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Safety evaluation of the food enzyme chymosin from the genetically modified *Kluyveromyces lactis* strain CIN

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Abstract

The food enzyme chymosin (EC 3.4.23.4) is produced with the genetically modified *Kluyveromyces lactis* strain CIN by DSM Food Specialties B.V. The genetic modifications do not give rise to safety concerns. The food enzyme is free from viable cells of the production organism and its recombinant 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 0.73 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 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 1,300. Similarity of the amino acid sequence of the food enzyme to those of known allergens was searched for 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, *Kluyveromyces lactis*, 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.

Five applications have been introduced by the companies "Roquette", "Novozymes A/S", "DSM Food Specialties B.V." and "Advanced Enzyme Technologies Ltd." for the authorisation of the food enzymes Beta-amylase from wheat (*Triticum spp*), Alpha-amylase from a genetically modified strain of *Bacillus licheniformis* (strain NZYM-AN); Chymosin from genetically modified strain of *Kluyveromyces lactis* (strain CIN); Polygalacturonase from a genetically modified strain of *Aspergillus niger* (strain FLYSC) and Pectinesterase from a genetically modified strain of *Aspergillus niger* (strain FLZSC).

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 five 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, 11.03.2011, 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 Beta-amylase from wheat (*Triticum spp*), Alpha-amylase from a genetically modified strain of *Bacillus licheniformis* (strain NZYM-AN); Chymosin from genetically modified strain of *Kluyveromyces lactis* (strain CIN); Polygalacturonase from a genetically modified strain of *Aspergillus niger* (strain FLYSC) and Pectinesterase from a genetically modified strain of *Aspergillus niger* (strain FLZSC) in accordance with Article 17.3 of Regulation (EC) No 1332/2008 on food enzymes.[1]

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 *K. lactis* (strain CIN) from DSM Food Specialties B.V.

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 *K. lactis* (strain CIN).

Additional information was spontaneously provided from the applicant on 20 March 2020.

Additional information was requested from the applicant during the assessment process on 7 December 2020 and subsequently 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
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-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 the genetically modified *K. lactis* strain CIN, which is deposited at the Westerdijk Fungal Biodiversity Institute (Netherlands), with deposit number [redacted][4] The production strain was identified as *K. lactis* [redacted][5]

[4] Technical dossier/Spontaneous data_submission May 20/2015_00085 Safe Deposit certificate.
[5] Technical dossier/Additional information Sept 21/Annex II 1.

The species *K. lactis* is included in the list of organisms for which the Qualified Presumption of Safety (QPS) may be applied (EFSA BIOHAZ Panel, 2022).

3.1.1. Characteristics of the parental and recipient microorganisms

[redacted][6]

3.1.2. Characteristics of introduced sequences

[redacted]

3.1.3. Description of the genetic modification process

The purpose of the genetic modification was to enable the production strain to synthesise prochymosin [redacted][7]

[6] Technical dossier/p. 95.
[7] Technical dossier/Additional info September 2021/Annex II-1.

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.

[redacted][7]

3.2. Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004[8], with food safety procedures based on hazard analysis and critical control points, and in accordance with current good manufacturing practice.[9]

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 supernatant containing the food enzyme. The filtrate containing the enzyme is then further purified and concentrated, including an ultrafiltration step in which enzyme protein is retained, while most of the low molecular mass material passes the filtration membrane and is discarded.[10] 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]

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 mature chymosin is a single polypeptide chain of 323 amino acids.[12] Chymosin is produced as a pro-peptide that is cleaved by low pH during downstream processing. The molecular mass of the mature protein, derived from the amino acid sequence, was calculated to be 36 kDa.[13] The food enzyme was analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis. A consistent protein pattern was observed across all batches examined. The gels showed a single major protein band migrating between 36.5 and 55.4 kDa, consistent with the expected mass of the enzyme.[14] No other enzymatic activities were reported.[15]

The in-house determination of chymosin activity is based on the clotting of reconstituted skimmed milk (reaction conditions: pH 6.5, 37°C). The enzymatic activity is determined spectrophotometrically at 600 nm by measuring the time from the addition of the enzyme to an increase in optical density of 0.833. The chymosin activity is quantified relative to an enzyme standard and expressed in International Milk Clotting Unit/g (IMCU/g).[16]

The food enzyme has a temperature optimum between 37°C and 45°C (pH 6.5) and a pH optimum around pH 5.8 (37°C), the lowest pH tested. Thermostability was tested after pre-incubation of the food enzyme for 10 min at different temperatures (pH 6.5). Enzyme activity decreased above 55°C, showing no residual activity above 65°C.[17]

[8] 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.
[9] Technical dossier/Annex I-6.
[10] Technical dossier/p. 50–58/Annex I-7.
[11] Technical dossier/Annex I-8.
[12] Technical dossier/p. 41/Annex I-5.
[13] Technical dossier/Section 3.2.1.1.2.3/p. 41/Annex I-5.
[14] Technical dossier/Section 3.2.1.1.2.3/p. 39.
[15] Technical dossier/Section 3.2.1.1.2.3/p. 42.
[16] Technical dossier/pg. 41/Annex I-2.
[17] Technical dossier/p. 43–44.

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches used for commercialisation and one batch produced for the toxicological tests (Table 1).[18] The mean total organic solids (TOS) of the three food enzyme batches for commercialisation was 11.9% and the mean enzyme activity/TOS ratio was 12.7 IMCU/mg TOS.

Table 1: Compositional data of the food enzyme

Parameters	Unit	Batch			
		1	2	3	4[(a)]
Chymosin activity	IMCU/g batch[(b)]	1,540	1,070	1,880	1,174
Protein	%	2.8	2.3	3.4	3.7
Ash	%	0.49	0.37	1.2	1.3
Water	%	89.8	88.5	84.0	87.7
Total organic solids (TOS)[(c)]	%	9.7	11.1	14.8	11.0
Activity/mg TOS	IMCU/mg TOS	15.9	9.6	12.7	10.7

(a): Batch used for the toxicological studies.
(b): IMCU: International Milk Clotting Units (see Section 3.3.1).
(c): TOS calculated as 100% – % water – % ash.

3.3.3. Purity

The lead content in the three commercial batches and in the batch used for toxicological studies was below 1 mg/kg[19,20] 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). No antimicrobial activity was detected in any of the tested batches (FAO/WHO, 2006).[19]

3.3.4. Viable cells and DNA of the production strain

The absence of viable cells of the production strain in the food enzyme was demonstrated [redacted][21]

The absence of recombinant DNA in the food enzyme was demonstrated [redacted][22]

3.4. Toxicological data

As the production strain qualifies for the QPS status and that no issues of concern arise from the production process, toxicological tests are not required. However, the applicant, following the guidance available at the time of the submission of the application provided a bacterial gene mutation assay (Ames test), an *in vitro* mammalian chromosomal aberration test, and a repeated dose 90-day oral toxicity study, performed with the food enzyme under assessment. The batch 4 (Table 1) used in these studies has a similar protein pattern and chemical purity as the batches used for commercialisation, and thus is considered suitable as a test item.

[18] Technical dossier/Additional information September 2021/Annex 1.
[19] Technical dossier/p. 71/Annex I-3/Annex I-4.
[20] LOD: Pb = 0.006 mg/L sample solution.
[21] Technical dossier/Additional information September 2021/Annex II-3.
[22] Technical dossier/Additional information September 2021/Annex II-2.

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).[23] Four strains of *Salmonella* Typhimurium (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* WP2uvrA(pKM101) were used in the presence or absence of metabolic activation (S9-mix), applying the standard plate incorporation method. A dose range finding test and three main experiments were carried out in triplicate. The dose range finding test was performed at eight concentrations of 3–5,000 μg TOS/plate with *S*. Typhimurium TA100 and *E. coli* WP2uvrA (pKM101) with and without S9-mix (5%). No cytotoxicity or precipitation were observed at any concentration level of the test substance.

Based on these results, a first experiment was performed at five concentrations of food enzyme (100, 333, 1,000, 3,330 and 5,000 μg TOS/plate) with the strains TA1535, TA1537 and TA98 with and without S9-mix (5%). An increase in revertant colony numbers was recorded at the concentrations of 100, 333 and 1,000 μg TOS/plate in *S*. Typhimurium TA98 without metabolic activation being 2.65, 2.70 and 1.98-fold, respectively).

A second main experiment was performed with five concentrations of food enzyme (100, 333, 1,000, 3,330 and 5,000 μg TOS/plate) with *S*. Typhimurium TA1535, TA1537, TA98, TA100 and *E. coli* WP2uvrA with and without S9-mix (10%). There was no increase in revertant colony numbers above the control values in any strain with or without S9-mix.

A confirmatory experiment was performed at five concentrations of food enzyme (100, 333, 1,000, 3,330 and 5,000 μg TOS/plate) with and without S9-mix (5%). There was no increase in revertant colony numbers above the control values.

Since the increase in revertant colony numbers in the strain *S*. Typhimurium TA98 was not reproducible in the second and third experiments, and there was no increase in revertant colony numbers above the control values in other strains 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.[24]

The dose-finding study was performed at concentrations ranging from 100 to 5,000 μg TOS/mL. Based on these results, in the first experiment the cells were exposed to the food enzyme at 1,000, 3,000 and 5,000 μg TOS/mL in the short-term treatment (3 h followed by 21 h recovery period) with and without metabolic activation (S9-mix). In the second experiment, the cells were exposed to the food enzyme at 1,000, 3,000 and 5,000 μg TOS/mL in the short-term treatment (3 h followed by 45 h recovery period) in the presence of S9-mix. In the continuous treatment in the absence of S9-mix, the cell cultures were treated at 100, 300 and 500 μg TOS/mL for 24 h and at 10, 100 and 300 μg TOS/mL for 48 h.

The frequency of structural and numerical chromosomal aberrations in treated cultures was comparable to the values detected in negative controls and within the range of the laboratory historical solvent control data.

The Panel concluded that the 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.[25] Groups of 10 male and 10 female Wistar (HsdHan™: WIST) rats received by gavage the food enzyme in doses of 100, 300 and 1,000 mg TOS/kg body weight (bw) per day for 91 consecutive days. Controls received the vehicle (MilliQ water).

No mortality was observed.

The body weight was statistically significantly increased in low-dose (week 6–13, average difference of 9%) and mid-dose (week 4–11, average difference of 9%) males. The Panel considered the

[23] Technical dossier/Annex I-11.
[24] Technical dossier/Annex I-12.
[25] Technical dossier/Annex I-13.

changes as not toxicologically relevant as they were only observed in one sex, they were recorded sporadically and the changes were without a statistically significant effect on the final body weight.

The body weight gain was statistically significantly increased in low-dose males [week 1 (+14%), 6 (+37%) and 11 (+68%)], mid-dose males (week 2, +28%), and in high-dose females (week 9, +205%) and decreased in high-dose males (week 12, −41%), in mid-dose females (week 12, −27%) and high-dose females (week 12, −20%). The Panel considered the changes as not toxicologically relevant as they were recorded sporadically and the changes were without a statistically significant effect on the final body weight.

The feed consumption was statistically significantly increased in mid-dose males during the 3rd (+13%) and 4th (+12%) week. The Panel considered the changes as not toxicologically relevant as they were only observed in one sex, they were recorded sporadically, there was no dose–response relationship and there was no statistically significant change in the final body weight and body weight gain.

In the functional observations, some statistically significant differences to controls were observed. The Panel considered the changes as not toxicologically relevant as they were minimal or not dose related.

The haematological investigation revealed a statistically significant increase in platelet count (PLT) in high-dose males, (+23%), a decrease in mean corpuscular haemoglobin concentration (MCHC) in mid- (−2%) and high-dose (−3%) males, a decrease in activated partial thromboplastin time (APTT, −18%) in mid-dose males, an increase in mean platelet volume (MPV, +10%) in mid-dose males, an increase in red blood cells (RBC) count in mid- and high-dose females (+5%, +7%, respectively), an increase in haematocrit (HCT) in mid- and high-dose females (+6%, +5%, respectively), an increase in platelet volume (MPV) in mid- and high-dose females (+10%, +13%, respectively), and a decrease in mean corpuscular haemoglobin concentration (MCHC) in the mid-dose females (−3%). The Panel considered the changes as not toxicologically relevant as they were only observed in one sex (PLT, APTT, RBC, HCT), the changes were small (MCHC, RBC, HTC) and there was no dose–response relationship (ATTP, MPV in males, HTC).

The clinical chemistry investigation revealed a statistically significant increase in sodium concentration in all treated groups of males (+2%, +3%, +3%), a decrease in albumin concentration (−6%) and an increase in globulin concentration (+13%), and a lower albumin to globulin ratio (−16%) in high-dose males. The Panel considered the changes as not toxicologically relevant as they were only observed in one sex (all parameters), the changes were small (sodium) and there were no changes in the total protein concentration (albumin, globulin, albumin to globulin ratio).

Statistically significant changes in organ weight included an increase in absolute thymus weight and in thymus to brain weight ratio in mid-dose males (+30%, +29%, respectively), and an increase in pituitary weight to brain weight ratio in mid-dose females (+16%). 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 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 the chymosin produced with the genetically modified *K. lactis* strain CIN 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, four matches were found.[26] The matching allergens were pepsin A from wild boar (*Sus scrofa)*, aspartic protease-like protein Bla g2 from German cockroach (*Blattella germanica)*, aspergillopepsin Asp f10 from the filamentous fungus *Aspergillus fumigatus* and protease CPA63 from Japanese cedar (*Cryptomeria japonica)*.

[26] Technical dossier/p. 74/Annex I-14.

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., 2005). 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). 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.

According to the information provided, substances or products that may cause allergies or intolerances (Regulation (EU) No 1169/2011[27]) are used as raw materials [redacted]. In addition, [redacted] all known allergens, are also present in the media fed to the microorganisms.[11] 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 yeast 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(c)

Food manufacturing process(a)	Raw material (RM)	Recommended dosage of the food enzyme (mg TOS/kg RM)(b)
Milk processing for cheese production	Milk	2.2–**3.7**
Milk processing to production of fermented milk product	Milk	0.07–**0.22**

TOS: total organic solids.
(a): The name has been harmonised 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): Numbers in bold were used for calculation.
(c): Technical dossier/p. 65.

In cheese production, the food enzyme is added to milk together with the starter culture.[28] 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 N. 7), in which residual enzyme activity is expected. Whey produced during cheese making may be used in a variety of foods including infant

[27] 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.
[28] Technical dossier/p. 63.

and follow-on formula or food for special medical purposes.[29] 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 after pasteurisation.[30] Chymosin performs the same function as in cheese, making the viscosity of the fermented dairy products to increase.[31] 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 CEP Panel, 2021b). Exposure from all FoodEx categories was subsequently summed up, averaged over the total survey period (days) and normalised for body weight. This was done for all individuals across all surveys, resulting in distributions of individual average exposure. Based on these distributions, the mean and 95th percentile exposures were calculated per survey for the total population and per age class. Surveys with only one day per subject were excluded and high-level exposure/intake was calculated for only those population groups in which the sample size was sufficiently large to allow calculation of the 95th percentile (EFSA, 2011).

Table 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 0.727 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)	0.017–0.326 (11)	0.018–0.147 (15)	0.008–0.020 (19)	0.004–0.027 (21)	0.002–0.023 (22)	0.002–0.007 (22)
Min–max 95th (number of surveys)	0.082–0.727 (9)	0.059–0.334 (13)	0.018–0.067 (19)	0.011–0.028 (20)	0.007–0.072 (22)	0.005–0.018 (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.

[29] Additional data September 2021.
[30] Technical dossier/p. 64.
[31] Technical dossier/p. 89.

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 0.002–0.326 mg TOS/kg bw per day at the mean and from 0.005 to 0.727 mg TOS/kg bw per day at the 95th percentile, resulted in margin of exposure of at least 1,376.

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 *K. lactis* strain CIN 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 its recombinant DNA.

5. Documentation as provided to EFSA

1) Dossier "Application for authorisation of chymosin from a genetically modified strain of *Kluyveromyces lactis* in accordance with Regulation (EC) No 1331/2008".December 2014. Submitted by DSM Food Specialties B.V.
2) Additional information. March 2020. Submitted by DSM Food Specialties B.V.
3) Additional information. May 2020. Submitted by DSM Food Specialties B.V.
4) Summary report on genetically modified microorganism part. January 2016. Delivered by Technical University of Denmark (Lyngby, Denmark).
5) Summary report on technical data and dietary exposure. August 2016. Delivered by Hylobates Consulting (Rome, Italy) and BiCT (Lodi, Italy).
6) Summary report on genotoxicity and subchronic toxicity. March 2016. Delivered by FoBiG GmbH, (Freiburg, Germany).
7) "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

APTT	activated partial thromboplastin time
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
HCT	haematocrit
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 corpuscular haemoglobin concentration
MPV	mean platelet volume
OECD	Organisation for Economic Cooperation and Development
PCR	total organic solids polymerase chain reaction
PLT	platelet count
QPS	qualified presumption of safety
RBC	red blood cell haematocrit (HCT) in mid- and high-dose females (+6%, +5%, respectively), an increase in platelet volume (MPV)
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TOS	total organic solids
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.2022.7461#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).