyethylenimine) used to immobilize the enzyme. For the other two it was mainly to confirm a safe level of secondary metabolites formed through microbial fermentation

In general, the safety evaluation of enzymes is covered under the headings shown in Table 1 taken from the JECFA (JECFA, 2006) requirements.

Table 1. Considerations for enzyme preparations	
i.	Classification and Nomenclature
ii.	Enzyme preparations
iii.	Active components
iv.	Source materials (source and production strain details)
v.	Formulation (total organic solids, TOS)
vi.	Other considerations
	a. Microorganism safety
	b. rDNA modifications and products
vii.	Allergenicity
viii.	Exposure
ix.	Toxicology
x.	Additional information for consideration

4.3 Enzyme preparations and Total Organic Solids (TOS)

The product of fermentation that is of interest for the safety assessment is the article of commerce which may contain other excipients, stabilizers, etc. in addition to the enzyme in question. If the secondary components have been previously assessed and are of food grade quality, then focus is on the enzyme itself and any carryover from the culture medium and the microorganism. This material is termed Total Organic Solids (TOS) and is the sum of the enzyme protein together with other carryover

materials. The same material is routinely used in in vitro and in vivo toxicological testing, however, its relationship to the final article(s) of commerce is required for the safety evaluation.

4.4 Proteins and allergenic potential

Proteins are an important part of the daily diet and are present in common foods. In foods consumed raw, enzymes are ingested in an active conformation whereas most are inactivated by food processing or cooking. Proteins are usually digested in the gastrointestinal tract to form peptides and amino acids. Therefore, enzymes added to food are unlikely to be absorbed in their native forms in significant amounts. However, most food allergens survive degradation in order to initiate or elicit an immunological response.

In 2001, the FAO and WHO convened a workshop to discuss the 'Current Approach to Determine the Allergenicity of Genetically Modified Foods (Decision Tree Approach)'. The workshop report states that "food allergies are adverse reactions to an otherwise harmless food or food component that involves an abnormal response of the body's immune system to specific protein(s) in foods. True food allergies may involve several types of immunological responses. However, the most common types of food allergies are mediated by allergen-specific immunoglobulin E (IgE) antibodies.

Almost all food allergens are proteins, although the possibility exists that other food components may act as haptens. While the crops from which staple foods are derived contain tens of thousands of different proteins, relatively few are allergenic. However, altered dietary preferences can have significant implications for the development of food allergies. For example, allergy to peanut (groundnut) occurs at a significant frequency in North America and Western Europe but not in other countries where peanuts are less commonly eaten. Also, recent food introductions such as kiwi fruit have proven to be additional sources of food allergens. These observations provide confidence that there are not a large number of potential allergens in the food supply, but show that new allergenic foods are sometimes introduced into the marketplace. Because of the above, a clear need exists to pay particular attention to allergenicity when assessing the safety of foods produced through genetic modification.

The Codex Alimentarius Commission has adopted a list of the most common allergenic foods associated with IgE-mediated reactions on a world-wide basis that includes peanuts, soybeans, milk, eggs, fish, crustacea, wheat, and tree nuts. These commonly allergenic foods account for over 90% of all moderate to severe allergic reactions to foods, although an extensive literature search has revealed more that 160 foods associated with sporadic allergic reactions' (FAO/WHO 2001).

Since some enzymes used in food applications are extracted from natural sources or produced by native microorganisms by fermentation, their historic and regular use has been considered sufficient to exempt them from consideration as allergens. There

have been no reports of allergenic reactions from finished foods using GMM enzymes in their production. Only adverse reactions following direct inhalation or skin or eye contact with high levels of work-place exposure to food enzymes have been reported (Ladics & Sewalt, 2018).

The amino acid sequence of an enzyme can be probed in silico, using algorithms such as FAST-All (FASTA) and Basic Local Alignment Search Tool (BLAST), against various databases (e.g. AllergenOnline, and National Center for Biotechnology Information (NCBI) that contain sequences of known allergens (Ladics et al., 2011). Both bioinformatic methods (FASTA and BLAST) rely on assessing the probability that an alignment between a query sequence (the unknown protein) and a sequence in the database occurs by chance. Full length alignment, 80 amino acid alignments and 8 amino acid exact matches may be performed. However, Allergen Online states:

> "In our experience, isolated identity matches of 8 contiguous amino acids occur by chance alone at some modest rate, matches of 7 and 6 occur more commonly. Experience (published and unpublished) demonstrates that two proteins sharing only a single short identity match of from 6 to 8 contiguous amino acids do not share IgE binding in the absence of more extensive identity alignments (at least >35% identity over 80 or more amino acids). And that sequences sharing less than 50% identity over their full-lengths are rarely cross-reactive. Thus we recommend not using these short identity matches as there is no scientific evidence that they predict IgE cross-reactivity and they do not predict shared clinical activities."

A negative sequence homology result indicates that a newly expressed protein is not a known allergen and is unlikely to be cross-reactive to known allergens. A result indicating absence of significant sequence homology should be considered along with other data (e.g. resistance to protease digestion) in assessing the allergenic potential of microbial expressed enzymes. In deciding about allergenic potential, there is always the possibility of the creation of a *de novo* allergen through enzyme protein digestion.

4.5 Use levels and dietary exposure

Use levels of enzymes in food production are very low (g enzyme/kg ingredients). Enzymes are added to carry out a specific function in the production of a variety of food products. In some cases, they are added at early stages of production to breakdown starch or other macromolecular precursors of a food ingredient such as corn syrup or distilled alcohol. In these cases, the enzyme is removed from the final product during different manufacturing steps. In other cases, such as bakery applications, the enzyme is expected to be heat inactivated with the possibility of still being intact and a potential allergen in the final product. Calculating the level of enzyme use in a food is dependent on the process used, the recipe/formulation and the number of different ingredients present in the enzyme preparation. In the calculation of dietary exposure, it is also assumed that all of the enzyme used will appear in the final food, and all of a specific food type contains the enzyme at the

maximum use level. This provides the basis for an estimate of dietary exposure to be very conservative.

Though a number of different approaches have been considered over the years, the Budget Method has been most often used and accepted. Recently, as an attempt to add more reliability to the exposure calculation, EFSA has launched a new database to collect data from Member States (EFSA, 2018):

> "The Food Enzyme Intake Model (FEIM) is a tool for estimating chronic dietary exposure to food enzymes used in food processes. FEIM follows the methodology recommended in the CEF Panel's Statement on Exposure assessment of food enzymes. It has been developed on the basis of summary statistics of food consumption data collected from Member States (stored in the EFSA Comprehensive European Food Consumption Database).
>
> FEIM comprises process-specific calculators, such as FEIM-baking or FEIM-brewing, which allow estimation of dietary exposure to food enzymes used in individual food manufacturing processes. Exposure results are reported at mean and high level for different population groups (e.g. infants, toddlers, adults, etc.) in different countries."

These individual food application databases are in early stages of development and may only be of use once completed by the relevant organizations in the EU member states. Other national bodies may use the Budget Method, consumption data and use levels when determining an estimate of dietary intake. The US FDA uses a similar approach to EFSA in calculating the dietary exposure to enzyme preparations added to food by considering publicly available food consumption databases. US FDA also uses market disappearance and annual poundage data to approximate per capita exposure estimates.

4.6 Current toxicological considerations

A small number of tests have been routinely applied to enzymes of microbiological origins. OECD recommends results from two in vitro assays (bacterial reverse mutation assay, in vitro chromosomal aberration assay, micronucleus test or mouse lymphoma tk assay) and a 90-day sub-chronic oral toxicity study are performed (OECD 408, 471 and 487). It has been proposed that some or all of this testing may be avoided if the enzyme and its production strain are well-characterized and sufficient test results are available for the host strain or for a closely related production strain. Since it is likely that the enzyme itself is not the cause of any irregular test results, these tests primarily provide information about the balance of the organic material in the TOS. Since many of the newly developed microorganism have been modified in such a way as to delete genes responsible for the production of toxins and are cultivated under conditions that do not cause stress and the production of toxins, it has been proposed that this type of testing may not be required and could be replaced

by direct analysis for toxins and a weight-of-evidence approach (Ladics & Sewalt, 2018) considering data generated in related production strains.

Data from numerous enzymes preparations have been submitted for review by EFSA, JECFA and US FDA GRAS, wherein safety has been assured by data from in vitro and in vivo studies that in most cases show negative findings at the highest doses tested. Hence a Margin of Exposure (calculated as: mg TOS/kg bw per day (calculated dietary exposure) vs NOAEL or NOEL obtained from a 90-day oral toxicity study) can be large.

5. Review of the safety evaluation of food enzymes

In 2006, JECFA published its most recent guidance on the evaluation of enzymes for use in food. A literature search indicated very few advances in the consideration of food enzyme safety since that time. The main focus has been on the assurance of safety of microorganisms used in enzyme production. However, the publications of Pariza, et al. EFSA, US EPA and ETA have contributed newer considerations of the approach to dealing with enzyme safety.

The present JECFA Priority list for safety evaluation contains 29 enzyme preparations. To reduce this backlog, it is thought necessary to evaluate the current process and determine if a more streamlined process can be implemented. Considering the current state of the art of enzyme production by microorganisms, there are a number of ways in which the process could be improved.

Certain factors indicate possible ways to consider enzyme safety that may lead to greater progress in enzyme safety evaluations (Table 2). A number of these factors are described below:

Table 2. Possible factors affecting Enzyme Assessment

Drivers for simplification	**Constraints**
Safe strain lineage concept	DNA stability/instability, toxigenicity and pathogenicity
Enzymes with the same specificity	Strain performance/productivity Similarities in primary sequence
Enzymes from the same source organism	Scale up Enzyme activity and TOS
GMP production	Toxin production Specifications Enzyme purity in TOS
Absence of production strain in final product	Secondary metabolites and residual medium components

Read-across for safety evaluation	Requirement for whole genome sequencing and modification identification Allergenicity Prediction of toxicity Digestibility Heat stability
Dietary exposure level	A consistent method of assay using generally-available substrates Use and use level dependent

5.1 Similar catalytic activities/specificity

A number of enzymes have been developed by various industry players to meet the needs of food ingredient processors and formulators. Such innovations include the development of enzymes with the same catalytic specificity but with different tolerances to conditions such as heat or pH and yield.

For Discussion: Is it possible to assume that all enzymes with the same catalytic specificity would be equally safe from a food safety evaluation perspective? If so, what additional data would be required to satisfy the food safety assessor?

5.2 Safe Strain Lineage

The development of lists of microorganisms that have a history of safe use as production strains is a step in the right direction in the assurance of safety of use. A number of cases have been presented to both EFSA and the US FDA (GRAS submission) that use a "Read-Across" approach in providing safety data for stable host strains and also for other individual production strains modified to produce different enzymes to support the safety of a newly derived production strain and its enzyme product.

For Discussion: If a history of testing can be established and assurance that the strain in question does not produce toxins as a result of the most recent modification, can this information provide sufficient assurance that the enzyme preparation is safe under normally accepted conditions of use?

5.3 Protein sequence data

Knowledge of the amino acid sequence of the enzyme is required for the *in silico* determination of potential allergenicity.

For Discussion: Taken together with a determination of process stability (heat, pressure, etc.), digestibility and catalytic activity, can sufficient data be accumulated to assure the safety of the enzyme when used in appropriate levels?

5.4 DNA sequence data

As part of the knowledgebase on a particular production strain, a full DNA sequence of the organism is preferred. However, a DNA sequence of the inserted gene(s) is required and can be used to confirm the primary structure of the enzyme protein. Where synthetic DNA has been used to create the gene, the chances of unintended consequences is reduce compared with the use of DNA extracted from another microorganism.

For Discussion: Is there a difference in risk if the inserted gene(s) are synthesized *de novo* or extracted from other microorganisms? Gene insertion is only of relevance if it is possible for the inserted gene to affect the production of toxic metabolites.

6. Possible requirements for the future review the safety of food enzymes

In evaluating the list of options for the simplification of the enzyme review process it is apparent that there are a number of items that parallel the safety assessment of flavors (see Table 3). These include:

Table 3.

Flavors	**Enzymes**
Structural similarities	Similar catalytic activities
Extremely low use levels	Low use levels defined by function
Read-across approach for toxicology	Safe Strain Lineage and industry knowledge
Multiple isomers	Secondary activities
Residual synthetic byproducts and/or contaminants	TOS Toxic metabolites

It was also noted that there are similarities between food enzyme evaluation and the definition of Modified Starches where products are manufactured to meet a functional requirement; structures are related and of little toxicological concern; data gaps may be filled by “Read-Across” from related materials.

Therefore, it is proposed that many of these similarities are applied to the safety assessment of food enzymes in order to demonstrate their safety. Information required for assessment would be modified, but the approach would be “fill in the boxes” and consider toxicological evidence.

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Table 4. Details of the information required for enzyme safety assessment	
information required	**Detail**
i. Classification and Nomenclature	According to IUBMB including full name and synonyms
ii. Enzyme preparation(s) (total organic solids, TOS)	Description of enzyme material and any carryover from the fermentation as used in toxicological testing
iii. Active components	Enzyme activity or activities present, definition of catalytic activity
iv. Source materials (source and production strain details)	Definition of the production strain organism and the origins of the expressed enzyme
v. Formulation	Other components added to the enzyme preparation for commercial use, such as excipients and stabilizers
vi. Other considerations	
a. Microorganism safety	History of derivation of the microorganism and development of host strain(s), including names and any name changes during the development of the host strain and production strain
b. rDNA modifications and products	Details of DNA modifications made and techniques used, sources of DNA added and identification of DNA removed
vii. Allergenicity	Results of comparison of primary amino acid structure with allergen databases
viii. Exposure	Levels of use in food ingredient and final food production
ix. Toxicology	Results of recommended toxicological testing for the enzyme including results from other members of the same family production strains and the host strain
x. Additional information for consideration	
xi. Test method and units	A universally usable test method to define enzyme activity present in the preparation

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Appendix 1: Information required for safety assessment when foods are or include organisms obtained through recombinant DNA techniques.

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WHO/FAO 2001 Decision Tree Approach to the Evaluation of the Allergenicity of Genetically Modified Foods (http://www.fao.org/docrep/007/y0820e/y0820e05.htm)

Exhibit 1.

Outline of ILSI China Workshop , June 2018:
"In China, the criteria of safety assessment and approval pathway for food additives including food enzymes derived from GMMs have been discussed for years and still no clear clarification. All impacted food additives including enzymes are pending for approval since 2009.

It was clear that Ministry of Health and the Ministry of Agriculture (MOA) had discussed the matter, and industry started to make GMM dossier submissions in 2013 under the existing Agri-GMO guidelines, mainly for evaluating GMO crops. The MOA apparently received numerous GMM dossiers, including some for food enzymes, and reacted in 2014 by rejecting its responsibility for GMM oversight for food enzymes, and announcing it would no longer receive GMM dossiers for food enzymes.

As of June 2017, the NHFPC (National Health and Family Planning Commission; successor of MOH) and the MOA reached an agreement under which the MOA will serve a technical reviewer of a GMM dossier as part of the overall review and approval process by the NHFPC. Therefore, MOA tasked a technical expert committee to develop GMM evaluation guidance appropriate for food ingredients. Since August of 2017, industry has engaged with the committee in several workshops, resulting in a draft guidance that distinguishes 3 categories of food GMMs based on living GMMs level, which is similar to EFSA Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use. MOA is conducting a technical review on the draft guidance.

Industry partners are focused on getting the MOA to move on approving the draft guidance covering 3 categories below:

1) Purified products made with GMMs, but living GMMs and inserted genes removed;
2) Composite products made with GMMs, but living GMMs removed;
3) Products containing living GMMs.

Based on the latest alignment between MOA and NHFPC, the door of the registration for the food enzymes produced from GMMs are expected to be re-opened soon. However, the door for many other food ingredients such as vitamins, amino acids, steviol glycosides and oligosaccharides (GOS and HMO) derived from GMMs, which are under Category 1 is still closed. Actually, the inserted GM genes and microorganisms are totally removed from the final products. Those food ingredients are chemically defined purified compounds and not evaluated as GM-crop process in general in the EU and US. GMM-derived food enzymes produced under containment do not contain the GMM used to produce them, hence they are not genetically modified organisms (GMOs), unlike Agri-GMO intended for deliberate release.

We hope that the workshop will be able for Chinese regulators and experts to clarify the different safety risks between GMMs derived products and GM crops, so the future safety assessment and approval pathway can to be straightforward and practical."

Exhibit 2.
IECG Charter

Charter – International Enzyme Coordination Group

Background

Companies that manufacture, formulate and/or sell enzymes and enzyme products are organized in professional enzyme associations and advocacy groups based in various regions of the world.

These enzyme associations/advocacy groups have identified the need for a structured and continuous exchange of information and coordination of initiatives amongst themselves, in order to achieve stronger, common positions and better harmonization in safety, regulatory and technical matters.

As a result, these various global associations/advocacy groups have established the "International Enzyme Coordination Group", further referred to as the "IECG".

Mandate

The IECG itself does not act other than through the individual enzyme associations.

The aim of the IECG is to identify and seek consensus on relevant regulatory, legislative, safety and technical issues that are global or are expected to have an effect across regions, with the purpose to:

- Establish global standards for the safety and quality of enzyme products.
- Support each regional enzyme association with unified positions in order to maximize the interaction with the regional governmental authorities and to maintain a globally balanced governance.
- Promote coherence between activities and positions towards international standard setting bodies, such as Codex and JECFA.
- Stimulate global regulatory harmonization.
- Seek participation of other geographically distinct associations.

Composition of the IECG

The current enzyme associations and advocacy groups represented in the IECG are:

- Amfep – The Association of Manufacturers and Formulators of Enzyme Products – www.amfep.org
- E. A. – The Enzyme Technical Association – www.enzymeassociation.org
- JEA – Japanese Enzyme Association – http://j-enzyme.com/index_Eng.html
- ABIAM – Associação Brasileira da Industria e Comercio de Ingredientes e Aditivos para Alimentos – www.abiam.com.br
- CERF – China Enzyme Regulatory Forum.

Other enzyme associations or advocacy groups that may develop in geographically distinct regions will be considered for IECG membership (e.g. advocacy groups in India, Latin American countries, Australia / New Zealand), provided that their charters are in compliance with competition laws.

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Annex 2. **Definitions for Safe Food Enzyme Production Strain and Presumed Safe Progeny Strain (Class I Type iii)**

A Safe Food Enzyme Production Strain is a non-pathogenic, non-toxigenic microbial strain with a demonstrated history of safe use in the manufacture 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.

A Presumed Safe Progeny Strain is developed from a safe food enzyme production strain through specific non-random modifications to its genome; the modifications must be thoroughly characterized, must not encode any harmful substances and not result in adverse effects. This concept also applies to multiple generations of progeny. Evidence supporting its safety includes knowledge of taxonomy, genetic background, and toxicological testing.

Annex 3. **Information required for the safety assessment of enzyme preparation for use in foods**

Class I: Enzymes derived from sources which 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 derived from micro-organisms that are traditionally accepted as constituents of foods or are normally used in the preparation of foods I(ii).

Type iii: Enzymes derived from a Safe Food Enzyme Production Strain or a Presumed Safe Progeny Strain I(iii).

Class II: Enzymes derived from sources which are NOT considered safe for consumption and are not in any of the sub-categories listed above

No.	Class/es	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 catalyzed 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 Total Organic Solids (TOS) in mg/kg food
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/source of genetic material	e.g., identify source of genetic material by genus, species (native, modified or synthetic)
13.	I (iii), II	Details of genetic modification: i. To host genome ii. Addition of rDNA (gene of interest from another microorganism) to host microorganism through mobile genetic elements	 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 Donor/ source 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 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, recombinant DNA technology, etc.
15.	I (iii), II	Description of intended and non-specific 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 non-specific 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
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 derived from plants and animals, manufacturing details are required. For enzymes in Class I(iii) and Class II produced by micro-organisms, 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 source organism and prevent the introduction of microorganisms that

			could be the source of toxic or other undesirable substances.
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 derived from the source material as opposed to that contributed by diluents and other additives and ingredients, individual specifications require a statement of percentage Total Organic Solids (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. TOS content is usually expressed in milligrams or micrograms TOS per kilogram body weight per day.
Specifications & 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 5 batches) are required.

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 5 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
25.	I (iii), II	Evidence for absence of recombinant DNA and production organisms in the enzyme concentrate	
Assessment of Potential Allergenicity of the Enzyme			
26.	I (iii), II	Comparison of the amino acid sequence of the enzyme to 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 (SGF) studies, etc.
Toxicology			
28.	II	Results of toxicological testing of the enzyme concentrate.	It is necessary to conduct toxicological studies in order to establish an ADI: (a) 90-day oral toxicity test in a rodent species; (b) Two short-term genotoxicity tests (mutagenicity and clastogenicity) 1. A test for gene-mutations in bacteria, 2. A test for chromosomal aberrations (preferably in vitro).
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 total organic solids (TOS). Separate dietary exposure situations may need to be considered with respect to the	Express the dietary exposure as mg TOS/kg bw per day; provide an explanation of the methodology used to derive the estimated dietary exposure

		enzymes described in Classes I (iii) and II, depending on whether they are: a) enzyme preparations added directly to food and not removed; b) enzyme preparations added to food but removed from the final product according to Good Manufacturing Practice (GMP); or c) immobilized enzyme preparations that are in contact with food only during processing.	
31		Additional information and comments	Additional items considered helpful in the safety assessment

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Annex 4. **Terms and definitions**

Terms	Definitions
Source/Donor Organism	The animal, plant or microorganism that provides the genetic material used to modify the Host/Recipient organism that will express the enzyme or enzymes of interest. It is typically described by genus, species and strain.
Host/Recipient Organism	The animal, plant or microorganism that receives the genetic material from the Source/Donor organism. It is typically described by genus, species and strain.
Production Organism/Strain	The animal, plant or microorganism that is used to express the enzyme or enzymes of interest. It is typically described by genus, species and strain.
Enzyme	The identity of the specific biologically active protein used to catalyze the reaction of interest. It is typically characterized by a specific amino acid sequence and described using EC/IUBMB nomenclature.
Enzyme concentrate	The product after manufacturing (typically from fermentation) and before formulation of the enzyme preparation; it contains the enzyme of interest along with other components from the manufacturing process and its composition is expressed in Total Organic Solids (TOS). This is the material typically used for toxicological studies.
Formulation ingredients	Food-grade materials, e. g. stabilizers, pH adjustment agents, carriers, diluents, preservatives, etc., that are added to the enzyme concentrate to make the enzyme preparation.
Enzyme preparation	Consists of the enzyme concentrate and formulation ingredients; it represents the article of commerce used in food production.

Annex 5. **List of experts**

Members of the JECFA Expert Working Group established to consider the evaluation of enzyme preparations used in the manufacture of foods

- Maria Beatriz de Abreu GLORIA, Brazil
- Daniel FOLMER, USA
- G. J. Benoit GNONLONFIN, Nigeria
- Suzanne JEURISSEN, The Netherlands
- Kristie LAURVICK, USA
- Madduri V. RAO, India
- Joel ROTSTEIN, Canada
- Mohammad SHOJAEE, IRAN
- Jannavi SRINIVASAN, USA
- Atsuko TADA, Japan
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