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SCIENTIFIC OPINION

Safety evaluation of the food enzyme mucorpepsin from the non-genetically modified *Rhizomucor miehei* strain FRO

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Abstract

The food enzyme mucorpepsin (EC 3.4.23.23) is produced with the non-genetically modified *Rhizomucor miehei* strain FRO by DSM Food Specialties B.V. The enzyme can be chemically modified to produce a thermolabile form. The food enzyme is free from viable cells of the production organism. It is intended to be used in three food manufacturing processes: processing of dairy products for the production of (1) cheese, (2) edible rennet casein, (3) fermented dairy products. Dietary exposure to the food enzyme–total organic solids (TOS) was estimated to be up to about 0.072 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 2000 mg TOS/kg bw per day, the highest dose tested, which, when compared with the estimated dietary exposure, results in a margin of exposure of at least 27,778. A search for the similarity of the amino acid sequence of the food enzyme to known allergens was made and five matches were found. The Panel considered that a risk of allergic reactions upon dietary exposure to this food enzyme cannot be excluded, but is considered low, except for individuals sensitised to mustard proteins, for whom the risk will not exceed that of mustard 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

aspartic endopeptidase, EC 3.4.23.23, food enzyme, microbial rennet, mucorpepsin, non-genetically modified microorganism, *Rhizomucor miehei*

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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 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.

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

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

1.1.1 | Background as provided by the European Commission

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

Six applications have been introduced by the companies "Decernis, LLC", "Keller and Heckman LLP", "the Association of Manufacturers and Formulators of Enzyme Products (AMFEP)" and "Novozymes A/S" for the authorisation of the food enzymes Cyclomaltodextrin glucanotransferase from *Geobacillus stearothermophilus*, Dextranase from *Chaetomium gracile*, Subtilisin from *Bacillus licheniformis*, Mucorpepsin from *Rhizomucor miehei*, Animal rennet consisting of chymosin and pepsin from the abomasum of *Bos primigenius* (cattle), *Bubalus bubalis* (buffalo), *Capra aegagrus hircus* (goat) and *Ovis aries* (sheep), and Lipase from a genetically modified strain of *Aspergillus niger* (strain NZYM-DB), respectively.

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 six 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 requested the EFSA to carry out the safety assessments on the food enzymes Cyclomaltodextrin glucanotransferase from *Geobacillus stearothermophilus*, Dextranase from *Chaetomium gracile*, Subtilisin from *Bacillus licheniformis*, Mucorpepsin from *Rhizomucor miehei*, Animal rennet consisting of chymosin and pepsin from the abomasum of *Bos primigenius* (cattle), *Bubalus bubalis* (buffalo), *Capra aegagrus hircus* (goat) and *Ovis aries* (sheep), and Lipase from a genetically modified strain of *Aspergillus niger* (strain NZYM-DB) in accordance with Article 17.3 of Regulation (EC) No 1332/2008 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, pp. 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 food enzyme mucorpepsin from *Rhizomucor miehei* from AMFEP.

Initially, the application was submitted as a joint dossier[4] identified as EFSA-Q-2015-00233. During a meeting between EFSA, the European Commission and the Association of Manufacturers and Formulators of Enzyme Products (AMFEP),[5] it was agreed that joint dossiers will be split into individual data packages.

The current opinion addresses one data package originating from the joint dossier EFSA-Q-2015-00233. This data package, identified as EFSA-Q-2023-00017, concerns the food enzyme mucorpepsin that is produced with a strain of *R. miehei* (FRO) and was submitted by DSM.

2 | DATA AND METHODOLOGIES

2.1 | Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme mucorpepsin from a non-genetically modified *R. miehei* FRO.

Additional information was requested from the applicant during the assessment process on 12 May 2023 and received on 7 July 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, 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	Mucorpepsin
Systematic name	Aspartic endopeptidase
Synonyms	Microbial rennet, Mucor rennin
IUBMB no	EC 3.4.23.23
CAS no	148465-73-0
EINECS no	642-981-3

Mucorpepsins catalyse the hydrolysis of proteins, including the peptide bond Phe105-Met106 of κ-casein in milk, resulting in the destabilisation of casein micelles and causing milk to clot.

The enzyme under assessment is intended to be used in three food manufacturing processes: processing of dairy products for the production of (1) cheese, (2) edible rennet casein, (3) fermented dairy products.

3.1 | Source of the food enzyme

The mucorpepsin is produced with the non-genetically modified filamentous fungus *R. miehei* strain FRO, which is deposited at the [redacted] with the deposit number [redacted].[6] The production strain was identified as *R. miehei* by [redacted].[7]

[4]Commission Implementing Regulation (EU) No 562/2012 of 27 June 2012 amending Commission Regulation (EU) No 234/2011 with regard to specific data required for risk assessment of food enzymes Text with EEA relevance. OJ L 168, 28.6.2012, p. 21–23.

[5]The full detail is available at the https://www.efsa.europa.eu/en/events/event/ad-hoc-meeting-industry-association-amfep-joint-dossiers-food-enzymes

[6]Technical dossier/Annex 15.

[7]Technical dossier/Annex 14.

The genome of the production strain was searched for gene clusters with known functions and no cluster was found involved in the synthesis of compounds with known toxicity.[8]

3.2 | Production of the food enzyme

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 grown as a pure culture using a typical industrial medium in a submerged, fed-batch fermentation system with conventional process controls in place. After completion of the fermentation, the solid biomass is removed from the fermentation broth by filtration. The filtrate containing the food enzyme is then further purified and concentrated, including an ultrafiltration step in which food enzyme protein is retained, while most of the low molecular mass material passes through the filtration membrane and is discarded.[11]

The food enzyme concentrate may be used in an unmodified form or treated with [redacted] to increase its heat sensitivity.[12] In the latter case, [redacted] is subsequently added to ensure complete removal of the [redacted] The enzyme may also be further treated by [redacted] or separated by chromatography to obtain a more purified form of the mucorpepsin.[13] The food enzyme is then filtered and formulated.[14]

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.[15]

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 mucorpepsin is a single polypeptide chain of [redacted] amino acids.[16] The molecular mass of the protein, calculated from the amino acid sequence, is around [redacted] kDa.[17] The food enzyme was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.[18] A consistent protein pattern was observed across all batches. The gels showed a major band of [redacted] kDa, corresponding to the expected mass of the food enzyme. No other enzyme activities were reported.[19]

The in-house determination of mucorpepsin activity is based on the hydrolysis of casein resulting in milk clotting (reaction conditions: pH 6.2, 37°C), measuring the time needed for visual flocculation of a standard milk substrate. The mucorpepsin activity is quantified relative to a reference standard with known milk-clotting activity and expressed in International Milk-Clotting Units (IMCU)/g.[20]

The native food enzyme has a temperature optimum around 50°C (pH 6.6), while the thermolabile form of the food enzyme has a temperature optimum between 40°C and 45°C (pH 6.6). The native and the thermolabile forms of the food enzyme have a pH optimum around pH 6 (32°C). Thermostability was tested after a pre-incubation of the food enzyme for 20 s at different temperatures (pH 6). The native form showed decreased enzyme activity above 68°C, with low residual activity above 72°C, the thermolabile form decreased activity above 60°C, with no activity detected above 70°C.[21]

3.3.2 | Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches of each of the forms in which the food enzyme is obtained (Table 1).[22] The mean total organic solids (TOS) of the three batches of the native food enzyme was 14.8% and the mean enzyme activity/TOS ratio was 35.9 IMCU/mg TOS. The mean total organic solids (TOS) of the three

[8]Technical dossier/Additional data July 2023/Annex 1.
[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/pp. 8–9, 40–41 and Annex 5.
[11]Technical dossier/pp. 41–50 and Annex 6.
[12]Technical dossier/pp. 44–47 and Additional data July 2023/Annex 2.
[13]Technical dossier/pp. 45–47, Annex 6, Annex 7 and Additional data July 2023/Annex 2.
[14]Technical dossier/pp. 47, 48 and Annex 6.
[15]Technical dossier/Annex 7 and Additional data July 2023/Annex 2.
[16]Technical dossier/Annex 14/pp. 11, 12.
[17]Technical dossier/pp. 8, 30.
[18]Technical dossier/pp. 27–28.
[19]Technical dossier/pp. 7, 31–32.
[20]Technical dossier/p. 31 and Annex 2.
[21]Technical dossier/pp. 32–34.
[22]Technical dossier/pp. 25, 62–63, Annex 3 and Annex 12.

batches of the thermolabile form of the food enzyme for commercialisation was 3.6% and the mean enzyme activity/mg TOS ratio was 19.6 IMCU/mg TOS.

TABLE 1 Composition of the food enzyme.

		Batches					
		Native enzyme			Thermolabile form of the enzyme		
Parameters	**Unit**	**1**	**2**	**3**[a]	**4**	**5**	**6**
Mucorpepsin activity	IMCU/g[b]	4685	6035	5220	707	696	694
Protein	%	10.2	12.2	9.8	1.3	1.3	1.4
Ash	%	1.8	0.5	0.4	18.4	18.4	18.6
Water	%	84.8	83.4	84.8	77.9	78.1	77.9
Total organic solids (TOS)[c]	%	13.4	16.1	14.8	3.7	3.5	3.5
Activity/TOS ratio	IMCU/mg TOS	35.0	37.5	35.3	19.1	19.8	19.8

[a]Batch used for the toxicological studies.

[b]IMCU: International Milk-Clotting Units (see Section 3.3.1).

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

3.3.3 | Purity

The lead content in the six batches 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).[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). No antimicrobial activity was detected in any of the tested batches.[22]

Strains of *Rhizomucor*, in common with most filamentous fungi, have the capacity to produce a range of secondary metabolites. The applicant did not provide analytical data on potential secondary metabolites produced under the conditions of fermentation which might contribute to the food enzyme–TOS. This issue 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 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. For each sample, 1 mL of product was incubated in 100 mL of selective medium at 30°C for 6 days. From these, 10 µL were inoculated on selective agar plates and incubated at 30°C for 6 days. No colonies were produced. A positive control was included.[25]

3.4 | Toxicological data

A battery of toxicological tests was provided, including a bacterial reverse mutation test (Ames test), an in vitro mammalian micronucleus test and a repeated dose 90-day oral toxicity study in rats. The test item (batch 3) used in all toxicological studies was the native enzyme.

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, 2020) and following Good Laboratory Practice (GLP).[26]

[23]Technical dossier/p. 30, Annex 3 and Annex 4.

[24]LoD: Pb = 0.001 mg/kg.

[25]Technical dossier/p. 40 and Annex 16.

[26]Technical dossier/Annex 10.

Four strains of Salmonella Typhimurium (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* WP2uvrA(pKM101) were used with or without metabolic activation (S9-mix), applying the standard plate incorporation method. The experiment was carried out in triplicate, using eight concentrations of the food enzyme ranging from 3 to 5000 μg TOS/plate.

No cytotoxicity was observed at any concentration of the test item. A 2.2-fold increase in the number of revertant colonies was reported in TA98 strain with S9-mix at 5000 μg TOS/plate, exceeding the historical negative control range.

As the author attributed this increase to histidine release from the protein fraction of the test-item, a second experiment was carried out, applying the 'treat and wash' method to exclude the effect of free amino acids. It was performed in triplicate, with or without S9-mix, in the TA98 strain at seven enzyme concentrations ranging from 100 to 5000 μg TOS/plate, and in the remaining strains of *S*. Typhimurium, and *E. coli*, at seven concentrations ranging from 33 to 5000 μg TOS/plate. An additional confirmatory experiment was carried out in the TA98 strain with S9-mix at five concentrations of the food enzyme, ranging from 1000 to 5000 μg TOS/plate. No increase of revertant colony numbers was observed in any of these tests.

The Panel concluded that the food enzyme mucorpepsin 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, 2016) and following GLP.[27]

A dose range-finding and two separate experiments were performed with duplicate cultures of human peripheral whole blood lymphocytes. In the dose range-finding test, no cytotoxicity above 50% was seen at any concentration tested up to 5000 μg TOS/mL with and without metabolic activation (S9-mix). In the first experiment, the cells were exposed to the food enzyme and scored for the frequency of bi-nucleated cells with micronuclei (MNBN) at concentrations of 1633, 2857 and 5000 μg TOS/mL in a short-term treatment (4 h exposure and 16 h recovery period), either with or without S9-mix. In the second experiment, the cells were exposed to the food enzyme and scored for MNBN at concentrations of 1633, 2857 and 5000 μg TOS/mL in a long-term treatment (20 h exposure) without S9-mix.

In the short-term treatment with S9-mix, a cytotoxicity of 12.9% (based on the reduction of replication index) was reported at the highest concentration tested of 5000 μg TOS/mL.

The frequency of MNBN was not statistically significantly different to the negative controls in all conditions of treatment.

The Panel concluded that the food enzyme mucorpepsin 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 following GLP[28] and in accordance with the OECD Test Guideline 408 (OECD, 2018), with the deviation that blood urea nitrogen determination was not performed. The Panel considered that this deviation is minor and does not impact on the evaluation of the study.

Groups of 10 male and 10 female Wistar rats (Crl: WI(Han)) received by gavage the food enzyme in doses of 500, 1000 or 2000 mg TOS/kg body weight (bw) per day. Controls received the vehicle (sterile water).

No mortality was observed.

The body weight was statistically significantly decreased (–13%) in low-dose males from day 64 of administration to the end of the study; this change was associated with statistically significant decrease in body weight gain on weeks 7, 10 and 11, and overall body weight gain (–22%); moreover, statistically significant decrease in body weight gain was observed in week 10, and in the total body weight gain (–14%) in high-dose males. The Panel considered the changes as not toxicologically relevant, as they were only recorded sporadically, they were only observed in one sex, there was no dose–response relationship, and the changes were without a statistically significant effect on the final body weight in the high-dose males.

Decreased feed consumption over the entire period of study (–9%) as well as in weeks 2, 3, 5 and 6 of administration (–9%, –10%, –12% and – 11%, respectively) was statistically significant in high-dose females. The Panel considered these changes as not toxicologically relevant, as they were only recorded sporadically, they were only observed in one sex and there were no statistically significant associated changes in the body weight and the body weight gain.

Haematological investigations revealed a statistically significant decrease in low-, mid- and high-dose males in total white blood cells (WBC) (–31%, –30% and – 26%, respectively), in lymphocyte (–37%, –35%, –28%, respectively) and monocyte (–29%, –40%, –36%, respectively) counts, and in basophil counts in mid-dose males (–64%). A statistically significant decrease was observed in red cell distribution width (RDWG) in low-dose males (–4%), while increase in haemoglobin (HGB) was observed in mid- and high-dose females (+5% and + 6%, respectively). The Panel considered the changes in red blood cell parameters (HGB and RDWG) as not toxicologically relevant, as there was no dose–response, they were small, within the historical control values and there were no corroborative changes in other relevant parameters (red blood cells counts, haematocrit). Changes in WBC will be considered in conjunction with changes observed in lymphoid organ weights.

Clinical chemistry investigations revealed a statistically significant increase in urea in mid-dose males (+22%), a decrease in glucose in mid-dose males (–15%) and a decrease in high-density lipoproteins (HDL) in high-dose males (–13%).

[27]Technical dossier/Annex 11.

[28]Technical dossier/Annex 12/ p. 9.

Statistically significant increases in serum electrolytes were observed for sodium in males at all doses (+1%, +3% and + 2%, respectively) and in low-dose females (+1%), for chloride in low- and mid-dose males (+2% and + 3%, respectively) as well as for calcium in high-dose males (+10%) and low- and mid-dose females (+6% both). The Panel considered the changes as not toxicologically relevant, as they were only observed in one sex (urea, glucose, HDL and chloride), there was no dose–response relationship (all), there were no corroborative changes in other relevant parameters (no changes in creatinine associated with increased urea), there were no histopathological changes in the kidneys (urea) and the changes were within the historical control values (urea, glucose, HDL, chloride).

Statistically significant changes in organ weights included a decrease in the absolute thyroid/parathyroid weight in males at all doses (–16% at low- and mid-dose, –21% at high-dose), in low- and high-dose females (–19%, –21%), and in relative thyroid/parathyroid weight to body weight in females at low- and high-doses (–20%, –21%). Statistically significant organ weight changes were also observed in lymphoid organs, including a decrease in absolute spleen weight in males at all doses (–22%, –14%, –18%, respectively) and relative to body weight in high-dose males (–12%) as well as a decrease in absolute thymus weight in low- and high-dose males (–19%, –20%, respectively). Other statistically significant organ weight changes were: decrease in absolute kidney weight in low- and mid-dose males (–10% both), decrease in absolute epididymides weight in low- and mid-dose males (–9%, –10%), decrease in absolute pituitary and liver weights in low-dose males (–16%, –13%) and increase in relative brain weight in low- and high-dose males (+12%, +10%).

Decreases in lymphoid organ weights (spleen and thymus) were variably observed in males at all doses, which correlated with reduced peripheral WBC, lymphocyte, basophil and monocyte counts and reduced body weight. Moreover, a minimal increase in adipocytes was observed in the bone marrow in 2/10 males and 3/10 females at the high dose. The Panel regarded these changes as not toxicologically relevant, considering: (i) there was no dose–response, (ii) haematology and organ weight changes were only present in males, (iii) there were no histopathological changes in lymphoid organs (spleen and thymus) and the severity of the finding in the bone marrow was minimal and (iv) all haematology and organ weight parameters were within historical control values.

A decrease in absolute and relative thyroid weights was observed in both sexes, with a weak dose–response in males. Therefore, the Panel considered that a relationship to treatment could not be excluded. Nevertheless, these changes were regarded by the Panel as not adverse taking into consideration that: (i) there was no effect on thyroid function (total triiodothyronine, thyroxine and thyroid stimulating hormone), (ii) follicular cell hypertrophy observed at microscopic examination in high-dose males (with no differences in incidence and severity compared with controls) was not compatible with a reduction of organ weight, hence no correlation with microscopic findings and (iii) absolute weights were within historical control values in males.

The remaining statistically significant changes in organ weights (kidneys, epididymides, pituitary, liver and brain) were considered not toxicologically relevant, as the changes were small, they were only observed in one sex (all), there was no dose–response relationship (all), there were no associated histopathological changes (all) and the changes were within the historical control values (kidneys, liver and brain).

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

The Panel identified a no observed adverse effect level (NOAEL) of 2000 mg TOS/kg bw per day, the highest dose tested.

3.4.3 | Allergenicity

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 mucorpepsin produced with the non-genetically modified *R. miehei* strain FRO was assessed by comparing its amino acid sequence with those of known allergens according to the 'Scientific opinion on the assessment of allergenicity of genetically modified 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, five matches were found.[29] The matching allergens were P00791.3, pepsin A from *Sus scrofa* (pig); Aed a 11, lysosomal aspartic protease from *Aedes aegypti* (yellow fever mosquito); Rhi o 1, aspartyl endopeptidase from *Rhizopus oryzae*; Asp f 10, an aspergillopepsin I from *Aspergillus fumigatus* and Sin a 3, non-specific lipid transfer protein type 1 from *Sinapis alba* (yellow mustard).

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

Mustard is an allergenic food and is listed as a food allergen in the Annex II of the Regulation (EU) No 1169/2011.[30] Sin a 3, one of the allergens in mustard, is not the major allergen.

The lysosomal aspartic protease from yellow fever mosquito *A. aegypti* is associated with allergic reactions to insect bites (Cantillo et al., 2017), but allergic reactions after oral exposure have not been reported.

[29]Technical dossier/pp. 63-69/Annex 13.

[30]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.

Occupational allergy to respiratory allergens, such as pepsin, including that in microbial rennet, was described (van Kampen et al., 2013). Aspergillopepsin and aspartyl endopeptidases are also respiratory allergens. However, several studies have shown that adults sensitised to respiratory allergens are able to ingest these allergens without acquiring clinical symptoms of food allergy (Armentia et al., 2009; Brisman, 2002; Poulsen, 2004). In addition, no allergic reactions upon dietary exposure to any mucorpepsin have been reported in the literature.

[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 cannot be excluded, but is considered low, except for individuals sensitised to mustard proteins, for which the risk will not exceed that of mustard consumption.

3.5 | Dietary exposure

3.5.1 | Intended use of the food enzyme

The food enzyme is intended to be used in three 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.[c]

Food manufacturing process[a]	Raw material (RM)	Recommended use level (mg TOS/kg RM)[b]
Processing of dairy products		
• Production of cheese	Milk	0.1–**1.7**
• Production of edible rennet casein	Milk	0.1–**1.7**
• Production of fermented dairy products	Milk	0.01–**0.8**

Abbreviation: TOS, total organic solids.

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

[b]The numbers in bold were used for calculation.

[c]Technical dossier/p. 55.

In cheese production, the food enzyme is added to milk during the coagulation step to hydrolyze κ-casein.[31] Whey is separated from the curd during the draining step and the curd is further processed into different types of cheese. Whey is used in the production of several foods, including bakery products and beverages. The majority of the food enzyme–TOS partitions into the whey and is mostly removed during the draining of the whey. Only a small portion of the food enzyme–TOS remains in the curd (~ 10%). The food enzyme–TOS remains in the final foods.

The treatment of milk with this food enzyme can lead to the production of edible rennet casein.[32] According to the Directive (EU) 2015/2203,[33] *'edible rennet casein means a milk product obtained by separating, washing and drying the coagulum of skimmed milk and/or of other products obtained from milk; the coagulum is obtained through the reaction of rennet or other coagulating enzymes'*. The food enzyme–TOS remains in the final foods.

For the production of fermented milk products, the food enzyme is added to raw or pasteurised milk during the coagulation/fermentation step.[34] The food enzyme–TOS remains in the fermented milk products.

Based on data provided on thermostability (see Section 3.3.1), the food enzyme may remain active in fermented dairy products. In cheese, it may remain active, depending on the production process and the form of food enzyme used (native or thermolabile).

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

[31]Technical dossier/p. 53.

[32]Technical dossier/p. 53.

[33]https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32015L2203

[34]Technical dossier/p. 54.

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

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

Population group	Estimated exposure (mg TOS/kg body weight per day)					
	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)	0.002–0.020 (12)	0.008–0.024 (15)	0.005–0.014 (19)	0.002–0.007 (21)	0.002–0.005 (22)	0.001–0.004 (23)
Min–max 95th (number of surveys)	0.009–0.072 (11)	0.020–0.062 (14)	0.011–0.031 (19)	0.006–0.012 (20)	0.004–0.012 (22)	0.004–0.010 (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	
Exposure to food enzyme–TOS was always calculated based on the recommended maximum use level	+
Selection of broad FoodEx categories for the exposure assessment	+
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.

The conservative approach applied to the exposure estimate to 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 (2000 mg TOS/kg bw per day) from the 90-day rat study with the derived exposure estimates of 0.001–0.024 mg TOS/kg bw per day at the mean and from 0.004 to 0.072 mg TOS/kg bw per day at the 95th percentile resulted in a margin of exposure (MOE) of at least 27,778.

4 | CONCLUSIONS

Based on the data provided and the derived margin of exposure, the Panel concluded that the food enzyme mucorpepsin produced with the non-genetically modified *R. miehei* strain FRO does not give rise to safety concerns under the intended conditions of use.

5 | DOCUMENTATION AS PROVIDED TO EFSA

Application for authorization of Mucorpepsin from *Rhizomucor miehei* in accordance with Regulation (EC) No 1331/2008. December 2022. Submitted by DSM Food Specialties B.V.

Additional information. July 2023. Submitted by DSM Food Specialties B.V.

ABBREVIATIONS

ANI	average nucleotide identity
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 Organization of the United Nations
GLP	good laboratory practice
GMO	genetically modified organism
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kDa	kiloDalton
LoD	limit of detection
MNBN	bi-nucleated cells with micronuclei
MOE	margin of exposure
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Co-operation and Development
TOS	total organic solids
WGS	whole genome sequence
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[b]	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 included 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).