Adopted: 13 March 2024

DOI: 10.2903/j.efsa.2024.8717

SCIENTIFIC OPINION

efsa JOURNAL

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

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Abstract

The food enzyme leucyl aminopeptidase (EC 3.4.11.1) is produced with the genetically modified *Aspergillus oryzae* strain NZYM-BU by Novozymes A/S. The genetic modifications do not give rise to safety concerns. The food enzyme is free from viable cells of the production organism and its DNA. It is intended to be used in five food manufacturing processes. Dietary exposure to the food enzyme TOS was estimated to be up to 1.508 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 4,928 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 3,268. A search for the 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 the food enzyme does not give rise to safety concerns under the intended conditions of use.

KEYWORDS

A. oryzae, EC 3.4.11.1, Food enzyme, genetically modified microorganism, leucine aminopeptidase, leucyl aminopeptidase

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; and
- its use does not mislead the consumer.

All food enzymes currently on the EU 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.

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 on food enzymes.

An application has been introduced by the applicant "Novozymes A/S" for the authorisation of the food enzyme Leucyl aminopeptidase from a genetically modified strain of *Aspergillus oryzae* (strain NZYM-BU).

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 application falls within the scope of the food enzyme Regulation and contains all the elements required under Chapter II of that Regulation.

1.1.2 | Terms of Reference

In accordance with Article 29(1)(a) of Regulation (EC) No 178/2002, the European Commission requests the European Food Safety Authority to carry out the safety assessment on the following food enzyme: Leucyl aminopeptidase from a genetically modified strain of *Aspergillus oryzae* (strain NZYM-BU) in accordance with Regulation (EC) No 1331/2008 establishing a common authorisation procedure for food additives, food enzymes and flavourings.

2 | DATA AND METHODOLOGIES

2.1 | Data

The applicant has submitted a dossier in support of the application for the authorisation of the food enzyme leucyl aminopeptidase from a genetically modified *Aspergillus oryzae* (strain NZYM-BU). The dossier was updated on 30 April 2021.

Additional information was requested from the applicant during the assessment process on 20 October 2021 and received on 17 December 2021 (see 'Documentation provided to EFSA').

[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.

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), the 'Statement on characterisation of microorganisms used for the production of food enzymes' (EFSA CEP Panel, 2019) and following the relevant existing guidance documents of EFSA Scientific Committees.

The 'Guidance on the submission of a dossier on food enzymes for safety evaluation' (EFSA, 2009) and the 'Food manufacturing processes and technical data used in the exposure assessment of food enzymes' (EFSA CEP Panel, 2023) have been followed for the evaluation of the application.

3 | ASSESSMENT

IUBMB nomenclature	Leucyl aminopeptidase
Systematic name	–
Synonyms	Leucine aminopeptidase; peptidase S; cytosol aminopeptidase
IUBMB No	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, resulting in the release of amino acids. The food enzyme under assessment is intended to be used in five food manufacturing processes as described in the EFSA guidance (EFSA CEP Panel, 2023): processing of cereals and other grains for the production of (1) baked products and (2) brewed products; (3) processing of meat and fish products for the production of protein hydrolysates; (4) processing of plant- and fungal-derived products for the production of protein hydrolysates; and (5) processing of yeast and yeast products.

3.1 | Source of the food enzyme

The leucyl aminopeptidase is produced with the genetically modified filamentous fungus *A. oryzae* strain NZYM-BU, which is deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany), with the deposit number [redacted].[3]

The production strain was identified as *A. oryzae* by [redacted].[4]

3.1.1 | Characteristics of the parental and recipient microorganisms

The parental microorganism is [redacted].

The recipient strain was [redacted].[5]

[redacted].[6]

[3]Technical dossier/2nd submission/Annex A2.

[4]Technical dossier/2nd submission/Annex A1.

[5]Technical dossier/2nd submission/Annexes C1-C9.

[6]Technical dossier/2nd submission/Annex D2.

3.1.2 | Characteristics of introduced sequences

The sequence encoding the leucyl aminopeptidase is [redacted].[7]

3.1.3 | Description of the genetic modification process

The purpose of genetic modification was to increase the production of leucyl aminopeptidase, [redacted].

- [redacted]
- [redacted]

[redacted][8]

3.1.4 | Safety aspects of the genetic modification

The technical dossier contained all necessary information on the recipient microorganism, the donor organism and the genetic modification process.

The production strain *A. oryzae* NZYM-BU differs from the recipient strain in its capacity to express high levels of leucyl aminopeptidase [redacted].

[redacted][9]

No issues of concern arising from the genetic modifications were identified by the Panel.

3.2 | Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004,[10] with food safety procedures based on Hazard Analysis and Critical Control points, and in accordance with current Good Manufacturing Practice.[11]

The production strain is grown as a pure culture using a typical industrial medium in a submerged, [redacted] fermentation system with conventional process controls in place. After the completion of the fermentation, the solid biomass is removed from the fermentation broth by filtration, leaving a filtrate containing the food enzyme. 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. The applicant provided

[7]Technical dossier/2nd submission/Annex B1.
[8]Technical dossier/2nd submission/Annex D1.
[9]Technical dossier/2nd submission/Annex D2.
[10]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.
[11]Technical Dossier/2nd submission/p. 48.

information on the identity of the substances used to control the fermentation and 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 leucyl aminopeptidase is a single polypeptide chain of [redacted] amino acids.[13] The molecular mass of the mature protein, calculated from the amino acid sequence, is around [redacted] kDa.[14] The food enzyme was analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis. A consistent protein pattern was observed across all batches. The gels showed a major protein band corresponding to a molecular mass of about 66 kDa, assigned to the enzyme.[15] The food enzyme was tested for α-amylase, lipase and protease activities, and none were detected.[16]

The in-house determination of leucyl aminopeptidase 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 50°C (pH 8) and a pH optimum around pH 9.0 (30°C).[18] After 15 min pre-incubation at various temperatures, the leucyl aminopeptidase activity was found to be stable up to 50°C but decreased at higher temperatures. No residual activity was detected after pre-incubation at 70°C.[19]

3.3.2 | Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches intended for commercialisation and the two batches produced for the toxicological tests (Table 1).[20] The mean total organic solids (TOS) of the three batches intended for commercialisation was 10.6% and the mean enzyme activity/TOS ratio was 18.4 LAPU/mg TOS.[21]

TABLE 1 Composition of the food enzyme.

		Batches				
Parameters	**Unit**	**1**	**2**	**3**	**4[a]**	**5[b]**
Leucyl aminopeptidase activity	LAPU/g[c]	1,990	1,910	1,910	1,166	2,801
Protein	%	7.4	7.5	7.4	5.0	12.5
Ash	%	0.3	0.4	0.3	1.0	1.7
Water	%	87.7	89.3	90.2	89.1	75.8
Total organic solids (TOS)[d]	%	12.0	10.3	9.5	9.9	22.5
Activity/TOS ratio	LAPU/mg TOS	16.6	18.5	20.1	11.8	12.5

[a]Batch used for the genotoxicity studies (an Ames test, two in vitro micronucleus assays, an in vitro MultiFlow™ DNA Damage Test and a combined in vivo micronucleus assay and comet assay).

[b]Batch used for the repeated dose 90-day oral toxicity study in rats.

[c]LAPU: Leucine Aminopeptidase Unit (see Section 3.3.1).

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

[12]Technical Dossier/2nd submission/p. 51/Annex 6.

[13]Technical dossier/2nd submission/p. 35/Annex 1.

[14]Technical dossier/2nd submission/p. 35/Annex 1.

[15]Technical dossier/2nd submission/p. 37.

[16]Technical dossier/2nd submission/p. 42/Annex 3.02, Annex 3.03, Annex 3.04.

[17]Technical dossier/2nd submission/p. 40/Annex 3.01.

[18]Technical dossier/2nd submission/p. 11, p. 78/Annex 9.

[19]Technical dossier/2nd submission/p. 78, p. 79/Annex 9.

[20]Technical dossier/2nd submission/p. 36, p. 64/Annex 7.01–7.07, Annex 10; Additional data December 2021/Annex 1.

[21]Technical dossier/2nd submission/p. 36/Annex 10; Additional data December 2021/Annex 1.

3.3.3 | Purity

The lead content in the three commercial batches and in the two batches used for toxicological studies was below 0.5 mg/kg,[22] 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, the concentrations of arsenic, cadmium and mercury were below the limit of detection (LoD) of the employed methods.[23,24]

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

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, 3-nitropropionic acid, cyclopiazonic acid and kojic acid was examined in the three commercial and the two toxicological food enzyme batches.[27,28] All were below the LoD of the applied methods. Adverse effects caused by the possible presence of other secondary metabolites are 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 and DNA of the production strain

The absence of viable cells of the production strain in the food enzyme was demonstrated [redacted]. No colonies were produced. [redacted].[29]

The absence of recombinant DNA in the food enzyme was demonstrated [redacted].[30]

3.4 | Toxicological data

A battery of toxicological tests has been provided, including an in vitro bacterial gene mutation assay, two in vitro mammalian cell micronucleus assays, an in vitro MultiFlow™ assay including caspase activity quantification, a combined in vivo micronucleus and comet assay, and a repeated dose 90-day oral toxicity study in rats. The batches 4 and 5 (Table 1) used in these studies have similar protein patterns as the batches used for commercialisation and lower activity/TOS values, and thus were considered suitable as test items.

3.4.1 | Genotoxicity

3.4.1.1 | *In vitro genotoxicity tests*

3.4.1.1.1 | *Bacterial reverse mutation test*

A bacterial reverse mutation assay (Ames test) was performed according to the Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 (OECD, 1997a, 1997b) and following Good Laboratory Practice (GLP).[31]

Four strains of Salmonella Typhimurium (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* WP2uvrA (pKM101) were used in the presence or absence of metabolic activation, applying the 'treat and plate' assay. Two separate experiments were carried out, using six concentrations of the food enzyme (16, 50, 160, 500, 1,600 and 5,000 µg TOS/mL in the first experiment and 160, 300, 625, 1,250, 2,500 and 5,000 µg TOS/mL in the second experiment).

[22]Technical dossier/2nd submission/p. 38, p. 64/Annex 10; Additional data December 2021/Annex 1.

[23]LoDs: Pb = 0.5 mg/kg; As = 0.3 mg/kg; Cd, Hg = 0.05 mg/kg each.

[24]Technical dossier/2nd submission/p. 38, p. 64/Annex 10; Additional data December 2021/Annex 1.

[25]Technical dossier/2nd submission/p. 39, p. 64/Annex 10; Additional data/Annex 1.

[26]Technical dossier/2nd submission/p. 38, p. 64/Annex 10; Additional data December 2021/Annex 1.

[27]Technical dossier/2nd submission/p. 38, p. 64/Annex 10; Additional data December 2021/Annex 1.

[28]LoDs: aflatoxin B1 = 0.0003 mg/kg; 3-nitropropionic acid = 0.29/0.3/0.35/0.4835/1.5762 mg/kg (LOD is matrix-dependent); cyclopiazonic acid = 0.003 mg/kg; kojic acid = 0.024/0.0277/0.0239/0.028/0.033 mg/kg (LOD is matrix-dependent).

[29]Technical dossier/2nd submission/Annex E1; Additional data December 2021/Annex E1.

[30]Technical dossier/2nd submission/Annex E2; Additional data December 2021.

[31]Technical dossier/Annex 7.02.

No cytotoxicity was observed at any concentration level 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 employed in this study.

3.4.1.1.2 | *In vitro mammalian cell micronucleus assay*

The in vitro mammalian cell micronucleus test was carried out according to the OECD Guideline 487 (OECD, 2016a, 2016b) and following GLP.[32] The cell cultures were treated with the food enzyme with or without metabolic activation (S9-mix).

Two separate experiments were performed in duplicate cultures of human peripheral whole blood lymphocytes. In the first experiment, the cells were exposed to the test substance (3,000, 4,000 and 5,000 µg TOS/mL) in the short-term treatment (3 h of exposure and 21 h of recovery period) in the presence or absence of S9-mix. Additionally, the food enzyme was tested at 400, 500, 600 and 800 µg TOS/mL in the long-term treatment (24 h of exposure with 24 h of recovery period) without S9-mix. A second experiment was conducted applying the long-term 24-h treatment without S9-mix with 24 h of recovery time, in which the cells were exposed to 400, 500, 600 and 800 µg TOS/mL, and treated for 24 h without recovery time in which the cells were exposed to 500, 700, 1,000 and 1,500 µg TOS/mL.

In the first experiment, a decrease in the Replication Index (RI), as a measure of cytotoxicity, was observed in the continuous 24-hs treatment (6%, 23%, 35% and 52% at 400, 500, 600 and 800 µg TOS/mL, respectively). After the short-term treatment in the presence or absence of S9-mix, a weak but statistically significant increase in the frequency of binucleated cells with micronuclei (MNBN), poorly reproduced between replicate cultures, was recorded for some concentrations. After 24-h treatment in the absence of S9-mix, a statistically significant, concentration-dependent increase in the frequency of MNBN was observed for all concentrations analysed (1.85%, 2.05%, 2.20% and 3.45% vs. 0.65% in the control; $p \leq 0.001$). In the second experiment, a decrease in RI was observed in the long-term treatment (24 h + 24 h recovery time) (23%, 29%, 37% and 41% at 400, 500, 600 and 800 µg TOS/mL, respectively) and in the long-term treatment (24 h without recovery time) (12%, 23%, 28% and 30% at 500, 700, 1,000 and 1,500 µg TOS/mL, respectively). The 24-h treatment with 24-h recovery time resulted in a statistically significant increase in the frequencies of MNBN cells at all the concentrations analysed (5.75%, 5.25%, 7.55% and 7.10% vs. 0.30% in the control; $p \leq 0.001$). The 24-h treatment without recovery time resulted in a statistically significant increase in the frequency of MNBN cells only at 500 µg TOS/mL (1.5% vs. 0.35% in the control; $p \leq 0.001$).

To determine the mechanism of micronuclei formation in this treatment, an *in situ* hybridisation (FISH) with pan-centromeric DNA probes was conducted applying an extended 24-h treatment with 24 h of recovery time without S9-mix at 500 and 600 µg TOS/mL. The mean percentages of centromere negative micronuclei (C-) observed for the concentrations analysed (91% and 89%, respectively) allowed the conclusion that the micronuclei were induced via a clastogenic mechanism.

The Panel concluded that, under the test conditions employed in this study, the food enzyme leucyl aminopeptidase induced an increase in the frequency of MNBNs in cultured human peripheral blood lymphocytes with a clastogenic mode of action.

3.4.1.1.3 | *In vitro mammalian cell micronucleus assay*

A second in vitro mammalian cell micronucleus test was carried out according to the OECD Guideline 487 (OECD, 2016a, 2016b, 2016c) and following GLP.[33] The cell cultures were treated with the food enzyme in the absence of metabolic activation (S9-mix). In addition to the batch 4 described in Table 1, a heat-treated version of batch 4 and the purified enzyme were used as test items.

Human peripheral whole blood cells were exposed to the test substances for 24 h without S9-mix with 24 h of recovery time. The food enzyme was tested at 300, 400, 600 and 800 µg TOS/mL. The purified aminopeptidase was tested at 7.443, 15.19 and 31.0 LAPU/mL. The heat-treated leucyl aminopeptidase was tested at 800, 1,000 and 1,200 µg TOS/mL. No cytotoxicity was observed at any concentration of these test substances. After extended treatment with the food enzyme aminopeptidase, a statistically significant increase in MNBN was observed at all concentrations analysed (0.65% [$p \leq 0.05$], 1.45%, 1.20%, 1.75% [$p \leq 0.001$] vs. 0.20% in the control). After extended treatment (24 h + 24 h of recovery time) with purified and heat-treated aminopeptidase the frequency of MNBN was comparable to the negative controls at all concentrations tested.

The Panel concluded that, under the test conditions employed in this study, the food enzyme leucyl aminopeptidase induced an increase in the frequency of MNBNs in cultured human peripheral blood lymphocytes.

3.4.1.1.4 | *In vitro MultiFlow™ DNA Damage Test*

To investigate the mechanism of micronucleus induction by the aminopeptidase, MultiFlow™ DNA Damage Test was performed in cultured human lymphoblastoid TK6 cells. Caspase-3/7 Activity Quantification was included in this study to further investigate the effect of confounding factor (apoptosis) for micronucleus assessment.[34] The TK6 cells were exposed

[32]Technical dossier/Annex 7.03.

[33]Technical dossier/Annex 7.04.

[34]Technical dossier/Annex 7.05.

to the food enzyme at a range of concentrations from 5.699 to 5,000 μg TOS/mL for 24 h in the absence of the S9-mix. Potential genotoxic mode of action was analysed by flow cytometric analysis and evaluated by a combination of global evaluation factors (GEFs) and algorithm-based machine learning models. The food enzyme leucyl aminopeptidase demonstrated genotoxicity with a clastogenic signature, as indicated by the machine learning models. No increases in nuclear p53, γH2AX, phospho-Histone H3 or polyploidy compared to the concurrent vehicle control were observed that met the GEF criteria for a clastogenic or aneugenic signature. However, a concentration-related increase in nuclear p53 and γH2AX at both the 4- and 24-h sampling times were observed, which may be indicative of a clastogenic response. Analysis of caspase-3/7 activity showed a clear concentration-related increase in activity, indicating that the food enzyme induced apoptosis in this test system. However, because of the clear clastogenic signature from MultiFlow™ analysis, this apoptotic response was not considered to have been a confounding factor in the genotoxic conclusion in the previously performed in vitro mammalian cell micronucleus study.

3.4.1.2 | *In vivo genotoxicity tests*

3.4.1.2.1 | *Combined in vivo micronucleus assay and comet assay*

The genotoxic potential of the food enzyme aminopeptidase (batch 4) was assessed in vivo using the combined in vivo micronucleus assay in the bone marrow and a comet assay in the liver, duodenum and glandular stomach of Crl:CD(SD) rats. The study was carried out according to the OECD Test Guideline 474 (OECD, 2016a, 2016b), the OECD Test Guideline 489 (OECD, 2016a, 2016b) and following GLP.[35]

Since there were no differences in toxicity between sexes in the preliminary toxicity test, only males were used in the main study. Five male rats were dosed once daily by oral gavage with the food enzyme at 500, 1,000 and 2,000 mg TOS/kg body weight (bw) per day for three consecutive days with a 24-h interval after the first dose and a 21-h interval after the second dose (approximately 3 h before sampling). Three animals of the positive control group for micronucleus assay were dosed with 20-mg cyclophosphamide (CP)/kg bw. Three animals of the positive control group for the Comet assay were administered a single dose of 200 mg ethyl methane sulfonate (EMS)/kg bw per day, approximately 3 h before sampling.

No mortality and statistically significant differences in bw were observed.

Three hours after the final administration, the bone marrow, liver, duodenum and glandular stomach samples were collected, and slides were prepared.

3.4.1.2.2 | *Micronucleus assay*

No decrease in the ratio of polychromatic erythrocytes (PCEs) to normochromatic erythrocytes (NCEs) compared to the concurrent vehicle control was recorded in treated animals. Rats treated with the food enzyme exhibited mean frequencies of micronucleated PCEs (MNPCE) that were not statistically different from those observed in the concurrent vehicle control. The food enzyme did not induce micronuclei in the bone marrow when tested up to 2,000 mg TOS/kg bw per day under the experimental conditions employed. However, these results were considered of limited relevance due to the lack of demonstration of bone marrow exposure.

3.4.1.2.3 | *Comet assay*

Increases in the hedgehog cells were observed in all tissues analysed in high-dose animals. No statistically significant increase in the median % tail intensity values for animals treated with the food enzyme was observed in the liver of any treated group compared with the concurrent vehicle control group. A weak, but statistically significant, increase in the median % tail intensity values was observed in the duodenum and glandular stomach of high-dose males compared to the extremely low values of the concurrent vehicle control group; however, the values were within the 95 confidence limits of the current historical control range. The increases in the % tail intensity observed in the duodenum and glandular stomach of animals treated with aminopeptidase at 2,000 mg/kg bw per day were considered secondary to treatment-related cell toxicity, as indicated by the concurrent increase in the hedgehog cells (+14.8% in duodenum and +7% in glandular stomach compared to 0% in the concurrent controls and in the groups treated with lower doses of food enzyme). Therefore, the Panel considered them as not biologically relevant.

The food enzyme leucyl aminopeptidase did not induce DNA damage in the liver, duodenum and glandular stomach of the rats administered via oral gavage, as analysed by the Comet assay. The negative results obtained in the Comet assay, including at the first site of contact (duodenum, glandular stomach), removed the concern for clastogenicity.

3.4.1.3 | *Conclusions on genotoxicity*

The food enzyme was assessed in a battery of genotoxicity studies. The bacterial reverse mutation assay (Ames test) showed clearly negative results both in the absence and presence of metabolic activation (S9-mix). In the in vitro micronucleus test,

[35]Technical dossier/Annex 7.06.

induced increases in micronuclei were seen in two independent studies in cultured human peripheral blood lymphocytes following extended treatment (24 h of treatment in the presence or absence of a 24-h recovery period) in the absence of S9-mix. Mechanistic analysis with the use of FISH with pan-centromeric DNA probes demonstrated that micronuclei were generated via clastogenicity. The results of the MicroFlow assay confirmed the clastogenic mode of action of the food enzyme. The Comet assay did not show any induction of DNA strand breaks at the first sites of contact (duodenum and glandular stomach).

On the basis of the in vitro and in vivo studies, the Panel concluded that there is no concern for genotoxicity for the food enzyme leucyl aminopeptidase.

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, 2018) and following GLP.[36] Groups of 10 male and 10 female Crl:WI(Han) rats received by gavage 2,464, 3,696 or 4,928 mg TOS/kg bw per day (for all groups, the total daily dose volume was administered on two occasions). Controls received the vehicle (reverse osmosis water).

No mortality was observed.

Bw gain was statistically significantly increased on Days 64–71 in mid- and high-dose males (+67% and +61%, respectively) and in mid-dose females (+2100%) and decreased on Days 85–91 in low-dose females (–336%). The Panel considered the changes as not toxicologically relevant, as they were only recorded sporadically, there was no dose response, and the changes were without a statistically significant effect on the final bw and total bw gain.

In the functional observations, statistically significant increases were observed in the first 10-min interval in basic movements in low- and high-dose males (+25% and +18%, respectively) and in low-dose females (+21%), in fine movements in low-dose males (+25%) and in low-dose females (+30%), in total ambulations in low- and high-dose males (+25 and +32%, respectively) and in high-dose females (+18%), and in total distance travelled in low- and high-dose males (+26% and +35%, respectively) and in high-dose females (+19%). The Panel considered the changes as not toxicologically relevant, as they were only recorded in the first recording interval and there was no dose-response relationship (basic movements, fine movements, total ambulations in males, total distance travelled in males).

Haematological investigation revealed a statistically significantly increase in activated partial thromboplastin time in low-dose males (+8%). The Panel considered the change as not toxicologically relevant, as it was only recorded in one sex, there was no dose–response relationship, there were no changes in other coagulation parameters (prothrombin time and fibrinogen) and the change was within the historical control values.

Clinical chemistry investigation in males revealed statistically significantly lower aspartate aminotransferase (AST, –17%; –21%) and lower phosphorus levels (–14%; –14%) in low- and high-dose groups; lower creatine phosphokinase (CPK) activity in all treated males (–41%, –36%, –43%); and lower sodium levels in mid- and high-dose groups (–1.4%; –1.4%). In females, statistically significantly higher chloride levels in low- and mid-dose groups (+1%; +2%); higher urea (+18%) and blood urea nitrogen concentration (+18%) in high-dose group; lower CPK activity (–36%) in high-dose group, lower total cholesterol (Tchol, –26%), lower high density lipoprotein (HDL) levels (–28%), lower total protein (–6.7%) and lower albumin levels (–8.9%) in low-dose group; and higher glucose levels in mid-dose group (+13%) were reported. The Panel considered the changes as not toxicologically relevant, as they were only recorded in one sex (all parameters except for CPK), there was no dose–response relationship (AST, phosphorus, chloride, Tchol, HDL, total protein, albumin and glucose), the changes were small (AST, CPK, sodium, chloride, total protein and albumin) and all the changes were within the relevant historical control values.

Statistically significant changes in organ weight detected were increases in kidney to brain weight ratio in mid- and high-dose males (+16%; +15%) and in kidney to body weight ratio in high-dose males (+8%), in heart to body weight ratio in low- and mid-dose males (+9%; +10%), in absolute ovary weight (+30%), ovary to bodyweight ratio (+33%) and ovary to brain weight ratio (+29%) in high-dose females, a decrease in absolute thyroid/parathyroid weight (–24%),thyroid/parathyroid to body weight ratio (–23%) and thyroid/parathyroid to brain weight ratio (–22%) in mid-dose females. The Panel considered the changes as not toxicologically relevant, as they were only recorded in one sex (all parameters), there was no dose–response relationship (heart, thyroid/parathyroid), the changes were small (kidney and heart weights relative to bw), there were no histopathological changes in the organs and there were no supportive functional or morphological adverse correlates (for ovary: in oestrus cycle, in weight and morphology of uterus, for thyroid: no changes in thyroid hormones).

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

The Panel identified the no observed adverse effect level (NOAEL) of 4,928 mg TOS/kg bw per day, the highest dose tested.

3.4.3 | Allergenicity

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

[36]Technical dossier/Annex 7.07.

The potential allergenicity of the leucyl aminopeptidase produced with *A. oryzae* strain NZYM-BU 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.[37]

No information was available on oral and respiratory sensitisation or elicitation reactions of this leucyl aminopeptidases. In addition, no allergic reactions after oral exposure to leucyl aminopeptidases have been reported in the literature.[38]

[redacted], a known source of allergens, is present in the media fed to the microorganisms. However, during the fermentation process, this product will be degraded and utilised by the microorganisms for cell growth, cell maintenance and the 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 this source are present in the food enzyme.

The Panel considered that the risk of allergic reactions upon dietary exposure 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.

TABLE 2 Intended uses and recommended use levels of the food enzyme as provided by the applicant.[39]

Food manufacturing process[a]	Raw material (RM)	Recommended use level (mg TOS/kg RM)[b,c]
Processing of cereals and other grains		
• Production of baked products	Flour	2.7–**10.9**
• Production of brewed products	Cereals (malted or not)	2.7–**27.2**
Processing of meat and fish products		
• Production of protein hydrolysates from meat and fish proteins	Meat and fish protein	1,630.4–**2,391.3**
Processing of plant- and fungal-derived products		
• Production of protein hydrolysates from plants and fungi	Plant protein	1,630.4–**2,391.3**
Processing of yeast and yeast products	Yeast extract	543.5–**815.2**

[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]Based on mean activity/mg TOS of 18.4 LAPU/mg TOS.

[c]Numbers in bold were used for calculation.

In the production of baked products, the food enzyme is added to flour during the preparation of dough or batter.[40] The leucyl aminopeptidase is used to weaken the gluten structures and consequently to improve the rheology of the dough.[41] The food enzyme–TOS remains in the baked products.

In the production of brewed products, the food enzyme is added to cereal grist at the beginning of mashing.[42] The hydrolysis by leucyl aminopeptidase releases free amino acids as nitrogen sources for the yeast.[43] The food enzyme–TOS remains in the beer.

[37]Technical dossier/2nd submission/pp. 69–72/Annex 8.

[38]Additional data December 2021/Annex 2.

[39]Additional data December 2021/p. 8.

[40]Technical dossier/p. 84.

[41]Technical dossier/p. 83.

[42]Technical dossier/p. 85.

[43]Technical dossier/pp. 84–85.

In the production of protein hydrolysates, the food enzyme is added with other peptidases to a variety of partially purified proteins from plants (e.g. soybean meal or wheat gluten) and animals (e.g. meat and fish trimmings).[44] The action of the leucyl aminopeptidase results in extensive protein hydrolysis that 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 and dressings).

In the processing of yeast and yeast products, the food enzyme is added to yeast extract.[45] The hydrolysis intensifies the flavour of yeast extracts, which are then added in small amounts to various savoury foods, ready-to-eat vegetable meals, soups, bouillons and sauces.[46] 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 was expected that the food enzyme is inactivated in all the 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 bw. This was done for all individuals across all surveys, resulting in the 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 one 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 1.508 mg TOS/kg bw per day in children 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)	0.026–0.384 (12)	0.103–0.498 (15)	0.147–0.497 (19)	0.044–0.319 (21)	0.038–0.212 (22)	0.022–0.205 (23)
Min–max 95th percentile (number of surveys)	0.046–0.948 (11)	0.327–1.500 (14)	0.485–1.508 (19)	0.133–1.188 (20)	0.116–0.770 (22)	0.052–0.710 (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.

[44] Additional data December 2021/Answer to question 5.

[45] Technical dossier/p. 82.

[46] Additional data December 2021/Answer to question 6.

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	+
For yeast processing, although the food enzyme is not used to treat yeast cell wall, the food categories chosen for calculation cover also those containing mannoproteins resulting from the treatment of yeast cell wall	+
Use of recipe fractions to disaggregate FoodEx categories	+/–
Use of technical factors in the exposure model	+/–

Abbreviations: +, uncertainty with potential to cause the overestimation of exposure; –, uncertainty with potential to cause the 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

A comparison of the NOAEL (4,928 mg TOS/kg bw per day) from the 90-day study in rats with the derived exposure estimates of 0.022–0.498 mg TOS/kg bw per day at the mean and of 0.046–1.508 mg TOS/kg bw per day at the 95th percentile resulted in a margin of exposure (MoE) of at least 3,268.

4 | CONCLUSIONS

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

The EFSA Panel on Food Contact Materials, Enzymes and Processing Aids Panel considered the food enzyme free from viable cells of the production organism and recombinant DNA.

5 | DOCUMENTATION AS PROVIDED TO EFSA

Leucyl aminopeptidase produced by a genetically modified strain of *Aspergillus oryzae* (strain NZYM-BU). June 2021. Submitted by Novozymes A/S.

Additional data. December 2021. Submitted by Novozymes A/S.

ABBREVIATIONS

bw	body weight
CAS	Chemical Abstracts Service
EINECS	European Inventory of Existing Commercial Chemical Substances
FISH	Fluorescence *in situ* hybridisation
GLP	Good Laboratory Practice
IUBMB	International Union of Biochemistry and Molecular Biology
LoD	limit of detection
MoE	margin of exposure
OECD	Organisation for Economic Co-operation and Development
PCR	polymerase chain reaction
TOS	total organic solids
WHO	World Health Organization

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.

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European Commission

QUESTION NUMBER
EFSA-Q-2021-00224

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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.

How to cite this article: EFSA CEP Panel (EFSA Panel on Food Contact Materials, Enzymes and Processing Aids), Lambré, C., Barat Baviera, J. M., Bolognesi, C., Cocconcelli, P. S., Crebelli, R., Gott, D. M., Grob, K., Lampi, E., Mengelers, M., Mortensen, A., Rivière, G., Steffensen, I.-L., Tlustos, C., Van Loveren, H., Vernis, L., Zorn, H., Andryszkiewicz, M., Boinowitz, E., … Chesson, A. (2024). Safety evaluation of the food enzyme leucyl aminopeptidase from the genetically modified *Aspergillus oryzae* strain NZYM-BU. *EFSA Journal*, *22*(4), e8717. https://doi.org/10.2903/j.efsa.2024.8717

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, 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, 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*, 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*, 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*, 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).