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

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Abstract

The food enzyme containing chymosin (EC 3.4.23.4) and pepsin (EC 3.4.23.1) is produced from the abomasum of *Bos taurus* purchased from different rennet manufacturers by Laboratorios Arroyo S.A. The food enzyme is intended to be used in milk processing for cheese production. As no concerns arise from the 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 not required, and no exposure assessment was necessary. On the basis of literature data, the Panel considered that, under the intended conditions of use, the risk of allergic sensitisation and elicitation reactions by dietary exposure could not be excluded, but the likelihood for this to occur was considered to be 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: Chymosin, EC 3.4.23.4, pepsin, EC 3.4.23.1, abomasum, calf, cow, rennet

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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 applicants "Intertek Scientific & Regulatory Consultancy" for the authorization of the food enzymes Triacylglycerol lipase from *Aspergillus niger* (strain NL 151), Aspergillopepsin I from *Aspergillus niger* (strain AP 233) and Pectinase from *Rhizopus oryzae* (strain MC3-3-9), "Alpha Ingredients S.r.l" for the authorization of the food enzyme Transglutaminase from *Streptomyces mobaerensis* (strain DSM40587) and "Laboratorios Arroyo S.A." for chymosin and pepsin from stomachs of calves and cows.

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 of the following food enzymes Triacylglycerol lipase from *Aspergillus niger* (strain NL 151), Aspergillopepsin I from *Aspergillus niger* (strain AP 233), Pectinase from *Rhizopus oryzae* (strain MC3-3-9), Transglutaminase from *Streptomyces mobaerensis* (strain DSM40587) and chymosin and pepsin from stomachs of calves and cows 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 and pepsin from stomachs (abomasum) of calves and cows.

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 and pepsin from the abomasum of calves and cows.

Additional information was requested from the applicant during the assessment process on 2 April 2020 and was consequently provided (see 'Documentation provided to EFSA').

2.2. Methodologies

The assessment was conducted in line with the principles described in the EFSA 'Guidance on transparency in the scientific aspects of risk assessment' (EFSA, 2009b) and following the relevant existing guidances of EFSA Scientific Committees.

The current '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 to the methodology described in the CEF Panel 'Statement on the exposure assessment of food enzymes' (EFSA CEF Panel, 2016).

3. Assessment

The food enzyme under application contains two declared activities:

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

Chymosin is an aspartic endopeptidase that catalyses the hydrolysis of the 104-Ser-Phe-/-Met-Ala-107 bonds of k-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, an endopeptidase, breaks down peptide bonds in protein 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.

3.1. Source of the food enzyme

The food enzyme preparation is a blend of calf and bovine rennet obtained from the abomasum of cows and calves (*Bos taurus)* purchased from different rennet manufacturers and relies on the good practice of their suppliers to ensure that the raw material meets current legal standards.

In the EU, according to EC 1774/2002[4], 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[5].

3.2. Production of the food enzyme

The blending of the food enzyme is done according to the Food Hygiene Regulation (EC) No 852/2004[6], with food safety procedures based on Hazard Analysis and Critical Control Points, and in accordance with current Good Manufacturing Practice.[7]

The food enzyme is an aqueous extract obtained from the abomasum of calves and cows produced by and purchased from different suppliers. Based on the activity and composition of the material obtained from the suppliers, two or more batches are mixed in order to obtain a food enzyme with the desired enzymatic activities. If necessary, authorised preservatives are added, or the preparation is standardised with [redacted].[8]

3.3. Characteristics of the food enzyme

3.3.1. Properties of the food enzyme

The chymosin is a single polypeptide chain of [redacted] amino acids.[9] The molecular mass, derived from the amino acid sequence, was calculated to be [redacted] kDa.[10] The pepsin is a single polypeptide chain of [redacted] amino acids.[11] The molecular mass, derived from the amino acid sequence, was calculated to be [redacted] kDa.[12] The food enzyme was analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis. The gel showed two main bands consistent with the expected apparent molecular masses.[13] No other enzymatic activities were reported.

The determination of chymosin and pepsin activities is based on the milk-clotting activity of each enzyme, after separation of both enzymes on an ion exchange column (IDF standard 110/ISO 15163; edition 2012-05-15). The enzymatic activity is based on hydrolysis of the substrate casein (reaction conditions: pH 6.5, 18–22°C). The enzymatic activity is determined by measuring the time needed for visual flocculation of a standard milk substrate. The chymosin and pepsin activities are quantified relative to an internal enzyme standard and expressed in International Milk-Clotting Units/g (IMCU/g).[14]

The temperature optimum of the food enzyme is around 40–42°C and the pH optimum is between 5.0 and 6.0. Activity began to decrease above 42°C. Thermostability was tested at different temperatures. Under the conditions (pH 6.0) of the applied temperature stability assay, enzyme activity decreased 90% at 67°C.[15]

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme were provided for four batches of the food enzyme; two batches of rennet at dilution 1:10,000 and two at dilution 1:15,000 (Table 1).[16] The average total organic solids (TOS) of the two food enzyme batches for commercialisation 1:10,000 was

[4] 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.
[5] Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for on the hygiene of foodstuffs. OJ L139/55, 30.4.2004.
[6] 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.
[7] Technical dossier/Additional data February 2021/Annex 1.
[8] Technical dossier/Additional data February 2021/Annex 2.
[9] Technical dossier/p. 10/ http://www.uniprot.org/uniprot/P00794.
[10] Technical dossier/p. 10.
[11] Technical dossier/p. 11/ http://www.uniprot.org/uniprot/P00792.
[12] Technical dossier/p. 11.
[13] Technical dossier/Annex 2 and Additional data/February 2021/Annex 4.
[14] Technical dossier/p.16.
[15] Technical dossier/Additional data February 2021/Annex 3.
[16] Technical dossier/Additional data February 2021/Annex 4.

10.7% and that for the two batches 1:15,000 was 11.6%. The average enzyme activity/TOS ratio of the two batches 1:10,000 was 1.4 and for the two batches 1:15,000 it was 1.95. Chymosin activity contributed approximately [redacted] of the milk coagulating activity and pepsin [redacted].

Table 1: Composition of the food enzyme

Parameter	Unit	Batches			
		1[(a)]	2[(a)]	3[(b)]	4[(b)]
Total peptidase activity	IMCU/g batch[(c)]	122.54	145.02	171.03	177.07
Protein	%	1.12	1.58	1.44	1.49
Ash	%	16.77	16.39	16.76	16.48
Water	%	76.77	68.75	66.16	77.39
TOS[(d)]	%	6.46	14.86	17.08	6.13
Activity/mg TOS	IMCU/mg TOS	1.9	1.0	1.0	2.9

(a): Batch obtained with a 1:10,000 dilution of the food enzyme.
(b): Batch obtained with a 1:15,000 dilution of the food enzyme.
(c): IMCU: International Milk-Clotting Units (see Section 3.3.1).
(d): TOS calculated as 100% – % water – % ash.

3.3.3. Purity

The lead content in six commercial batches was below 0.5 mg/kg which complies with the specification for lead (≤ 5 mg/kg) as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006). In addition, the levels of arsenic, cadmium and mercury were below the limits of quantification of the employed methodologies.[11,17]

The food enzyme complies with the microbiological criteria (for *Escherichia coli* and *Salmonella*) as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006). Additionally, aerobic bacteria, yeasts and moulds, Enterobacteriaceae, *Staphylococcus, Clostridium* spp. and *Listeria* were tested, and their levels did not raise safety concern.[18]

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

3.4. Toxicological data

According to the Commission Implementing Regulation (EU) No 562/2012[19], 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, 2009a).

The Panel considered that these requirements are fulfilled, because:

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

[17] LoQ: Pb = 0.5 mg/kg; As = 0.5 mg/kg; Cd = 0.2 mg/kg; Hg = 0.02 mg/kg.
[18] Technical dossier/p. 13 and Annex 5.
[19] 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.

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 chymosin and pepsin derived from abomasum of calves and cows was not assessed by comparing its amino acid sequence with those of known allergens. Occupational respiratory allergies and skin sensitisation to dust of chymosin and pepsin 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 to an enzyme 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.

Cattle are not a source included in the list of substances or products causing allergies or intolerances (Reg. (EU) No 1169/2011). Proteins from bovine abomasum are not known to be food allergens.

The Panel considered that the likelihood of food allergic reactions to this food enzyme obtained from stomachs of calves and cows is low.

3.5. Dietary exposure

3.5.1. Intended use of the food enzyme

The food enzyme is intended to be used in milk processing for cheese production at a recommended use level of up to 94.8 mg TOS/kg milk.[20]

The food enzyme is added to milk to separate milk into solid curd and liquid whey (coagulation). The majority of the food enzyme–TOS partitions into the whey. The food enzyme is mostly removed during the draining of the whey and only a small portion remains in the curd (approximately 6%). The remaining food enzyme contributes to the ripening of cheese due to its general proteolytic activity.[21]

3.5.2. Dietary exposure estimation

The technology of extracting enzymes from animal abomasum and the technology of using animal rennet for cheese making have remained the same for thousands of years and cheese remains the major source of human exposure to the food enzyme. Cheese and by-products of cheese making 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 and in line with the weight of evidence approach, dietary exposure estimation is not required.

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 containing chymosin and pepsin from the abomasum of calves and cows does not give rise to safety concerns under the intended conditions of use.

5. Documentation as provided to EFSA (if appropriate)

1) Chymosin and pepsine from stomachs of calves and cows. March 2015. Submitted by Laboratorios Arroyo S.A.
2) Additional information. February 2021. Submitted by Laboratorios Arroyo S.A.

[20] Technical dossier/Additional data February 2021/Annex 7.
[21] Technical dossier/p. 18 and Additional data February 2021/Annex 6.

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Abbreviations

CAS	Chemical Abstracts Service
CEF	EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CEP	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
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
LoQ	limit of quantification
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TOS	total organic solids
WHO	World Health Organization