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SCIENTIFIC OPINION

Safety evaluation of the food enzyme leucyl aminopeptidase from the non-genetically modified *Aspergillus* sp. strain AE-MB

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Abstract

The food enzyme leucyl aminopeptidase (EC 3.4.11.1) is produced with the non-genetically modified *Aspergillus* sp. strain AE-MB by Amano Enzyme Inc. The food enzyme is considered free from viable cells of the production organism. It is intended to be used in five food manufacturing processes: processing of dairy products for the production of (1) flavouring preparations; processing of plant- and fungal-derived products for the production of (2) protein hydrolysates; processing of meat and fish products for the production of (3) protein hydrolysates, (4) modified meat and fish products and processing of (5) yeast and yeast products. Dietary exposure to the food enzyme–total organic solids (TOS) was estimated to be up to 2.273 mg TOS/kg body weight (bw) per day in European populations. Genotoxicity tests did not indicate a safety concern. The systemic toxicity was assessed by means of a repeated dose 90-day oral toxicity study in rats. The Panel identified a no observed adverse effect level of 183 mg TOS/kg bw per day. The calculated margin of exposure for each age group was 135 (infants), 81 (toddlers), 83 (children), 109 (adolescents), 160 (adults) and 184 (the elderly). A search for the similarity of the amino acid sequence of the food enzyme to known allergens was made and no matches were found. The Panel considered that the risk of allergic reactions by dietary exposure cannot be excluded, but the likelihood is low. The safety of the food enzyme could not be established given the derived margins of exposure. Therefore, the Panel concluded that this food enzyme could not be considered safe under the intended conditions of use.

KEYWORDS

Aspergillus sp., EC 3.4.11.1, food enzyme, leucine aminopeptidase, Leucyl aminopeptidase, non-genetically modified organism

CONTENTS

1 | INTRODUCTION

Article 3 of the Regulation (EC) No 1332/2008[1] provides definition for 'food enzyme' and 'food enzyme preparation'.

'Food enzyme' means a product obtained from plants, animals or microorganisms or products thereof including a product obtained by a fermentation process using microorganisms: (i) containing one or more enzymes capable of catalysing a specific biochemical reaction; and (ii) added to food for a technological purpose at any stage of the manufacturing, processing, preparation, treatment, packaging, transport or storage of foods.

'Food enzyme preparation' means a formulation consisting of one or more food enzymes in which substances such as food additives and/or other food ingredients are incorporated to facilitate their storage, sale, standardisation, dilution or dissolution.

Before January 2009, food enzymes other than those used as food additives were not regulated or were regulated as processing aids under the legislation of the Member States. On 20 January 2009, Regulation (EC) No 1332/2008 on food enzymes came into force. This Regulation applies to enzymes that are added to food to perform a technological function in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food, including enzymes used as processing aids. Regulation (EC) No 1331/2008[2] established the European Union (EU) procedures for the safety assessment and the authorisation procedure of food additives, food enzymes and food flavourings. The use of a food enzyme shall be authorised only if it is demonstrated that:

- it does not pose a safety concern to the health of the consumer at the level of use proposed;
- there is a reasonable technological need;
- its use does not mislead the consumer.

All food enzymes currently on the European Union market and intended to remain on that market, as well as all new food enzymes, shall be subjected to a safety evaluation by the European Food Safety Authority (EFSA) and approval via an EU Community list.

The 'Guidance on submission of a dossier on food enzymes for safety evaluation' (EFSA, 2009a) lays down the administrative, technical and toxicological data required.

1.1 | Background and Terms of Reference as provided by the requestor

1.1.1 | Background as provided by the European Commission

Only food enzymes included in the Union list may be placed on the market as such and used in foods in accordance with the specifications and conditions of use provided for in Article 7 (2) of Regulation (EC) No 1332/2008[1] on food enzymes.

Three applications have been introduced by the company Amano Enzyme Inc. for the authorization of the food enzymes Triacylglycerol lipase from *Rhizopus oryzae* (strain AE-TL), Triacylglycerol lipase from *Candida cylindracea* (strain AE-LAYH), and Leucyl aminopeptidase from *Aspergillus oryz*ae (strain AE-MB).

Following the requirements of Article 12.1 of Regulation (EC) No 234/2011[3] implementing Regulation (EC) No 1331/2008, the Commission has verified that the three applications fall within the scope of the food enzyme Regulation and contain all the elements required under Chapter 11 of that Regulation.

1.1.2 | Terms of Reference

The European Commission requests the European Food Safety Authority to carry out the safety assessments on the food enzymes Triacylglycerol lipase from *Rhizopus oryzae* (strain AE-TL), Triacylglycerol lipase from *Candida cylindracea* (strain AE-LAYH), and Leucyl aminopeptidase from *Aspergillus oryzae* (strain AE-MB) in accordance with Article 17.3 of Regulation (EC) No. 1332/2008 on food enzymes.

1.2 | Interpretation of the Terms of Reference

The present scientific opinion addresses the European Commission's request to carry out the safety assessment of the food enzyme leucyl aminopeptidase from the non-genetically modified *Aspergillus oryzae* strain AE-MB.

Recent data identified the production microorganism as *Aspergillus* sp. (Section 3.1). Therefore, this name will be used in this opinion instead of *A. oryzae*.

[1]Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on Food Enzymes and Amending Council Directive 83/417/EEC, Council Regulation (EC) No 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/97. OJ L 354, 31.12.2008, pp. 7–15.

[2]Regulation (EC) No 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 354, 31.12.2008, pp. 1–6.

[3]Regulation (EC) No 234/2011 of 10 March 2011 implementing Regulation (EC) No 1331/2008 of the European Parliament and of the Council establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 64, 11.3.2011, p 15–24.

2 | DATA AND METHODOLOGIES

2.1 | Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme leucyl aminopeptidase from a non-genetically modified *A. oryzae* (strain AE-MB).

Additional information was requested from the applicant during the assessment process on 20 January 2023 and was consequently provided (see 'Documentation provided to EFSA').

2.2 | Methodologies

The assessment was conducted in line with the principles described in the EFSA 'Guidance on transparency in the scientific aspects of risk assessment' (EFSA, 2009b) and following the relevant guidance documents of the EFSA Scientific Committee.

A data package originated from a joint dossier should fulfil the data requirements in the 'Submission of a Dossier on Food Enzymes for Safety Evaluation' (EFSA, 2009a). During the evaluation, the Panel applied, whenever possible, the updated current 'Scientific Guidance for the submission of dossiers on Food Enzymes' (EFSA CEP Panel, 2021) and the guidance on the 'Food manufacturing processes and technical data used in the exposure assessment of food enzymes '(EFSA CEP Panel, 2023).

3 | ASSESSMENT

IUBMB nomenclature	Leucyl aminopeptidase
Systematic name	–
Synonyms	Cytosol aminopeptidase, leucine aminopeptidase, peptidase S
IUBMB No	EC 3.4.11.1
CAS No	9001-61-0
EINECS No	232-618-3

Leucyl aminopeptidases catalyse the hydrolysis of the peptide bonds of N-terminal amino acid residues of proteins or peptides, with a preference for leucine residues, resulting in the release of free amino acids. The enzyme under application is intended to be used in five food manufacturing processes: processing of dairy products for the production of (1) flavouring preparations; processing of plant- and fungal-derived products for the production of (2) protein hydrolysates; processing of meat and fish products for the production of (3) protein hydrolysates, (4) modified meat and fish products and processing of (5) yeast and yeast products.

3.1 | Source of the food enzyme

The leucyl aminopeptidase is produced with the non-genetically modified filamentous fungus *Aspergillus* sp. strain AE-MB, which is deposited at the [redacted] with the deposit number [redacted].[4] The production strain was derived from [redacted].[5] The production strain was identified as belonging to *Aspergillus* section *Flavi*. Data provided on the sequence analysis of [redacted] did not allow the assignation to *A. flavus* and *A. oryzae*.[6] Consequently, in this opinion the production strain is referred as *Aspergillus* sp.

3.2 | Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004,[7] with food safety procedures based on Hazard Analysis and Critical Control Points and in accordance with current Good Manufacturing Practice.[8]

[4]Additional information July 2023/ Annex 2.

[5]Technical dossier/second submission/Annex 1.

[6]Additional information July 2023/ Annex 1–1.

[7]Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of food additives. OJ L 226, 25.6.2004, pp. 3–21.

[8]Technical dossier/First submission/Annex 4.

The production strain is grown as a pure culture using a typical industrial medium as a solid-state fermentation in trays with conventional process controls in place. After completion of the fermentation, the fermentation medium is extracted with water and the solid biomass is removed by filtration. The filtrate containing the enzyme is further purified and concentrated, including an ultrafiltration step in which enzyme protein is retained, while most of the low molecular mass material passes the filtration membrane and is discarded. Finally, the food enzyme is dried prior to analysis.[9] The applicant provided information on the identity of the substances used to control the fermentation and in the subsequent downstream processing of the food enzyme.[10,11]

The Panel considered that sufficient information has been provided on the manufacturing process and the quality assurance system implemented by the applicant to exclude issues of concern.

3.3 | Characteristics of the food enzyme

3.3.1 | Properties of the food enzyme

The leucyl aminopeptidase is a single polypeptide chain of [redacted] amino acids.[12] The molecular mass of the mature protein, calculated from the amino acid sequence, is [redacted] kDa. The food enzyme was analysed by size exclusion chromatography.[13] The chromatograms of the three food enzyme batches for commercialisation showed similar patterns.[14] The food enzyme was tested for protease and α-amylase activities, and both were detected.[15]

The in-house determination of leucyl aminopeptidase activity is based on the hydrolysis of a L-leucyl *p*-nitroanilide hydrochloride solution (reaction conditions: pH 7, 37°C, 15 min), measuring the release of *p*-nitroaniline spectrophotometrically at 405 nm. The enzyme activity is expressed in Units/g (U/g). One U is defined as the amount of enzyme required to release one µmol of *p*-nitroaniline per minute under the conditions of the assay.[16]

The food enzyme has a temperature optimum around 70°C (pH 7) and a pH optimum around pH 7 (37°C). Thermostability was tested after a pre-incubation of the food enzyme for 1 hour at different temperatures (pH 7). The enzyme activity decreased above 60°C, showing no residual activity above 75°C.[17,18]

3.3.2 | Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches used for commercialisation and one batch produced for the toxicological tests (Table 1)[19,20] The mean total organic solids (TOS) of the three batches for commercialisation was 93.4% and the mean enzyme activity/TOS ratio was 4.9 UNIT/mg TOS.

TABLE 1 Composition of the food enzyme.

		Batches			
Parameters	**Unit**	**1**	**2**	**3**	**4[a]**
Leucyl aminopeptidase activity	U/g[b]	4730	4550	4480	4150
Protein	%	64.3	63.7	65.6	61.6
Ash	%	2.0	1.7	1.8	2.9
Water	%	6.0	2.1	6.1	5.4
Total organic solids (TOS)[c]	%	92.0	96.2	92.1	91.7
Activity/TOS ratio	U/mg TOS	5.1	4.7	4.9	4.5

[a]Batch used for the toxicological studies.

[b]U: UNIT (see Section 3.3.1).

[c]TOS calculated as 100% – % water – % ash.

[9]Technical dossier/First submission/Annex 5.
[10]Technical dossier/First submission/Annex 6.
[11]Additional information July 2023/Answer to question 6.
[12]Additional information July 2023/Answer to question 7.
[13]Additional information July 2023/Answer to question 8.
[14]Additional information July 2023/Answer to question 8.
[15]Technical dossier/Add information 10072014.
[16]Technical dossier/first submission/ Annex 2.
[17]Technical dossier/Dossier p.27.
[18]Additional information July 2023/Answer to question 9.
[19]Technical dossier/first submission/Annex 3.
[20]Additional information July 2023/Answer to question 5 and Annex 4.

3.3.3 | Purity

The lead content in the three commercial batches and in the batch used for toxicological studies was below 5 mg/kg, which complies with the specification for lead as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006).[21,22] For arsenic, cadmium and mercury, the mean concentrations determined in the commercial batches were 0.14, 0.55 and 0.002 mg/kg, respectively. The Panel considered these concentrations as not of concern.

The food enzyme complies with the microbiological criteria for total coliforms, *Escherichia coli* and *Salmonella* as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006). No antimicrobial activity was detected in any of the tested batches.[23]

Strains of *Aspergillus*, in common with most filamentous fungi, have the capacity to produce a range of secondary metabolites (Frisvad et al., 2018). The presence of aflatoxins B1, B2, G1 and G2, ochratoxin A, sterigmatocystin, HT-2 toxin, T-2 toxin, deoxynivalenol and zearalenone was examined in three food enzyme batches and all were below the limit of quantification (LoQ) of the applied methods.[24,25] Adverse effects caused by the possible presence of other secondary metabolites is addressed by the toxicological examination of the food enzyme–TOS.

The Panel considered that the information provided on the purity of the food enzyme was sufficient.

3.3.4 | Viable cells of the production strain

The absence of viable cells of the production strain in the food enzyme was demonstrated in three independent batches analysed in triplicate. [redacted]. No colonies derived from the production strain were produced. Colonies morphologically distinct from the production strain were found. A positive control was included.[26]

3.4 | Toxicological data

A battery of toxicological tests, including a bacterial reverse mutation test (Ames test), an *in vitro* mammalian chromosomal aberration test and a repeated dose 90-day oral toxicity study in rats, has been provided.

The batch 4 (Table 1) was considered suitable as a test item.

3.4.1 | Genotoxicity

3.4.1.1 | *Bacterial reverse mutation test*

A bacterial reverse mutation test (Ames test) was performed according to the Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 (OECD, 1997) and following Good Laboratory Practice (GLP).[27]

Two independent experiments were carried out with four strains of Salmonella Typhimurium (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* WP2*uvr*A, either with or without metabolic activation (S9-mix), applying the pre-incubation or the 'treat and wash' method, all with triplicate plating.

In a pre-test, using the pre-incubation method, where the bacteria were tested up to 5000 µg/plate, a doubling or more of revertant colonies were seen for TA1535, with and without S9-mix and for TA98 without S9-mix, compared to the vehicle control. For TA1537, a doubling was seen at 50 µg/plate with S9-mix only. A concentration-related stimulation of the background growth was seen for all strains with or without S9-mix. No precipitate was observed.

In the main experiment, six concentrations of the food enzyme were tested, ranging from 156 to 5000 µg/plate, corresponding to 143, 287, 573, 1145, 2292 and 4585 µg TOS/plate, with or without S9-mix. The same trend as in the pre-test was observed with a doubling of revertant colonies for TA98 and TA1535 with S9-mix at the highest concentration tested. All remaining strains did not show a biologically relevant increase in revertant colony number. The increase in revertant counts was attributed by the author to the presence of free amino acids in the food enzyme. Therefore, in a second experiment, the main test was reiterated for TA98 and TA1535 using the 'treat and wash' method. No biologically relevant increase in revertant counts were seen in this experiment, with and without S9-mix.

The Panel concluded that the food enzyme leucyl aminopeptidase did not induce gene mutations under the test conditions applied in this study.

[21]LoQs: Pb = 0.005 mg/kg; As = 0.002 mg/kg; Cd = 0.001 mg/kg; Hg = 0.001 mg/kg.
[22]Technical dossier/first submission/Annexes 1 & 3.
[23]Technical dossier/first submission/Annex 3.
[24]LoQ: aflatoxins B1, B2, G1 and G2 = 0.2 µg/kg each; ochratoxin A = 0.5 µg/kg; sterigmatocystin = 10 µg/kg; HT-2 toxin, T-2 toxin and zearalenone = 10 mg/kg each; Deoxynivalenol = 20 mg/kg.
[25]Technical dossier/first submission/Annex 3.
[26]Additional information July 2023/Annex 3–1 and Annex 3–2.
[27]Technical dossier/4th submission, Annex 2.

3.4.1.2 | *In vitro mammalian chromosomal aberration test*

The *in vitro* mammalian chromosomal aberration test was carried out according to the OECD Test Guideline 473 (OECD, 2014) and following GLP.[28]

A main and a confirmative experiment were performed with duplicate cultures of Chinese hamster lung fibroblast cells, where cell cultures were treated with the food enzyme either with or without metabolic activation (S9-mix). In a growth inhibition test where cells were tested up to 5000 µg/mL (corresponding to 4585 µg TOS/mL) in a short-term treatment (3 hours exposure with 21 hours recovery period) with or without S9-mix, and in a long-term treatment (24 h without recovery) without S9-mix, severe cytotoxicity was observed. In the short-term treatments, 50% growth inhibition was calculated to be at 170 and 410 µg/mL, with and without S9-mix, respectively. In the long-term treatment, the relative cell growth rate was 40% at the lowest concentration tested (39.1 µg/mL).

In the main experiment, cells were exposed to the food enzyme and scored for chromosomal aberrations in the short-term treatment at concentrations of 54.4, 90.7 and 151 µg/mL (corresponding to 49.9, 83 and 138.5 µg TOS/mL) with S9-mix and 151, 420 and 700 µg/mL (corresponding to 138.5, 385 and 642 µg TOS/mL) without S9-mix, and in the long-term treatment at concentrations of 19.6, 32.7 and 54.4 µg/mL (corresponding to 18, 30 and 50 µg TOS/mL).

In the short-term treatment without S9-mix, a concentration-related statistically significant increase in structural chromosomal aberrations was observed. At the highest concentration, the increase was above the historical control range, accompanied by a cytotoxicity of 64%. The results from the short-term treatment (with S9-mix) and for the long-term treatment were not statistically significantly different when compared with the negative controls at any of the concentrations tested. A confirmatory test was conducted for the short-term treatment without S9-mix at revised concentrations of 207, 413 and 510 µg/mL (corresponding to 190, 379 and 468 µg TOS/mL). This test did not show any statistically significant increase in the number of structural chromosomal aberrations compared to the controls and a cytotoxicity of 57.5% at the highest concentration tested.

The frequency of structural and numerical chromosomal aberrations was not statistically significant different when compared to the negative controls at any of the concentrations tested, with or without S9-mix, in any of the experiments. All results were within the historical control range.

The Panel concluded that the food enzyme leucyl aminopeptidase did not induce an increase in the frequency of structural and numerical aberrations under the test conditions applied in this study.

3.4.2 | Repeated dose 90-day oral toxicity study in rodents

The repeated dose 90-day oral toxicity study was performed following GLP[29] and in accordance with guidelines of the Japanese Ministry of Health and Welfare (1996 and 1999). The study is in accordance with the OECD Test Guideline 408 (OECD, 1998) with the following deviations: detailed clinical observations and functional observations were not performed, urea was not determined in the clinical chemistry investigation, epididymides were not weighed and the regions of the brain examined were not specified. The Panel considered that these deviations are minor and do not have an impact on the evaluation of the study.

Groups of 10 male and 10 female Sprague–Dawley (Crl:CD(SD)) rats received by gavage the food enzyme in doses of 200, 650 or 2000 mg/kg body weight (bw) per day, corresponding to 183, 596 or 1834 mg TOS/kg bw per day. Controls received the vehicle (water for injection).

No mortality was observed.

The haematological investigation revealed a statistically significant increase in the ratio and number of eosinophils in high-dose males (+50% and +67%, respectively) and a decrease in the basophil ratio in mid-dose females. The Panel considered the changes as not toxicologically relevant, as they were only observed in one sex (both parameters), there was no dose–response relationship (basophil ratio), the changes were small (both parameters), there was no change in the number of basophils (basophil ratio) and there was no change in the total white blood cell count.

The clinical chemistry investigation revealed a statistically significant increase in the β-globulin ratio in high-dose males (+7%) and a decrease in the aspartate aminotransferase (AST) level (–22%) in mid-dose females and in the calcium level (–3%) in mid-dose males. The Panel considered the changes as not toxicologically relevant, as they were only observed in one sex (all parameters), there was no dose–response relationship (AST, calcium) and the changes were small (all parameters).

The urinalysis revealed a statistically significant decrease in the potassium level in mid-dose males (–31%). The Panel considered the change as not toxicologically relevant, as it was only observed in one sex and there was no dose–response relationship.

Statistically significant changes in organ weights included an increase in the relative weight of the salivary glands in high-dose females (+12%). The Panel considered the change as not toxicologically relevant, as it was only observed in one sex, the change was small and there were no histopathological changes in the salivary glands of high-dose animals, the only treated group examined.

[28]Technical dossier/4th submission, Annex 3.

[29]Technical dossier/4th submission/Annex 2/pg.7.

The microscopic examination revealed noteworthy histopathological changes in the forestomach and glandular stomach. On the limiting ridge of the forestomach, mucosal oedema (males: 0/10, 1/10, 3/10, 9/10; females: 0/10, 0/10, 1/10, 8/10 in the control, low-, mid- and high-dose groups, respectively) and eosinophilic material on mucosal surface (male: 0/10, 1/10, 4/10, 9/10; females: 4/10, 2/10, 3/10, 7/10 in the control, low-, mid- and high-dose groups, respectively) were seen. In the glandular stomach, infiltration of globule leukocytes in mucosa (males: 0/10, 0/10, 5/10, 8/10; females: 0/10, 0/10, 0/10, 7/10 in the control, low-, mid- and high-dose groups, respectively) and inflammatory cell infiltration of the submucosa (males: 0/10, 0/10, 4/10, 5/10; females: 0/10, 0/10, 0/10, 1/10 in the control, low-, mid- and high-dose groups, respectively) were observed. The severity of these changes was minimal in the low- and mid-dose groups and slight in the control and high-dose groups. The Panel noted that the incidence of the changes in forestomach of the low-dose group was within the historical control ranges for the laboratory. The Panel considered the changes in the forestomach and in glandular stomach in mid- and high-dose groups as test-item-related, since their incidences increased dose-dependently.

No other statistically significant or biologically relevant differences to controls were reported.

The Panel identified a no observed adverse effect level (NOAEL) of 183 mg TOS/kg bw per day, based on the changes in the forestomach and glandular stomach in the mid- and high- doses.

Although the findings are of minimal severity, they are indicative of development of inflammation and there is a trend to increasing incidence with dose. The Panel considered that the low-dose was a clear NOAEL and that the high dose was an effect level. Whilst there was uncertainty about the mid-dose, the Panel considered it prudent based on the available data to regard the mid-dose as a lowest observed adverse effect level.

3.4.3 | Allergenicity

The allergenicity assessment considers only the food enzyme and not carriers or other excipients that may be used in the final formulation.

The potential allergenicity of the leucyl aminopeptidase produced with the *Aspergillus* sp. strain AE-MB was assessed by comparing its amino acid sequence with those of known allergens according to the 'Scientific opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed of the Scientific Panel on Genetically Modified Organisms' (EFSA GMO Panel, 2010). Using higher than 35% identity in a sliding window of 80 amino acids as the criterion, no match was found.[30]

No information was available on oral and respiratory sensitisation or elicitation reactions of this leucyl aminopeptidase.

No allergic reactions upon dietary exposure to any leucyl aminopeptidase have been reported in the literature.[31]

[redacted], products that may cause allergies or intolerances (listed in the Regulation (EU) No 1169/2011[32]) are used as raw materials. However, during the fermentation process, these products will be degraded and utilised by the microorganisms for cell growth, cell maintenance and production of enzyme protein. In addition, the fungal biomass and fermentation solids are removed. Taking into account the fermentation process and downstream processing, the Panel considered that no potentially allergenic residues from these sources are present in the food enzyme.

The Panel considered that the risk of allergic reactions upon dietary exposure to this food enzyme cannot be excluded, but the likelihood is low.

3.5 | Dietary exposure

3.5.1 | Intended use of the food enzyme

The food enzyme is intended to be used in five food manufacturing processes at the recommended use levels summarised in Table 2.

[30]Technical dossier/ Additional information July 2023/Annex 7–1.

[31]Technical dossier/ Additional information July 2023/Annex 7–1 and Annex 7–2.

[32]Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004.

TABLE 2 Intended uses and recommended use levels of the food enzyme as provided by the applicant.[33,34]

Food manufacturing process[a]	Raw material (RM)	Recommended use level (mg TOS/kg RM)[b]
Processing of dairy products		
• Production of flavouring preparations from dairy products	Cheese, cream, butter, etc.	Up to **32.8**
Processing of plant- and fungal-derived products		
• Production of protein hydrolysates from plants and fungi	Plant proteins	343–**3427**
Processing of meat and fish products		
• Production of protein hydrolysates from meat and fish proteins	Animal proteins	343–**3427**
• Production of modified meat and fish products	Meat or fish	Up to **32.8**
Processing of yeast and yeast products	Yeast extract	Up to **32.8**

Abbreviation: TOS, total organic solids.

[a]The name has been harmonised by EFSA according to the 'Food manufacturing processes and technical data used in the exposure assessment of food enzymes' (EFSA CEP Panel, 2023).

[b]Numbers in bold were used for calculation.

In all intended food manufacturing processes, the leucyl aminopeptidase hydrolyses peptide bonds in proteins and releases free amino acids.[35]

In the production of flavouring preparations from dairy products, the food enzyme is added to the cheese slurry[36] and other milk components (e.g. cream, butter)[37] after the heat treatment. The food enzyme–TOS remains in the enzyme-modified dairy ingredients, which are added to a variety of final foods (e.g. processed cheese, soups, snacks, dressings, sauces).

In the production of protein hydrolysates, the food enzyme is added to a variety of partially purified proteins from plant (e.g. legumes and cereals) and animal (e.g. meat, collagen) materials during hydrolysis.[38] The hydrolysis can reduce bitterness of the protein hydrolysates. The food enzyme–TOS remains in the final foods.

In the production of modified meat and fish products, the food enzyme is added to the meat or fish broth to obtain meat and fish extracts.[39] The hydrolysis intensifies the flavour of meat and fish extracts. The food enzyme–TOS remains in these extracts.

In yeast processing, the food enzyme is added to the yeast extract.[40] The hydrolysis intensifies the flavour of yeast extracts, which are added in small amounts to enhance the flavour of various savoury foods, ready-to-eat vegetable meals, soups, bouillons and sauces. The food enzyme–TOS remains in the yeast extract.

Based on data provided on thermostability (see Section 3.3.1) and the downstream processing step applied in the food manufacturing processes, it is expected that the food enzyme is inactivated in all the relevant final foods.

3.5.2 | Dietary exposure estimation

Chronic exposure to the food enzyme–TOS was calculated by combining the maximum recommended use level with individual consumption data (EFSA CEP Panel, 2021). The estimation involved selection of relevant food categories and application of technical conversion factors (EFSA CEP Panel, 2023). Exposure from all FoodEx categories was subsequently summed up, averaged over the total survey period (days) and normalised for body weight. This was done for all individuals across all surveys, resulting in distributions of individual average exposure. Based on these distributions, the mean and 95th percentile exposures were calculated per survey for the total population and per age class. Surveys with only 1 day per subject were excluded and high-level exposure/intake was calculated for only those population groups in which the sample size was sufficiently large to allow calculation of the 95th percentile (EFSA, 2011).

Table 3 provides an overview of the derived exposure estimates across all surveys. Detailed mean and 95th percentile exposure to the food enzyme–TOS per age class, country and survey, as well as contribution from each FoodEx category to the total dietary exposure are reported in Appendix A – Tables 1 and 2. For the present assessment, food consumption data were available from 48 dietary surveys (covering infants, toddlers, children, adolescents, adults and the elderly), carried out in 26 European countries (Appendix B). The highest dietary exposure was estimated to be 2.273 mg TOS/kg bw per day in toddlers at the 95th percentile.

[33]Technical dossier/1 submission/updated info/technical dossier/pp. 37–38.

[34]Additional information July 2023/Answers 15, 16 and 17.

[35]Technical dossier/1 submission/updated info/technical dossier/p. 35.

[36]Technical dossier/1 submission/updated info/technical dossier/Annex 7/p.1.

[37]Additional information July 2023/Answer 15.

[38]Technical dossier/1 submission/updated info/technical dossier/Annex 7/p.1.

[39]Technical dossier/1 submission/updated info/technical dossier/Annex 7/p.2.

[40]Technical dossier/1 submission/updated info/technical dossier/Annex 7/p.3.

TABLE 3 Summary of the estimated dietary exposure to food enzyme–TOS in six population groups.

	Estimated exposure (mg TOS/kg body weight per day)					
Population group	**Infants**	**Toddlers**	**Children**	**Adolescents**	**Adults**	**The elderly**
Age range	3–11 months	12–35 months	3–9 years	10–17 years	18–64 years	≥65 years
Min–max mean (number of surveys)	0.072–0.555 (12)	0.214–0.705 (15)	0.252–0.708 (19)	0.061–0.457 (21)	0.046–0.308 (22)	0.026–0.269 (22)
Min–max 95th percentile (number of surveys)	0.159–1.355 (11)	0.563–2.273 (14)	0.734–2.193 (19)	0.197–1.674 (20)	0.152–1.141 (22)	0.101–0.996 (22)

3.5.3 | Uncertainty analysis

In accordance with the guidance provided in the EFSA opinion related to uncertainties in dietary exposure assessment (EFSA, 2006), the following sources of uncertainties have been considered and are summarised in Table 4.

TABLE 4 Qualitative evaluation of the influence of uncertainties on the dietary exposure estimate.

Sources of uncertainties	Direction of impact
Model input data	
Consumption data: different methodologies/representativeness/underreporting/misreporting/no portion size standard	+/–
Use of data from food consumption surveys of a few days to estimate long-term (chronic) exposure for high percentiles (95th percentile)	+
Possible national differences in categorisation and classification of food	+/–
Model assumptions and factors	
Exposure to food enzyme–TOS was always calculated based on the recommended maximum use level	+
Selection of broad FoodEx categories for the exposure assessment	+
For yeast processing, although the food enzyme is not used to treat yeast cell wall,[41] the food categories chosen for calculation covers also those containing mannoproteins resulted from the treatment of yeast cell wall.	+
Use of recipe fractions in disaggregation FoodEx categories	+/–
Use of technical factors in the exposure model	+/–

Abbreviation: TOS, total organic solids.

+: uncertainty with potential to cause overestimation of exposure.

–: uncertainty with potential to cause underestimation of exposure.

The conservative approach applied to estimate the exposure to the food enzyme–TOS, in particular assumptions made on the occurrence and use levels of this specific food enzyme, is likely to have led to an overestimation of the exposure.

3.6 | Margin of exposure

The comparison of the NOAEL (183 mg TOS/kg bw per day) from the 90-day rat study with the mean exposure estimates of 0.026–0.708 mg TOS/kg bw per day and 0.101–2.273 mg TOS/kg bw per day at the 95th percentile resulted in margins of exposure (MoEs) for infants, toddlers, children, adolescents, adults and the elderly of at least 135, 81, 83, 109, 160 and 184, respectively.

4 | CONCLUSIONS

Based on the data provided and the derived margins of exposure in all age groups, the Panel concluded that the food enzyme leucyl aminopeptidase produced with the non-genetically modified *Aspergillus* sp. strain AE-MB could not be considered safe under the intended conditions of use.

5 | DOCUMENTATION AS PROVIDED TO EFSA

Technical dossier "Application for authorisation of Leucyl aminopeptidase from *Aspergillus oryzae* AE-MB. 30 October 2013. Submitted by Amano Enzyme Inc.

Additional information. July 2023. Submitted by Amano Enzyme Inc.

[41] Additional information July 2023/Answer 17.

ABBREVIATIONS

bw	body weight
CAS	Chemical Abstracts Service
CEF	EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CEP	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
EC	European Commission
EINECS	European Inventory of Existing Commercial Chemical Substances
EU	European Union
FAO	Food and Agricultural Organization of the United Nations
GLP	Good Laboratory Practice
GMO	genetically modified organism
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kDa	kiloDalton
LoD	limit of detection
LoQ	limit of quantification
MoE	margin of exposure
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Co-operation and Development
TOS	total organic solids
WHO	World Health Organization

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CONFLICT OF INTEREST

If you wish to access the declaration of interests of any expert contributing to an EFSA scientific assessment, please contact interestmanagement@efsa.europa.eu.

REQUESTOR

European Commission

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NOTE

The full opinion will be published in accordance with Article 12 of Regulation (EC) No 1331/2008 once the decision on confidentiality will be received from the European Commission.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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APPENDIX A

Dietary exposure estimates to the food enzyme–TOS in details

Appendix A can be found in the online version of this output (in the 'Supporting information' section). The file contains two sheets, corresponding to two tables.

Table 1: Average and 95th percentile exposure to the food enzyme–TOS per age class, country and survey.

Table 2: Contribution of food categories to the dietary exposure to the food enzyme–TOS per age class, country and survey.

APPENDIX B

Population groups considered for the exposure assessment

Population	Age range	Countries with food consumption surveys covering more than 1 day
Infants	From 12 weeks on up to and including 11 months of age	Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Portugal, Slovenia, Spain
Toddlers	From 12 months up to and including 35 months of age	Belgium, Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Hungary, Italy, Latvia, the Netherlands, Portugal, Republic of North Macedonia*, Serbia*, Slovenia, Spain
Children	From 36 months up to and including 9 years of age	Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, the Netherlands, Portugal, Republic of North Macedonia*, Serbia*, Spain, Sweden
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Bosnia and Herzegovina*, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Montenegro*, the Netherlands, Portugal, Romania, Serbia*, Slovenia, Spain, Sweden
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Bosnia and Herzegovina*, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Montenegro*, the Netherlands, Portugal, Romania, Serbia*, Slovenia, Spain, Sweden
The elderly[a]	From 65 years of age and older	Austria, Belgium, Cyprus, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Montenegro*, the Netherlands, Portugal, Romania, Serbia*, Slovenia, Spain, Sweden

*Consumption data from these pre-accession countries are not reported in Table 3 of this opinion, however, they are included in Appendix B for testing purpose.

[a]The terms 'children' and 'the elderly' correspond, respectively, to 'other children' and the merge of 'elderly' and 'very elderly' in the Guidance of EFSA on the 'Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment' (EFSA, 2011).