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Safety evaluation of the native and thermolabile forms of the food enzyme mucorpepsin from *Rhizomucor miehei* strain MMR 164

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Abstract

The food enzyme mucorpepsin (aspartic endopeptidase, EC 3.4.23.23) is produced with the non-genetically modified microorganism *Rhizomucor miehei* strain MMR 164 by Takabio. The enzyme is 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 milk processing for cheese production. The dietary exposure to the food enzyme–total organic solids (TOS) was estimated to be up to 0.98 mg TOS/kg body weight (bw) per day in European populations. Genotoxicity tests did not raise 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 1,320 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 1,300. Similarity of the amino acid sequence of the food enzyme to those of known allergens was searched and five matches were found. The Panel considered that, under the intended conditions of use, the risk of allergic sensitisation and elicitation reactions upon dietary exposure to this food enzyme cannot be excluded, but is considered low except for individuals sensitised to mustard proteins, but this 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.

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[†] Deceased.

Table of contents

Abstract.....	1
1. Introduction.....	4
1.1. Background and Terms of Reference as provided by the requestor.....	4
1.1.1. Background as provided by the European Commission.....	4
1.1.2. Terms of Reference.....	4
1.2. Interpretation of the Terms of Reference.....	5
2. Data and methodologies.....	5
2.1. Data.....	5
2.2. Methodologies.....	5
3. Assessment.....	5
3.1. Source of the food enzyme.....	6
3.2. Production of the food enzyme.....	6
3.3. Characteristics of the food enzyme.....	6
3.3.1. Properties of the food enzyme.....	6
3.3.2. Chemical parameters.....	7
3.3.3. Purity.....	8
3.3.4. Viable cells of the production strain.....	9
3.4. Toxicological data.....	9
3.4.1. Genotoxicity.....	9
3.4.1.1. Bacterial reverse mutation test.....	9
3.4.1.2. <i>In vitro</i> mammalian chromosomal aberration test.....	10
3.4.2. Repeated dose 90-day oral toxicity study in rodents.....	10
3.4.3. Allergenicity.....	11
3.5. Dietary exposure.....	11
3.5.1. Intended use of the food enzyme.....	11
3.5.2. Dietary exposure estimation.....	12
3.5.3. Uncertainty analysis.....	12
3.6. Margin of exposure.....	13
4. Conclusions.....	13
5. Documentation as provided to EFSA.....	13
References.....	13
Abbreviations.....	14
Appendix A – Dietary exposure estimates to the food enzyme–TOS in details.....	16
Appendix B – Population groups considered for the exposure assessment.....	17

1. Introduction

Article 3 of the Regulation (EC) No 1332/2008¹ 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 1332/2008¹ 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 1331/2008² established the European Union (EU) procedures for the safety assessment and the authorisation procedure of food additives, food enzymes and food flavourings. The use of a food enzyme shall be authorised only if it is demonstrated that:

- it does not pose a safety concern to the health of the consumer at the level of use proposed;
- there is a reasonable technological need;
- its use does not mislead the consumer.

All food enzymes currently on the European Union market and intended to remain on that market, as well as all new food enzymes, shall be subjected to a safety evaluation by the European Food Safety Authority (EFSA) and approval via an EU Community list.

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

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

1.1.1. Background as provided by the European Commission

Only food enzymes included in the European Union (EU) Community list may be placed on the market as such and used in foods, in accordance with the specifications and conditions of use provided for in Article 7 (2) of Regulation (EC) No 1332/2008¹ on food enzymes.

Three applications have been submitted by the companies "Takabio" and "DSM Food Specialties B.V" of the food enzymes microbial rennet from *Rhizomucor miehei*, acid prolyl endopeptidase from a genetically modified strain of *Aspergillus niger* (strain GEP) and beta-galactosidase from a genetically modified strain of *Aspergillus niger* (strain TOL).

Following the requirements of Article 12.1 of Regulation (EC) No 234/2011³ implementing Regulation (EC) No 1331/2008², the Commission has verified that the three 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 of the food enzymes microbial rennet from *Rhizomucor miehei*, acid prolyl endopeptidase from a genetically modified strain of *Aspergillus niger* (strain GEP) and beta-galactosidase from a

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.

genetically modified strain of *Aspergillus niger* (strain TOL) in accordance with Article 17.3 of Regulation (EC) No 1332/2008¹ on food enzymes.

1.2. Interpretation of the Terms of Reference

EFSA received two applications of the use of mucorpepsin from *R. miehei* as a food enzyme. The native mucorpepsin is produced from the *R. miehei* strain MMR 164. Chemical treatment of the native mucorpepsin results in a thermolabile form. The first applicant Takabio manufactures the native enzyme and the thermolabile form (EFSA-Q-2014-00851). The second applicant DuPont Nutrition Biosciences (now IFF) obtains the native enzyme from Takabio and then manufactures and seeks the authorisation only for the thermolabile form (EFSA-Q-2016-00030).

The present scientific opinion addresses the European Commission's request to carry out the safety assessment of the native and thermolabile forms of the food enzyme mucorpepsin from *R. miehei* strain MMR 164 from Takabio.

2. Data and methodologies

2.1. Data

The applicant has submitted a dossier in support of an application for authorisation of the microbial rennet thermostable and thermolabile from *R. miehei*. The dossier ('Microbial rennet thermostable and thermolabile from *Rhizomucor miehei*') was updated on 28 September 2021.

Additional information was requested from the applicant during the assessment process on 19 May 2020 and was received on 28 September 2021 (see 'Documentation provided to EFSA').

Following the request for additional data sent by EFSA on 19 May 2020, the applicant requested a clarification teleconference on 16 October 2020, after which the applicant provided additional data on 28 September 2021.

2.2. Methodologies

The assessment was conducted in line with the principles described in the EFSA 'Guidance on transparency in the scientific aspects of risk assessment' (EFSA, 2009) as well as in the 'Statement on characterisation of microorganisms used for the production of food enzymes' (EFSA CEP Panel, 2019) and following the relevant guidance documents of EFSA Scientific Committee.

The current 'Guidance on the submission of a dossier on food enzymes for safety evaluation' (EFSA CEP Panel, 2009) 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 with the exception of the exposure assessment, which was carried out in accordance to the updated 'Scientific Guidance for the submission of dossiers on food enzymes' (EFSA CEP Panel, 2021a).

3. Assessment⁴

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 is intended to be used in milk processing for cheese production.⁵

⁴ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 4–5, 8, 27, 69–70.

⁵ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 5.

3.1. Source of the food enzyme⁶

The food enzyme mucorpepsin is produced with a non-genetically modified filamentous fungus *R. miehei* strain MMR 164, which is deposited at the Biological Resource Center, National Institute of Technology and Evaluation (NBRC, Japan)⁷ with deposit number NITE SD 00394. The production strain was identified as *R. miehei* by analysis of the internal transcribed spacer (ITS) 1 and 2 regions, including the 5.8S ribosomal DNA and the large subunit D1 and D2 region.⁸

3.2. Production of the food enzyme⁹

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004¹⁰, with food safety procedures based on hazard analysis and critical control points, and in accordance with current good manufacturing practice.¹¹

The production strain is

[REDACTED]

The native food enzyme

[REDACTED]

The applicant provided information on the identity of the substances used to control the fermentation and in the subsequent downstream processing of the food enzyme.¹²

The Panel considered that sufficient information has been provided on the manufacturing process and the quality assurance system implemented by the applicant to exclude issues of concern.

3.3. Characteristics of the food enzyme

3.3.1. Properties of the food enzyme¹³

The mucorpepsin is a single polypeptide chain of 430 amino acids.¹⁴ The molecular mass of the mature protein, derived from the amino acid sequence, was calculated to be 43.9 kDa.¹⁵ The sodium

⁶ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 4, 9, 36, 69; Technical dossier/Additional data, 28 September 2021/other annexes join Dossier MMR 164 - Q 2014-00851_update/Annex F, Annex G.

⁷ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 37; Technical dossier/Additional data, 28 September 2021/other annexes join Dossier MMR 164 - Q 2014-00851_update/Annex G.

⁸ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 37; Technical dossier/Additional data, 28 September 2021/other annexes join Dossier MMR 164 - Q 2014-00851_update/Annex F.

⁹ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 9–10, 15–16, 41–49; Technical dossier/Additional data, 28 September 2021/Annex H, Annex K, Annex Q; Technical dossier/Additional data, 28 September 2021/other annexes join Dossier MMR 164 - Q 2014-00851_update/Annex I.

¹⁰ Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of food additives. OJ L 226, 25.6.2004, pp. 3–21.

¹¹ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 10, 36, 40; Technical dossier/Additional data, 28 September 2021/Annex H.

¹² Technical dossier/Additional data, 28 September 2021/other annexes join Dossier MMR 164 - Q 2014-00851_update/Annex I.

¹³ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 7–8; Technical dossier/Additional data, 28 September 2021/other annexes join Dossier MMR 164 Q 2014-00851_update/Annex J.

¹⁴ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 42; Technical dossier/Additional data, 28 September 2021/Annex Q.

¹⁵ Technical dossier/Additional data, 28 September 2021/Annex Q; Technical dossier/Additional data, 28 September 2021/other annexes join Dossier MMR 164 - Q 2014-00851_update/Annex J.

¹⁶ Technical dossier/Additional data, 28 September 2021/Annex K, Annex L.

¹⁷ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 28; Technical dossier/Additional data, 28 September 2021/other annexes join Dossier MMR 164 - Q 2014-00851_update/Annex B, Annex C; Technical dossier/Additional data, 28 September 2021/Annex U.

¹⁸ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 29, 31; Technical dossier/Additional data, 28 September 2021/Annex U.

¹⁹ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 31.

dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels showed a major protein band migrating to the position corresponding to about 43 kDa. The protein profile also included bands with lower molecular masses of 34, 33 and 25 kDa.²⁰ A consistent protein pattern was observed across all batches. No other enzymatic activities were reported.²¹

The determination of milk-clotting activity²² of the native food enzyme is based on measurement of the coagulation time of milk by the food enzyme compared to a standard enzyme. One unit (U) of milk-clotting activity is defined as the amount of enzyme required to clot 1 mL of skim milk within 40 min at 35°C.

The native food enzyme has a temperature optimum around 50–60°C (pH 6.5) and a pH optimum around pH 5.0 (35°C).²³ Thermostability of the native food enzyme²⁴ was tested after a pre-incubation of the food enzyme for 15 min at different temperatures (pH 6.5). Enzyme activity decreased above 50°C, showing low residual activity at 60°C.²⁵

The activity of the thermolabile form of the enzyme²⁶ is measured following the International Standard ISO 15174: 2012 [IDF 176:2012]. The activity is expressed in International Milk-clotting Units (IMCU)/mL. The method is based on the comparison of the total milk-clotting activity of mucorpepsin with the milk-clotting activity of an international microbial coagulant reference standard on a standard milk substrate. The thermolabile food enzyme²⁷ exhibits activity from pH 5.5 to 7.0 and a temperature optimum around 50°C (at pH 6.5). No enzyme activity is left at temperatures above 70°C.

3.3.2. Chemical parameters²⁸

Data on the chemical parameters of the food enzyme were provided for three batches of the native food enzyme, three batches of the thermolabile food enzyme used for commercialisation and a batch of the native enzyme produced for the toxicological tests (Table 1).²⁸ The mean total organic solids (TOS) of the three batches of the native food enzyme used for commercialisation is 15.5% and the mean enzyme activity/mg TOS ratio is 4,832 Unit (U)/mg TOS. The mean total organic solids (TOS) of the three batches of the thermolabile form of the food enzyme for commercialisation is 2.5% and the mean enzyme activity/mg TOS ratio is 14.3 IMCU/mg TOS.

²⁰ Technical dossier/Additional data, 28 September 2021/p. 29, 31; Technical dossier/Additional data, 28 September 2021/other annexes join Dossier MMR 164 - Q 2014-00851_update/Annex C.

²¹ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 8, 29, 31, 35; Technical dossier/Additional data, 28 September 2021/other annexes join Dossier MMR 164 - Q 2014-00851_update/Annex C.

²² Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 32; Technical dossier/Additional data, 28 September 2021/other annexes join Dossier MMR 164 - Q 2014-00851_update/Annex B; Technical dossier/Additional data, 28 September 2021/Annex V.

²³ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 33; Technical dossier/Additional data, 28 September 2021/other annexes join Dossier MMR 164 - Q 2014-00851_update/Annex B; Technical dossier/Additional data, 28 September 2021/Annex R.

²⁴ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 32–34; Technical dossier/Additional data, 28 September 2021/other annexes join Dossier MMR 164 - Q 2014-00851_update/Annex B; Technical dossier/Additional data, 28 September 2021/Annex R.

²⁵ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 33.

²⁶ Technical dossier/Additional data, 28 September 2021/other annexes join Dossier MMR 164 - Q 2014-00851_update/Annex B; Technical dossier/Additional data, 28 September 2021/Annex V.

²⁷ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 34; Technical dossier/Additional data, 28 September 2021/other annexes join Dossier MMR 164 - Q 2014-00851_update/Annex B; Technical dossier/Additional data, 28 September 2021/Annex R.

²⁸ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 28, 61; Technical dossier/Additional data, 28 September 2021/other annexes join Dossier MMR 164 - Q 2014-00851_update/Annex A, Annex B, Annex O; Technical dossier/Additional data, 28 September 2021/Annex D.

Table 1: Composition of the native and thermolabile form of the food enzyme

Parameters	Unit	Batches			
		Native enzyme			
		1	2	3	4 ^(a)
Mucorpepsin activity	U/g batch ^(b)	750,000	752,000	743,000	740,000
Protein	%	9.2	9.3	9.4	6.6
Ash	%	10.13	10.35	10.14	2.3
Water	%	73.9	74.3	74.7	84.5
Total organic solids (TOS)^(c)	%	15.97	15.35	15.16	13.2
Activity/mg TOS	U/mg TOS	4,696	4,899	4,901	5,606
		Thermolabile form of the enzyme			
		1	2	3	
Mucorpepsin activity	IMCU/g batch ^(d)	196.5	763.9	197.4	
Protein	%	0.6	1.7	< 0.5	
Ash	%	17.30	15.79	17.61	
Water	%	81.0	80.0	80.9	
Total organic solids (TOS)^(c)	%	1.7	4.21	1.49	
Activity/mg TOS	IMCU/mg TOS	12	18	13	

(a): Batch used for the toxicological studies.

(b): U: Unit (see Section 3.3.1).

(c): TOS calculated as 100% – % water – % ash.

(d): IMCU: International Milk-clotting Unit (see Section 3.3.1).

3.3.3. Purity²⁹

The lead content in all batches described in Table 1 was below 5 mg/kg which complies with the specification for lead (≤ 5 mg/kg) as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006).³⁰

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).³¹ In addition, the absence of filamentous fungi and yeasts was demonstrated (colony-forming unit (CFU) < 1).³² No antimicrobial activity was detected in any of the batches tested.²⁹

Strains of *Rhizomucor*, in common with most filamentous fungi, have the capacity to produce a range of secondary metabolites. The presence of aflatoxin B₁, B₂, G₁, G₂, sterigmatocystin, zearalenone, ochratoxin A, fumonisin B1 and fumonisin B2 was examined in all batches tested. All were below the limits of detection (LOD) of the applied analytical methods.³³ The possible presence of other 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 is sufficient.

²⁹ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 9, 31, 61, 69; Technical dossier/Additional data, 28 September 2021/Annex D; Technical dossier/Additional data, 28 September 2021/other annexes join Dossier MMR 164 - Q 2014-00851_update/Annex E.

³⁰ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 9, 31, 61, 69; Technical dossier/Additional data, 28 September 2021/Annex D; Technical dossier/Additional data, 28 September 2021/other annexes join Dossier MMR 164 - Q 2014-00851_update/Annex O; Technical dossier/Additional data, 28 September 2021/other annexes join Dossier MMR 164 - Q 2014-00851_update/Annex E; LOD: Pb = 5 mg/kg.

³¹ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 9, 31, 61, 69; Technical dossier/2nd submission/Additional data, 28 September 2021/Annex D; Technical dossier/Additional data, 28 September 2021/other annexes join Dossier MMR 164 - Q 2014-00851_update/Annex O; Annex E.

³² Technical dossier/Additional data, 28 September 2021/Annex D; Technical dossier/Additional data, 28 September 2021/other annexes join Dossier MMR 164 - Q 2014-00851_update/Annex E.

³³ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 9, 31, 61, 69; Technical dossier/Additional data, 28 September 2021/Annex D; Technical dossier/Additional data, 28 September 2021/other annexes join Dossier MMR 164 - Q 2014-00851_update/Annex J; LOD: aflatoxins B₁, B₂, G₁ and G₂ = 0.5 µg/kg each; ochratoxin A = 0.5 µg/kg; sterigmatocystin = 20 µg/kg; T2-toxin = 100 µg/kg; zearalenone = 50 µg/kg.

3.3.4. Viable cells of the production strain³⁴

The absence of the production strain was confirmed in 1 g of three batches of the food enzyme, taken after sterile filtration by incubating on plates for 3 and 6 days at 30°C.³⁴

3.4. Toxicological data³⁵

A battery of toxicological tests, including a bacterial gene mutation assay (Ames test), an *in vitro* mammalian chromosomal aberration test and a repeated dose 90-day oral toxicity study in rats, has been provided. The batch 4 (Table 1) used in these studies has a similar protein pattern as the native batches used for commercialisation and is thus considered suitable as a test item. In addition, in the view of the Panel the chemical modification of the native enzyme is unlikely to modify its toxicological profile and thus it is also considered as a suitable test item for the thermolabile form of the food enzyme.

3.4.1. Genotoxicity

3.4.1.1. Bacterial reverse mutation test

A bacterial reverse mutation assay (Ames test) was performed according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 (OECD, 1997a) and following Good Laboratory Practice (GLP).³⁶ Four strains of *Salmonella* Typhimurium (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* WP2uvrA were used with or without metabolic activation (S9-mix). Five separate experiments (preliminary, dose-finding, main study applying the preincubation method, main study and confirmation study applying the treat and wash method) were performed.

The preliminary experiment was carried out using seven concentrations of the food enzyme (from 0.074 to 74,000 U/plate, corresponding to 0.0132, 0.132, 1.32, 13.2, 132, 1,320 and 13,200 µg TOS/plate). An increase in revertant colony numbers above the control values was observed at 13,200 µg TOS/plate in *S. Typhimurium* TA98, TA100, TA1535 and TA1537 without S9-mix, at 13,200 µg TOS/plate in *S. Typhimurium* TA98, TA100 and TA1535 with S9-mix and at 1,320 and 13,200 µg TOS/plate in *S. Typhimurium* TA1537 with S9-mix.

The dose-finding study was carried out using eight concentrations of the food enzyme (from 33.8 to 74,000 U/plate, corresponding to 6.0, 18.2, 54.4, 163, 489, 1,466, 4,406 and 13,200 µg TOS/plate). An increase in revertant colony numbers above the control values was observed at 13,200 µg TOS/plate in *S. Typhimurium* TA98, TA100, TA1535 and TA1537 without S9-mix and in strains TA100 and TA1535 in the presence of S9-mix.

The main study applying the preincubation method was carried out using six concentrations of the food enzyme (from 2,310 to 74,000 U/plate, corresponding to 412.1, 825.9, 1,650, 3,300, 6,600.1 and 13,200 µg TOS/plate) in *E. coli* WP2uvrA in the absence of S9-mix, and in *E. coli* WP2uvrA and *S. Typhimurium* TA98 and TA1537 in the presence of S9-mix. An increase in revertant colony numbers above the control values was observed at 13,200 µg TOS/plate in *S. Typhimurium* TA98 in the presence of S9-mix.

The increase in revertant colony number recorded in the experiments applying the preincubation method was attributed to growth stimulation due to the presence of free amino acids in the test item.

The main study applying the treat and wash method was carried out using eight concentrations of the food enzyme (from 33.8 to 74,000 U/plate, corresponding to 6.0, 18.2, 54.4, 163, 489, 1,466, 4,406 and 13,200 µg TOS/plate) in *S. Typhimurium* TA98 in the presence and absence of S9-mix. No cytotoxicity was observed at any concentration of the test substance. 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.

The confirmation study applying the treat and wash method was carried out using seven concentrations of the food enzyme (from 1,160 to 74,000 U/plate, corresponding to 207, 412.1, 825.9, 1,650, 3,300, 6,600.1 and 13,200 µg TOS/plate) in *S. Typhimurium* TA100, TA1535 and TA98 in the presence of S9-mix and in *S. Typhimurium* TA100, TA1535, TA1537 and TA98 in the absence of S9-mix.

³⁴ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 40; Technical dossier/Additional data, 28 September 2021/other annexes join Dossier MMR 164 - Q 2014-00851_update/Annex N.

³⁵ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 12; Technical dossier/Additional data, 28 September 2021/other annexes join Dossier MMR 164 - Q 2014-00851_update/Annex O, Annex P.

³⁶ Technical dossier/Additional data, 28 September 2021/other annexes join Dossier MMR 164 - Q 2014-00851_update/Annex P.

No cytotoxicity was observed at any concentration level of the test substance. Upon treatment with the food enzyme, there was no biologically relevant increase in the number of revertant colonies above the control values, in any strain tested, with or without S9-mix.

The Panel concluded that the food enzyme 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 OECD Test Guideline 473 (OECD, 1997b) and following GLP.³⁶ A single experiment was performed with duplicate cultures of Chinese hamster lung fibroblast cell line (CHL/IU).

The cell growth inhibition test was performed at concentrations ranging from 1,160 to 74,000 U/mL, and no inhibition of cell growth by 50% or more was observed. Based on these results, the cell cultures were treated with the food enzyme at 18,500, 37,000 and 74,000 U/mL (corresponding to 3,300, 6,600.1 and 13,200 µg TOS/mL) in the short-term treatment (6 h exposure and 18 h recovery period) either with or without metabolic activation (S9-mix) and in the long-term treatment (24 h) without S9-mix. In the long-term treatment, a dose-dependent decrease in the relative cell growth rate was observed up to 42.3% at the highest concentration of 13,200 µg TOS/mL. The frequency of structural and numerical chromosomal aberrations in treated cultures was not statistically significantly different to the negative controls at all concentrations tested.

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

3.4.2. Repeated dose 90-day oral toxicity study in rodents

The repeated dose 90-day oral toxicity study was performed in accordance with OECD Test Guideline 408 (OECD, 1998) and following GLP.³⁶ Groups of 10 male and 10 female Sprague–Dawley CrI:CD(SD) rats received the food enzyme by gavage in doses of 74,000, 740,000 and 7,400,000 U/kg, corresponding to 13.2, 132 and 1,320 mg TOS/kg body weight (bw) per day for 13 weeks. Controls received the vehicle (water for injection).

No mortality was observed.

The body weight of high-dose females was statistically significantly decreased on three occasions (–11% on days 78, 85 and 90). In this group also, an overall body weight gain (–18%) and feed intake (at the mean 87% of the control in days 1–90) were decreased. The Panel noted that the changes in body weight of high-dose females were related to lower feed intake but were not accompanied by any clinical or post-mortem pathology observations. Therefore, the Panel considered the changes as not toxicologically relevant.

The haematological investigation revealed a statistically significant decrease in haematocrit (–4%) in mid-dose males, an increase in absolute neutrophil count (+57%), a decrease in percentage of large unstained cells (–25%) in low-dose males and a decrease in percentage of basophils in low- and high-dose females (–36% and –36%). The Panel considered the changes as not toxicologically relevant as the changes were small (haematocrit, basophils), there were no changes in total number of white blood cells (neutrophils), there was no dose–response relationship (all parameters) and the changes were only observed in one sex (all parameters).

The urinalysis revealed a statistically significant increase in concentrations of sodium in high-dose males (+81%) and females (+142%) and of chloride in high-dose females (+56%). The Panel noted that the highest dose tested would be accompanied by a high concentration of minerals as shown by the ash content (approximately 230 mg ash/kg bw). Therefore, the increased excretion of sodium and chloride was considered probably related to NaCl presence in the test compound. The Panel considered the changes as not toxicologically relevant as there were no correlates in clinical chemistry and there were no histopathological changes in kidneys.

Statistically significant changes in organ weights included an increase in the relative kidney weight in high-dose males (+10%). The Panel considered the change as not toxicologically relevant as it was small, there were no histopathological changes in kidneys and the change was only observed in one sex.

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

The Panel identified a no observed adverse effect level (NOAEL) of 1,320 mg TOS/kg bw per day, the highest dose tested.

3.4.3. Allergenicity³⁷

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

The potential allergenicity of the mucorpepsin produced with the *R. miehei* strain MMR 164 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 pepsin A from *Sus scrofa*, lysosomal aspartic protease from yellow fever mosquito *Aedes aegypti*, aspartyl endopeptidase from *Rhizopus oryzae*, aspergillopepsin i from *Aspergillus fumigatus* and Sin a 3 allergen from yellow mustard *Sinapis alba*.

No information is available on oral and respiratory sensitisation or elicitation reactions of this mucorpepsin.³⁸

Mustard is an allergenic food and is listed as a food allergen in the Annex II of the Regulation (EU) No 1169/2011³⁹. Sin a 3, one of the allergens in mustard, is not the major allergen.

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

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

A product that may cause allergies or intolerances (Regulation (EU) No 1169/2011³⁹) is used as raw material (██████████).⁴⁰ In addition, ██████████ a known source of allergens, is also present in the media fed to the microorganisms. However, during the fermentation process, these products will be degraded and utilised by the microorganisms for cell growth, cell maintenance and production of enzyme protein. In addition, the fungal biomass and fermentation solids are removed. Taking into account the fermentation process and downstream processing, the Panel considered that potentially allergenic residues of these foods employed as protein sources are not expected to be present in the food enzyme.

The Panel also notes that ██████████ is used during the downstream processing of the thermolabile food enzyme and is likely to be present in the final product. Respiratory sensitisation to ██████████ 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, under the intended conditions of use, the risk of allergic sensitisation and elicitation reactions upon dietary exposure to this food enzyme cannot be excluded, but is considered low except for individuals sensitised to mustard proteins, but this risk will not exceed that of mustard consumption.

3.5. Dietary exposure

3.5.1. Intended use of the food enzyme⁴¹

The food enzyme is intended to be used in milk processing for cheese production at a recommended use level of up to 5 mg TOS/kg milk.⁴²

³⁷ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 12–13, 62–63; Technical dossier/Additional data, 28 September 2021/Annex S, Annex T, Annex U.

³⁸ Technical dossier/Additional data, 28 September 2021/Annex T.

³⁹ 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.

⁴⁰ Technical dossier/Additional data, 28 September 2021/Annex K.

⁴¹ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 5, 11, 52–54, 75; Technical dossier/Additional data, 28 September 2021/other annexes join Dossier MMR 164 - Q 2014-00851_update/Annex M.

⁴² Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 54.

In cheese production, the food enzyme is added to the milk during the coagulation step to hydrolyse κ -casein. Whey, a by-product, is separated from the curd during the draining step.⁴³ Curd is further processed into different types of cheese, whereas whey is used in the production of several foods, including bakery products and beverages. The food enzyme partitions differentially in curd and whey with a ratio of approximately 1:9 (Guinee and Wilkinson, 1992). The food enzyme TOS remains in the final foods.

Based on thermostability data (see Section 3.3.1), the enzyme is expected to be inactivated during the pasteurisation of whey.

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, 2021a). The estimation involved selection of relevant food categories and application of technical conversion factors (EFSA CEP Panel, 2021b). 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 2 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 41 dietary surveys (covering infants, toddlers, children, adolescents, adults and the elderly), carried out in 22 European countries (Appendix B). The highest dietary exposure to the food enzyme–TOS was estimated to be about 0.982 mg TOS/kg bw per day in infants.

Table 2: Summary of estimated dietary exposure to food enzyme–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)	0.022–0.440 (11)	0.023–0.198 (15)	0.010–0.026 (19)	0.006–0.036 (21)	0.003–0.031 (22)	0.003–0.010 (22)
Min–max 95th (number of surveys)	0.108–0.982 (9)	0.078–0.451 (13)	0.022–0.091 (19)	0.015–0.037 (20)	0.009–0.097 (22)	0.007–0.024 (21)

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 3.

⁴³ Technical dossier/Additional data, 28 September 2021/Dossier MMR 164 Q 2014-00851_update28SEP21/p. 53.

Table 3: 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	
FoodEx categories included in the exposure assessment were assumed to always contain the food enzyme-TOS	+
Exposure to food enzyme-TOS was always calculated based on the recommended maximum use level	+
Assuming that whey protein concentrate is used in all milk-based infant formulae and follow-on formulae	+
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	+/-

TOS: total organic solids.

+: uncertainty with potential to cause overestimation of exposure.

-: uncertainty with potential to cause underestimation of exposure.

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 overestimation of the exposure.

3.6. Margin of exposure

A comparison of the NOAEL (1,320 mg TOS/kg bw per day) from the 90-day study in rats with the derived exposure estimates of 0.003–0.440 mg TOS/kg bw per day at the mean and from 0.007 to 0.982 mg TOS/kg bw per day at the 95th percentile, resulted in margin of exposure (MOE) of at least 1,344.

4. Conclusions

Based on the data provided, and the derived margin of exposure, the Panel concluded that the native and thermolabile forms of the food enzyme mucorpepsin produced with the *R. miehei* strain MMR 164 does not give rise to safety concerns under the intended conditions of use.

5. Documentation as provided to EFSA

Technical dossier 'Application for authorisation of Microbial rennet thermostable and thermolabile from *Rhizomucor miehei* in accordance with Regulation (EC) No 1331/2008'. The dossier was updated on 28 September 2021. Submitted by Takabio and IFF.

Additional information. 28 September 2021. Submitted by Takabio and IFF.

Summary report on technical data and dietary exposure. 12 May 2016. Delivered by contractor Hylobates Consulting and BiCT (Roma, Italy).

Summary report genotoxicity and subchronic toxicity. May 2016. Delivered by contractor FoBiG GmbH (Freiburg, Germany).

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Abbreviations

bw	body weight
CAS	Chemical Abstracts Service
CFU	colony forming unit
EFSA CEF Panel	EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
EFSA CEP Panel	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids

EFSA GMO Panel	EFSA Panel on Genetically Modified Organisms
CHL/IU	Chinese hamster lung fibroblast cell line
EINECS	European Inventory of Existing Commercial Chemical Substances
FAO	Food and Agricultural Organization of the United Nations
FoodEx	the food classification and description system
GLP	Good Laboratory Practice
GM	genetically modified
IMCU	International Milk-clotting Unit
IUBMB	International Union of Biochemistry and Molecular Biology
ISO	International Organisation for Standardisation
ITS	Internal Transcribed Spacer
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kDa	kiloDalton
LOD	limit of detection
MOE	margin of exposure
NBRC	National Institute of Technology and Evaluation
NOAEL	no-observed-adverse-effect level
OECD	Organisation for Economic Cooperation and Development
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TOS	total organic solids
U	Unit
WHO	World Health Organization

Appendix A – Dietary exposure estimates to the food enzyme–TOS in details

Information provided in this appendix is shown in an excel file (downloadable <https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2022.7459#support-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
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, 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, Spain, Sweden
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Netherlands, Portugal, Romania, Slovenia, Spain, Sweden
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Netherlands, Portugal, Romania, 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, Netherlands, Portugal, Romania, Slovenia, Spain, Sweden

(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).