Food and Agriculture Organization of the United Nations

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LIPASE (JECFA 95-7) FROM *THERMOMYCES LANUGINOSIS* AND *FUSARIUM OXYSPORUM* EXPRESSED IN *ASPERGILLUS ORYZAE* (JECFA95-7)

Chemical and Technical Assessment (CTA)

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1. Summary

This Chemical and Technical Assessment summarizes data and information that was submitted to JECFA for the safety assessment of lipase enzyme preparation from *Thermomyces lanuginosis* and *Fusarium oxysporum expressed Aspergillus oryzae* (lipase enzyme preparation).[1] This document also discusses published information relevant to the safety of the *A. oryzae* production organism, the production strain, details related to the manufacturing, specifications, use, and use levels of lipase enzyme preparation in food. This document uses the expression "lipase" to refer to the modified enzyme and its amino acid sequence, and the expression "lipase enzyme preparation" to refer to the product formulated for commercial use.

Lipase catalyses the hydrolysis of ester linkages of triacylglycerides and phospholipids. The lipase enzyme preparation is intended to be used as a processing aid to hydrolyse lipids in (to-be) baked goods to improve dough strength, stability, and characteristics during the baking process and to ensure a uniform and increased volume, and improved crumb structure.

The production organism, *A. oryzae*, can be found in soil and on decaying plant material (Barbesgaard et al., 1991). It has a history of safe use in industrial applications, including as sources of enzymes used in food processing (Barbesgaard et. al, 1992; Cook et al., 1994, and Beckhorn et al. 1965). *A. oryzae* has been used to produce koji, miso, and sake for more than 2000 years (Barbesgaard et al., 1992).

A. oryzae is designated as a Group 1 microorganism according to European Union Directive 2000/54/EC of the European Parliament (EC, 2000). A Group 1 microorganism is unlikely to cause human disease. *A. oryzae* is not included on the list of pathogens in Annex III of Directive 2000/54/EC on the protection of workers from risks related to exposure to biological agents at work or on the list of pathogens in Belgium (EC, 2000; Belgian Biosafety Server, 2008). *A. oryzae* production strains have been traditionally regarded as non-pathogenic and non-toxigenic based on its historical uses in the production of fermented foods (Olempska-Beer et al. 2006). The production strain, *A. oryzae* was obtained by transforming the recipient organism, *A. oryzae* JaL830, with an expression plasmid consisting of a fusion lipase gene obtained from *T. lanuginosis* CBS596.94 and *F. oxysporum* DSM2672.

The lipase enzyme preparation is manufactured by submerged controlled fermentation of the *A. oryzae* produciton strain in accordance with Good Manufacturing Practices (GMP). The lipase enzyme is released into the fermentation medium and subsequently recovered and concentrated using multiple

[1] The sponsor considers the identity of the *F. oxysporum* production strain to be confidential; however, the enzyme preparation is the subject of GRAS Notice 103, which is publicly available at: GRAS Notices (fda.gov).

filtration techniques; the enzyme is formulated into a powder preparation. The lipase enzyme preparation complies with the General Specifications and Considerations for Enzyme Preparations Used in Food Processing (FAO/WHO, 2006).

The sponsor examined the potential for the lipase enzyme to be a food allergen by comparing its amino acid sequence to sequences of known allergens contained within the AllergenOnline and Allergen.org databases using internationally accepted search criteria. No meaningful identity with known allergens was observed. Based on the results obtained, oral intake of lipase is not anticipated to pose any allergenicity concern.

2. Description

Off-white powder.

3. Method of manufacture

3.1 A. oryzae

A. oryzae belongs to the genus *Aspergillus*, under the family Trichocomaceae. Aspergilli are filamentous fungi that are found in cereals, cereal grains, and spoiled foods (Cook et al., 1994). Aspergilli used in fermented foods are considered to have been domesticated by cultivation for thousands of years and may differ from their wild type predecessors morphologically and physiologically (Barbesgaard et al., 1992 and Cook et al., 1994).

The taxonomic classification of this microorganism is as follows:

Kingdom:	Fungi
Phylum:	Ascomycota
Class:	Eurotiomycetes
Order:	Eurotiales
Family:	Trichocomaceae
Genus:	*Aspergillus*
Section:	Flavus (= *Aspergillus flavus* group)
Species:	*oryzae*

A. oryzae has long been a recognized source organism for production of enzymes intended for use in food processing. *A. oryzae* is designated as a Group 1 microorganism according to European Union Directive 2000/54/EC of the European Parliament (EC, 2000). A Group 1 microorganism is unlikely to cause human disease. *A. oryzae* is not included on the list of pathogens in Annex III of Directive 2000/54/EC on the protection of workers from risks related to exposure to biological agents at work or on the list of pathogens in Belgium (Belgian Biosafety Server, 2008). *A. oryzae* is confirmed to be non-pathogenic by its long history of safety use. *A. oryzae* strains have been used to produce koji, miso, and sake for more than 2000 years (Barbesgaard et al., 1992). The parental strain, *A. oryzae* A1560, was obtained from Institute for Fermentation Osaka; its identity was confirmed by Centraalbureau voor Schimmelcultures (Netherlands) and by DNA sequencing and comparison with published DNA sequences. The parental strain was subjected to genetic recombination techniques and chemical mutagenesis to eliminate mycotoxins and reduce kojic acid production. The resulting recipient strain *A. oryzae* JaL830, was transformed with a plasmid containing a fusion of lipase genes from *T. lanuginosis* CBS596.94 and *F. oxysporum* DSM2672. The recipient strain was developed from a lineage that has been used to produce food substances since 1995; it has an extensive history of commercial safe use for production of recombinant enzymes used in food.[2] The host strain is regarded as a Good Industrial

[2] *A. oryzae* strains are known to be capable of producing the secondary metabolites; however, the strain lineage used to develop the production strain was modified to delete genes required to produce cyclopiazonic acid aflatoxin and to drastically reduce kojic acid production.

Large Scale Practice (GILSP) organism belonging to class 1. The identity of the parental strain was confirmed by Centraalbureau voor Schimmelcultures (Netherlands) and by DNA sequencing and comparison with published DNA sequences.

A. oryzae has long been known as a safe source for food enzyme preparations. Lipase enzymes from genetically modified *A. oryzae* strains have been evaluated by regulatory authorities around the world. Enzymes from *A. oryzae* have been positively evaluated by JECFA (1987). FDA did not question manufacturers' Generally Recognized as Safe (GRAS) conclusions for the intended uses of the following enzyme preparations produced by *A. oryzae* strains: pectin esterase (1999 and 2021); phospholipase A1 (2019); asaparaginase (2006); phospholipase (2004); laccase (2003); lipase (2000, 2001, 2002 and 2003); glucose oxidase (2002); carbohydrase (2002), and aspartic proteinase (2000) (U.S. FDA).[3]

3.2 A. oryzae production strain

The production strain, *A. oryzae* NZYM-LH, was obtained by a combination of chemical mutagenesis and genetic modifications of the parental strain. The parental strain was obtained from the Institute for Fermentation, Osaka, Japan. The host strain, *A. oryzae* JaL830, was obtained by inactivation of genes encoding for a major secreted protein, deletion of genes encoding for three proteases, and disruption of genes responsible for production of kojic acid and mycotoxins. The production strain, *A. oryzae* NZYM-LH, was developed by transforming the host strain, *A. oryzae* JaL830, with a plasmid containing a lipase gene created from portions of the lipase genes from *T. lanuginosis* CBS596.94 and *F. oxysporum* DSM2672, an optimized promoter from *Aspergillus niger* strain BO-1, a terminator from *A. niger* BO-1, and a selectable marker. The stability of the introduced sequences was confirmed by morphological and genomic comparison via Southern blot between the production culture and sporulated samples grown in liquid culture and cultivated at the end of three independent fermentation runs. The host strain, *A. oryzae* JaL830, does not contain genetic elements which can confer antibiotic resistance, nor were any genetic sequences conferring antibiotic resistance introduced during the development of the production strain, *A. oryzae* NZYM-LH.

3.3 Fermentation, recovery, and formulation

The lipase enzyme is produced by controlled submerged fermentation of a pure culture of *A. oryzae* NYZM-LH. The manufacture of the lipase enzyme preparation consists of three steps: fermentation (pre, seed and main fermentation), recovery, and formulation. Control measures are in place for physical and chemical quality control during fermentation. Samples are tested for identity, viability, and microbial purity. All raw materials used in the manufacture of lipase enzyme preparation are food-grade.

Following fermentation, the culture broth containing the enzyme is separated from the biomass that consists of the production organism and spent fermentation medium; this is followed by several filtration steps including germ filtration to ensure removal of the production strain and insoluble substances. The liquid filtrate containing the enzyme is stabilised with sodium chloride and the pH is adjusted with acetic acid or NaOH. The liquid filtrate is then dried to a powder. The entire process is performed in accordance with current Good Manufacturing Practices using raw materials of food grade quality. The final powdered enzyme preparation was tested for absence of any major food allergens from the fermentation medium. The enzyme concentrate was also tested to be free from the production organism and any antibiotic activity. The enzyme preparation conforms to the General Specification for Enzyme Preparations used in Food Processing (FAO/WHO, 2006).

[3] Lipase enzyme preparation from *Aspergillus oryzae* carrying a gene constructed from a modified *Thermomyces lanuginosis* lipase gene and a portion of the *Fusarium oxysporum* lipase gene is the is the subject of GRAS Notice 103. In a letter dated August 19, 2002, the stated that it had no questions in response to GRAS Notice 103.

4. Identity and Characterization

4.1 Lipase

The lipase enzyme catalyses the hydrolysis of ester linkages at the fatty acid at the sn-1 position of triacylglycerides and phospholipids. It is classified by the Enzyme Commission of the International Union of Biochemistry and Molecular Biology (IUBMB) as follows:

Accepted name:	Lipase
Other name(s):	triglyceride lipase; tributyrase; butyrinase; glycerol ester hydrolase; tributyrinase
Reaction:	triacylglyceride + HOH → fatty acid + diacylglyceride Also hydrolyses the sn-1 ester bond of diacylphospholipids to form 2-acyl-1-lysophospholipd and free fatty acid
Systematic name:	triacylglycerol acylhydrolase
EC No.:	3.1.1.3
CAS No.	9001-62-1

The lipase enzyme does not contain side activities that occur in amounts relevant for the intended food uses. The primary sequence of the lipase enzyme has been determined to consist of 317 amino acids; its molecular weight by calculation from the determined amino acid sequence is 35 kDa.

Lipase activity is determined by measuring the rate at which the enzyme hydrolyses the tributyrin substrate at pH 7.0 to release butyric acid, in Lipase Units (LU). The released butyric acid is then titrated with sodium hydroxide, and the consumption of sodium hydroxide is recorded as a function of time. The mean activity of lipase enzyme from four batches is 107 LU/g.

4.2 Lipase Enzyme Preparation

The lipase enzyme preparation consists of the enzyme, lipase, and substances from the fermentation process; these constitute proteins, peptides, amino acids, carbohydrates, lipids and salt. The components of fermentation are referred to as Total Organic Solids (TOS).

The TOS content of an enzyme preparation is calculated according to the following equation (NAS/NRC, 1981; FAO/WHO, 2006):

$$TOS\ (\%) = 100 - (A + W + D)$$

Where

A is the % ash,
W is the % water and
D is the % diluents and/or other formulation ingredients.

The lipase enzyme preparation is marketed as a powder formulation.

A typical composition of the lipase enzyme preparation is provided below:

Enzyme TOS:	9.1 – 10.3 %
Ash:	1.3 – 1.6 %
Water:	88.1 – 89.4 %
Activity/mg TOS:	95 -116 LU/mg TOS

The specifications for the commercial lipase enzyme preparation include activity (9200-11300 LU/g), lead (< 5 mg/kg), arsenic (< 1 mg/kg), mercury (< 0.3 mg/kg), cadmium (≤ 0.5 mg/kg), coliforms (≤ 30 CFU/g),

Salmonella (negative in 25 g), *E. coli* (negative in 25 g), antimicrobial activity (absent by test), and mycotoxins (absent by test).

The lipase enzyme preparation also complies with the General Specifications for Enzyme Preparations used in Food Processing as established by the 67th meeting of the Joint Expert Committee on Food Additives (FAO/WHO, 2006).

5. Functional Uses

The lipase enzyme preparation is intended to be used as a processing aid to hydrolyse lipids in baked goods to improve dough strength, stability, and characteristics during the baking process and to ensure a uniform and slightly increased volume and improved crumb structure. The lipase enzyme in all the applications will be inactivated by heat treatment prior to use of the final foods. The lipase enzyme preparation is used at a maximum level of 20 mg TOS per kg of flour.

6. Fate in food

Lipase enzyme is a naturally occurring substance in microorganisms, plants and animal tissues that are commonly ingested by humans. In addition to lipase enzyme, the enzyme preparation will contain proteins, peptides, carbohydrates, and salts from the fermentation process that are common to the human diet.

Lipase enzyme preparation is intended to be used in the manufacture of baked goods that are intended to be consumed by the general population. While it is assumed that the lipase enzyme is carried over to the final food, the enzyme is inactivated and denatured during processing by treatment at high temperatures and is not expected to have any technical effect on the final food. If present, lipase enzyme will be digested, as would any other protein occurring in food. Therefore, use of lipase enzyme in the processing of food categories described will not have a significant effect on the human body.

7. References

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