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## Safety evaluation of the food enzyme containing chymosin and pepsin from the abomasum of suckling lambs

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### Abstract

The food enzyme rennet containing chymosin (EC 3.4.23.4) and pepsin (EC 3.4.23.1) is prepared from the abomasum (stomach) of suckling lambs, by Productos Nievi, SA. The food enzyme is intended to be used in milk processing for cheese production. 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 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 concludes that this food enzyme does not give rise to safety concerns under the intended conditions of use.

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## Table of contents

|  |    |
|--|----|
| Abstract.....  | 1  |
| 1. Introduction.....   | 4  |
| 1.1. Background and Terms of Reference as provided by the requestor..... | 4  |
| 1.1.1. Background as provided by the European Commission.....            | 4  |
| 1.1.2. Terms of Reference.....   | 5  |
| 1.2. Interpretation of the Terms of Reference.....                       | 5  |
| 2. Data and methodologies.....   | 5  |
| 2.1. Data.....   | 5  |
| 2.2. Methodologies.....  | 5  |
| 3. Assessment.....   | 5  |
| 3.1. Source of the food enzyme.....                                      | 6  |
| 3.2. Production of the food enzyme.....                                  | 6  |
| 3.3. Characteristics of the food enzyme.....                             | 6  |
| 3.3.1. Properties of the food enzyme.....                                | 6  |
| 3.3.2. Chemical parameters.....  | 7  |
| 3.3.3. Purity.....   | 7  |
| 3.4. Toxicological data.....   | 7  |
| 3.4.1. Allergenicity.....  | 8  |
| 3.5. Dietary exposure.....   | 8  |
| 3.5.1. Intended use of the food enzyme.....                              | 8  |
| 3.5.2. Dietary exposure estimation.....                                  | 8  |
| 3.6. Margin of exposure.....   | 8  |
| 4. Conclusion.....   | 9  |
| 5. Documentation as provided to EFSA.....                                | 9  |
| References.....  | 9  |
| Abbreviations.....   | 10 |

## 1. Introduction

Article 3 of the Regulation (EC) No 1332/2008<sup>1</sup> 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<sup>1</sup> 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<sup>2</sup> 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 CEF Panel, 2009) 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<sup>1</sup> on food enzymes.

Five applications have been introduced by the companies 'Productos Nievi, SA' for the authorisation of the food enzyme rennet consisting of chymosin and pepsin from stomachs of young calves and sheep, 'Avances Bioquímicos Alimentación, SL' for the authorisation of the food enzyme plant coagulant from the flowers of *Cynara cardunculus*, 'Mitsubishi-Kagaku Foods Corporation' and 'Kikkoman Biochemifa Company' for the authorisation of the food enzyme tannase from *Aspergillus oryzae* (strains NBRC 110971 and No. 11-5, respectively) and from 'Danisco US Inc.' for the authorisation of the food enzymes alpha-amylase from *Aspergillus niger* (DP-Azb60) and catalase from a genetically modified strain of *Aspergillus niger* (DP-Azw58).

Following the requirements of Article 12.1 of Commission Regulation (EU) No 234/2011<sup>3</sup> implementing Regulation (EC) No 1331/2008<sup>2</sup>, the Commission has verified that the five applications fall within the scope of the food enzyme Regulation and contains all the elements required under Chapter II of that Regulation.

<sup>1</sup> 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.

<sup>2</sup> 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.

<sup>3</sup> 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 (EC) requests the European Food Safety Authority (EFSA) to carry out the safety assessments on the food enzymes rennet consisting of chymosin and pepsin from stomachs of young calves and sheep, plant coagulant from the flowers of *Cynara cardunculus*, tannase from *Aspergillus oryzae* (strains NBRC 110971 and No. 11-5), alpha-amylase from *Aspergillus niger* (DP-Azb60) and catalase from a genetically modified strain of *Aspergillus niger* (DP-Azw58) in accordance with Article 17.3 of Regulation (EC) No 1332/2008<sup>1</sup> 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 rennet consisting of chymosin and pepsin from abomasum. EFSA limits the assessment to rennet from lambs, as in 2021 the applicant informed EFSA that it now produces the lamb liquid rennet only.<sup>4</sup>

## 2. Data and methodologies

### 2.1. Data

The applicant initially submitted a dossier in support of the application for authorisation of the food enzyme rennet containing chymosin and pepsin from abomasum of suckling calves and lambs. According to the additional information provided by the applicant in September 2021, the manufacture of rennet is now limited to a liquid product obtained from lamb abomasum. Consequently, EFSA used only data from the lamb rennet in this assessment.

Additional information was requested from the applicant during the assessment process on 28 April 2021 and received on 28 September 2021 (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, 2009) and following the relevant existing guidance documents of EFSA Scientific Committee.

The current 'Guidance on the submission of a dossier on food enzymes for safety evaluation' (EFSA CEF Panel, 2009) has been followed for the evaluation of the application with the exception of the exposure assessment, which was carried out in accordance with the methodology described in the CEF Panel 'Statement on the exposure assessment of food enzymes' (EFSA CEF Panel, 2016).

## 3. Assessment<sup>5</sup>

The food enzyme under application contains two declared activities:

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

Chymosin catalyses the hydrolysis of a single peptide bond between amino acid residues 105 and 106, phenylalanine and methionine (Ser-Phe<sup>105</sup>/Met-Ala) in  $\kappa$ -casein. This results in the precipitation of milk protein and curd formation.

|                    |  |
|--------------------|--|
| IUBMB nomenclature | Pepsin   |
| Synonyms           | Pepsin A; lactated pepsin; pepsin fortior; fundus-pepsin |
| IUBMB No           | EC 3.4.23.1  |

<sup>4</sup> Technical dossier/Additional information, 28 September 2021.

<sup>5</sup> Technical dossier/p. 3, 6, 10, 16, 17.

|           |           |
|-----------|-----------|
| 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.<sup>6</sup>

### 3.1. Source of the food enzyme<sup>7</sup>

The food enzyme is extracted from the abomasum of suckling lambs (*Ovis aries*) selected and prepared for human consumption and only fed with milk.<sup>8</sup> The raw material (dry abomasa) are obtained from a certified European supplier, surveyed and approved by the competent authorities.<sup>9</sup> 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<sup>10</sup> and Regulation (EC) No 853/2004<sup>11</sup>.

In EU, according to Regulation (EC) 1774/2002<sup>12</sup>, the stomach (abomasum) of lambs is edible offal as defined in Regulation (EC) No 853/2004<sup>11</sup>.

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

### 3.2. Production of the food enzyme<sup>13</sup>

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004<sup>10</sup>, with food safety procedures based on Hazard Analysis and Critical Control Points, and in accordance with current Good Manufacturing Practice.<sup>14</sup>

The food enzyme produced by the applicant is extracted from the abomasum of suckling lambs. Dried stomachs are sliced into small pieces, mixed with an aqueous extracting solution consisting of 5% sodium chloride, 0.6% potassium sorbate and 0.4% sorbic acid (at pH 4.5). Extraction continues for several days during which time the salt concentration is increased to around 17–18% (pH 5.5–5.7). At the end of the extraction period, the food enzyme is recovered by decantation and then filtered to give a clear solution. The extract so obtained is monitored for its specific activity and the chymosin/pepsin ratio.<sup>15</sup> The applicant provided information on the identity of the substances used in the extraction and in the subsequent downstream processing of the food enzyme.

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<sup>16</sup>

Data from literature indicates that the chymosin from the abomasum of suckling lambs is a single polypeptide chain of 381 amino acids with a molecular mass of 36.5 kDa (Kumar et al., 2010). The pepsin A from the abomasum of suckling lambs is a single polypeptide chain of 386 amino acids with a molecular mass of 35 kDa (Munoz et al., 2004).

<sup>6</sup> Technical dossier/p. 6.

<sup>7</sup> Technical dossier/p. 8; Technical dossier/Additional information, 28 September 2021.

<sup>8</sup> Technical dossier/Summary/p. 8.

<sup>9</sup> Technical dossier/Additional information, 28 September 2021/Annex 1.

<sup>10</sup> 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.

<sup>11</sup> 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, p. 22, 25/6/2004.

<sup>12</sup> 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. OJ L 273, p. 209, 10.10.2002.

<sup>13</sup> Technical dossier/Summary/p. 9–10; Technical dossier/Additional information, 28 September 2021.

<sup>14</sup> Technical dossier/2nd submission/Annex 7; Technical dossier/Additional information, 28 September 2021/ Annex 2 and Annex 2.1.

<sup>15</sup> Technical dossier/2nd submission/Summary/p. 9–10.

<sup>16</sup> Technical dossier/p. 3, 6.

The determination of chymosin and pepsin activities is based on the official method ISO 11815|IDF 157:2007.<sup>17</sup> 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 known milk-clotting activity. The clotting time of a rennet sample is compared under identical chemical and physical conditions to that of a reference standard with known milk-clotting activity and having the same enzyme composition as the sample, determined using the International Dairy Federation (IDF) standard 110 (IDF110B). The total milk-clotting activity is expressed in International Milk-Clotting Units (IMCU).

The applicant also uses a Milk Clot Unit analytical method (MCU).<sup>18</sup> The assay is based on the hydrolysis of a buffered milk at 40°C. Enzymatic activity is related to the time required to clot 25 mL of milk substrate. The enzymatic activity is then compared to an enzyme standard.

The food enzyme chymosin has a pH optimum around pH 6.5.<sup>4</sup> Chymosin activity was stable up to 50°C.<sup>19</sup>

### 3.3.2. Chemical parameters<sup>20</sup>

Data on the enzyme activity<sup>21</sup> were provided for three batches of lamb liquid rennet (the mean enzymatic activity is 105 ± 5 IMCU/g batch). No information was provided on the ash, water or protein content. However, in the case of animal rennet, these data are used only to judge consistency of product and are not otherwise used in the safety assessment.

### 3.3.3. Purity<sup>22</sup>

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). In addition, the results of the analyses of aerobic mesophilic total count, sulfite-reducing clostridia, yeast and moulds, *Enterobacteriaceae*, *Listeria monocytogenes* and coagulase-positive staphylococci, were reported in the three commercial batches, all met the specification set and did not raise any safety concern.<sup>23,24</sup>

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

## 3.4. Toxicological data<sup>25</sup>

According to the Commission Implementing Regulation (EU) No 562/2012<sup>26</sup>, 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 CEF Panel, 2009).

The Panel considers that these requirements are fulfilled, because:

- i) rennet obtained from lamb abomasum has been safely used in the production of cheese and related products for many centuries;
- ii) the abomasum from suckling lambs is consumed throughout the EU and elsewhere in the world as meat product<sup>27</sup>;

<sup>17</sup> Technical dossier/2nd submission/Annex 5.3; Technical dossier/Additional information, 28 September 2021.

<sup>18</sup> Technical dossier/2nd submission/Annex 1; Technical dossier/Additional information, 28 September 2021.

<sup>19</sup> Technical dossier/2nd submission/p. 6; Technical dossier/Additional information, 28 September 2021.

<sup>20</sup> Technical dossier/2nd submission/Annex 6; Annex 7; Technical dossier/Additional information, 28 September 2021.

<sup>21</sup> Technical dossier/Additional information, 28 September 2021/Annex 3.

<sup>22</sup> Technical dossier/2nd submission/Annex 6, Annex 7; Technical dossier/Additional information, 28 September 2021/Annex 3, Annex 3.1, Annex 4 and Annex 4.1.

<sup>23</sup> Technical dossier/Annex 6; Technical dossier/Additional information, 28 September 2021/Annex 3.

<sup>24</sup> LODs: *Enterobacteriaceae*: 1 CFU/g; staphylococci: 1 CFU/g; sulphite-reducing clostridia: 1 CFU/g; yeasts and moulds: 4 CFU/g.

<sup>25</sup> Technical dossier/2nd submission/p. 13.

<sup>26</sup> 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.

<sup>27</sup> Technical dossier/Additional data, 17 February 2021/3. Allergenicity.

- iii) the manufacturing process of the food enzyme is not considered to introduce substances that could raise safety concerns;
- iv) the data on microbial quality provided on the food enzyme are considered sufficient.<sup>28</sup>

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<sup>29</sup>

The potential allergenicity of the food enzyme containing chymosin and pepsin derived from the abomasum of suckling lambs was not assessed by comparing its amino acid sequence with those of known allergens and was not considered relevant in this case.

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).

No information is available on oral sensitisation or elicitation reactions to chymosin and pepsin obtained from the abomasum of suckling lambs under evaluation. There are no reports in the literature on adverse reactions upon ingestion of these enzymes in individuals sensitised through the respiratory route.

The Panel considers that the likelihood of food allergic reactions to this food enzyme obtained from the abomasum of suckling lambs is low and, therefore, does not give rise to safety concerns under the intended conditions of use.

## 3.5. Dietary exposure

### 3.5.1. Intended use of the food enzyme<sup>6</sup>

The food enzyme (liquid extract) is intended to be used in milk processing for cheese production at the recommended use level between 1 and 2.5 L per 10,000 L of milk.<sup>6</sup>

Animal rennet is added to milk to separate milk into solid curd and liquid whey (coagulation).<sup>6</sup> Both chymosin and pepsin contribute to the milk-clotting activity. 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 6–12%).

Approximately, 1 kg of cheese contains about 0.0002–0.0004 g of rennet enzymes (Holmes et al., 1977).<sup>30</sup> The remaining rennet contributes to the ripening of cheese due to its general proteolytic activity.

### 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 over thousands of years and remain 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 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.

<sup>28</sup> Technical dossier/p. 13.

<sup>29</sup> Technical dossier/p. 13; Technical dossier/Additional information, 28 September 2021.

<sup>30</sup> Technical dossier/p. 12, 18.



## 4. Conclusion

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

## 5. Documentation as provided to EFSA

- 1) Technical dossier 'Rennet from stomachs of young calves and sheep.' 22 July 2016. Submitted by Productos Nievi, SA.
- 2) Additional information. 28 September 2021. Submitted by Productos Nievi, SA.

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## Abbreviations

|                |  |
|----------------|--|
| CAS            | chemical abstracts service   |
| CFU            | colony forming unit  |
| EFSA CEF Panel | EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids |
| EFSA CEP Panel | EFSA Panel on Food Contact Materials, Enzymes and Processing Aids              |
| EC             | European Commission  |
| EINECS         | European Inventory of Existing Commercial Chemical Substances                  |
| FAO            | Food and Agricultural Organization of the United Nations                       |
| IDF            | International Dairy Federation   |
| ISO            | International Organisation for Standardization                                 |
| IMCU           | International Milk-Clotting Units  |
| IUBMB          | International Union of Biochemistry and Molecular Biology                      |
| JECFA          | Joint FAO/WHO Expert Committee on Food Additives                               |
| LOD            | limit of detection   |
| MCU            | milk clot unit analytical method   |
| TOS            | total organic solids   |
| WHO            | World Health Organization  |