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Safety evaluation of the food enzyme rennet containing chymosin and pepsin A from the abomasum of calves and cows

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Abstract

The food enzyme containing chymosin (EC 3.4.23.4) and pepsin A (EC 3.4.23.1) is prepared from the abomasum of calves and cows (*Bos taurus*) by Chr. Hansen. The food enzyme is intended to be used in milk processing for cheese production and in milk processing for the production of fermented milk products. As no concerns arise from the animal source of the food enzyme, from its manufacture, and based on the history of safe use and consumption, the Panel considered that toxicological data were unnecessary and an estimation of dietary exposure was not required. A search for the similarity of the amino acid sequences of the two proteins (chymosin and pepsin A) to those of known allergens was made and one match with pig pepsin, a respiratory allergen, was found. The Panel considered that, under the intended conditions of use, the risk of allergic reactions upon dietary exposure cannot be excluded, but the likelihood is 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, pepsin, EC 3.4.23.1, abomasum, calf, cow, rennet

Requestor: European Commission

Question number: EFSA-Q-2022-00482

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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 Union 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[1] on food enzymes.

Six applications have been introduced by the companies "Decernis, LLC", "Keller and Heckman LLP", the "Association of Manufacturers and Formulators of Enzyme Products (AMFEP)" and "Novozymes A/S" for the authorisation of the food enzymes Cyclomaltodextrin glucanotransferase from *Geobacillus stearothermophilus*, Dextranase from *Chaetomium gracile*, Subtilisin from *Bacillus licheniformis*, Mucorpepsin from *Rhizomucor miehei*, Animal rennet consisting of chymosin and pepsin from the abomasum of *Bos primigenius* (cattle), *Bubalus bubalis* (buffalo), *Capra aegagrus hircus* (goat) and *Ovis aries* (sheep), and Lipase from a genetically modified strain of *Aspergillus niger* (strain NZYM-DB) respectively.

Following the requirements of Article 12.1 of Regulation (EC) No 234/2011[3] implementing Regulation (EC) No 1331/2008[2], the Commission has verified that the six applications fall within the scope of the food enzyme Regulation and contains 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, p. 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 Cyclomaltodextrin glucanotransferase from *Geobacillus stearothermophilus*, Dextranase from *Chaetomium gracile*, Subtilisin from *Bacillus licheniformis*, Mucorpepsin from *Rhizomucor miehei*, Animal rennet consisting of chymosin and pepsin from the abomasum of *Bos primigenius* (cattle), *Bubalus bubalis* (buffalo), *Capra aegagrus hircus* (goat) and *Ovis aries* (sheep), and Lipase from a genetically modified strain of *Aspergillus niger* (strain NZYM-DB) 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 animal rennet consisting of chymosin and pepsin A from the abomasum of calves and cows submitted by AMFEP.

The application was submitted initially as a joint dossier[4] and identified as the EFSA-Q-2015-00237. During a meeting between EFSA, the European Commission and the Association of Manufacturers and Formulators of Enzyme Products (AMFEP),[5] it was agreed that joint dossiers will be split into individual data packages.

The current opinion addresses one data package originating from the joint dossier EFSA-Q-2015-00237. This data package, identified as EFSA-Q-2022-00482, concerns the food enzyme rennet containing chymosin and pepsin A from calves and cows and submitted by Chr. Hansen.

2. Data and Methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme rennet containing chymosin and pepsin A from the abomasum of calves and cows.

Additional information was requested from the applicant during the assessment process on 20 January 2023 and received on 15 February 2023 (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 existing guidance documents of EFSA Scientific Committee.

The 'Guidance on the submission of a dossier on food enzymes for safety evaluation' (EFSA, 2009a) has 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, 2021).

3. Assessment

The food enzyme under application contains two declared activities: chymosin and pepsin A activities:

IUBMB nomenclature	Chymosin
Synonyms	Rennin
IUBMB No.	3.4.23.4
CAS No.	9001-98-3
EINECS No.	232-645-0

[4] Commission Implementing Regulation (EU) No 562/2012 of 27 June 2012 amending Commission Regulation (EU) No 234/2011 with regard to specific data required for risk assessment of food enzymes Text with EEA relevance. OJ L 168, 28.6.2012, p. 21–23.

[5] The full detail is available at the https://www.efsa.europa.eu/en/events/event/ad-hoc-meeting-industry-association-amfep-joint-dossiers-food-enzymes

Chymosin is an aspartic endopeptidase that catalyses the hydrolysis of the 104-Ser-Phe−/−Met-Ala-107 bonds of κ-casein, resulting in the destabilisation of casein micelles and causing milk to clot.

IUBMB nomenclature	Pepsin A
Synonyms	Pepsin; lactated pepsin; pepsin fortior; fundus-pepsin
IUBMB No.	3.4.23.1
CAS No.	9001-75-6
EINECS No.	232-629-3

Pepsin A is also an aspartic endopeptidase which hydrolyses peptide bonds in proteins and peptide molecules with the formation of shorter peptides and free amino acids. It preferably cleaves peptide bonds between hydrophobic and aromatic amino acids.

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 is an aqueous extract obtained from the abomasum of cows and calves (*Bos taurus*) purchased from different sources. It relies on the good practice of their suppliers to ensure that the raw material meets current legal standards.[6] As confirmed by the applicant,[7] the food enzyme is exclusively obtained from healthy animals slaughtered under the supervision of official health authorities, following the requirements of the relevant EU hygiene regulations, the Food Hygiene Regulation (EC) No 852/2004[8] and Regulation (EC) No 853/2004[9].

In the EU, according to EC 1774/2002[10], the abomasum of calves and cows is considered fit for human consumption and is an edible offal as defined in Regulation (EC) No 853/2004[9].

No issues of concern arising from the safety of the source material were identified by the Panel.

3.2. Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004[8], with food safety procedures based on Hazard Analysis and Critical Control Points, and in accordance with current Good Manufacturing Practice.[11]

The food enzyme is extracted from the abomasa of calves and cows. After the animals are slaughtered, the abomasa are trimmed to remove unwanted tissues, emptied and then frozen before processing. The abomasa are minced and macerated in salt water to extract the enzymes. The liquid fraction is recovered by decantation and then sieved and centrifuged to remove any insoluble biomass and obtain a crude extract containing the food enzymes. Salt and acid are added to the clarified solution to reach a pH of [redacted], which activates the two declared enzymatic activities. The extract is further clarified by flocculation and neutralised [redacted]. After drum filtration that removes remaining insoluble material, the extract is concentrated by ultrafiltration.[12]

Authorised preservatives and salts may be added to the enzyme concentrate. [13]

The applicant provided information on the identity of the substances used in the extraction and in the subsequent downstream processing.[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.

[6] Technical dossier/Annex 20.
[7] Technical dossier/Additional information February 2023/Annex Q1.1.
[8] Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs. OJ L 139, 30.4.2004, pp. 54.
[9] Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. OJ L226, 25.6.2004, p. 22.
[10] Regulation (EC) No 1774/2002 of the European Parliament and of the Council of 3 October 2002 laying down health rules concerning animal by-products not intended for human consumption.
[11] Technical dossier/Annexes 17–19 & 21.
[12] Technical dossier/Dossier p. 47–54.
[13] Technical dossier/Dossier p. 52–53.
[14] Technical dossier/Annexes 22–32.
[15] Technical dossier/Additional information February 2023.

3.3. Characteristics of the food enzyme

3.3.1. Properties of the food enzyme

Chymosin from the abomasum of calves is a single polypeptide chain of 381 amino acids.[16] The molecular mass of the mature protein was calculated to be 36.5 kDa (Kumar et al., 2010).[17] Pepsin A from the abomasum of calves is a single polypeptide chain of 386 amino acids.[18] The molecular mass of the mature protein was calculated to be 35 kDa (Munoz et al., 2004).[19] The food enzyme was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. A consistent pattern was observed with two major bands consistent with the expected apparent molecular masses of the declared activities. No other enzymatic activities were reported.

The determination of chymosin and pepsin A activities is based on the official method ISO 11815|IDF157:2007.[20] The time needed for visual flocculation of a standard milk substrate prepared with a calcium chloride solution of 0.5 g per litre (pH ≈ 6.5) is determined. The clotting time of the rennet sample is compared to that of a bovine rennet reference standard with a known milk-clotting activity. The total milk-clotting activity is expressed in International Milk-Clotting Units (IMCU).[21]

The relative contents of chymosin and pepsin present in the rennet were determined by chromatographic analysis, based on the recognised official method ISO 15163|IDF 110:2012 for milk and milk products, indicated for calf rennet and adult bovine rennet.

The rennet milk clotting activity has a pH optimum around 6.5 and a temperature optimum around 45°C. This activity decreased above 50°C, showing no residual activity above 55°C.[22]

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches used for commercialisation (Table 1).[23] The mean total organic solids (TOS) was 5.5% and the mean enzyme clotting activity/mg TOS ratio was 4.4 IMCU/mg TOS.

Table 1: Composition of the food enzyme rennet from the abomasum of calves and cows

Parameters	Unit	Batches		
		1	2	3
Milk clotting activity	IMCU/g[(a)]	246	225.5	244
Protein	%	1.4	1.6	2
Ash	%	16.1	16.2	15.7
Water	%	77.8	78.0	79.6
Total organic solid (TOS)[(b)]	%	6.1	5.8	4.7
Chymosin	(% of total activity)	78.8	77.1	68.4
Milk clotting activity/TOS	IMCU/mg TOS	4.0	3.9	5.2

(a): IMCU: International Milk Clotting Unit (see Section 3.3.1).
(b): TOS calculated as 100% – % water – % ash.

3.3.3. Purity

The lead content in the three commercial batches was below 0.2 mg/kg[24,25] which complies with the specification for lead as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006).

The microbiological analyses of three commercial batches were reported. The food enzyme complies with the microbiological criteria, for total coliforms, *Escherichia coli* and *Salmonella* as laid

[16] Technical dossier/Dossier p. 39 & Annex 13.
[17] Technical dossier/Dossier p. 39.
[18] Technical dossier/Dossier p. 39 and Annex 12.
[19] Technical dossier/Dossier p. 39.
[20] Technical dossier/Annex 14.
[21] Technical dossier/Dossier p. 40 and Annex 15.
[22] Technical dossier/p. 40–42 & Annex 16.
[23] Technical dossier/Additional information February 2023/Annex Q4.1.
[24] Technical dossier/Dossier tables 4, 7–8 & Annexes 5–7/Additional information February 2023/Annex Q2.1.
[25] LoQ: Pb = 0.05 mg/kg.

down in the general specifications for enzymes used in food processing (FAO/WHO, 2006). Additional microbiological parameters (*E. coli* STEC, *Campylobacter species*, filamentous fungi and yeasts) were also reported and raised no concern.[26]

The Panel considered that the information provided on the purity of the food enzyme is sufficient.

3.4. Toxicological data

According to the Commission Implementing Regulation (EU) No 562/2012[27], an application for the safety evaluation of a food enzyme does not need to include toxicological data if the food enzyme is obtained from edible parts of animals intended or reasonably expected to be ingested by humans.

According to the EFSA Guidance on the submission of a dossier on food enzymes for safety evaluation, the justification for not supplying toxicological data may include a documented history on the safety of the source of the food enzyme, the composition and the properties of the food enzyme, as well as its use in foods, demonstrating no adverse effects on human health when consumed in a comparable way (EFSA CEP Panel, 2021).

The Panel considers that these requirements are fulfilled, because:

i) rennet obtained from the abomasum of calves and cows has been safely used in the production of cheese and related products for many centuries;
ii) the abomasum from calves and cows is consumed throughout the EU and elsewhere in the world as a meat product;
iii) the manufacturing process of the food enzyme is not considered to introduce substances that could raise safety concerns;
iv) the compositional and purity data provided on the food enzyme are considered sufficient.

The Panel considered that sufficient information has been provided on the animal source, its history of safe use and consumption, and the manufacturing process. Therefore, the need for toxicological data is waived.

3.4.1. Allergenicity

The potential allergenicity of the food enzyme containing chymosin and pepsin A derived from the abomasum of calves and cows was assessed by comparing both amino acid sequences 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, one match was found. The matching allergen was pig pepsin, which is known as an occupational respiratory allergen.[28]

Cattle are not a source included in the list of substances or products causing allergies or intolerances (Reg. (EU) No 1169/2011[29]). Proteins from bovine abomasum are not known to be food allergens. No information is available on oral sensitisation or elicitation reactions to chymosin or pepsin A obtained from the abomasum of calves and cows under evaluation.

Occupational respiratory allergies and skin sensitisation to dust of chymosin and pepsin A have been described in workers upon industrial exposure and in medical laboratory technicians (Cartier et al., 1984; Jensen et al., 2006; van Kampen et al., 2013; Gómez Torrijos et al., 2018; Khan and Selamoglu, 2020). However, several studies have shown that adults with occupational asthma can commonly ingest the corresponding respiratory allergens without acquiring clinical symptoms of food allergy (Cullinan et al., 1997; Brisman, 2002; Poulsen, 2004; Armentia et al., 2009). There are no reports in the literature on adverse reactions upon ingestion of these enzymes in individuals sensitised through the respiratory route.

The Panel considered that allergic reactions to this food enzyme obtained from the abomasum of calves and cows cannot be excluded but the likelihood is low.

[26] Technical dossier/ Dossier tables 4, 7–8 & Annexes 8–10.
[27] Commission Implementing Regulation (EU) No 562/2012 of 27 June 2012 amending Commission Regulation (EU) No 234/2011 with regard to specific data required for risk assessment of food enzymes. OJ L 168, 28.6.2012, p. 21–23.
[28] Technical dossier/pp. 11–12; pp. 20–21; pp. 69-74/Annexes: 37, 38.
[29] 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.

3.5. Dietary exposure

3.5.1. Intended use of the food enzyme

The food enzyme is intended to be used in two food manufacturing 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[30]

Food manufacturing process[(a)]	Raw material (RM)	Recommended use level (mg TOS/L RM)[(b)]
Milk processing for cheese production	Milk	6.8–13.6
Milk processing for production of fermented milk products	Milk	0.5–1.6

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): Recalculated by EFSA, based on the average value of 4.4 IMU/mg TOS (see Section 3.3.2).

Animal rennet is added to milk to separate milk into solid curd and liquid whey (coagulation). Both chymosin and pepsin contribute to the milk-clotting activity.[31] The majority of the food enzyme–TOS partitions into the whey and is mostly removed during the draining of the whey. Only a small portion of the food enzyme–TOS remains in the curd (approximately 10%). The remaining rennet contributes to the ripening of cheese due to its proteolytic activity.[32] The food enzyme may remain active in cheese, depending on the cheese-making process.

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[33]. Rennet performs in milk products the same function as in cheese, increasing the viscosity of the fermented dairy products. The food enzyme–TOS remains in the fermented milk products, in which residual enzyme activity is expected.

3.5.2. Dietary exposure estimation

The technology of extracting enzymes from animal abomasum and the technology of using animal rennet for making cheese and fermented milk products have remained the same over thousands of years, and is the major source of human exposure to the food enzyme. Cheese and fermented milk products have been consumed by humans in Europe and many other parts of the world for millennia. In addition, abomasum from ruminants is consumed in some European countries, which constitutes a minor fraction of the overall exposure to the food enzyme in the EU.

In the view of the Panel, dietary exposure estimation is not required.

3.6. Margin of exposure

Since no toxicological assessment and no dietary exposure estimation were considered necessary by the Panel, the margin of exposure was not calculated.

4. Conclusion

Based on the data provided, the origin of the food enzyme and its history of safe use, the Panel concluded that the food enzyme rennet containing chymosin and pepsin A obtained from the abomasum of calves and cows does not give rise to safety concerns under the intended conditions of use.

[30] Technical dossier/Additional information February 2023/Annex Q4.1/table 2.
[31] Technical dossier/ Figure 7.
[32] Technical dossier/pp. 56–57.
[33] Technical dossier/ Figure 8.

5. Documentation as provided to EFSA

Dossier "Animal rennet from the abomasum of calves and cows (*Bos taurus*)". July 2022. Submitted by Chr. Hansen.

Additional information. February 2023. Submitted by Chr. Hansen and Schill+Seilacher "Struktol".

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Abbreviations

CAS	Chemical Abstracts Service
EC	European Commission

EFSA CEP Panel	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
EFSA GMO Panel	EFSA Panel on Genetically Modified Organisms
EINECS	European Inventory of Existing Commercial Chemical Substances
EU	European Union
FAO	Food and Agricultural Organization of the United Nations
IDF	International Dairy Federation
IMCUS	International Milk-Clotting Units
ISO	International Organization for Standardization
IUBMB	International Union of Biochemistry and Molecular Biology
kDa	kiloDalton
LOQ	limit of quantification
TOS	total organic solids
WHO	World Health Organization