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SCIENTIFIC OPINION

Safety evaluation of the food enzyme leucyl aminopeptidase from non-genetically modified *Aspergillus oryzae* strain NZYM-EX

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Abstract

The food enzyme leucyl aminopeptidase (EC 3.4.11.1) is produced with the non-genetically modified microorganism *Aspergillus oryzae* strain NZYM-EX by Novozymes A/S. The food enzyme is free from viable cells of the production organism. It is intended to be used in eight food manufacturing processes: processing of dairy products for the production of (1) flavouring preparations, (2) modified milk proteins; processing of plant- and fungal-derived products for the production of (3) protein hydrolysates, (4) soy sauce; processing of meat and fish products for the production of (5) protein hydrolysates; processing of cereals and other grains for the production of (6) baked products, (7) brewed products; (8) processing of yeast and yeast products. Dietary exposure to the food enzyme total organic solids (TOS) was estimated to be up to 0.577 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 440 mg TOS/kg bw per day, the highest dose tested, which, when compared with the estimated dietary exposure, resulted in a margin of exposure of at least 763. A search for similarity of the amino acid sequence of the food enzyme to known allergens was made and no match was found. The Panel considered that the risk of allergic reactions by dietary exposure cannot be excluded, but the likelihood is low. Based on the data provided, the Panel concluded that this food enzyme does not give rise to safety concerns under the intended conditions of use.

KEYWORDS

Aspergillus oryzae, EC 3.4.11.1, food enzyme, leucyl aminopeptidase, non-genetically modified microorganism

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[1] 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 CEF Panel, 2009) 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 European Union (EU) Community 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.

Five applications have been introduced by the companies 'Meiji Seika Pharma Co., Ltd.' for the authorisation of the food enzyme cellulase from *Talaromyces cellulolyticus*/*Talaromyces pinophilus* (strain *Acremonium cellulolyticus*), 'Danisco US Inc.' for the authorisation of the food enzymes aspergillopepsin I from a genetically modified strain of *Trichoderma reesei* (strain DP-Nzq40) and triacylglycerol lipase from a genetically modified strain of *Hansenula polymorpha* (strain Dp-Jzk33), 'Neova Technologies Inc.' for the authorisation of the food enzyme trypsin and chymotrypsin from porcine pancreatic glands, and 'Novozymes A/S' for the authorisation of the food enzyme peptidase from a strain of *Aspergillus oryzae* (strain NZYM-EX).

Following the requirements of Article 12.1 of Regulation (EC) No 234/2011[3] implementing Regulation (EC) No 1331/2008,[2] the Commission has verified that the five applications fall within the scope of the food enzyme Regulation and contain all the elements required under Chapter II 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 cellulase from *Talaromyces cellulolyticus*/*Talaromyces pinophilus* (strain *Acremonium cellulolyticus*), aspergillopepsin I from a genetically modified strain of *Trichoderma reesei* (strain DP-Nzq40), triacylglycerol lipase from a genetically modified strain of *Hansenula polymorpha* (strain Dp-Jzk33), trypsin and chymotrypsin from porcine pancreatic glands, and peptidase from a strain of *Aspergillus oryzae* (strain NZYM-EX) in accordance with Article 17.3 of Regulation (EC) No 1332/2008[1] on food enzymes.

[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]Commission Regulation (EU) 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.

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 NZYM-EX.

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 NZYM-EX).

Additional information was requested from the applicant during the assessment process on 22 September 2022 and received on 23 March 2023 (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, 2009) and following the relevant guidance documents of the EFSA Scientific Committee.

The 'Guidance on the submission of a dossier on food enzymes for safety evaluation' (EFSA CEF Panel, 2009) has been followed for the evaluation of the application. Additional information was requested in accordance with the updated '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[4]

IUBMB nomenclature	Leucyl aminopeptidase
Systematic name	Not assigned
Synonyms	Leucine aminopeptidase; peptidase S; cytosol aminopeptidase
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 acids of proteins or peptides, with a preference for leucine, resulting in the release of free amino acids. The food enzyme under application is intended to be used in eight food manufacturing processes: processing of dairy products for the production of (1) flavouring preparations, (2) modified milk proteins; processing of plant- and fungal-derived products for the production of (3) protein hydrolysates, (4) soy sauce; processing of meat and fish products for the production of (5) protein hydrolysates; processing of cereals and other grains for the production of (6) baked products, (7) brewed products; (8) processing of yeast and yeast products.

3.1 | Source of the food enzyme[5]

The enzyme is produced with the non-genetically modified filamentous fungus *A. oryzae* strain NZYM-EX, which is deposited [redacted] with the deposit number [redacted].[6]

The production strain was [redacted] and identified as *A. oryzae* [redacted].[7]

[4]Technical dossier/p. 7, 35, 81.
[5]Technical dossier/p. 12, 45–50; Technical dossier/Additional information, 23 March 2023/Annex 1; Annex 2.
[6]Technical dossier/Additional information, 23 March 2023/Annex 1.
[7]Technical dossier/Additional information, 23 March 2023/Annex 2.

3.2 | Production of the food enzyme[8]

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004,[9] with food safety procedures based on Hazard Analysis and Critical Control Points and in accordance with current Good Manufacturing Practice.[10]

The production strain is [redacted].[11] 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.[12]

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 enzyme is a single polypeptide chain of [redacted] amino acids.[13] The molecular mass of the mature protein, calculated from the amino acid sequence, is [redacted] kDa.[14] The food enzyme was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.[15] A consistent protein pattern was observed across all batches. Carboxypeptidase, endopeptidase and α-amylase activities were also detected in the food enzyme.[16]

The in-house determination of the enzyme activity is based on the hydrolysis of L-leucine-*p*-nitroanilide (reaction conditions: pH 8.0, 37°C, 5 min) and determined by measuring the release of *p*-nitroaniline spectrophotometrically at 405 nm. The leucyl aminopeptidase activity is quantified relative to an internal enzyme standard and expressed in Leucine Aminopeptidase Units/g (LAPU/g).[17]

The food enzyme has a temperature optimum around 70°C (pH 7.0) and a pH optimum around pH 8.5 (30°C). Thermostability was tested after a pre-incubation of the food enzyme for 15 min at different temperatures (pH 7.0). The leucyl aminopeptidase activity was stable up to 75°C. With increasing temperature, the activity was reduced, with no activity detected after pre-incubation at 90°C.[18]

3.3.2 | Chemical parameters[19]

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).[20] The mean total organic solids (TOS) of the three food enzyme batches for commercialisation was 25.8% and the mean enzyme activity/TOS ratio was 9.4 LAPU/mg TOS.

[8]Technical dossier/p. 12, 22–23, 50–57; Technical dossier/Annex 3; Annex 4; Technical dossier/Additional information, 23 March 2023.
[9]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.
[10]Technical dossier/p. 12, 50; Technical dossier/Annex 3.
[11]Technical dossier/p. 50–57.
[12]Technical dossier/p. 51, 55; Technical dossier/Annex 4.
[13]Technical dossier/Additional information, 23 March 2023.
[14]Technical dossier/Additional information, 23 March 2023.
[15]Technical dossier/p. 37.
[16]Technical dossier/p. 11, 18, 44–45; Technical dossier/Annex 2.02; Annex 2.03; Annex 2.04.
[17]Technical dossier/p. 41–42; Technical dossier/Annex 2.01.
[18]Technical dossier/p. 12, 43–44, 84–85; Technical dossier/Annex 6.
[19]Technical dossier/p. 36, 70–71; Technical dossier/Annex 1.01; Annex 1.02; Annex 1.03; Technical dossier/Additional information, 23 March 2023/Annex 4.
[20]Technical dossier/p. 36, 70–71; Technical dossier/Annex 1; Annex 2.01.

TABLE 1 Composition of the food enzyme.[21]

		Batches			
Parameters	**Unit**	**1**	**2**	**3**	**4**[a]
Leucyl aminopeptidase activity	LAPU/g[b]	2260	2600	2430	2110
Protein	%	17.2	18.8	17.1	NA
Ash	%	0.6	0.5	0.6	0.5
Water	%	74.3	72.4	74.1	79.0
Total organic solids (TOS)[c]	%	25.1	27.1	25.3	20.5
Activity/TOS ratio	LAPU/mg TOS	9.0	9.6	9.6	10.3

Abbreviation: NA, Not analysed.

[a]Batch used for the toxicological studies.

[b]LAPU: Leucyl Aminopeptidase Units (see Section 3.3.1).

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

3.3.3 | Purity[22]

The lead content in the three commercial batches and in the batch used for toxicological studies was below 5 mg/kg,[23] which complies with the specification for lead as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006). In addition, arsenic, cadmium and mercury were below the limits of detection (LoD) of the employed methods.[24,25]

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).[26] No antimicrobial activity was detected in any of the tested batches.[27]

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 aflatoxin B1, kojic acid, 3-nitro propionic acid and cyclopiazonic acid was examined in all batches. All were below the LoD or limit of quantification (LoQ) of the applied analytical methods.[28,29] Adverse effects caused by the possible presence of other secondary metabolites was 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[30]

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 were produced. A positive control was included.

3.4 | Toxicological data[31]

A battery of toxicological tests, including a bacterial reverse mutation test (Ames test), an *in vitro* mammalian cell micronucleus test and a repeated dose 90-day oral toxicity study in rats, has been provided. The batch 4 (Table 1) used in these studies has similar protein pattern and activity/TOS value as the batches used for commercialisation and, thus, was considered suitable as a test item.

[21]Technical dossier/Additional information, 23 March 2023/Annex 4.

[22]Technical dossier/Additional information, 23 March 2023/Annex 4.

[23]Technical dossier/p. 11, 37–38, 40, 70; Technical dossier/Annex 1.04; Technical dossier/Additional information, 23 March 2023/Annex 4.

[24]Technical dossier/p. 11, 37–38, 40, 70; Technical dossier/Annex 1.04; Technical dossier/Additional information, 23 March 2023/Annex 4.

[25]Technical dossier/Additional information, 23 March 2023/Annex 4: LoDs: Pb = 0.5 mg/kg; As = 0.1 mg/kg; Cd = 0.05 mg/kg; Hg = 0.03 mg/kg.

[26]Technical dossier/p. 11, 40, 70; Technical dossier/Annex 1.09; Annex 1.10; Annex 1.11; Annex 1.12.

[27]Technical dossier/p. 11, 40, 70; Technical dossier/Annex 1.08; Technical dossier/Additional information, 23 March 2023/Annex 4.

[28]Technical dossier/p. 11, 37–38, 40, 70; Technical dossier/Annex 1.05; Annex 1.06; Technical dossier/Additional information, 23 March 2023/Annex 4.

[29]Technical dossier/Additional information, 23 March 2023/Annex 4: LoDs: aflatoxin B1 = 0.0003 mg/kg; kojic acid = 0.004 mg/kg; 3-nitro propionic acid: LoQ (matrix dependent) = 0.005–0.13 mg/kg; cyclopiazonic acid = 0.003 mg/kg.

[30]Technical dossier/Annex 1.09; Technical dossier/Additional information, 23 March 2023/Annex 3.

[31]Technical dossier/p. 16, 70–75; Technical dossier/Annex 5.01; Annex 5.02; Annex 5.03.

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).[32]

Four strains of *Salmonella* Typhimurium (TA98, TA100, TA1535 and TA1537) and *E. coli* WP2uvrA(pKM101) were used with or without metabolic activation (S9-mix), applying the 'treat and plate' assay. Two experiments were carried out in triplicate, using six concentrations ranging from 156 to 5000 µg dry matter/plate, corresponding to 152, 306, 610, 1220, 2440 and 4881 µg TOS/plate.

No cytotoxicity was observed at any concentration of the test substance. Upon treatment with the food enzyme, there was no biologically relevant increase in the number of revertant colonies above the control values in any strain tested, with or 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.

3.4.1.2 | *In vitro mammalian cell micronucleus test*

The *in vitro* mammalian cell micronucleus test was carried out according to the OECD Test Guideline 487 (OECD, 2010) and following GLP.[33]

An experiment was performed with duplicate cultures of human peripheral whole blood lymphocytes. The cell cultures were treated with the food enzyme with or without metabolic activation (S9-mix).

In a range-finding test, cytotoxicity above 50% was seen at 648 µg/mL and above in the long-term treatment without S9-mix. Based on these results, the cells were exposed to the food enzyme and scored for the frequency of bi-nucleated cells with micronuclei (MNBN) at concentrations of 3000, 4000 and 5000 µg/mL (corresponding to 615, 820 and 1025 µg TOS/mL) in a short-term treatment (3 h exposure and 21 h recovery period), either with or without S9-mix, and at concentrations of 125, 150, 175 and 200 µg/mL (corresponding to 25.6, 30.8, 35.9 and 41.0 µg TOS/mL) in a long-term treatment (24 h exposure and 24 h recovery period) without S9-mix.

In the long-term treatment, cytotoxicity of 57% (replication index) was observed at concentration of 200 µg/mL (corresponding to 41 µg TOS/mL). The frequency of MNBN was not statistically significantly different to the negative controls at all concentrations tested.

The Panel concluded that the food enzyme leucyl aminopeptidase did not induce an increase in the frequency of MNBNs 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 in accordance with the OECD Test Guideline 408 (OECD, 1998) and following GLP.[34] Groups of 10 male and 10 female Sprague–Dawley (Crl:CD(SD)) rats received by gavage the food enzyme in doses of 240, 340 and 440 mg TOS/kg bw per day. Controls received the vehicle (reverse osmosis water).

No mortality was observed.

The overall body weight gain was statistically significantly increased after 13 weeks of administration in low-, mid- and high-dose males (+10%, +13%, +7%, respectively) and in mid-dose females (+7%). The Panel considered the change as not toxicologically relevant, as there was no dose–response relationship and there was no statistically significant effect on the final body weight.

The overall water intake was statistically significantly increased after 13 weeks of administration in low- and high-dose males (+9% and +3%, respectively) and in mid-dose females (+8%). The Panel considered the change as not toxicologically relevant, as there was no dose–response relationship.

In the functional observations, statistically significant increases were observed in high beam scores in the 8th time-interval in high-dose males (21.0 vs. 0.5 in the control) and in the 9th time-interval in high-dose females (30 vs. 2 in the control) and in the 10th time-interval in low-, mid- and high-dose females (38, 21 and 32, respectively, vs. 3.0 in the control). In addition, low beam scores were statistically significantly increased in the 9th time-interval in the high-dose females (53 vs. 13.0 in the control) and in the 10th time-interval in low-, mid- and high-dose females (65, 41 and 52, respectively, vs. 13.0 in the control). The Panel considered the changes as not toxicologically relevant, as they were only recorded sporadically, there was no dose–response relationship (the 10th time-interval in females) and the changes were without a statistically significant effect on the total high- and low- beam scores.

Haematological investigation revealed a statistically significant decrease in mean cell haemoglobin (MCH) (–3%) and in mean cell volume (MCV) (–3%) in high-dose males, an increase in MCH (+3%) and in MCV (+2%) in high-dose females, an increase in neutrophil counts (N) (+105%) in low-dose females and an increase in prothrombin times (PT) in low-, mid- and high-dose females (+6%, +5%, +7%, respectively). The Panel considered the changes as not toxicologically relevant, as they

[32]Technical dossier/Annex 5.01.

[33]Technical dossier/Annex 5.02.

[34]Technical dossier/Annex 5.03; Technical dossier/Additional information, 23 March 2023/Annex 5.

were only observed in one sex (N, PT), there was no consistency between the change in males and females (MCH, MCV), there was no dose–response relationship (N), the changes were small (MCH, MCV, PT), there were no changes in other relevant parameters (i.e. for PT in platelet count and in activated partial thromboplastin time, for N in white blood cells) and the changes were within the historical control values.

Clinical chemistry investigation revealed a statistically significant increase in glucose in high-dose males (+22%), a decrease in albumin concentration in low-, mid- and high-dose males (−3%, −3%, −3%, respectively) and an increase in potassium concentration in high-dose females (+9%). 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 (albumin) and the changes were small (albumin) and within the historical control values.

Statistically significant changes in organ weights detected were an increase in absolute testes weight in high-dose males (+9%), an increase in relative brain weight in high-dose males (+4%) and a decrease in relative liver weight in low-dose males (−16%). The Panel considered the changes as not toxicologically relevant, as they were only observed in one sex (brain, liver), the changes were small (testes, brain), there was no dose–response relationship (liver), there were no histopathological changes in organs (testes, brain, liver) and the changes were within the historical control values.

The microscopic examination revealed an increased number of mucous secreting neck cells in the glandular stomach in 4/10 mid-dose males (severity minimal in one and slight in three) and in 6/10 high-dose males (severity slight in five and moderate in one). One of these high-dose males had also a slight muscularis and/or submucosal inflammation in the glandular stomach. The inflammatory effect was not observed in high-dose females. Furthermore, epithelial hyperplasia of minimal severity was observed in 2/10 mid-dose males. Increased mucous secreting neck cells in the glandular stomach were also observed in 3/10 high-dose females (severity minimal in two and slight in one). The Panel viewed the increased number of mucous secreting neck cells as an adaptive response to irritation within the stomach, which was test-item related, but not adverse, considering the low severity of the finding.

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

The Panel identified a no observed adverse effect level (NOAEL) of 440 mg TOS/kg bw per day, the highest dose tested.

3.4.3 | Allergenicity[35]

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

The potential allergenicity of the enzyme leucyl aminopeptidase produced with *A. oryzae* strain NZYM-EX 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.

No information was available on oral and respiratory sensitisation or elicitation reactions of this leucyl aminopeptidase.[36] In addition, no allergic reactions after oral exposure to leucyl aminopeptidases have been reported in the literature.

[redacted], a product that may cause allergies or intolerances (listed in the Regulation (EU) No 1169/2011[37]), is used as raw material. In addition, [redacted], a known source of allergens, is also present in the media fed to the microorganisms. 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 eight food manufacturing processes at the recommended use levels summarised in Table 2.[38]

[35]Technical dossier/p. 17, 76–77; Technical dossier/Additional information, 23 March 2023/Annex 6; Annex 7.

[36]Technical dossier/Additional information, 23 March 2023/Annex 7.

[37]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.

[38]Technical dossier/Additional information, 23 March 2023/Answers 10, 11 and 12.

TABLE 2 Intended uses and recommended use levels of the food enzyme as provided by the applicant.

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.	9–**27**
• Production of modified milk proteins	Whey proteins	66–**212**
Processing of plant- and fungal-derived products		
• Production of protein hydrolysates from plants and fungi	Plant proteins	66–**212**
• Production of soy sauce	Soya beans	74–**372**
Processing of meat and fish products		
• Production of protein hydrolysates from meat and fish proteins	Animal proteins	37–**106**
Processing of cereals and other grains		
• Production of baked products	Flour	4–**21**
• Production of brewed products	Cereals (malted or not)	10–**53**
Processing of yeast and yeast products	Yeast	10–**53**

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 of the intended food manufacturing processes, the leucyl aminopeptidase hydrolyses peptide bonds in proteins and releases free amino acids.[39]

In the production of flavouring preparations from dairy products, the food enzyme is added to the cheese slurry and other milk components (e.g. cream, butter) after pasteurisation.[40] The hydrolysis produces in the enzyme-modified dairy ingredients (EMDI) a distinctive cheese flavour. The food enzyme–TOS remains in the EMDI, which is an ingredient of a variety of final foods (e.g. processed cheese, soups, snacks, dressings, sauces).

In the production of whey protein hydrolysates, the food enzyme can be added with or without other peptidases to whey protein isolate or concentrate during mixing with water.[41] The hydrolysis improves the solubility and taste. The food enzyme–TOS remains in the whey protein hydrolysates, which are ingredients of a variety of final foods (e.g. sports products, nutritional bars). Whey protein hydrolysates, which are ingredients of infant formulae, follow-on formulae and food for special medical purposes, are further purified by ultra- and nanofiltration as well as activated carbon treatment.[42] These downstream processes are expected to remove the food enzyme–TOS. However, in the absence of analytical data to establish the extent of removal,[43] the Panel opted for a conservative scenario by considering the full remaining of the food enzyme–TOS in these formulae.

In the production of protein hydrolysates, the food enzyme is added with or without other peptidases to a variety of partially purified proteins from plant (e.g. legumes and cereals) and animal (e.g. meat, collagen) materials during hydrolysis.[44] The hydrolysis can reduce the bitterness of the protein hydrolysates. The food enzyme–TOS remains in the final hydrolysates, which are added to a variety of final foods (e.g. soups, bouillons, snacks, dressings).

In the production of soy sauce, the food enzyme is added to the fermented soybeans.[45] The hydrolysis increases the yield and the flavour intensity of the sauce.[46] The food enzyme–TOS remains in the soy sauce.

In the production of brewed products, the food enzyme is added to cereals grist at the beginning of the mashing.[47] The hydrolysis by leucyl aminopeptidase releases free amino acids as fermentable nitrogen sources.[48] The food enzyme–TOS remains in the beer.

In the production of baked products, the food enzyme is added to flour during the preparation of dough or batter.[49] The leucyl aminopeptidase is used to weaken the gluten structures and consequently to improve the rheology of the dough.[50] The food enzyme–TOS remains in the baked products.

[39]Technical dossier/p. 59.
[40]Technical dossier/Additional information, 23 March 2023/Annex 8/p. 2.
[41]Technical dossier/Additional information, 23 March 2023/Annex 8/p. 3.
[42]Technical dossier/Additional information, 23 March 2023/Annex 8/p. 5–6.
[43]Technical dossier/Additional information, 23 March 2023/Answer 13.
[44]Technical dossier/Additional information, 23 March 2023/Annex 8/p. 8.
[45]Technical dossier/p. 90.
[46]Technical dossier/p. 89.
[47]Technical dossier/p. 91.
[48]Technical dossier/p. 91.
[49]Technical dossier/p. 94.
[50]Technical dossier/p. 93.

In the processing of yeast and yeast products, the food enzyme is added to yeast to obtain yeast extract.[51] The hydrolysis intensifies the flavour of yeast extracts that 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 processes, it is expected that the food enzyme is inactivated in all food manufacturing processes listed in Table 2.

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 0.577 mg TOS/kg bw per day in infants at the 95th percentile.

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)	0.029–0.263 (12)	0.058–0.162 (15)	0.015–0.119 (19)	0.005–0.071 (21)	0.029–0.099 (22)	0.027–0.062 (23)
Min–max 95th percentile (number of surveys)	0.093–0.577 (11)	0.137–0.322 (14)	0.036–0.232 (19)	0.011–0.144 (20)	0.072–0.310 (22)	0.063–0.154 (22)

Abbreviation: TOS, total organic solids.

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	
Selection of broad FoodEx categories for the exposure assessment	+
Exposure to food enzyme–TOS always calculated based on the recommended maximum use level	+
In the absence of analytical data to demonstrate the removal of the food enzyme–TOS in infant formulae, follow-on formulae and foods for special medical purposes,[52] these highly regulated formulae were included in the calculation.	+/–
Use of recipe fractions to disaggregate FoodEx categories	+/–
Use of technical factors in the exposure model	+/–

+: Uncertainty with potential to cause overestimation of exposure.

–: Uncertainty with potential to cause underestimation of exposure.

Abbreviation: TOS, total organic solids.

[51]Technical dossier/Additional information, 23 March 2023/Annex 8/p. 9.

[52]Technical dossier/Additional information, 23 March 2023/Answer to question 13.

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 (440 mg TOS/kg bw per day) from the 90-day rat study with the derived exposure estimates of 0.005–0.263 mg TOS/kg bw per day at the mean and from 0.011–0.577 mg TOS/kg bw per day at the 95th percentile resulted in a margin of exposure (MoE) of at least 763.

4 | CONCLUSIONS

Based on the data provided and the derived margin of exposure, the Panel concluded that the food enzyme leucyl aminopeptidase produced with the non-genetically modified *A. oryzae* strain NZYM-EX does not give rise to safety concerns under the intended conditions of use.

5 | DOCUMENTATION AS PROVIDED TO EFSA

Technical dossier `Peptidase produced by a strain of *Aspergillus oryzae* (strain NZYM-EX). 11 February 2015. Submitted by Novozymes A/S.

Additional information. 23 March 2023. Submitted by Novozymes A/S.

ABBREVIATIONS

[redacted]	[redacted]
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
[redacted]	[redacted]
EINECS	European Inventory of Existing Commercial Chemical Substances
EMDI	enzyme-modified dairy ingredients
FAO	Food and Agricultural Organization of the United Nations
FoodEx	food description and classification system
GLP	Good Laboratory Practice
GM	genetically modified
GMO	genetically modified organism
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kDa	kiloDalton
LAPU	leucyl aminopeptidase unit
LoD	limit of detection
LoQ	limit of quantification
MCH	mean cell haemoglobin
MCV	mean cell volume
MNBN	bi-nucleated cells with micronuclei
MoE	margin of exposure
N	neutrophil counts
NA	not analysed
NOAEL	no observed adverse effect level
non-GM	non-genetically modified
OECD	Organisation for Economic Co-operation and Development
PT	prothrombin time
RM	raw material
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).