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## Safety evaluation of the food enzyme triacylglycerol lipase from the pregastric tissues of calves, young goats and lambs

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### Abstract

The food enzyme containing triacylglycerol lipase (triacylglycerol acylhydrolase; EC 3.1.1.3) is prepared from the pregastric tissues of calves, young goats and lambs by Caglifacio Clerici SpA. The food enzyme is intended to be used in the production of cheese. As no concerns arose 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. A search for the similarity of the amino acid sequence of the food enzyme to known allergens was made and no match was found. The Panel considered that a 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.

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<sup>†</sup> Deceased.

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## 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 microorganisms or products thereof including a product obtained by a fermentation process using microorganisms: (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<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, 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<sup>1</sup> on food enzymes.

Five applications have been introduced by the companies "Novozymes A/S", "DSM Food Specialties B.V.", "Advanced Enzyme Technologies Ltd" and the "Association of Manufacturers and Formulators of Enzyme Products (AMFEP)" for the authorisation of the food enzymes Pullulanase from a genetically modified strain of *Bacillus subtilis* (strain NZYM-AK), Glucoamylase from a genetically modified strain of *Aspergillus niger* (strain NZYM-BW), Chymosin from a genetically modified strain of *Kluyveromyces lactis* (strain CHY), Pectin lyase from a genetically modified strain of *Aspergillus niger* (strain FLOSC) and Triacylglycerol lipase from pregastric tissues of cattle, goat and sheep, respectively.

Following the requirements of Article 12.1 of Regulation (EC) 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, 11.3.2011, 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 Pullulanase from a genetically modified strain of *Bacillus subtilis* (strain NZYM-AK), Glucoamylase from a genetically modified strain of *Aspergillus niger* (strain NZYM-BW), Chymosin from a genetically modified strain of *Kluyveromyces lactis* (strain CHY), Pectin lyase from a genetically modified strain of *Aspergillus niger* (strain FLOSC) and Triacylglycerol lipase from pregastric tissues of cattle, goat and sheep 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 the food enzyme triacylglycerol lipase from the pregastric tissues of cattle, goat and sheep submitted by AMFEP.

The application was submitted initially as a joint dossier<sup>4</sup> and identified as the EFSA-Q-2015-00131. During a meeting between EFSA, the European Commission and representatives from AMFEP.<sup>5</sup> 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-2015-00131. This data package, identified as EFSA-Q-2022-00367, concerns the food enzyme triacylglycerol lipase from the pregastric tissues of calves, young goats and lambs and submitted by Caglificio Clerici SpA.

## 2. Data and methodologies

### 2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme triacylglycerol lipase from the pregastric tissues of cattle, goats and sheep.

Additional information was requested from the applicant during the assessment process on 20 January 2023 and 8 May 2023 and received on 15 March 2023 and on 16 May 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) 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, 2021).

## 3. Assessment

IUBMB nomenclature	Triacylglycerol lipase
Systematic name	Triacylglycerol acylhydrolase
Synonyms	Lipase, triglyceride lipase, glycerol ester hydrolase
IUBMB No	EC 3.1.1.3
CAS No	9001-62-1
EINECS No	232-619-9

Triacylglycerol lipases catalyse, in the presence of water, the hydrolysis of the ester linkages in triacylglycerols, resulting in the generation of glycerol, free fatty acids, diacylglycerols and monoacylglycerols. The food enzyme under application is intended to be used in the production of cheese.

<sup>4</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 Text with EEA relevance. OJ L 168, 28.6.2012, p. 21–23.

<sup>5</sup> 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>

### 3.1. Source of the food enzyme

The food enzyme is obtained from the pregastric tissues<sup>6</sup> of calves, young goats and lambs from certified European and non-European slaughterhouses<sup>7</sup>, surveyed and approved by the competent authorities. 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>8</sup> and Regulation (EC) No 853/2004<sup>9</sup>. Examples of related certificates from non-European slaughterhouses were provided by the applicant, confirming that animal tissues used for the preparation of the food enzyme comply with meat inspection requirements and are handled in accordance with good hygiene practice.<sup>10</sup>

In EU, according to Regulation (EC) 1774/2002<sup>11</sup>, pregastric tissues of calves, young goats and lambs are considered fit for human consumption. It is an edible offal as defined in Regulation (EC) No 853/2004<sup>9</sup>.

No issues of concern arising from 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<sup>8</sup>, with food safety procedures based on Hazard Analysis and Critical Control Points, and in accordance with current Good Manufacturing Practice.<sup>12</sup>

The food enzyme is extracted from pregastric tissues, which are sourced frozen from the supplier and stored at  $-18^{\circ}\text{C}$  before processing. The tissues are thawed and then trimmed and ground to a suitable particle size to release the enzyme. The ground tissue is then mixed with salt and a drying agent, after which the material is freeze-dried, milled and homogenised. The obtained food enzyme concentrate is monitored for its specific activity and further standardised with [REDACTED] and [REDACTED].

Traditionally, a batch of the food enzyme is obtained from a single animal species, but the food enzyme may also be obtained by mixing triacylglycerol lipases from two species.<sup>13</sup>

The applicant provided information on the identity of the substances used in the extraction and in the subsequent downstream processing.<sup>14</sup>

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 triacylglycerol lipases from the pregastric tissues of calves, young goats and lambs are all single polypeptide chains of 397 amino acids.<sup>15</sup> The molecular mass of the mature proteins is 45.2 kDa.<sup>16</sup> No other enzyme activities were reported.<sup>17</sup>

The triacylglycerol lipase activity is determined according to the official method ISO 13082/IDF 218 (2011) that is based on the hydrolysis of tributyrin (reaction conditions: pH 6.2,  $42^{\circ}\text{C}$ ). The enzymatic activity is determined by back titration of the excess of sodium hydroxide after neutralisation of the butyric acid released from tributyrin. The amount of sodium hydroxide consumed within a defined period is used to calculate the activity in International Lipase Units (ILU) per mL or

<sup>6</sup> Pregastric tissues include the soft palate, the root of tongue, the glosso-epiglottic region, the epiglottis, the larynx and the apex of the arytenoids cartilage.

<sup>7</sup> Technical dossier/Dossier p. 42.

<sup>8</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>9</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, 25.6.2004, p. 22.

<sup>10</sup> Technical dossier/Annexes 24–25.

<sup>11</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.

<sup>12</sup> Technical dossier/Dossier p. 43/Annex 26, Annex 27; Additional data March 2023/Annex 24, Annex 25.

<sup>13</sup> Technical dossier/p. 44.

<sup>14</sup> Technical dossier/Annex 28.

<sup>15</sup> Technical dossier/p. 36; [https://www.ncbi.nlm.nih.gov/protein/XP\\_017900376.1](https://www.ncbi.nlm.nih.gov/protein/XP_017900376.1); [https://www.ncbi.nlm.nih.gov/protein/XP\\_027816132.2](https://www.ncbi.nlm.nih.gov/protein/XP_027816132.2); [https://www.ncbi.nlm.nih.gov/protein/NP\\_776528.1](https://www.ncbi.nlm.nih.gov/protein/NP_776528.1); Additional data March 2023.

<sup>16</sup> Technical dossier/p. 35; UniProtKB database.

<sup>17</sup> Technical dossier/p. 33.

per gram. One ILU is defined as the amount of lipase activity that releases butyric acid at a rate of 1.25  $\mu\text{mol}/\text{min}$  under the specified conditions.<sup>18</sup>

The triacylglycerol lipases have a pH optimum around pH 6.0 (30°C) and a temperature optimum around 40°C (pH 5.5). Activity decreased above 50°C, showing no residual activity above 65°C.<sup>19</sup>

### 3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme were provided for nine batches used for commercialisation, three from calves (Table 1), three from young goats (Table 2) and three from lambs (Table 3).<sup>20</sup> The mean total organic solids (TOS) were 24.0%, 28.4% and 28.9% respectively, and the mean enzyme activities/mg TOS ratio were 0.3, 0.2 and 0.2 ILU/mg TOS, respectively.

**Table 1:** Composition of the food enzyme preparation obtained from calves<sup>21</sup>

Parameters	Unit	Batches		
		1	2	3
<b>Triacylglycerol lipase activity</b>	ILU/g <sup>(a)</sup>	71.4	68.6	70.7
<b>Protein</b>	%	4.7	5.9	5.9
<b>Ash</b>	%	81.2	73.1	71.7
<b>Water</b>	%	0.8	0.5	0.6
<b>Total organic solids (TOS)<sup>(b)</sup></b>	%	18.0	26.4	27.7
<b>Triacylglycerol lipase activity/TOS</b>	ILU/mg TOS	0.40	0.26	0.26

(a): ILU: International Lipase Unit (see Section 3.3.1).

(b): TOS calculated as 100% – % water – % ash.

**Table 2:** Composition of the food enzyme preparation obtained from young goats<sup>22</sup>

Parameters	Unit	Batches		
		1	2	3
<b>Triacylglycerol lipase activity</b>	ILU/g <sup>(a)</sup>	64.3	62.4	64.3
<b>Protein</b>	%	3.9	4.0	4.8
<b>Ash</b>	%	72.2	73.7	67.1
<b>Water</b>	%	0.8	0.5	0.6
<b>Total organic solids (TOS)<sup>(b)</sup></b>	%	27.0	25.8	32.3
<b>Triacylglycerol lipase activity/TOS</b>	ILU/mg TOS	0.24	0.24	0.20

(a): ILU: International Lipase Unit (see Section 3.3.1).

(b): TOS calculated as 100% – % water – % ash.

**Table 3:** Composition of the food enzyme preparation obtained from lambs<sup>23</sup>

Parameters	Unit	Batches		
		1	2	3
<b>Triacylglycerol lipase activity</b>	ILU/g <sup>(a)</sup>	51.0	50.5	50.5
<b>Protein</b>	%	3.4	3.7	5.6
<b>Ash</b>	%	74.7	74.8	62.0
<b>Water</b>	%	0.6	0.6	0.7
<b>Total organic solids (TOS)<sup>(b)</sup></b>	%	24.7	24.6	37.3
<b>Triacylglycerol lipase activity/TOS</b>	ILU/mg TOS	0.21	0.21	0.14

(a): ILU: International Lipase Unit (see Section 3.3.1).

(b): TOS calculated as 100% – % water – % ash.

<sup>18</sup> Technical dossier/Annex 13.

<sup>19</sup> Technical dossier/pp. 38–39.

<sup>20</sup> Technical dossier/Annex 10, Annex 11, Annex 12.

<sup>21</sup> Technical dossier/Dossier p. 32/Annex 1-Annex 3.

<sup>22</sup> Technical dossier/Dossier p. 32/Annex 4-Annex 6.

<sup>23</sup> Technical dossier/Dossier p. 32/Annex 7-Annex 9.

### 3.3.3. Purity

The lead content in the nine commercial batches was  $\leq 0.22$  mg/kg<sup>24,25</sup> which complies with the specification for lead as laid down in the general specifications and considerations 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. In addition, data on *Enterobacteriaceae*, *Campylobacter spp.*, Shiga Toxin-producing *Escherichia coli*, filamentous fungi and yeasts were also reported and raised no concern.<sup>26</sup> *Listeria monocytogenes* was not detected in 25 g of nine representative commercial batches (ISO method 11290-1:2017).<sup>27</sup>

## 3.4. Toxicological data

According to the Commission Implementing Regulation (EU) No 562/2012<sup>28</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 CEP Panel, 2021).

The Panel considers that these requirements are fulfilled, because:

- i) triacylglycerol lipases obtained from the pregastric tissues of calves, young goats and lambs have been safely used in the production of cheese and related products for centuries (Nelson et al., 1977);
- ii) the tongue from cattle is consumed throughout the EU and elsewhere in the world;
- 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 as well as the manufacturing process. Therefore, the need for toxicological data is waived.

### 3.4.1. Allergenicity

The potential allergenicity of the food enzyme containing triacylglycerol lipase derived from the pregastric tissues of cattle, goat and sheep 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, no match was found.<sup>29</sup>

No information is available on oral sensitisation or elicitation reactions to these triacylglycerol lipases.

Occupational allergies to triacylglycerol lipases have been described (Elms et al., 2003; Lindstedt et al., 2005; Shin et al., 2008; Loureiro et al., 2009; Martel et al., 2010; Budnik et al., 2017). 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 triacylglycerol lipases in individuals sensitised through the respiratory route.

<sup>24</sup> Technical dossier/Dossier p. 34; Annexes 1–9, Annex 23; Additional data March 2023.

<sup>25</sup> LoQ: Pb = 0.01 mg/kg.

<sup>26</sup> Technical dossier/Dossier p. 34/Annexes 1–9, Annexes 14–20.

<sup>27</sup> Technical dossier/Dossier p. 34/ Annexes 1–9, Annexes 21–22.

<sup>28</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>29</sup> Technical dossier/Additional information March 2023.



The Panel considered that a risk of allergic reactions upon dietary exposure to this food enzyme cannot be excluded, but the likelihood is low.

### 3.5. Dietary exposure

#### 3.5.1. Intended use of the food enzyme

The food enzyme is intended to be used in the production of cheese at the recommended use level between 1 and 5.5 mg TOS/kg milk.<sup>30</sup>

In cheese making, the triacylglycerol lipase is added to milk together with the starter culture during coagulation.<sup>31</sup> The free fatty acids released by the hydrolysis of the triacylglycerols enhance flavours of the cheese (e.g. pecorino, provolone and feta). After the coagulation, a fraction of the food enzyme-TOS partitions into the whey and is removed during the draining of the whey. A fraction of the food enzyme TOS remains in the curd.<sup>30</sup>

Based on data provided on thermostability (see Section 3.3.1), it is expected that the food enzyme may remain active in cheese, depending on the cheese-making process.

#### 3.5.2. Dietary exposure estimation

The technology of extracting enzymes from pregastric tissues and the technology of using the food enzyme for cheese making have remained traditionally the same over many years and 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, tongues from ruminants are consumed in European countries, which contributes to the overall exposure to the food enzyme in the EU. Consequently, the Panel considered that a dietary exposure estimation was 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 concludes that the food enzyme triacylglycerol lipase obtained from the pregastric tissues of calves, young goats and lambs does not give rise to safety concerns under the intended conditions of use.

## 5. Documentation as provided to EFSA

- 1) Triacylglycerol lipase from pregastric tissues of cattle, goat and sheep. June 2022. Submitted by Caglificio Clerici SpA.
- 2) Additional information. March 2023. Submitted by Caglificio Clerici SpA.
- 3) Additional information. May 2023. Submitted by Caglificio Clerici SpA.

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<sup>30</sup> Technical dossier/p. 53.

<sup>31</sup> Technical dossier/Figure 5.

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## Abbreviations

AMFEP	Association of Manufacturers and Formulators of Enzyme Products
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
ILU	International Lipase Unit
ISO	International Organization for Standardization
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kDa	kiloDalton
LOQ	limit of quantification
TOS	total organic solids
WHO	World Health Organization