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SCIENTIFIC OPINION

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Safety evaluation of the food enzyme carboxypeptidase D from the genetically modified *Aspergillus oryzae* strain NZYM-MK

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Abstract

The food enzyme carboxypeptidase D (EC 3.4.16.6) is produced with the genetically modified *Aspergillus oryzae* strain NZYM-MK by Novozymes A/S. It is free from viable cells of the production organism and its DNA. The genetic modifications do not give rise to safety concerns. The food enzyme is intended to be used in five food manufacturing processes. Dietary exposure to the food enzyme-total organic solids (TOS) was estimated to be up to 0.908 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 2220 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 2445. A search for the similarity of the amino acid sequence of the food enzyme to known allergens was made and two matches were found, one with a food allergen (wheat). The Panel considered that a risk of allergic reactions upon dietary exposure to this food enzyme, particularly in individuals sensitised to wheat, cannot be excluded, but will not exceed that of wheat consumption. 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, carboxypeptidase D, cereal serine carboxypeptidase II, EC 3.4.16.6, food enzyme, genetically modified microorganism

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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.

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 Carboxypeptidase D from a genetically modified strain of *Aspergillus oryzae* (strain NZYM-MK).

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: Carboxypeptidase D from a genetically modified strain of *Aspergillus oryzae* (strain NZYM-MK) 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 authorisation of the food enzyme Carboxypeptidase D from the genetically modified *Aspergillus oryzae* strain NZYM-MK.

[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, 11.03.2011, pp. 15–24.

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').

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, 2009a) 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, 2009b) as well as the 'Statement on characterisation of microorganisms used for the production of food enzymes' (EFSA CEP Panel, 2019) have 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

IUBMB nomenclature	Carboxypeptidase D
Systematic name	–
Synonyms	Cereal serine carboxypeptidase II; carboxypeptidase KEX1
IUBMB No	EC 3.4.16.6
CAS No	153967-26-1
EINECS No	849-291-3

Carboxypeptidases D catalyse the hydrolysis of the peptide bond of C-terminal amino acid residues in proteins, with a preference for arginine and lysine, releasing peptides and free 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 carboxypeptidase D is produced with the genetically modified filamentous fungus *Aspergillus oryzae* strain NZYM-MK, which is deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany), with the deposit number [redacted].[4]

The production strain was identified as *A. oryzae* by [redacted].[5]

3.1.1 | Characteristics of the parental and recipient microorganisms

The parental microorganism is *A. oryzae* strain A1560.

The recipient strain was [redacted].

The genetic modifications were [redacted].[6]

[redacted]

[4]Technical dossier/2nd submission/ Annex 4 GMM dossier/Annex A2.

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

[6]Technical dossier/2nd submission/ Annex 4 GMM dossier/Annexes C1–C7.

[redacted][7].

3.1.2 | Characteristics of introduced sequences

The sequence encoding the carboxypeptidase D is [redacted].

[redacted][8].

3.1.3 | Description of the genetic modification process

The purpose of the genetic modifications was to increase the production of carboxypeptidase D, [redacted].

[redacted]

[redacted]

The production strain NZYM-MK contains [redacted][9], [redacted]

3.1.4 | Safety aspects of the genetic modification

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

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

[redacted][10].

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,[11] with food safety procedures based on Hazard Analysis and Critical Control Points, and in accordance with current good manufacturing practice.[12]

[7]Technical dossier/2nd submission/ Annex 4 GMM dossier/Annex D2.
[8]Technical dossier/2nd submission/Annex 4 GMM dossier/Annex B1.
[9]Technical dossier/2nd submission/ Annex 4 GMM dossier/Annex D1.
[10]Technical dossier/2nd submission/ Annex 4 GMM dossier/Annex D2.
[11]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.
[12]Technical dossier/p. 12.

The production strain is grown as a pure culture using a typical industrial medium in a [redacted] fermentation system with conventional process controls in place. After completion of the fermentation, the solid biomass is removed from the fermentation broth by filtration, leaving a supernatant 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 information on the identity of the substances used to control the fermentation and in the subsequent downstream processing of the food enzyme.[13]

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 carboxypeptidase D is a single polypeptide chain of [redacted] amino acids.[14] The molecular mass of the mature protein, calculated from the amino acid sequence, is [redacted] kDa.[15] The food enzyme was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A consistent protein pattern was observed across all batches. The gels showed the single major protein band migrating between the marker proteins of [redacted] in all batches, attributed to the mass of the glycosylated enzyme.[16] The food enzyme was tested for α-amylase, lipase and other serine-protease activities. Only α-amylase was detected.[17]

The in-house determination of carboxypeptidase D activity is based on the hydrolysis of the substrate *N*-(3-[2-furyl] acryloyl)-Ala-Lys (reaction conditions: pH 5.8, 37°C, 2 min). The enzymatic activity is proportional to the decrease in absorbance of the substrate at 340 nm. The enzyme activity is expressed in carboxypeptidase units A/g (CPDU(A)/g) and determined relative to an internal standard.[18]

The food enzyme has a temperature optimum around 45°C (pH 6) and a pH optimum around pH 5 (30°C).[19] Thermostability was tested after a pre-incubation of the food enzyme for 15 min at different temperatures (pH 6). Its activity was stable up to 45°C and then decreased sharply, with no residual activity after pre-incubation at 70°C.[20]

3.3.2 | Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches used for commercialisation and two batches produced for the toxicological tests (Table 1).[21] The mean total organic solids (TOS) of the three food enzyme batches for commercialisation was 12.1% and the mean enzyme activity/TOS ratio was 28.5 CPDU(A)/mg TOS.

TABLE 1 Composition of the food enzyme.

		Batches				
Parameters	**Unit**	**1**	**2**	**3**	**4**[a]	**5**[b]
Carboxypeptidase D activity	CPDU(A)/g[c]	3510	3250	3450	2370	5150
Protein	%	6.9	6.8	6.4	5.2	10.9
Ash	%	0.2	0.2	0.2	0.5	0.9
Water	%	85.6	89.4	88.1	89	78.6
Total organic solids (TOS)[d]	%	14.2	10.4	11.7	10.5	20.5
Activity/TOS ratio	CPDU(A)/mg TOS	24.7	31.3	29.5	22.6	25.1

[a]Batch used for Ames test and in vitro micronucleus study.
[b]Batch used for the repeated dose 90-day oral toxicity study in rats.
[c]CPDU(A): Carboxypeptidase Unit A (see Section 3.3.1)
[d]TOS calculated as 100% – % water – % ash.

[13]Technical dossier/pp. 49, 51 and Annex 6.
[14]Technical dossier/p. 33 and Annex 1.
[15]Technical dossier/p. 33 and Annex 1.
[16]Technical dossier/pp. 34–35.
[17]Technical dossier/p. 40 and Annexes 3.02–3.04.
[18]Technical dossier/p. 38 and Annex 3.01.
[19]Technical dossier/p. 39 and Annex 9.
[20]Technical dossier/Annex 9.
[21]Technical dossier pp. 34, 62, Annex 7, 10 and additional information 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 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). In addition, arsenic, cadmium and mercury concentrations were below the limits of detection (LoD) of the employed methods.[22,23]

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

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, cyclopiazonic acid, 3-nitropropionic acid and kojic acid was examined in the five food enzyme batches tested and all were below the LoD of the applied methods.[26,27] 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 in three independent batches analysed in triplicate. One gram of product was incubated in [redacted] of non-selective medium [redacted] for resuscitation. From this, [redacted]. No colonies were produced. A positive control was included.[28]

The absence of recombinant DNA in the food enzyme was demonstrated by PCR analysis of three batches in triplicate. No DNA was detected with primers that would [redacted], with an LoD of [redacted].[29]

3.4 | Toxicological data

A battery of toxicological tests, including a bacterial gene mutation assay (Ames test), an in vitro mammalian cell micronucleus test and a repeated dose 90-day oral toxicity study in rats, has been provided. The batches 4 and 5 (Table 1) used in these studies have similar compositions as the batches used for commercialisation and, thus, were considered suitable as test items.

3.4.1 | Genotoxicity

3.4.1.1 | *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, 1997) and following Good Laboratory Practice (GLP).[30]

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 (16, 50, 160, 500, 1600 and 5000 µg TOS/plate) in the first experiment and 160, 300, 625, 1250, 2500 and 5000 µg TOS/plate in the second experiment.

Toxic effects, evident as a reduction in the number of revertants or very thin background bacterial lawn, occurred in the second experiment in the absence of S9-mix in *S.* Typhimurium strain TA98 at 160 and 300 µg TOS/plate and in *S.* Typhimurium strain TA1535 at 1250 µg TOS/mL. Upon treatment with the food enzyme, there was no significant increase in revertant colony numbers above the control values in any strain with or without S9-mix. An exception was in the second experiment in *S.* Typhimurium strain TA98, in which a twofold increase in revertant colony numbers was recorded. To further investigate any potential mutagenic activity in this strain, a third experiment was performed using six concentrations of the food enzyme (160, 300, 625, 1250, 2500 and 5000 µg TOS/plate) in the presence of S9-mix. No cytotoxicity and no

[22]LoDs: Pb = 0.5 mg/kg; As = 0.3 mg/kg; Cd = 0.05 mg/kg; Hg = 0.05 mg/kg.
[23]Technical dossier pp. 36–37, Annex 10 and additional information December 2021/Annex 1.
[24]Technical dossier p. 37, Annex 10 and additional information December 2021/Annex 1.
[25]Technical dossier p. 36, Annex 10 and additional information December 2021/Annex 1.
[26]Technical dossier p. 36, Annex 10 and additional information December 2021/Annex 1.
[27]LoDs: aflatoxin B1 = 0.3 µg/kg; 3-nitropropionic acid = 0.3498 mg/kg; cyclopiazonic acid = 3 µg/kg; kojic acid = 0.0569 mg/kg.
[28]Technical dossier/ Annex 4 GMM dossier/Annex E1 and additional information December 2021/Annex E1_Version2.
[29]Technical dossier/ Annex 4 GMM dossier/Annex E2 and additional information December 2021/Annex_Answers p. 4.
[30]Technical dossier/Annex 7.01.

significant increase in revertant colony numbers above the control values were observed at any concentration level of the test substance in this experiment.

The panel concluded that the food enzyme carboxypeptidase D did not induce gene mutations under the test conditions employed 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 Guideline 487 (OECD, 2016) and following GLP.[31]

Two separate experiments were performed in duplicate cultures of human peripheral whole blood lymphocytes. The food enzyme was tested at 3000, 4000 and 5000 µg TOS/mL. In the first experiment, the cells were exposed for 3 h in the presence or absence of S9-mix and harvested 24 h after the beginning of treatment (3 h + 21 h of recovery time). Additionally, a continuous 24-h treatment without S9-mix was included with harvesting 24 h after removal of the test substance (24 h + 24 h of recovery time). No cytotoxicity was observed after the treatments, both in the presence and absence of S9-mix. The frequency of binucleated cells with micronuclei (MNBN) was comparable to the negative controls at all concentrations tested, with the exception of the 24-h continuous treatment in the absence of S9-mix at 4000 µg TOS/mL, where a statistically significant increase of the frequency of MNBN was observed (0.93% vs. 0.5% in the control; $p \leq 0.05$).

In the second experiment, the cells were exposed to the test substance at the same concentrations for 24 h in the absence of the S9-mix, with harvesting 24 h after removal of the test substance (24 h + 24-h of recovery time). No cytotoxicity or increased frequency of MNBNs was observed at any concentration level of the test substance.

The panel concluded that, under the test conditions employed in this study, the food enzyme carboxypeptidase D did not induce an increase in the frequency of MNBNs in cultured human peripheral blood lymphocytes.

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.[32] Groups of 10 male and 10 female Crl:WI(Han) rats received the food enzyme in doses of 1110, 1665 and 2220 mg TOS/kg bw per day by gavage. Controls received the vehicle (purified water—reverse osmosis water).

One control female was euthanised on day 8 due to mis-dosing.

The body weight gain for the entire interval (Day 1–91) was statistically significantly decreased in high-dose females (–20%). The panel considered the change as not toxicologically relevant, as it was without a statistically significant effect on the final body weight and it was only observed in one sex.

Clinical chemistry investigation revealed statistically significant decreases in the activity of aspartate aminotransferase in high-dose males (–22%) and of alkaline phosphatase in high-dose females (–32%). The panel considered the changes as not toxicologically relevant, as they were small (both parameters), they were only observed in one sex (both parameters) and the changes were within the historical control values.

Statistically significant organ weight changes detected were decreases in adrenal weight in high-dose males (absolute –19%, relative to brain –18%, relative to body weight –19%) and in mid-dose males (relative to body weight –18%) and decreases in thyroid/parathyroid weight in low-dose females (absolute –26%, relative to brain –26% and relative to body weight –24%). The panel considered the changes as not toxicologically relevant as they were only observed in one sex, there was no dose–response relationship (thyroid/parathyroid) and there were no histopathological changes in the adrenals and in the thyroid/parathyroid glands.

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

The panel identified the no observed adverse effect level (NOAEL) of 2220 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.

The potential allergenicity of the carboxypeptidase D produced with the *A. oryzae* strain NZYM-MK 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, two matches were found: a serine carboxypeptidase produced by wheat (*Triticum aestivum*) and Api m 9.0101, a serine carboxypeptidase produced by honeybee (*Apis mellifera*).[33]

No information was available on oral and respiratory sensitisation or elicitation reactions of this caboxypeptidase D.

[31]Technical dossier/Annex 7.02.

[32]Technical dossier/Annex 7.03.

[33]Technical dossier/pp. 70–73 and Annex 8.01.

Serine carboxypeptidase is one of the allergens of wheat, although not the major allergen. Wheat is an allergenic food listed in the Annex II of the Regulation (EU) No 1169/2011.[34] No allergic reactions after oral exposure to the serine carboxypeptidase from honeybee has been reported. There are no reports in the literature on allergenicity of carboxypeptidases from other sources.[35]

[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 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 a risk of allergic reactions upon dietary exposure to this food enzyme, particularly in individuals sensitised to wheat, cannot be excluded, but will not exceed that of wheat consumption.

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 as described in the EFSA guidance (EFSA CEP Panel, 2023) 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.[36]

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	Flours	2.1–**10.9**
• Production of brewed products	Cereals (malted or not)	3.5–**26.3**
Processing of meat and fish products		
• Production of protein hydrolysates from meat and fish proteins	Meat and fish protein	877.2–**1403.5**
Processing of plant- and fungal-derived products		
• Production of protein hydrolysates from plants and fungi	Plant protein	877.2–**1403.5**
Processing of yeast and yeast products	Yeast extract	350.9–**473.7**

[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 28.5 CPDU(A)/mg TOS.

[c]Numbers in bold represent the maximum recommended use levels, which were used for calculation.

In the production of baked products, the food enzyme is added to flour during the preparation of dough or batter.[37] The carboxypeptidase D is used to weaken the gluten structures and consequently to improve the rheology of the dough.[38] The food enzyme–TOS remains in the baked products.

In the production of brewed products, the food enzyme is added to grains during the mashing step.[39] The proteolytic reaction of the food enzyme aids the release of free amino acids as assimilable nitrogen sources for the yeast.[40] The food enzyme–TOS remains in the beer.

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. soybean meal and vital wheat gluten) and animal (e.g. meat and fish trimmings) materials during hydrolysis. Hydrolysis can reduce the bitterness of the protein hydrolysates.[41] The food enzyme–TOS remains in the final hydrolysates, which are added to a variety of final foods (e.g. soups, bouillons, snacks, dressings) to enhance the flavour.

[34]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.

[35]Technical dossier/Annex 8.02 and additional information December 2021/Annex 2.

[36]Technical dossier/Additional information December 2021/Annex-Answers p. 8.

[37]Technical dossier/p. 86.

[38]Technical dossier/p. 85.

[39]Technical dossier/p. 87.

[40]Technical dossier/pp. 86–87.

[41]Technical dossier/p. 83 and Additional information December 2021/Answer to question 5.

In the processing of yeast and yeast products, the food enzyme is added to yeast extract.[42] The hydrolysis intensifies the flavour of yeast products that are added to various savoury foods, ready-to-eat vegetable meals, soups, bouillons and sauces.[43] 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, it is 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 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 0.908 mg TOS/kg bw per day in toddlers 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)	0.016–0.226 (12)	0.061–0.310 (15)	0.100–0.308 (19)	0.034–0.198 (21)	0.030–0.132 (22)	0.019–0.132 (23)
Min–max 95th percentile (number of surveys)	0.027–0.556 (11)	0.192–0.908 (14)	0.293–0.906 (19)	0.093–0.707 (20)	0.082–0.464 (22)	0.045–0.425 (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	
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 resulted from the treatment of yeast cell wall.	+
Use of recipe fractions to disaggregate FoodEx categories	+/–
Use of technical factors in the exposure model	+/–

Note: +: uncertainty with potential to cause overestimation of exposure; –: uncertainty with potential to cause underestimation of exposure.

[42]Technical dossier/p. 84.

[43]Technical dossier/Additional information December 2021/Answer to question 6.

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 (2220 mg TOS/kg bw per day) identified from the 90-day rat study with the derived exposure estimates of 0.016–0.310 mg TOS/kg bw per day at the mean and from 0.027–0.908 mg TOS/kg bw per day at the 95th percentile resulted in a margin of exposure of at least 2445.

4 | CONCLUSIONS

Based on the data provided and the derived margin of exposure, the panel concluded that the food enzyme carboxypeptidase D produced with the genetically modified *Aspergillus oryzae* strain NZYM-MK does not give rise to safety concerns under the intended conditions of use.

The CEP panel considered the food enzyme free from viable cells of the production organism and recombinant DNA.

5 | DOCUMENTATION AS PROVIDED TO EFSA

Carboxypeptidase D produced by a genetically modified strain of *Aspergillus oryzae* (strain NZYM-MK). June 2021. Submitted by Novozymes A/S.

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

ABBREVIATIONS

bw	body weight
CAS	Chemical Abstracts Service
CEP	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
EINECS	European Inventory of Existing Commercial Chemical Substances
FAO	Food and Agricultural Organisation 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
MNBN	Bi-nucleated cells with micronuclei
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Cooperation and Development
PCR	polymerase chain reaction
TOS	total organic solids
WGS	Whole Genome Sequence
WHO	World Health Organisation

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, 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).