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SCIENTIFIC OPINION

Safety evaluation of the food enzyme mucorpepsin from the non-genetically modified *Rhizomucor miehei* strain M19-21

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Abstract

The food enzyme mucorpepsin (EC 3.4.23.23) is produced with the non-genetically modified *Rhizomucor miehei* strain M19-21 by Meito Sangyo Co., Ltd. The enzyme is chemically modified to produce a thermolabile form. The food enzyme was considered free from viable cells of the production organism. It is intended to be used in the processing of dairy products for the production of cheese and fermented dairy products. Based on the maximum use levels, dietary exposure was estimated to be up to 0.108 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 226 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 2093. A search for the similarity of the amino acid sequence of the food enzyme to known allergens was made and four matches to respiratory allergens and one match to a food allergen (mustard) were found. The Panel considered that the risk of allergic reactions upon dietary exposure to this food enzyme, particularly in individuals sensitised to mustard proteins, cannot be excluded. 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, *Rhizomucor miehei*

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 micro-organisms or products thereof including a product obtained by a fermentation process using micro-organisms: (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 I foods, in accordance with the specification and condition of use provided for in Article 7 (2) of Regulation (EC) No 1332/2008 on food enzyme.[3]

Six applications have been introduced by the companies "Decernis, LLC", "Keller and Heckman LLP", the "Association of Manufacturers and Formulation of Enzyme Products (AMFEP)", and "Novozymes A/S" for the authorization 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), *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[4] 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 requests the European Food Safety Authority 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), *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.

[4]Regulation (EC) No 234/2011.

1.2 | Interpretation of the Terms of Reference

The present scientific opinion addresses the European Commission's request to carry out the safety assessment of the food enzyme Mucorpepsin from *Rhizomucor miehei* from AMFEP.

The application was submitted initially as a joint dossier[5] and identified as the EFSA-Q-2015-00233. During a meeting between EFSA, the European Commission and representatives from the Association of Manufacturers and Formulators of Enzyme Products (AMFEP),[6] it was agreed that joint dossiers will be split into individual data packages.

The current opinion addresses one data package originating from the former joint dossier EFSA-Q-2015-00233. This data package is identified as EFSA-Q-2022-00201 and concerns the food enzyme mucorpepsin produced with the *R. miehei* strain M19-21 and submitted by Meito Sangyo Co., Ltd.

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* (strain M19-21). The dossier was updated on 9 March 2022 (see 'Documentation provided to EFSA').

2.2 | Methodologies

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

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 food enzyme under assessment is intended to be used in the processing of dairy products for the production of cheese and fermented dairy products.

3.1 | Source of the food enzyme

The mucorpepsin is produced with the non-genetically modified filamentous fungus *Rhizomucor miehei* strain M19-21, which is deposited at the National Institute of Technology and Evaluation (NITE) Biological Resource Center (Japan) with the deposit number [redacted].[7]

The production strain was identified as *R. miehei* by a phylogenetic analysis of the seven closest related strains, including the type strain [redacted], selected by blast analysis of the ITS region.[8]

[5]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.

[6]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

[7]Technical Dossier/ADD DATA_SEPTEMBER 2023/Attachment Request 1.

[8]Technical Dossier/Annex 5.

R. miehei strain M19-21 was obtained by conventional mutagenesis of an original isolate and selection for high mucorpepsin production.

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 batch or 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 enzyme is stabilised and then 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.[11]

The food enzyme concentrate is modified by treatment with [redacted] to increase the heat sensitivity of the enzyme.[12] Additionally, the food enzyme concentrate is treated with acid to inactivate the esterase (lipase) activity.[13] After treatment, the remaining [redacted] is removed by addition of catalase.[14,15] The food enzyme concentrate is then filtered and formulated. 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.

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].[16] The molecular mass of the mature protein, calculated from the amino acid sequence, is around [redacted]. The food enzyme was analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. A consistent protein pattern was observed across all batches. The gels showed a major protein band corresponding to an apparent molecular mass of about [redacted], consistent with the expected mass of the enzyme.[17] No other enzymatic activities were reported.

The in-house determination of mucorpepsin activity is based on the hydrolysis of casein resulting in milk clotting (reaction conditions: pH 5.5, 32°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.[18]

The food enzyme has a temperature optimum around 50°C, the highest temperature tested, and a pH optimum around pH 5.5, the lowest pH value tested. Thermostability was tested after a pre-incubation of the food enzyme for 15 s at different temperatures (pH 5.5). Enzyme activity decreased above 50°C, showing no residual activity at 75°C.[19]

3.3.2 | Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches used for commercialisation and one batch produced for the toxicological tests (Table 1). The mean total organic solids (TOS) of the three food enzyme batches for commercialisation was 4.9% and the mean enzyme activity/TOS ratio was 25.7 IMCU/mg TOS.

[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/Annex 6.
[11]Technical Dossier/Annex 7.
[12]Technical Dossier/Dossier file, section B.5. Chemical treatment, p. 50.
[13]Technical Dossier/Dossier file, section B.5. Chemical treatment, p. 50.
[14]Technical dossier/Additional information December 2023/Attachment request 1/Catalase information.
[15]The catalase used in the manufacturing process of the mucorpepsin was assessed by EFSA (EFSA Q-2016-00532).
[16]Technical dossier/Annex 14.
[17]Technical dossier/Dossier p. 33.
[18]Technical dossier/Annex 2.
[19]Technical dossier/Dossier p. 37.

TABLE 1 Composition of the food enzyme.[d]

Parameters	Unit	Batches			
		1	2	3	4[a]
Mucorpepsin activity	IMCU/g[b]	1291	1234	1271	1231
Protein	%	3.0	3.3	2.5	3.0
Ash	%	14.4	14.3	14.5	3.3
Water	%	80.8	80.6	80.6	92.1
Total organic solids (TOS)[c]	%	4.8	5.1	4.9	4.6
Activity/TOS ratio	IMCU/mg TOS	26.9	24.2	25.9	26.8

[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.
[d]Technical dossier/Annex 1, 3 & Dossier p. 32, 67.

3.3.3 | Purity

The lead content in the three commercial batches and in the batch used for toxicological studies was below 5 mg/kg, which complies with the specification for lead as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006).[20,21,22]

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

Strains of *Rhizomucor*, in common with most filamentous fungi, have the capacity to produce a range of secondary metabolites. The possible presence of metabolites of concern is addressed by the toxicological examination of the food enzyme–TOS.

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

3.3.4 | Viable cells of the production strain

The absence of viable cells of the production strain in the food enzyme was demonstrated in three independent batches analysed in triplicate. Ten mL of product were diluted in 90 mL of phosphate buffer, 30 mL of which was filtered through a 0.45 µm membrane. [redacted]. No colonies were produced. A positive control was included.[24]

3.4 | Toxicological data

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

The batch 4 (Table 1) used in these studies was considered suitable as a test item.

3.4.1 | Genotoxicity

3.4.1.1 | *Bacterial reverse mutation test*

A bacterial reverse mutation test (Ames test) was performed according to the Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 (OECD, 1997a) and following Good Laboratory Practice (GLP).[25]

Four strains of *Salmonella* Typhimurium (TA98, TA100, TA1535 and TA1537) and *E. coli* WP2uvrA were used with or without metabolic activation (S9-mix), applying the standard plate incorporation method. Two experiments were carried out in triplicate, using five concentrations of the food enzyme, ranging from 62 to 5000 µg/plate (corresponding to 2.8, 8.4, 25.3,

[20]LoDs: Pb = 5 mg/kg.
[21]Technical dossier/Annex 4.
[22]Technical Dossier/ADD DATA_SEPTEMBER 2023/Attachment Request 9.
[23]Technical dossier/Annex 4.
[24]Technical Dossier/ADD DATA_SEPTEMBER 2023/Attachment Request 2.
[25]Technical dossier/Annex 11.

75.8 and 227.5 μg TOS/plate) in the first experiment and from 313 to 5000 μg/plate (corresponding to 14.2, 28.4, 56.9, 113.7 and 227.5 μg TOS/plate) in the second experiment.

No cytotoxicity was observed at any concentration of the test substance. In the second experiment, a twofold increase in the number of revertant colonies above the control values was observed at the lowest concentration (14.2 μg TOS/plate) in *Salmonella* Typhimurium strain TA1537 without S9-mix, however, the increase was not observed in the first experiment and, therefore, the change was not considered to be of biological relevance.

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 chromosomal aberration test*

The in vitro mammalian chromosomal aberration test was carried out according to the OECD Test Guideline 473 (OECD, 1997b) and following GLP.[26]

Two separate experiments were performed with duplicate cultures of human peripheral whole blood lymphocytes. The cell cultures were treated with the food enzyme either with or without metabolic activation (S9-mix). In the first experiment, cells were exposed to the food enzyme and scored for chromosomal aberrations at concentrations of 1250, 2500 and 5000 μg/mL (corresponding to 56.88, 113.75 and 227.5 μg TOS/mL) in a short-term treatment (4 hours exposure and 20 hours recovery period), either with or without S9-mix, and in a long-term treatment (24 hours exposure without recovery period) without S9-mix. In the second experiment, cells were exposed to the food enzyme and scored for chromosomal aberrations at concentrations of 3000, 4000 and 5000 μg/mL (corresponding to 136.5, 182 and 227.5 μg TOS/mL) in a short-term treatment (4 hours exposure and 44 hours recovery period) with S9-mix and in a long-term treatment (48 hours exposure and 0 hours recovery period) without S9-mix.

No cytotoxicity was seen either in the short-term (with or without S9-mix) or in the long-term treatment. The frequency of structural and numerical aberrations was not statistically significantly different to the negative controls at all concentrations tested.

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

3.4.1.3 | *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. A range finding test and a main experiment were carried out with duplicate cultures of human lymphoblastoid (TK6) cells. The cell cultures were treated with the food enzyme with or without metabolic activation (S9-mix).

A range-finding test was performed with the highest concentration level set at 5170 μg TOS/mL in a short-term treatment (4 h exposure and 20 h recovery period) with and without S9-mix and in a long-term treatment (24 h exposure without recovery period). The 50% cell-growth inhibition concentration (IC_{50}) was 1660 μg TOS/mL in the short-term treatment without S9-mix, 1370 μg TOS/mL in the short-term treatment with S9-mix and 1470 μg TOS/mL in the long-term treatment. On the basis of these results, cells were exposed to the food enzyme and scored for micronuclei frequency at concentrations of 1000, 1250, 1500 and 1750 μg TOS/mL in the short-term treatment with and without S9-mix and in the long-term treatment.

A cytotoxicity of 50% was observed at the highest concentration tested in all the treatment conditions. The frequency of MNBN was not statistically significantly different to the negative controls at all concentrations tested in the short- or long-term 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 followed the OECD Test Guideline 408 (OECD, 1998) and GLP.[27] Groups of 20 male and 20 female Sprague–Dawley rats received by gavage the food enzyme in doses of 10, 20, and 50% (1220, 2450 and 6110 IMCU/kg bw per day, respectively), corresponding to 45.1, 90.5 and 226 mg TOS/kg bw per day, respectively. Controls received the vehicle (distilled water). In the following text, all changes in measured parameters are reported in the order low-, mid- and high-dose.

No mortality was observed.

The body weight was slightly (+4 to +6%), but statistically significantly increased in low-dose females on several occasions. The Panel considered the change as not toxicologically relevant, as it was only recorded sporadically, it was only observed in one sex, there was no dose–response relationship, the changes were small and without a statistically significant effect on the final body weight.

The feed consumption was statistically significantly decreased on the following days (D) of administration: D38–42 (−5%) and D42–45 (−5%) in low-dose males; D45–49 in low-, mid- and high-dose males (−6%, −6% and −6%); D52–56 (−5%, −4%)

[26]Technical dossier/Annex 12.

[27]Technical dossier/Annex 13.

and D56–59 (–5%, –4%) in low- and high-dose males; D59–63 (–5%) and D70–73 (–5%) in high-dose males and D80–84 in low-, mid- and high-dose males (–6%, –5% and –7%). In mid-dose females, the feed consumption was statistically significantly decreased on D7–10 of administration (–6%). The Panel considered the changes as not toxicologically relevant, as they were only recorded sporadically, there was no dose–response relationship, the changes were small and there was no statistically significant change in the final feed consumption and the body weight.

In the functional observations, a statistically significant decrease in the number of rears was observed during week –1 in low-, mid- and high-dose males (–28%, –36%, –19%); during week 2 in low- and mid-dose males (–32%, –63%); during week 6 and 11 in low-dose males (–30% and –28%). A statistically significant increase in the number of rears was observed during week 4 in low-dose males (+48%) and during week 5 in high-dose males (+50%). In females, a statistically significant decrease in the number of rears was observed during week 1 and 4 in mid-dose females (–27% and –26%, respectively) and during week 8 and 11 in low-dose females (–13% and –26%, respectively). The Panel considered the changes as not toxicologically relevant, as they were only recorded sporadically and there was no dose–response relationship.

Haematological investigations revealed a statistically significant decrease in red blood cell counts (RBC) in low-dose males (–4%) and in mid-dose females (–3%), and a decrease in lymphocytes (lymph) in low-, mid- and high-dose males (–20%, –23%, –24%). Furthermore, there were statistically significant increases in the following parameters: mean cell volume (MCV) in low-dose males (+2%), mean cell haemoglobin (MCH) in low-dose males (+4%) and in mid- and high-dose females (+3%, +3%), mean cell haemoglobin concentration (MCHC) in low-dose males (+2%) and in high-dose females (+2%), haemoglobin (Hb) in high-dose females (+3%), reticulocytes (Reti) in low-, mid- and high-dose males (a dose-dependent increase: +3% and +5% in the low- and mid-dose groups, with statistical significance in high-dose group: +11%) and in mid-dose females (+19%), and white blood cells (WBC, +43%), lymphocytes (Lymph, +39%), monocytes (Mono, +80%) and eosinophils (Eos, +41%) in low-dose females. A statistically significant increase in fibrinogen (Fb, +7%) and a decrease in activated partial thromboplastin time (APTT, –4%) in mid-dose males were also reported. The Panel considered the changes as not toxicologically relevant, as they were only observed in one sex (MCV, MCHC, Hb, WBC, Mono, Eos, Fb, APTT), there was no consistency between the changes in males and females (lymph), there was no dose–response relationship (RBC, MCV, MCHC, WBC, Mono, Eos, Fb, APTT), there were no changes in other relevant parameters (for Lymph in the total count of WBC) and there were no histopathological changes in bone marrow, spleen, liver and lymph nodes.

Clinical chemistry investigations revealed a statistically significant decrease in aspartate aminotransferase (AST, –12%) and lactate dehydrogenase (LDH, –25%) in low-dose males, an increase in chloride in mid-dose males (+1%), an increase in urea in mid- and high-dose males (+13% and +11%) and in high-dose females (+8%), a decrease in glucose in high-dose males (–7%) and an increase in bilirubin (+14%) in high-dose females. The Panel considered the changes as not toxicologically relevant, as they were only observed in one sex (AST, LDH, chloride, glucose, bilirubin), there was no dose–response relationship (AST, LDH, chloride) and there were no histopathological changes in liver and kidneys.

The urinalysis revealed a statistically significant decrease in urine volume in high-dose males (–30%) and in high-dose females (–31%), an increase in refractive index in high-dose males (+69%) and in high-dose females (+106%), an increase in incidence of ketones in high-dose males (9/20 vs. 0/20 in the control) and in high-dose females (8/20 vs. 2/20 in the control), a dose-dependent increase in urine pH in low-, mid- and high-dose females non-significant increase: +6% in low dose and significant increase: +17% and +21%, in mid- and high-dose, an increase in incidence of urates in the urine sediment in mid- and high-dose females (8/20, 11/20 vs. 2/20 in the control) and an increase in incidence of urine proteins in low-dose females (15/20 vs. 12/20 in the control). The Panel considered the changes as not toxicologically relevant, as they were only observed in one sex (urine pH, urates, urine proteins), there was no dose–response relationship (urine proteins), there were no macroscopic and histopathological changes in kidneys.

Statistically significant increases in the absolute organ weights included: empty caecum in high-dose males (+8%), kidneys in mid-dose males (+7%) and in high-dose females (+7%), liver in mid-dose males (+8%), mesenteric lymph nodes (+13%) and spleen (+10%) in mid-dose females. Statistically significant decrease in the absolute organ weights included: thymus in low-, mid- and high-dose males (–15%, –14%, –12%) and the mean ovary weight in low-dose females (–14%).

Statistically significant increases in the relative organ weights included: empty caecum in high-dose males (+8%), kidneys in mid- and high-dose males (+5%, +4%) and in high-dose females (+5%), liver in mid- and high-dose males (+6%, +5%) and spleen in mid-dose females (+7%). Statistically significant decreases in the relative organ weights included: heart in high-dose males (–4%), thymus in low-, mid- and high-dose males (–16%, –14%, –12%), brain in low-dose females (–5%) and ovaries in low-dose females (–18%). The Panel considered the changes as not toxicologically relevant, as they were only observed in one sex (caecum, liver, mesenteric lymph nodes, spleen, thymus, heart, brain), there was no dose–response relationship (liver, mesenteric lymph nodes, spleen, thymus, ovaries, brain) and there were no histopathological changes in caecum, kidneys, liver, lymph nodes, spleen, thymus, ovaries, heart 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 226 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 *R. miehei* strain M19-21 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, five matches were found. The matching allergens were P00791.3, pepsin A from *Sus scrofa* (pig); Aed a 11, a lysosomal aspartic protease from *Aedes aegypti*; Rhi o 1, an endopeptidase from *Rhizopus oryzae*; Asp f 10, an aspergillopepsin from *Aspergillus fumigatus*, and Sin a 3, a non-specific lipid transfer protein type 1 from *Sinapis alba* (mustard), a known food allergen.[28]

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

Pepsin A from *Sus scrofa* is associated with occupational asthma and rhinitis (Añíbarro Bausela & Fontela, 1996; Cartier et al., 1984). Also Rhi o 1 and Asp f 10 are respiratory allergens. However, several studies have shown that adults respiratorily sensitised may be able to ingest these allergens without acquiring clinical symptoms of food allergy (Armentia et al., 2009; Brisman, 2002; Poulsen, 2004). Aspartic protease is associated with allergic reactions to insect bites, but allergic reactions after oral exposure have not been reported (Cantillo et al., 2017). Mustard is a food allergen and listed in Annex II of the Regulation (EU) No 1169/2011.

[redacted], a substance that may cause allergies or intolerances (listed in the Regulation (EU) No 1169/2011[29]) is used as raw material. In addition, [redacted], a known source of allergens, is present in the media fed to the microorganisms. However, during the fermentation process, these products will be degraded and utilised by the microorganisms for cell growth, cell maintenance and production of enzyme protein. In addition, the microbial/fungal biomass and fermentation solids are removed. Taking into account the fermentation process and downstream processing, the Panel considered that no potentially allergenic residues from these sources are present in the food enzyme.

The Panel noted that a catalase is used during the downstream processing of the thermolabile food enzyme and is likely to be present in the final product. Respiratory sensitization to catalases has been reported, but as indicated above, sensitised individuals are usually able to ingest respiratory allergens without acquiring food allergic reactions.

The Panel considered that the risk of allergic reactions upon dietary exposure to this food enzyme, particularly in individuals sensitised to mustard, cannot be excluded but the likelihood will not exceed that of consumption of mustard.

3.5 | Dietary exposure

3.5.1 | Intended use of the food enzyme

The food enzyme is intended to be used in two 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–**3**
• Production of fermented dairy products	Milk	0.03–**1.2**

Abbreviation: TOS, total organic solids.

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

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

[c]Technical dossier/p. 58.

In cheese production, the food enzyme is added to milk together with the starter culture during coagulation step to hydrolyse κ-casein. Whey, a by-product, is separated from the curd during the draining step.[30] Curd is further processed into different types of cheese, whereas whey is further processed to produce several foods, including bakery products and beverages. The food enzyme partitions between curd and whey, with a ratio of approximately 10:90 (Guinee and Wilkinson, 1992). The food enzyme–TOS remains in the final foods.

In the production of fermented milk products, the food enzyme is added to milk during the coagulation/fermentation step.[31] The food enzyme–TOS remains in the fermented milk products.

Based on data provided on thermostability (see Section 3.3.1), it is expected that this enzyme may remain active in the final foods (cheese or fermented milk products), depending on the respective food manufacturing processes.

[28]Technical dossier/pp. 12–13; pp. 67-70/Annex 14.

[29]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.

[30]Technical dossier/p.56.

[31]Technical dossier/p.57.

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 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 to the food enzyme–TOS was estimated to be 0.108 mg TOS/kg bw per day in infants at the 95th percentile.

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

	Estimated exposure (mg TOS/kg body weight per day)					
Population group	**Infants**	**Toddlers**	**Children**	**Adolescents**	**Adults**	**The elderly**
Age range	3–11 months	12–35 months	3–9 years	10–17 years	18–64 years	≥ 65 years
Min–max mean (number of surveys)	0.002–0.030 (12)	0.012–0.039 (15)	0.006–0.020 (19)	0.003–0.010 (21)	0.002–0.007 (22)	0.002–0.007 (22)
Min–max 95th percentile (number of surveys)	0.011–0.108 (11)	0.032–0.103 (14)	0.016–0.051 (19)	0.009–0.020 (20)	0.006–0.020 (22)	0.005–0.017 (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 in disaggregation FoodEx categories	+/–
Use of technical factors in the exposure model	+/–

Abbreviations: +, uncertainty with potential to cause overestimation of exposure; –, uncertainty with potential to cause underestimation of exposure; TOS, total organic solids.

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

3.6 | Margin of exposure

The comparison of the NOAEL (226 mg TOS/kg bw per day) from the 90-day rat study with the derived exposure estimates of 0.002–0.039 mg TOS/kg bw per day at the mean and of 0.005–0.108 mg TOS/kg bw per day at the 95th percentile resulted in a margin of exposure of at least 2093.

4 | CONCLUSIONS

Based on the data provided and the derived margin of exposure, the Panel concluded that the food enzyme mucorpepsin produced with the *Rhizomucor miehei* strain M19-21 does not give rise to safety concerns under the intended conditions of use.

5 | REMARK

The use of the catalase from the non-genetically modified *Aspergillus niger* strain CTS 2093 as a raw material in the manufacture of the mucorpepsin under evaluation, is not considered to raise a safety concern when used only for this purpose. However, the Panel noted that an assessment of the same catalase for food enzyme use (EFSA Q-2016-00532) could not exclude safety concerns when used in the manufacture of food.

6 | DOCUMENTATION AS PROVIDED TO EFSA

Technical dossier 'Application for authorization of Mucorpepsin from *Rhizomucor miehei* M19-21 in accordance with Regulation (EC) No 1331/2008'. 09 March 2022. Submitted by Meito Sangyo Co., Ltd. (Japan).

Additional information. 03 October 2023. Submitted by Meito Sangyo Co., Ltd.

Additional information. 21 December 2023. Submitted by Meito Sangyo Co., Ltd.

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 Organization of the United Nations
GLP	Good Laboratory Practice
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kDa	kiloDalton
LoD	limit of detection
MNBN	binucleated cells with micronuclei
OECD	Organisation for Economic Cooperation and Development
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.

REQUESTOR

European Commission

QUESTION NUMBER

EFSA-Q-2022-00201

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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 one 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[a], Serbia[a], 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[a], Serbia[a], Spain, Sweden
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Bosnia and Herzegovina[a], Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Montenegro[a], Netherlands, Portugal, Romania, Serbia[a], Slovenia, Spain, Sweden
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Bosnia and Herzegovina[a], Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Montenegro[a], Netherlands, Portugal, Romania, Serbia[a], 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[a], Netherlands, Portugal, Romania, Serbia[a], Slovenia, Spain, Sweden

[a]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.

[b]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).