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Safety evaluation of the food enzyme chymosin from the genetically modified *Aspergillus niger* strain DSM 29544

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Abstract

The food enzyme chymosin (EC 3.4.23.4) is produced with the genetically modified *Aspergillus niger* strain DSM 29544 by Chr. Hansen. The genetic modifications do not give rise to safety concerns. The food enzyme was considered free from viable cells of the production organism and its DNA. It is intended to be used in milk processing for cheese production and for production of fermented milk products. Based on the maximum use levels, dietary exposure to the food enzyme total organic solids (TOS) was estimated to be up to 0.09 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 84.1 mg TOS/kg bw per day, the highest dose tested, which when compared with the estimated dietary exposure, results in a margin of exposure above 930. A search for similarity of the amino acid sequence of the food enzyme to known allergens was made and two matches with respiratory allergens were found. The Panel considered that, under the intended conditions of use, the risk of allergic sensitisation and elicitation reactions by dietary exposure cannot be excluded, but the likelihood for this to occur is considered low. 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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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 1332/2008 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 1331/2008² 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, 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 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 1332/2008 on food enzymes.

Six applications have been introduced by the companies "Amano Enzyme Inc." for the authorisation of the food enzymes Alpha-L-rhamnosidase from *Penicillium decumbens* (strain AE-I IP) and Acylglycerol lipase from a genetically modified strain of *Penicillium camemberti* (strain AE-LGS), and "Chr. Hansen" for the authorisation of the food enzymes Chymosin from a genetically modified strain of *Aspergillus niger* var. *awamori* (strain DSM 29544), Chymosin from a genetically modified strain of *Aspergillus niger* var. *awamori* (strain DSM 29545), Chymosin from a genetically modified strain of *Aspergillus niger* var. *awamori* (strain DSM 29546) and Mucorpepsin from *Rhizomucor miehei* (strain DSM 29547).

Following the requirements of Article 12.1 of Regulation (EC) No 234/2011³ 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 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.

1.1.2. Terms of Reference

The European Commission requests the European Food Safety Authority to carry out the safety assessments on the food enzymes Alpha-L-rhamnosidase from *Penicillium decumbens* (strain AE-HP), Acylglycerol lipase from a genetically modified strain of *Penicillium camemberti* (strain AE-LGS), Chymosin from a genetically modified strain of *Aspergillus niger* var. *awamori* (strain DSM 29544), Chymosin from a genetically modified strain of *Aspergillus niger* var. *awamori* (strain DSM 29545), Chymosin from a genetically modified strain of *Aspergillus niger* var. *awamori* (strain DSM 29546) and Mucorpepsin from *Rhizomucor miehei* (strain DSM 29547) in accordance with Article 17.3 of Regulation (EC) No 1332/2008 on food enzymes.

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 chymosin from a genetically modified *A. niger* var. *awamori* strain DSM 29544.

2. Data and methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme chymosin from a genetically modified *A. niger* var. *awamori* strain DSM 29544.

Additional information was spontaneously provided from the applicant on 23 January 2019.

Additional information was requested from the applicant during the assessment process on 21 July 2020 and 7 June 2021.

Following the request for additional data sent by EFSA on 21 July 2020, the applicant requested a clarification teleconference on 15 September 2020, after which the applicant provided additional data on 19 March 2021.

Following the request for additional data sent by EFSA on 7 June 2021, the applicant requested a clarification teleconference on 25 June 2021, after which the applicant provided additional data on 16 May 2022.

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, 2009a) 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 with the updated 'Scientific Guidance for the submission of dossiers on food enzymes' (EFSA CEP Panel, 2021a).

3. Assessment

IUBMB nomenclature	Chymosin
Systematic name	–
Synonyms	Rennin, chymase
IUBMB No	EC 3.4.23.4
CAS No	9001-98-3
EINECS No	232-645-0

Chymosins catalyse the hydrolysis of a single peptide bond between amino acid residues 105 and 106, phenylalanine and methionine (Ser-Phe¹⁰⁵/Met¹⁰⁶-Ala) in κ -casein. This results in precipitation of milk protein and curd formation. The food enzyme is intended to be used in milk processing for cheese production and for the production of fermented milk products.

3.1. Source of the food enzyme

The chymosin is produced with a genetically modified filamentous fungus *A. niger* var. *awamori* [redacted], which is deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany), with deposit number DSM [redacted]⁴ The production strain was identified as *A. niger* [redacted]⁵ It was found to produce ochratoxin A and fumonisin B2.⁶

3.1.1. Characteristics of the parental and recipient microorganisms

The parental strain is [redacted]

The recipient strain [redacted]

[redacted]

3.1.2. Characteristics of introduced sequences

The sequence encoding the chymosin [redacted]

[redacted]

3.1.3. Description of the genetic modification process

The purpose of the genetic modification was to enable the production strain to synthesise chymosin

[redacted]⁸

The production strain *A. niger* var. *awamori* DSM 29544

[redacted]⁹ [redacted]¹⁰

⁴ Technical Dossier/Annex 17.

⁵ Technical dossier/Additional data March 2021/Annex Q1.

⁶ Technical Dossier/Annex 36.

⁷ Technical dossier/Additional data December 2020/Annex Q1.

⁸ Technical Dossier/Annex 20.

⁹ Technical Dossier/Annex 22.

¹⁰ Technical Dossier/Annex 21.

3.1.4. Safety aspects of the genetic modification

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

The production strain *A. niger* var. *awamori* DSM 29544 differs from the recipient strain in its capacity to produce chymosin

¹¹

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

3.2. Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004¹², with food safety procedures based on hazard analysis and critical control points, and in accordance with current good manufacturing practice.¹³

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 cells of the production strain are [REDACTED] and separated from the fermentation broth by filtration, leaving a filtrate containing the food enzyme. The filtrate containing the enzyme is then further purified and concentrated, including a chromatographic separation step in which the chymosin is adsorbed to a specific resin and then eluted. After this step, [REDACTED] is added to the solution to protect the chymosin [REDACTED]. Finally, the food enzyme concentrate is sterile filtered and standardised.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 chymosin is a single polypeptide chain of 323 amino acids.¹⁶ The molecular mass of the mature protein, calculated from the amino acid sequence, is 36 kDa.¹⁶ The food enzyme was analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The gels showed a single major protein band corresponding to an apparent molecular mass of about 38 kDa, consistent with the expected mass of the enzyme, and a minor band of about 42 kDa, corresponding to the glycosylated form of the enzyme.¹⁷ The food enzyme was tested for amylase and lipase activities and none were detected.¹⁸ No other enzymatic activities were reported.

The determination of chymosin activity is based on clotting of reconstructed skim milk (reaction conditions: pH 6.5, 32°C, max. 20 min). The enzymatic activity is determined by measuring the time from the addition of the enzyme to the formation of visible flakes. The chymosin activity is quantified relative to a reference enzyme standard and expressed in International Milk Clotting Units/mL (IMCU/mL).¹⁹

The food enzyme has a temperature optimum between 45°C and 47°C (pH 6.5) and a pH optimum around pH 6.3 (32°C). Thermostability was tested after an incubation of the food enzyme at different temperatures (51–57°C) at different times (pH 6.6). At 57°C, enzyme activity decreased logarithmically with the incubation time, showing no residual activity after about 3 min of incubation.²⁰

¹¹ Technical Dossier/Additional data March 2021/Annex Q4.

¹² 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.

¹³ Technical dossier/Annexes 23–24.

¹⁴ Technical dossier/pp. 63–71/Annex 26/Additional data May 2022.

¹⁵ Technical dossier/2nd submission/Annex 6.

¹⁶ Technical dossier/pp. 33.

¹⁷ Technical Dossier/Additional information March 2021/Annex Q8.

¹⁸ Technical dossier/Annexes 8–10.

¹⁹ Technical dossier/Annex 7.

²⁰ Technical Dossier/Additional information March 2021/Annex Q11.

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches used for commercialisation and two batches produced for the toxicological tests (Table 1).²¹ The mean total organic solids (TOS) of the three food enzyme batches for commercialisation is 4.3% and the mean enzyme activity/TOS ratio is 144.0 IMCU/mg TOS.

Table 1: Compositional data of the food enzyme

Parameters	Unit	Batches				
		1	2	3	4 ^(a)	5 ^(b)
Chymosin activity	IMCU/mL batch ^(c)	6,166	6,543	5,873	1,858	1,378
Protein	%	3.2	3.2	3.3	NA	0.5
Ash	%	11.0	10.6	11.1	<0.1	0.1
Water	%	84.7	85.0	84.7	98.6	95.7
Total organic solids (TOS)^(d)	%	4.3	4.4	4.2	1.4	4.2
Activity/mg TOS	IMCU/mg TOS	143.4	148.7	139.8	132.7	32.8

NA: not available.

(a): Batch used for the bacterial reverse mutation test and the 90-day repeated dose oral toxicity study.

(b): Batch used for the *in vitro* micronucleus test.

(c): IMCU: International Milk Clotting Units (see Section 3.3.1).

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

3.3.3. Purity

The lead content in three commercial batches and in the two batches used for toxicological studies was below 5 mg/kg²² which complies with the specification for lead as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006). In addition, the levels of heavy metals arsenic, cadmium and mercury were below the limits of detection of the employed methodologies.^{18,23}

The food enzyme complies with the microbiological criteria (for total coliforms, *Escherichia coli* and *Salmonella*), as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006).¹⁸ The batches were also tested for *Clostridia* (< 1 CFU/g), *Listeria* (absent in 25 g) and *Staphylococcus aureus* (absent in 1 g). No antimicrobial activity was detected in any of the tested batches (FAO/WHO, 2006).²⁴

Strains of *Aspergillus*, in common with most filamentous fungi, have the capacity to produce a range of secondary metabolites (Frisvad et al., 2018). In fact, the production strain *A. niger* var. *awamori* DSM 29544 is able to produce ochratoxin A and fumonisin B2 (Section 3.1). The presence of these two mycotoxins was examined in the three food enzyme batches for commercialisation.²⁵ All were below the limits of detection (LODs) of the applied analytical methods.²⁶ The potential presence of other secondary metabolites 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.

3.3.4. Viable cells and DNA of the production strain

The absence of the production strain in the food enzyme was demonstrated [redacted] No colonies were observed.²⁷

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

²¹ Technical dossier/pp. 30/Annexes 1–3, 8–10, 37; Spontaneous data submission January 2019/Annex 2.

²² Technical dossier/pp. 32; Annexes 8–10.

²³ LODs: Pb = 0.02 mg/kg; As = 0.05 mg/kg; Cd = 0.01 mg/kg; Hg = 0.005 mg/kg.

²⁴ Technical dossier/Annexes 14–16.

²⁵ Technical dossier/Annexes 11–13.

²⁶ LODs: Fumonisin B1, B2: 20 µg/kg each; Ochratoxin A: 0.2 µg/kg.

²⁷ Technical Dossier/Additional data March 2021/Annex Q5.

3.4. Toxicological data²⁹

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. Batches 4 and 5 (Table 1) used in these studies have similar protein pattern and similar or lower chemical purity as the batches used for commercialisation, and thus are considered suitable as test items.

3.4.1. Genotoxicity

3.4.1.1. Bacterial reverse mutation test

A bacterial reverse mutation assay (Ames test) was performed according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 (OECD, 1997) and following Good Laboratory Practice (GLP).³⁰ Four strains of *Salmonella* Typhimurium (TA1535, TA1537, TA98 and TA100) and *Escherichia coli* WP2uvrA (pKM 101), were used with or without metabolic activation (S9-mix), applying the 'treat and wash' assay. Two separate experiments were carried out in triplicate using seven concentrations of the food enzyme protein (from 5 to 5,000 µg/plate, corresponding to 17.4, 52.1, 173.7, 521.1, 1,737, 5,211 and 17,370 µg TOS/plate)³¹ in the first experiment and five concentrations of the food enzyme protein (from 50 to 5,000 µg/plate, corresponding to 173.7, 521.1, 1,737, 5,211 and 17,370 µg TOS/plate) in the second experiment. No cytotoxicity was observed at any concentration tested. 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 chymosin 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 OECD Test Guideline 487 (OECD, 2016) and following GLP.³² An experiment was performed with duplicate cultures of human peripheral whole blood lymphocytes. The cell cultures were treated with the food enzyme with or without metabolic activation (S9-mix). In a range-finding test, no cytotoxicity above 50% was seen at any concentration tested up to 10% of food enzyme with and without metabolic activation (S9-mix). The cells were exposed to the food enzyme and scored for the frequency of binucleated cells with micronuclei (MNBN) at concentrations of 6, 8 and 10%, corresponding to 2,508, 3,344 and 4,180 µg TOS/mL in a short-term treatment (3 h exposure and 21 h recovery period) either with or without S9-mix and in a long-term treatment (24 h exposure and 24 h recovery period) without S9-mix.

No cytotoxicity was seen either in the short-term with and/or without S9-mix or in the long-term treatment. The frequency of MNBN was not statistically significant different to the negative controls at all concentrations tested.

The Panel concluded that the food enzyme chymosin did not induce an increase in the frequency of MNBN 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.³³ Groups of 10 male and 10 female Sprague–Dawley Crl:CD (SD) rats received by gavage the food enzyme in doses of 0.967, 4.84 or 24.2 mg food enzyme protein/kg body weight (bw) per day, corresponding to 3.4, 16.8 and 84.1 mg TOS/kg bw per day. Controls received the vehicle (chlorine-free water).

No mortality was observed.

²⁸ Technical dossier/Additional data March 2021/Annex Q6.

²⁹ Technical dossier/Spontaneous data submission 2019 Annex 1 and Annex 2.

³⁰ Technical dossier/Annex 30; Technical dossier/Spontaneous data submission 2019/ Annex 1 and Annex 2.

³¹ Technical dossier/Additional data May 2022/Annex Q14.1.

³² Technical dossier/Additional data May 2022/Annex Q14.4.

³³ Technical dossier/Annex 32; Technical dossier/Spontaneous data submission 2019/Annex 1 and Annex 2.

In the functional observations, a statistically significant increase in the forelimb grip strength in high-dose males (+13%), decreases in rearing activity at a 12-min interval in low-, mid- and high-dose males (−28%, −18% and −20%, respectively) and in low-, mid- and high-dose females (−28%, −20% and −24%, respectively), and at 30-min interval in low-, mid- and high-dose females (−66%, −43% and −43%, respectively), an increase in rearing activity at a 54-min interval in high-dose males (+381%) and decreases in cage floor activity in low-, mid- and high-dose females (−48%, −41% and −41%, respectively) were observed. The Panel considered the changes as not toxicologically relevant as they were only observed in one sex (forelimb grip strength, cage floor activity), they were only recorded sporadically (rearing and cage floor activities), there were no differences to control in the total scores (1-h recording period) (rearing and cage floor activities) and group mean scores for concurrent controls were towards the higher end of the normal range of historical control data for motor activity.

The haematological investigation revealed a statistically significant decrease in mean corpuscular haemoglobin (MCH) (−4.9%) in mid-dose males, mean corpuscular haemoglobin concentration (MCHC) in low-, mid- and high-dose males (−2%, −3%, −1%) and a decrease in prothrombin time (PT) in mid-dose (−12%) and high-dose (−8%) females. The Panel considered the changes as not toxicologically relevant as they were only observed in one sex (all parameters), there was no dose–response relationship (all parameters), the changes were small (MCH, MCHC) and there were no changes in other relevant parameters (for MCH and MCHC in haemoglobin, haematocrit and red blood cell count; for PT in activated partial thromboplastin time and platelet count).

The clinical chemistry investigation revealed a statistically significant increase in the chloride concentration in mid-dose males (+2%) and an increase in the sodium concentration in low-, mid- and high-dose females (+1%, +1% and +2%). The Panel considered the changes as not toxicologically relevant as they were only observed in one sex (both parameters), there was no dose–response relationship (both parameters), there were no histopathological changes in the kidneys, there were no changes in other relevant parameters (i.e. water intake).

Statistically significant changes in organ weights included increase in adjusted mean weights of the heart (+7%) and testes (+7%) in high-dose males. The Panel considered the changes as not toxicologically relevant as they were only observed in one sex (heart), the changes were small (both parameters), there were no histopathological changes in the heart and testes and the changes were within the historical control values.

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

The Panel identified a no observed adverse effect level (NOAEL) of 84.1 mg TOS/kg bw per day, the highest dose tested.

3.4.3. Allergenicity

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

The potential allergenicity of the chymosin produced with the genetically modified strain *A. niger* var. *awamori* DSM 29544 was assessed by comparing its amino acid sequence (including the pro-peptide) with those of known allergens according to the 'Scientific Opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed of the Scientific Panel on Genetically Modified Organisms' (EFSA GMO Panel, 2010). Using higher than 35% identity in a sliding window of 80 amino acids as the criterion, two matches were found.³⁴ The matching allergens were pepsin A from wild boar (*Sus scrofa*) and aspartic protease-like protein Bla g2 from German cockroach (*Blattella germanica*).³⁵

Pepsin is a known respiratory allergen causing occupational asthma and rhinitis in cheese workers (Cartier et al., 1984; Añibarro Bausela and Fontela, 1996; Marques et al., 2006). Bla g2 protease from the German cockroach has also been described as a respiratory allergen (Arruda et al., 1995; Gustchina et al., 2010). However, several studies have shown that adults with respiratory allergy can ingest respiratory allergens without acquiring clinical symptoms of food allergy (Brisman, 2002; Poulsen, 2004; Armentia et al., 2009).

No information is available on oral and respiratory sensitisation or elicitation reactions of this chymosin. The applicant provided a comprehensive literature search, in which no reports were found regarding allergenic reactions to chymosin upon oral exposure.³⁶

³⁴ Technical dossier/Annexes 33–35.

³⁵ Technical dossier/Annex 33.

³⁶ Technical dossier/Additional data March 2021/Annex Q13.

products that may cause allergies or intolerances (listed in the Regulation (EU) No 1169/2011³⁷), are used as raw materials 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.

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 the likelihood of such reactions to occur is considered low.

3.5. Dietary exposure

3.5.1. Intended use of the food enzyme

The food enzyme is intended to be used in two food 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^(d)

Food manufacturing process ^(a)	Raw material (RM)	Recommended dosage of the food enzyme (mg TOS/kg RM) ^{(b),(c)}
Milk processing for cheese production	Milk	0.02– 0.46
Milk processing to production of fermented milk products	Milk	0.05

TOS: total organic solids.

(a): The name has been harmonised by EFSA according to the 'EC working document describing the food processes in which food enzymes are intended to be used' – not yet published at the time of adoption of this opinion.

(b): Based on 131.6 IMCU/mg TOS.

(c): Numbers in bold were used for calculation.

(d): Technical dossier/pp. 78–79.

In cheese production, the food enzyme is added to the milk together with the starter culture.³⁸ The addition of chymosin causes the milk to coagulate and to form curd. By separating the liquid whey from the solid curd, 80–90% of the added enzyme will be found in the whey fraction and 10–20% is retained in the cheese (Documentation provided to EFSA No 6), in which residual enzyme activity is expected. Whey produced during cheese making may be used in a variety of foods including infant and follow-on formula or food for special medical purposes. The food enzyme–TOS remains in cheese and whey.

In the production of fermented milk products such as yoghurt, the food enzyme is added to milk before pasteurisation; alternatively following the pasteurisation it is added together with the lactic acid bacteria cultures.³⁹ Chymosin performs the same function as in cheese making, causing the viscosity of the fermented dairy products to increase. The food enzyme–TOS remains in the fermented milk products, in which residual enzyme activity is expected.

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 CEF 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

³⁷ 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.

³⁸ Technical dossier/Figure 3.2–10.

³⁹ Technical dossier/Figure 3.2–11.

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 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 0.09 mg TOS/kg bw per day in infants.

Table 3: 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)	0.002–0.041 (11)	0.002–0.018 (15)	0.001–0.003 (19)	0.001–0.003 (21)	0–0.003 (22)	0–0.001 (22)
Min–max 95th (number of surveys)	0.011–0.090 (9)	0.007–0.041 (13)	0.002–0.008 (19)	0.001–0.003 (20)	0.001–0.009 (22)	0.001–0.002 (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 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	
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 (84.1 mg TOS/kg bw per day) from the 90-day rat study with the derived exposure estimates of 0–0.041 mg TOS/kg bw per day at the mean and from 0.001 to 0.09 mg TOS/kg bw per day at the 95th percentile, resulted in margin of exposure (MoE) of at least 934.

4. Conclusions

Based on the data provided and the derived margin of exposure, the Panel concluded that the food enzyme chymosin produced with the genetically modified *A. niger* var. *awamori* strain DSM 29544 does not give rise to safety concerns under the intended conditions of use.

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

5. Documentation as provided to EFSA

- 1) Application for authorisation of Chymosin from a genetically modified strain of *Aspergillus niger* var. *awamori*. December 2015. Submitted by Chr. Hansen.
- 2) Additional information. January 2019 Submitted by Chr. Hansen.
- 3) Additional information. March 2021. Submitted by Chr. Hansen.
- 4) Additional information. July 2022. Submitted by Chr. Hansen.
- 5) Summary report on genetically modified microorganism part. July 2018. Delivered by Technical University of Denmark (Lyngby, Denmark).
- 6) "Transfer of food enzymes into whey and cheese during dairy processing". January 2019. Provided by the Association of Manufacturers and Formulators of Enzyme Products.

References

- Añibarro Bausela and Fontela JL, 1996. Occupational asthma in a cheese worker. *Allergy*, 51, 960–961.
- Armentia A, Dias-Perales A, Castrodeza J, Dueñas-Laita A, Palacin A and Fernández S, 2009. Why can patients with baker's asthma tolerate wheat flour ingestion? Is wheat pollen allergy relevant? *Allergologia et Immunopathologia*, 37, 203–204.
- Arruda LK, Vailes LD, Mann BJ, Shannon J, Fox JW, Vedvick TS, Hayden ML and Chapman MD, 1995. Molecular cloning of a major cockroach (*Blattella germanica*) allergen, Bla g 2. Sequence homology to the aspartic proteases.
- Brisman J, 2002. Baker's asthma. *Occupational and Environmental Medicine*, 59, 498–502.
- Cartier A, Malo J-L, Pineau L and Dolovich J, 1984. Occupational asthma due to pepsin. *Journal of Allergy and Clinical Immunology*, 73, 574–577.
- EFSA (European Food Safety Authority), 2006. Opinion of the Scientific Committee related to uncertainties in dietary exposure assessment. *EFSA Journal* 2006;5(1):438, 54 pp. <https://doi.org/10.2903/j.efsa.2007.438>
- EFSA (European Food Safety Authority), 2009a. Guidance of EFSA prepared by the Scientific Panel of Food Contact Material, Enzymes, Flavourings and Processing Aids on the Submission of a Dossier on Food Enzymes. *EFSA Journal* 2009;7(8):1305, 26 pp. <https://doi.org/10.2903/j.efsa.2009.1305>
- EFSA (European Food Safety Authority), 2009b. Guidance of the Scientific Committee on transparency in the scientific aspects of risk assessments carried out by EFSA. Part 2: general principles. *EFSA Journal* 2009;7(5):1051, 22 pp. <https://doi.org/10.2903/j.efsa.2009.1051>
- EFSA (European Food Safety Authority), 2011. Use of the EFSA Comprehensive European Food Consumption Database in exposure assessment. *EFSA Journal* 2011;9(3):2097, 34 pp. <https://doi.org/10.2903/j.efsa.2011.2097>
- EFSA CEP Panel (EFSA Panel on Food Contact Materials, Enzymes and Processing Aids), 2019. Statement on the characterisation of microorganisms used for the production of food enzymes. *EFSA Journal* 2019;17(6):5741, 13 pp. <https://doi.org/10.2903/j.efsa.2019.5741>
- EFSA CEP Panel (EFSA Panel on Food Contact Materials, Enzymes, and Processing Aids), Lambré C, Barat Baviera JM, Bolognesi C, Cocconcelli PS, Crebelli R, Gott DM, Grob K, Lampi E, Mengelers M, Mortensen A, Rivière G, Steffensen I-L, Tlustos C, Van Loveren H, Vernis L, Zorn H, Glandorf B, Herman L, Aguilera J, Andryszkiewicz M, Gomes A, Kovalkovicova N, Liu Y, Rainieri S and Chesson A, 2021a. Scientific Guidance for the submission of dossiers on Food Enzymes. *EFSA Journal* 2021;19(10):6851, 37 pp. <https://doi.org/10.2903/j.efsa.2021.6851>.

- EFSA CEP Panel (EFSA Panel on Food Contact Materials, Enzymes, and Processing Aids), Lambré C, Barat Baviera JM, Bolognesi C, Coconcelli PS, Crebelli R, Gott DM, Grob K, Lampi E, Mengelers M, Mortensen A, Rivière G, Steffensen I-L, Tlustos C, van Loveren H, Vernis L, Zorn H, Liu Y and Chesson A, 2021b. Statement on the process-specific technical data used in exposure assessment of food enzymes. *EFSA Journal* 2021;19(12):7010, 38 pp. <https://doi.org/10.2903/j.efsa.2021.7010>
- EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2010. Scientific Opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed. *EFSA Journal* 2010;8(7):1700, 168 pp. <https://doi.org/10.2903/j.efsa.2010.1700>
- FAO/WHO (Food and Agriculture Organization of the United Nations/World Health Organization), 2006. General specifications and considerations for enzyme preparations used in food processing in Compendium of food additive specifications. 67th meeting. FAO JECFA Monographs, 3, 63–67. Available online: <http://www.fao.org/3/a-a0675e.pdf>
- Frisvad JC, Møller LLH, Larsen TO, Kumar R and Arnau J, 2018. Safety of the fungal workhorses of industrial biotechnology: update on the mycotoxin and secondary metabolite potential of *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei*. *Applied Microbiology and Biotechnology*, 102, 9481–9515. <https://doi.org/10.1007/s00253-018-9354-1>
- Gustchina A, Li M, Wünschmann S, Chapman MD, Pomés A and Wlodawer A, 2010. Crystal structure of cockroach allergen Bla g 2, an unusual zinc binding aspartic protease with a novel mode of self-inhibition. *Journal of Molecular Biology*, 348, 433–444.
- Marques L, Lara S, Abos T and Bartolome B, 2006. Occupational rhinitis due to Pepsin. *J. Investig. Allergol. Clin. Immunol.*, 16(2), 136–137.
- OECD (Organisation for Economic Co-Operation and Development), 1997. OECD Guideline for the testing of chemicals, Section 4 Health effects, Test No. 471: Bacterial reverse mutation test. 21 July 1997. 11 pp. Available online: http://www.oecd-ilibrary.org/environment/test-no-471-bacterial-reverse-mutation-test_9789264071247-en;jsessionid=9zfgzu35paaq.x-oecd-live-01
- OECD (Organisation for Economic Co-Operation and Development), 1998. OECD Guideline for the testing of chemicals, Section 4 Health effects, Test No. 408: Repeated dose 90-day oral toxicity study in rodents. 21 September 1998. 10 pp. Available online: http://www.oecd-ilibrary.org/environment/test-no-408-repeated-dose-90-day-oral-toxicity-study-in-rodents_9789264070707-en
- OECD (Organisation for Economic Co-Operation and Development), 2016. OECD Guideline for the testing of chemicals, Section 4 Health effects, Test No 487: In vitro mammalian cell micronucleus test. July 29, 2016. Available online: <https://www.oecd.org/chemicalsafety/test-no-487-in-vitro-mammalian-cell-micronucleus-test-9789264264861-en.htm>
- Poulsen LK, 2004. Allergy assessment of foods or ingredients derived from biotechnology, gene-modified organisms, or novel food. *Molecular Nutrition & Food Research*, 48, 413–423.

Abbreviations

bw	body weight
CAS	Chemical Abstracts Service
CEP	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
CFU	colony forming units
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	binucleated cells with micronuclei
OECD	Organisation for Economic Cooperation and Development
PCR	polymerase chain reaction
QPS	qualified presumption of safety
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TOS	total organic solids
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.2022.7464#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).