SCIENTIFIC OPINION

EFSA Journal

ADOPTED: 7 July 2022

doi: 10.2903/j.efsa.2022.7465

Safety evaluation of the food enzyme chymosin from the genetically modified *Aspergillus niger* strain DSM 29546

EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (CEP), Vittorio Silano, José Manuel Barat Baviera, Claudia Bolognesi, Pier Sandro Cocconcelli, Riccardo Crebelli, David Michael Gott, Konrad Grob, Claude Lambré, Evgenia Lampi, Marcel Mengelers, Alicja Mortensen, Gilles Rivière, Inger-Lise Steffensen, Christina Tlustos, Henk Van Loveren, Laurence Vernis, Holger Zorn, Jaime Aguilera, Magdalena Andryszkiewicz, Rita Ferreira de Sousa, Yi Liu, Giulio di Piazza, Sandra Rainieri and Andrew Chesson

Abstract

The food enzyme chymosin (EC 3.4.23.4) is produced with the genetically modified *Aspergillus niger* strain DSM 29546 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 0.52 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 of 410 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 790. Similarity of the amino acid sequence of the food enzyme to those of known allergens was searched and four matches were found. The Panel considered that, under the intended conditions of use, the risk of allergic sensitisation and elicitation reactions by dietary exposure, although unlikely, cannot be excluded, particularly for individuals sensitised to cedar pollen allergens. 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, rennin, EC 3.4.23.4, *Aspergillus niger*, genetically modified microorganism

Requestor: European Commission

Question number: EFSA-Q-2015-00760

Correspondence: fip@efsa.europa.eu

Panel members: Vittorio Silano, José Manuel Barat Baviera, Claudia Bolognesi, Pier Sandro Cocconcelli, Riccardo Crebelli, David Michael Gott, Konrad Grob, Claude Lambré, Evgenia Lampi, Marcel Mengelers, Alicja Mortensen, Gilles Rivière, Inger-Lise Steffensen, Christina Tlustos, Henk Van Loveren, Laurence Vernis, Holger Zorn and Andrew Chesson.

Note: The full opinion will be published in accordance with Article 12 of Regulation (EC) No 1331/2008 once the decision on confidentiality will be received from the European Commission.

Declarations of interest: If you wish to access the declaration of interests of any expert contributing to an EFSA scientific assessment, please contact interestmanagement@efsa.europa.eu.

Suggested citation: EFSA CEP Panel (EFSA Panel on Food Contact Materials, Enzymes and Processing Aids), Silano V, Barat Baviera JM, Bolognesi C, Cocconcelli PS, Crebelli R, Gott DM, Grob K, Lambré C, Lampi E, Mengelers M, Mortensen A, Rivière G, Steffensen I-L, Tlustos C, Van Loveren H, Vernis L, Zorn H, Aguilera J, Andryszkiewicz M, de Sousa RF, Liu Y, di Piazza G, Rainieri S and Chesson A, 2022. Scientific Opinion on the safety evaluation of the food enzyme chymosin from the genetically modified *Aspergillus niger* strain DSM 29546. EFSA Journal 2022;20(8):7465, 17 pp. https://doi.org/10.2903/j.efsa.2022.7465

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, a European agency funded by the European Union.

Table of contents

1. Introduction

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

'Food enzyme' means a product obtained from plants, animals or micro-organisms or products thereof including a product obtained by a fermentation process using micro-organisms: (i) containing one or more enzymes capable of catalysing a specific biochemical reaction; and (ii) added to food for a technological purpose at any stage of the manufacturing, processing, preparation, treatment, packaging, transport or storage of foods.

'Food enzyme preparation' means a formulation consisting of one or more food enzymes in which substances such as food additives and/or other food ingredients are incorporated to facilitate their storage, sale, standardisation, dilution or dissolution.

Before January 2009, food enzymes other than those used as food additives were not regulated or were regulated as processing aids under the legislation of the Member States. On 20 January 2009, Regulation (EC) No 1332/2008 on food enzymes came into force. This Regulation applies to enzymes that are added to food to perform a technological function in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food, including enzymes used as processing aids. Regulation (EC) No 1331/2008[2] established the European Union (EU) procedures for the safety assessment and the authorisation procedure of food additives, food enzymes and food flavourings. The use of a food enzyme shall be authorised only if it is demonstrated that:

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

All food enzymes currently on the 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* (strain DSM 29544), Chymosin from a genetically modified strain of *Aspergillus niger* (strain DSM 29545), Chymosin from a genetically modified strain of *Aspergillus niger* (strain DSM 29546) and Mucorpepsin from *Rhizomucor miehei* (strain DSM 29547).

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

[1] 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* (strain DSM 29544), Chymosin from a genetically modified strain of *Aspergillus niger* (strain DSM 29545), Chymosin from a genetically modified strain of *Aspergillus niger* (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* strain DSM 29546.

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 29546.

Additional information was spontaneously provided from the applicant on 21 March 2019.

Additional information was requested from the applicant during the assessment process on 18 August 2020 and 7 June 2021 and were subsequently provided (see 'Information submitted to EFSA').

Following the request for additional data sent by EFSA on 18 August 2020, the applicant requested a clarification teleconference on 15 September 2020, after which the applicant provided additional data on 19 March 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, 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 chymosin is produced with a genetically modified filamentous fungus *A. niger* (internal names [redacted], [redacted], [redacted]), which is deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany), with the deposit number [redacted][4] The production strain was

[4] Technical Dossier/1st submission/Annex 14.

identified as *A. niger* [redacted][5] This strain was demonstrated to produce ochratoxin A and fumonisins B1 and B2.[6]

3.1.1. Characteristics of the parental and recipient microorganisms

The parental strain is [redacted]

The recipient strain [redacted][7]

3.1.2. Characteristics of introduced sequences

The sequence encoding the chymosin [redacted]

3.1.3. Description of the genetic modification process

The purpose of genetic modification was to enable the production strain to synthesise [redacted][8][9]

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 29546 differs from the recipient strain in its capacity to produce chymosin [redacted][10]

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

[5] Technical Dossier/Additional data March 2021/Annex Q1.
[6] Technical Dossier/1st submission/Annex 34.
[7] Technical dossier/Additional data December 2020/Annex Q1.
[8] Technical Dossier/1st submission/Annex 17.
[9] Technical Dossier/1st submission/Annex 18.
[10] Technical Dossier/Additional data March 2021/Annex Q4.

3.2. Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004[11], with food safety procedures based on hazard analysis and critical control points, and in accordance with current good manufacturing practice.[12]

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 fungal biomass is disrupted and killed by acid treatment and removed from the fermentation broth by filtration. The filtrate containing the enzyme is then further purified and concentrated, including a chromatographic separation step in which the chymosin protein is adsorbed to a specific resin and then eluted. [redacted] Finally, the food enzyme concentrate is sterile filtered and standardised.[13] 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.[14,15]

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 preparation was analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The SDS–PAGE gels showed a single major protein band corresponding to an apparent molecular mass of about 36 kDa, consistent with the expected mass of the enzyme.[17] No other enzyme activities were reported.[18]

The determination of chymosin activity is based on clotting of reconstituted 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 an reference enzyme standard and expressed in International Milk Clotting Units/mL (IMCU/mL).[19]

The food enzyme has a temperature optimum between 37°C and 47°C (pH 6.5) and a pH optimum around pH 6.3 (32°C).[20] Thermostability was tested after a pre-incubation of the food enzyme at different temperatures (from 51°C to 57°C) and for different times (pH 6.6). At 57°C, activity decreased logarithmically with the incubation time, showing no residual activity after ca. 3 min(s) of pre-incubation.[17]

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches used for commercialisation[21] and one batch produced for the toxicological tests[22] (Table 1). The mean total organic solids (TOS) of the three food enzyme batches for commercialisation is 4.2% and the mean enzyme activity/TOS ratio is 22.7 IMCU/mg TOS.

[11] 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.
[12] Technical dossier/1st submission/Annexes 23–24.
[13] Technical dossier/1st submission/pp. 64-72/Annexes 19 and 23–24.
[14] Technical dossier/2nd submission/Annex 6.
[15] Technical Dossier/Additional data March 2021/Annex Q7.
[16] Technical dossier/1st submission/pp. 34.
[17] Technical Dossier/Additional data March 2021/Annex Q8.
[18] Technical dossier/1st submission/pp. 35.
[19] Technical dossier/2nd submission/Annex 7.
[20] Technical dossier/1st submission/pp. 35–37.
[21] Technical dossier/1st submission/pp. 31, Annexes 1–3 and 33; Spontaneous information January 2019/Annex 2.
[22] Additional info March 2021/Annex Q12.

Table 1: Compositional data of the food enzyme

Parameters	Unit	Batches			
		1	2	3	4[(a)]
Chymosin activity	IMCU/mL batch[(b)]	1,035	1,069	943	1,427
Protein	%	1.1	1.0	1.0	NA[(c)]
Ash	%	10.8	10.8	10.8	< 0.2
Water	%	84.9	84.8	85.3	95.7
Total organic solids (TOS)[(d)]	%	4.3	4.4	3.9	4.1
Activity/mg TOS	IMCU/mg TOS	22.1	22.2	23.9	34.7

(a): Batch used for the toxicological testing.
(b): IMCU: International Milk Clotting Units (see Section 3.3.1).
(c): NA: not available.
(d): TOS calculated as 100% – % water – % ash.

3.3.3. Purity

The lead content in three commercial batches and in the batch used for toxicological studies was below 5 mg/kg[23] 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).

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). The three batches for commercialisation were also tested for yeast and filamentous fungi (< 5 CFU/g), *Listeria* (absent in 25 mL) and staphylococci (absent in 1 g).[24] No antimicrobial activity was detected in any of the tested batches (FAO/WHO, 2006).[25]

Strains of *Aspergillus*, in common with most filamentous fungi, have the capacity to produce a range of secondary metabolites (Frisvad et al., 2018). The production strain *A. niger* DSM 29546 is able to produce ochratoxin A and fumonisins B1 and B2 (Section 3.1). The presence of these mycotoxins was examined in the three food enzyme batches for commersialisation.[26] All were below the limits of detection (LODs) of the applied analytical methods.[27] Adverse effects due to the potential presence of other secondary metabolites are 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.[28]

The absence of recombinant DNA in the food enzyme was demonstrated [redacted][29]

3.4. Toxicological data

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 is a pilot-scale extract, produced following

[23] Technical Dossier/Additional data March 2021/Annex Q11.
[24] Technical dossier/1st submission/pp. 31.
[25] Technical dossier/1st submission/pp. 34/Annexes 11–13.
[26] Technical dossier/1st submission/pp. 33/Annexes 8–10 and 34.
[27] LODs: Fumonisin B1 and B2: 20 μg/kg each; Ochratoxin A: 0.2 μg/kg.
[28] Technical dossier/Additional data March 2021/Annex Q5.
[29] Technical dossier/Additional data March 2021/Annex Q6.

the same process as for the production batches described in Table 1.[30] Batch 4 has similar chemical purity as the batches used for commercialisation and thus is considered suitable as a test item.

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).[31] Four strains of *Salmonella* Typhimurium (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* WP2uvrA were used in the presence or absence of metabolic activation (S9-mix), applying the 'treat and wash' assay. The test was performed in triplicate using up to eight concentrations of the food enzyme protein; from 1.5 to 5,120 μg chymosin/plate, corresponding to 9.6, 32, 96, 320, 961, 3,204, 9,612 and 32,809 μg TOS/plate, for strains TA1535 and *E. coli* WP2uvrA in the absence of S9-mix and for strains TA100, TA1535 and TA1537 in the presence of S9-mix. Five concentrations of food enzyme protein; from 50 to 5,120 μg chymosin/plate, corresponding to 320, 960, 3,204, 9,612 and 32,809 μg TOS/plate, were applied for strains TA98, TA100 and TA1537 in the absence of S9-mix and for strains TA98 and *E. coli* WP2uvrA in the presence of S9-mix. No cytotoxicity was observed at any concentration level 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 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* mammalian chromosomal aberration test

The *in vitro* mammalian chromosomal aberration test was carried out in human peripheral blood lymphocytes according to OECD Test Guideline 473 (OECD, 1997b and following GLP.

The dose-finding study was performed at a range of concentrations up to 5,790 μg chymosin/mL. This was followed by two separate experiments carried out in duplicate in which the concentrations were selected based on the results of the dose-finding study and on the maximum feasible concentration, based on the percent of acetate buffer used as vehicle (pH 5.75).

In the first experiment. in a short-term treatment (3 h followed by 18 h recovery period) cells were exposed at 100, 300, 1,000 and 2,880 μg chymosin/mL (corresponding to 641, 1,922, 6,408 and 18,455 μg TOS/mL) with and without S9-mix and were scored for chromosomal aberrations at 1,922, 6,408 and 18,455 μg TOS/mL.

In the second experiment, cells in a short-term treatment with S9-mix and in a continuous treatment (21 h) without S9-mix cells were exposed to 300, 1,000, 2,000 and 2,880 μg chymosin/mL (corresponding to 1,920, 6,400, 12,800 and 18,450 μg TOS/mL) with S9-mix and at 15, 50, 150 and 320 (corresponding to 96, 320, 960 and 2,048 μg TOS/mL) without S9-mix. Cells were scored for chromosomal aberrations at 12.3, 41 and 118.1 μg TOS/mL in the presence of S9-mix and 0.61, 6.15 and 13.12 μg TOS/mL in the absence of S9-mix.

The frequency of structural and numerical chromosomal aberrations at any concentration and condition of treatment was comparable to the values detected in negative controls. The Panel concluded that food enzyme did not induce chromosome aberrations under the test conditions employed for 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.[32] Groups of 10 male and 10 female Han Wistar (Crl:WI (Han)) rats received by gavage the food enzyme in doses corresponding to 41.0, 128.2 and 410.3 mg TOS/kg body weight (bw) per day. Controls received the vehicle (sterile water).

One mid-dose female was sacrificed on day 62 after accidental trauma to the head.

The body weight gain was statistically significantly decreased in periods from week 11 to 12 in mid-dose males (−68%) and from week 9 to 10 in mid-dose females (−16%). The Panel considered the

[30] Technical dossier/p. 85.
[31] Technical dossier/2nd submission/Annex 25 and Spontaneous information submission March 2019/Annex 1.
[32] Technical dossier/2nd submission/Annex 2 and Spontaneous information submission March 2019/Annex 1.

changes as not toxicologically relevant as they were only recorded sporadically and the changes were without a statistically significant effect on the final body weight and the overall body weight gain.

In the functional observations, a statistically significant increase in movement count and distance travelled and a decrease in time of rest in the third time interval in mid- and high-dose males, an increase in movement count and distance travelled during the 1st time interval, a decrease in movement count and distance travelled during the second and a decrease in movement count during the fourth time interval in high-dose females were observed. The Panel considered the changes as not toxicologically relevant as they were only recorded sporadically, there was no consistency between the changes in males and females regarding intervals in which the changes were observed or in the direction of the change.

The haematological investigation revealed a statistically significant increase in the percentage of basophils in the high-dose males (+55%), a shorter activated partial thromboplastin time (APTT) in the high-dose males (−9%), a decrease in a percentage of reticulocytes in mid-dose females (−20%), an increase in percentage of eosinophils in all treated females (+83%, +62% and +37% at the low-, mid- and high-dose, respectively). The Panel considered the changes as not toxicologically relevant as they were only observed in one sex (all parameters), the changes were small (basophils, eosinophils), there was no dose–response relationship (reticulocytes) and there were no changes in other relevant parameters (e.g. in the total leucocyte count, in the total erythrocyte count, in the prothrombin time and in the platelet count).

The clinical chemistry investigation revealed a statistically significant decrease in calcium concentration in all treated males (−4%, −3%, −2% at the low-, mid- and high-dose, respectively), a decrease in aspartate aminotransferase (AST) activity in mid- and high-dose males (−15% and −17%, respectively), a decrease in the globulin concentration in all treated females (−10%, −10% and −5% at the low-, mid- and high-dose, respectively), an increase in the albumin to globulin ratio (A/G) in all treated females (+18%, +11% and +11% at the low-, mid- and high-dose, respectively), a decrease in the urea concentration in mid-dose females (−16%), an increase in the creatinine concentration in low-dose females (+12%). 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 (calcium, globulin, A/G, urea, creatinine), the changes were small (AST) and there were no changes in other relevant parameters (e.g. total protein).

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

The Panel identified the no observed adverse effect level (NOAEL) of 410.3 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* DSM 29546 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, four matches were found.[33] The matching allergens were Pepsin A from wild boar (*Sus scrofa*), protease from Japanese cedar (*Cryptomeria japonica*), aspartic protease-like protein Bla g2 from the German cockroach (*Blattella germanica*) and aspergillopepsin from *A. fumigatus*.

Pepsin is a known respiratory allergen causing occupational asthma and rhinitis in cheese workers (Cartier et al., 1984; Añíbarro Bausela and Fontela, 1996; Marques et al., 2006). Aspergillopepsin, which is also commonly used in food industry, is involved in aspergillosis (Lee and Kolattukudy, 1995; Reichard et al., 1995). Japanese cedar protease has been described as a pollen allergen (Ibrahim et al., 2010), and Bla g2 protease from the German cockroach has also been described as a respiratory allergen (Arruda et al., 1995; Gustchina et al., 2010). None of these proteins were reported to be oral allergens. Individuals sensitised to respiratory allergens usually can ingest the allergens without developing allergic reactions (Brisman, 2002; Poulsen, 2004; Armentia et al., 2009). Cedar pollen contain the respiratory allergen CPA63 (Ibrahim et al., 2010) and respiratory allergy to Cedar pollen is associated with the oral allergy syndrome (Midoro-Horiuti et al., 2003; Kiguchi et al., 2021).

[33] Technical dossier/1st submission/pp. 91-95/Annexes 28–32.

In this syndrome, allergic reactions are mainly in the mouth and seldomly lead to severe systemic anaphylaxis. However, oral allergy cannot be excluded after consumption.

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.[34]

[redacted] products that may cause allergies or intolerances (listed in the Regulation (EU) No 1169/2011[35]), 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, although unlikely, cannot be excluded, particularly for individuals sensitised to cedar pollen allergens.

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	0.09–**2.64**
Milk processing to production of fermented milk product[(d)]	Milk	**0.31**

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 22.7 IMCU/mg TOS.
(c): Numbers in bold were used for calculation.
(d): Technical dossier/p. 79–80.

In cheese production, the food enzyme is added to the milk together with the starter culture.[36] 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 5), 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.[37] 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.

[34] Technical dossier/Additional information March 2021/Annex Q13.
[35] 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.
[36] Technical dossier/1st submission/Figure 3.2–11.
[37] Technical dossier/1st submission/Figure 3.2–12.

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 0.519 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)	0.013–0.233 (11)	0.014–0.106 (15)	0.006–0.015 (19)	0.003–0.020 (21)	0.002–0.017 (22)	0.002–0.006 (22)
Min–max 95th (number of surveys)	0.061–0.519 (9)	0.043–0.238 (13)	0.013–0.048 (19)	0.008–0.020 (20)	0.005–0.052 (22)	0.004–0.014 (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	+

Sources of uncertainties	Direction of impact
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 (410.3 mg TOS/kg bw per day) from the 90-day rat study with the derived exposure estimates of 0.002–0.233 mg TOS/kg bw per day at the mean and from 0.004 to 0.519 mg TOS/kg bw per day at the 95th percentile, resulted in margin of exposure (MoE) of at least 790.

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 29546 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 authorisation of Chymosin from a genetically modified strain of *Aspergillus niger* var. *awamori*. December 2015. Submitted by Chr. Hansen.
2) Additional information. March 2019 Submitted by Chr. Hansen.
3) Additional information. March 2021. Submitted by Chr. Hansen.
4) Additional information. June 2022. Submitted by Chr. Hansen.
5) "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

Añíbarro 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 Fernándes 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, 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, 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.

Ibrahim ARN, Kawamoto S, Aki T, Shimada Y, Rikimaru S, Onishi N, Babiker EE, Oiso I, Hashimoto K, Hayashi T and Ono K, 2010. Molecular cloning and immunochemical characterization of a novel major Japanese Cedar pollen allergen belonging to the aspartic protease family. Int. Arch. Allergy Immunol., 152, 207–218.

Kiguchi T, Yamamoto-Hanada K, Saito-Abe M, Sato M, Irahara M and Ogita H, 2021. Pollen-food allergy syndrome and component sensitization in adolescents: A Japanese population-based study. PLoS ONE, 16(4), e0249649.

Lee JD and Kolattukudy PE, 1995. Molecular cloning of the cDNA and gene for an elastinolytic aspartic proteinase from *Aspergillus fumigatus* and evidence of its secretion by the fungus during invasion of the host lung. Infection and Immunity, 63, 3796–3803.

Marques L, Lara S, Abos T and Bartolome B, 2006. Occupational rhinitis due to Pepsin. J. Investig. Allergol. Clin. Immunol., 16(2), 136–137.

Midoro-Horiuti T, Mathura V, Schein CH, Braun W, Yu S, Watanabe M, Lee JC, Brooks EG and Goldblum RM, 2003. Major linear IgE epitopes of mountain cedar pollen allergen Jun a 1 map to the pectate lyase catalytic site. Molecular Immunology, 40, 555–562.

OECD (Organisation for Economic Co-Operation and Development), 1997a. 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), 1997b. OECD Guideline for the testing of chemicals, Section 4 Health effects, Test No. 473: *In vitro* mammalian chromosomal aberration test. 21 July 1997. 10 pp. Available online: https://www.oecd-ilibrary.org/environment/test-no-473-in-vitro-mammalian-chromosome-aberration-test_9789264071261-en

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. 10pp. Available online: http://www.oecd-ilibrary.org/environment/test-no-408-repeated-dose-90-day-oral-toxicity-study-in-rodents_9789264070707-en

Poulsen LK, 2004. Allergy assessment of foods or ingredients derived from biotechnology, gene-modified organisms, or novel food. Molecular Nutrition and Food Research, 48, 413–423.

Reichard U, Monod M and Rüchel R, 1995. Molecular cloning and sequencing of the gene encoding an extracellular aspartic proteinase from *Aspergillus fumigatus*. FEMS Microbioly Letters, 130, 69–74.

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
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.2022.7465#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 Da2atabase in Exposure Assessment' (EFSA, 2011).