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Safety evaluation of the food enzyme chymosin from the genetically modified *Aspergillus niger* strain DSM32805

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Abstract

The food enzyme chymosin (EC 3.4.23.4) is produced with the genetically modified *Aspergillus niger* strain by Chr. Hansen. The genetic modifications do not give rise to safety concerns. The food enzyme is 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 the production of fermented milk products. Dietary exposure was estimated to be up to 0.09 mg total organic solids (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 at the highest dose of 1,000 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 10,600. Similarity of the amino acid sequence of the food enzyme to those of known allergens was searched 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.

Keywords: Food enzyme, Chymosin, EC 3.4.23.4, rennin, *Aspergillus niger*, genetically modified microorganism

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1. Introduction

Article 3 of the Regulation (EC) No 1332/2008[1] provides definition for 'food enzyme' and 'food enzyme preparation'.

'Food enzyme' means a product obtained from plants, animals or 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 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.

An application has been introduced by the applicant "Chr. Hansen A/S" for the authorisation of the food enzyme Chymosin from a genetically modified strain of *Aspergillus niger* DSM 32805.

Following the requirements of Article 12.1 of Regulation (EC) No 234/2011[3] implementing Regulation (EC) No 1331/2008, the Commission has verified that the application falls within the scope of the food enzyme Regulation and contains all the elements required under Chapter II of that Regulation.

1.1.2. Terms of Reference

The European Commission requests the European Food Safety Authority to carry out the safety assessment of the food enzyme: Chymosin from a genetically modified strain of *Aspergillus niger* DSM 32805 in accordance with Article 17.3 of Regulation (EC) No 1332/2008 on food enzymes.

[1] Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on Food Enzymes and Amending Council Directive 83/417/EEC, Council Regulation (EC) No 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/97. OJ L 354, 31.12.2008, pp. 7–15.

[2] Regulation (EC) No 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 354, 31.12.2008, pp. 1–6.

[3] Commission Regulation (EU) No 234/2011 of 10 March 2011 implementing Regulation (EC) No 1331/2008 of the European Parliament and of the Council establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 64, 11.3.2011, pp. 15–24.

1.2. Interpretation of the Terms of Reference

The present scientific opinion addresses the European Commission's request to carry out the safety assessment of food enzyme chymosin from a genetically modified *A. niger* strain DSM32805.

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* strain DSM32805.

Additional information was spontaneously provided by the applicant on 13 August 2020.

Additional information was requested from the applicant during the assessment process on 20 October 2020 and was consequently provided (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, 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-Phe^{105}/Met^{106}-Ala) in κ-chain of casein. This results in extensive 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 food enzyme chymosin is produced with a genetically modified filamentous fungus *Aspergillus niger* strain, which is deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany) with deposit number DSM32805.[4]

3.1.1. Characteristics of the parental and recipient microorganisms

The parental strain is [redacted] This strain was identified as *A. niger* var. *awamori* and later as *A. niger* (Frisvad et al., 2018).

The recipient strain is [redacted][5]

The recipient strain [redacted]

[4] Technical dossier/1subm010319/Folder 1/Annex 8.
[5] Technical dossier/1subm010319/Folder 1/Annex 10.

[redacted]

[6]

3.1.2. Characteristics of introduced sequences

The sequence encoding the chymosin [redacted]

[7]

3.1.3. Description of the genetic modification process

The purpose of genetic modification was to enable the production strain to synthesise a modified chymosin derived from [redacted]

[8]

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* DSM 32805 differs from the recipient strain [redacted] in its capacity to produce a modified chymosin [redacted]

[9]

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

[6] Technical dossier/Additional data December 2020/Annex Q1.
[7] Technical dossier/1subm010319/Folder 1/Annex 11.
[8] Technical dossier/1subm010319/Folder 1/Annex 12.
[9] Technical dossier/1subm010319/Folder 1/Annex 13.

3.2. Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004[10], with food safety procedures based on hazard analysis and critical control points, and in accordance with current good manufacturing practice.

The production strain is grown as a pure culture using a typical industrial medium in a submerged, fed-batch fermentation system with conventional process controls in place. After completion of the fermentation the solid biomass is removed from the fermentation broth by filtration leaving a filtrate containing the food enzyme. The filtrate is stabilised and then further purified and concentrated. This is achieved using affinity chromatography in which the chymosin is first selectively bound to a modified resin column and then eluted. The eluent is decolourised if required, followed by an ultrafiltration step in which enzyme protein is retained, while most of the remaining low molecular mass material passes the filtration membrane and is discarded. The applicant provided information on the identity of the substances used to control the fermentation and in the subsequent downstream processing of the food enzyme,[11] including a safety assessment of the antifoam agent used and the affinity resin.[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 enzyme

Chymosin consists of a single polypeptide chain of 323 amino acids. The molecular mass of the mature protein derived from the amino acid sequence was calculated to be 35.5 kDa.[13] Five batches of the food enzyme were analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). A consistent protein pattern was observed across all batches with two bands of approximate masses of 39 and 43 kDa evident, which were ascribed to two glycosylation forms of the chymosin.[14] The food enzyme was tested for protease, glucoamylase, amylase, glucanase and lipase activities and all were detected.[15]

The in-house determination of chymosin activity is based on the ISO method 11,815 for the determination of total milk clotting activity.[16] Skimmed milk powder is dissolved in water and the pH adjusted to 6.5 and the temperature to 32°C. A sample of chymosin is introduced and the time to the observation of flocculation on the walls of the reaction vessel recorded. The enzyme activity is quantified relative to an internal enzyme standard and expressed in International Milk Clotting Units/g (IMCU/g).

The food enzyme has a temperature optimum around 50°C (pH 6.5), with no activity detected at 60°C. The effect of pH on activity was measured only between pH 6.2 and 6.7 (32°C). Activity at pH 6.7 was only about two-thirds of that at pH 6.2.[17] Thermostability was measured as a factor of pH in buffer solution (pH 5.9–6.7) and in whey (6.0–6.5) by exposing the enzyme to a temperature of 63°C for 30 min. No activity remained at any pH examined.[18]

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).[19] The mean total organic solids (TOS) of the three food enzyme batches for commercialisation is 5.1% and the mean enzyme activity/TOS ratio is 126.4 IMCU/mg TOS.

[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/1subm010319/Folder 1/Annex 03/02.
[12] Technical dossier/1subm010319/Folder 1/Annex 03/05, 06 and 09.
[13] Technical dossier/1subm010319/Folder 1/p. 27.
[14] Technical dossier/1subm010319/Folder 1/p. 30.
[15] Technical dossier/1subm010319/Folder 1/p. 36.
[16] Technical dossier/1subm010319/Folder 1/Annex 02/09.
[17] Technical dossier/1subm010319/Folder 1/p. 35.
[18] Technical dossier/1subm010319/Folder 1/Annex 17.
[19] Technical dossier/1subm010319/Folder 1/Annex 01.

Table 1: Compositional data of the food enzyme

Parameters	Unit	Batches				
		1	2	3	4[(a)]	5[(b)]
Chymosin activity	IMCU/g batch[(c)]	5,365	5,859	5,877	5,195	5,547
Protein	%	1.4	1.4	NA[(e)]	1.7	1.5
Ash	%	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Water	%	94.3	93.2	96.9	93.1	93.7
Total organic solids (TOS)[(d)]	%	5.6	6.7	3.0	6.8	6.2
Activity/mg TOS	IMCU/mg TOS	95.8	87.4	195.9	76.4	89.5

(a): Batch used for the genotoxicity studies.
(b): Batch used for the repeated dose 90-day oral toxicity study in rats.
(c): IMCU: International Milk Clotting Unit (see Section 3.3.1).
(d): TOS calculated as 100% – % water – % ash (taken as 0.1%).
(e): NA: not analysed.

3.3.3. Purity

The lead content in the commercial batches and in the batches used for toxicological studies was below 0.05 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). In addition, the levels of cadmium, mercury and arsenic were below the limits of detection (LODs) of the employed methods.[20,21]

The food enzyme complies with the microbiological criteria for *E. coli* and *Salmonella* as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006). Samples were also analysed for clostridia, staphylococci and *Listeria monocytogenes*, with negative results.[22] No antimicrobial activity was detected in any of the tested batches (FAO/WHO, 2006).[23]

Strains of *Aspergillus*, in common with most filamentous fungi, have the capacity to produce a range of secondary metabolites (Frisvad et al., 2018). The presence of fumonisins (B1, B2) and ochratoxin B was examined in all five food enzyme batches described in Table 1. Both were below the LODs of the applied analytical methods.[24,25] Adverse effects due to 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][26] [redacted] No colonies were produced.

The absence of recombinant DNA in the food enzyme was demonstrated [redacted][27] [redacted]

3.4. Toxicological data

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

[20] LODs: Pb = 0.05 mg/kg; As = 0.1 mg/kg; Cd = 0.01 mg/kg; Hg = 0.05 mg/kg.
[21] Technical dossier/1subm010319/Folder 1/Annex 01/1–1.
[22] Technical dossier/1subm010319/Folder 1/Annex 01/1–4, 2–3, 3–3, 4–3 and 5–3.
[23] Technical dossier/1subm010319/Folder 1/Annex 01/1–6, 2–5, 3–5, 4–6 and 5–5.
[24] Technical dossier/1subm010319/Folder 1/Annex 01/1–3, 2–3, 3–3, 4–3 and 5–3.
[25] LODs: fumonisins (B1, B2) = 40 μg/kg each; ochratoxin B = 0.2 μg/kg.
[26] Technical dossier/1subm010319/Folder 1/Annex 15.
[27] Technical dossier/1subm010319/Folder 1/Annex 16.

items used are described in Table 1 (batch 4 used for genotoxicity testing and batch 5 used for the repeated dose study) and are considered representative of the batches used for commercialisation.

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).[28] Four strains of *Salmonella* Typhimurium (TA98, TA100, TA1535 and TA1537) and *E. coli* WP2uvrA(pKM101) were used in the presence or absence of metabolic activation (S9-mix), applying the 'treat and plate' assay. Two separate experiments were carried out in triplicate. In the first experiment, seven concentrations of the food enzyme were tested (5, 15, 50, 150, 500, 1,500 and 5,000 μg TOS/plate in the absence and presence of S9-mix (10%). In the second experiment, five concentrations were applied (50, 150, 500, 1,500 and 5,000 μg TOS/mL) in the absence and presence of S9-mix (20%). No cytotoxicity was observed at any concentration tested. 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 Panel concluded that the food enzyme did not induce gene mutations under the test conditions employed in this study.

3.4.1.2. *In vitro* micronucleus assay

The *in vitro* micronucleus test was carried out according to OECD Draft Guideline 487 (OECD, 2016) and following GLP.[29] Two separate experiments were performed in duplicate cultures of human peripheral whole blood lymphocytes. On the basis of the results of a preliminary toxicity test, cell cultures were treated with the food enzyme at 1,250, 2,500 and 5,000 μg TOS/mL for 3 h in the presence or absence of S9-mix and harvested 20 h after the beginning of the treatment. No cytotoxicity was observed at any concentration tested. A continuous 20-h treatment without S9-mix at concentrations of 625, 2,500 and 5,000 μg TOS/mL was also included. A reduction (−34.9%) of the cytokinesis-block proliferative index (CBPI) was observed at 5,000 μg TOS/mL. The frequency of binucleated cells with micronuclei (MNBNs) was comparable to the negative controls at any concentration and condition tested.

The Panel concluded that the food enzyme did not induce an increase in the frequency of MNBNs in cultured human peripheral blood lymphocytes, under the test conditions employed 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.[30] Groups of 10 male and 10 female Wistar (RccHan, WIST) rats received by gavage the food enzyme in amounts corresponding to 250, 500 or 1,000 mg TOS/kg bw per day. Controls received the vehicle (water).

One male from the low-dose group was killed in week 7 for welfare reasons following a convulsive episode. Since this involved only one animal in a low-dose group this was not considered treatment-related.

In the functional observations, occasional statistically significant differences were observed (an increase in low beam scores in high-dose males, an increase in high beam scores in high-dose females and an increase in the grip strength in high-dose males). The Panel considered the changes as not toxicologically relevant as the changes were either transitory or small and they were only observed in one sex.

The haematological investigation revealed a statistically significant increase in mean cell haemoglobin concentration (MCHC, +3%) and a reduced red cell width distribution (RDW, −6%) in high-dose males. Prothrombin time (PT) was significantly increased in mid-dose males (+16%) and activated partial thromboplastin time (APTT) was increased in mid- and high-dose males (+18% and +3%, respectively). In females, PT was significantly reduced in low- and high-dose groups (−8% and −8%, respectively), while APTT significantly increased in the high-dose group (+8%). Significant increases in reticulocyte (Retic) counts (+23%) and in neutrophil (Neu) numbers (+72%) were also

[28] Technical dossier/1subm010319/Confidential/Folder 1/Annexes/Annex 04/ sub-annex 1.
[29] Technical dossier/1subm10319/confidential/Folder1/Annexes/Annex04/subannex1.
[30] Technical dossier/1subm010319/Confidential/Folder1/Annexes/annex04/subannex3.

noted in females receiving the high-dose. Although 5/9 animals in the high-dose group showed higher neutrophil counts than the mean control value, the statistical difference was driven by two individual values. The Panel considered the changes as not toxicologically relevant as the changes were only observed in one sex (MCHC, RDW, Retic, Neu), the changes were small (MCHC, RDW, Neu), there was no apparent dose–response relationship (PT, APTT), there was no consistency between the changes in males and females (PT) and there were no changes in other relevant parameters (e.g. erythrocyte count, white blood cell count and platelet count).

The clinical chemistry investigation revealed a statistically significant increase in aspartate aminotransferase (AST, +60%) and potassium (K, +14%) in the low-dose males, an increase in alkaline phosphatase (ALP) in high-dose males (+17%), a decrease in phosphorus (Phos) concentration in mid- and high-dose males (−8% at both doses), a decrease in total protein concentration (Tot Prot, −8%) and an increased albumin to globulin ratio (+24%) in low-dose females. The Panel considered the changes as not toxicologically relevant as the changes were only observed in one sex (all parameters), there was no dose–response relationship (all parameters except ALP) and the changes were small (ALP, Tot Prot).

Statistically significant changes in organ weights included increase in adjusted kidney weight (+10%) and the weight of seminal vesicles (+18%) in high-dose males. The Panel considered the changes as not toxicologically relevant as the changes were small (kidney) and there were no histopathological changes in these organs.

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

The Panel identified the no observed adverse effect level (NOAEL) of 1,000 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 chymosin produced with the genetically modified *Aspergillus niger* DSM 32805 was assessed by comparing its amino acid sequence with those of known allergens according to the 'Scientific Opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed of the Scientific Panel on Genetically Modified Organisms' (EFSA GMO Panel, 2010). Using higher than 35% identity in a sliding window of 80 amino acids as the criterion, two matches were found. The matching allergens were an aspartyl endopeptidase from *Rhizopus oryzae* (56.3% identity), described as a respiratory allergen, and Bla G 2 from *Blattella germanica* (36.2% identity), reported to elicit IgE formation and the development of asthma in genetically predisposed individuals (Arruda et al., 1995).

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

Several studies have shown that adults with respiratory allergy may be able to ingest the respiratory 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 chymosin have been reported in the literature.

[redacted] a substance that may cause allergies or intolerances (listed in the Regulation (EU) No 1169/2011[30]), is used as raw materials in the media fed to the microorganisms. However, during the fermentation process, this product will be degraded and utilised by the microorganisms for cell growth, cell maintenance and production of enzyme protein. In addition, the fungal biomass and fermentation solids are removed. Taking into account the fermentation process and downstream processing, the Panel considered that 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–**0.48**
Milk processing to production of fermented milk products	Milk	0.02–**0.06**

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 126 IMCU/mg TOS.
(c): Numbers in bold were used for calculation.
(d): Technical dossier/pp.74, 100, 101.

In cheese production, the food enzyme is added to the milk together with the starter culture.[31] 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 is found in the whey fraction and 10–20% is retained in the cheese (Documentation provided to EFSA No 3), 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.[32] Chymosin performs the same function as in cheese, making 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 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 0.094 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)	0.002–0.042 (11)	0.003–0.019 (15)	0.001–0.003 (19)	0.001–0.004 (21)	0–0.003 (22)	0–0.001 (22)
Min–max 95th (number of surveys)	0.011–0.094 (9)	0.008–0.043 (13)	0.002–0.009 (19)	0.001–0.004 (20)	0.001–0.009 (22)	0.001–0.002 (21)

TOS: total organic solids.

[31] Technical dossier/Figure 3.2–9.
[32] Technical dossier/Figure 3.2–10.

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 (1,000 mg TOS/kg bw per day) from the 90-day rat study with the derived exposure estimates of 0–0.042 mg TOS/kg bw per day at the mean and from 0.001 to 0.094 mg TOS/kg bw per day at the 95th percentile, resulted in margin of exposure (MoE) of at least 10,638.

4. Conclusions

Based on the data provided and the derived MoE, the Panel concluded that the food enzyme chymosin produced with the genetically modified *A. niger* strain DSM32805 does not give rise to safety concerns under the intended conditions of use.

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

5. Documentation as provided to EFSA

1) Application for authorization of chymosin from a genetically modified strain of *Aspergillus niger* (DSM32805). March 2019. Submitted by Chr. Hansen.
2) Additional information. December 2020. Submitted by Chr. Hansen.
3) "Transfer of food enzymes into whey and cheese during dairy processing". January 2019. Provided by the Association of Manufacturers and Formulators of Enzyme Products.

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Abbreviations

ALP	alkaline phosphatase
APTT	activated partial thromboplastin time
AST	aspartate aminotransferase
bw	body weight
CAS	Chemical Abstracts Service
CEP	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids

EINECS	European Inventory of Existing Commercial Chemical Substances
FAO	Food and Agricultural Organisation of the United Nations
GLP	good laboratory practice
GMO	genetically modified organism
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kDa	kiloDalton
LoD	limit of detection
MCHC	mean cell haemoglobin concentration
Neu	neutrophil
OECD	Organisation for Economic Cooperation and Development
PCR	polymerase chain reaction
QPS	qualified presumption of safety
Phos	phosphorus
PT	prothrombin time
RDW	red cell width distribution
Retic	reticulocyte
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TOS	total organic solids
Tot	Prot total protein concentration
WGS	whole genome sequencing
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.2022.7466#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).