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Safety evaluation of the food enzyme subtilisin from the non-genetically modified *Bacillus paralicheniformis* strain LMG S-30155

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Abstract

The food enzyme subtilisin (serine endopeptidase, EC 3.4.21.62) is produced with the non-genetically modified microorganism *Bacillus paralicheniformis* strain LMG S-30155 by ENMEX SA de CV, now part of Kerry Food Ingredients (Cork) Ltd. The food enzyme is intended to be used in oil production, hydrolysis of vegetable/microbial/animal proteins, yeast processing and production of flavouring preparations. The production strain of the food enzyme contains known antimicrobial resistance genes and genes involved in bacitracin biosynthesis. Consequently, it does not fulfil the requirements for the QPS approach to safety assessment. Bacitracin was detected in the food enzyme and the [redacted] The presence of bacitracin, a medically important antimicrobial, in the food enzyme represents a risk for the development of resistance in bacteria. Due to the presence of bacitracin, the Panel concluded that the food enzyme subtilisin produced with the non-genetically modified *Bacillus paralicheniformis* strain LMG S-30155 cannot be considered safe.

Keywords: food enzyme, subtilisin, serine endopeptidase, EC 3.4.21.62, alcalase, *Bacillus paralicheniformis*

Requestor: European Commission

Question number: EFSA-Q-2022-00366

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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 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[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 EU 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 I foods, in accordance with the specification and condition of use provided for in Article 7 (2) of Regulation (EC) No 1332/2008 on food enzyme.[3]

Six applications have been introduced by the companies 'Decernis, LLC', 'Keller and Heckman LLP', the Association of Manufacturers and Formulation of Enzyme Products (AMFEP), and 'Novozymes A/S' for the authorization 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), *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[4] implementing regulation (EC) No 1331/2008, the Commission has verified that the six 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, 11.3.2011, pp. 15–24.

[4] Regulation (EC) No 234/2011.

1.1.2. Terms of Reference

The European Commission requests the European Food Safety Authority (EFSA) 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), *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 the food enzyme Subtilisin from *Bacillus licheniformis* submitted by the Association of Manufacturers and Formulators of Enzyme Products (AMPEP).

The application was submitted initially as a joint dossier[5] and identified as EFSA-Q-2015-00232. During an ad hoc meeting between EFSA, the European Commission and representatives from the Association of Manufacturers and Formulators of Enzyme Products (AMFEP),[6] 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-2015-00232. This data package, identified as EFSA-Q-2022-00366, concerns the food enzyme subtilisin that is produced with a strain of *Bacillus licheniformis* and submitted by ENMEX SA de CV, now part of Kerry Food Ingredients (Cork) Ltd.

Recent data identified the production microorganism as *Bacillus paralicheniformis* (Section 3.1). Therefore, this name will be used in this opinion instead of *Bacillus licheniformis*.

2. Data and Methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme subtilisin from *Bacillus paralicheniformis* (strain LMG S-30155).

2.2. Methodologies

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

The current 'Scientific Guidance for the submission of dossiers on food enzymes' (EFSA CEP Panel, 2021) has been followed for the evaluation of the application.

3. Assessment

IUBMB nomenclature	Subtilisin
Systematic name	Serine endopeptidase
Synonyms	Alcalase, bacillopeptidase, alkaline proteinase, thermoase, subtilopeptidase
IUBMB No	EC 3.4.21.62
CAS No	9014-01-1
EINECS No	232–752-2

Subtilisins catalyse the hydrolysis of proteins with broad specificity for peptide bonds releasing peptides and amino acids. The enzyme under assessment is intended to be used in oil production, hydrolysis of vegetable/microbial/animal proteins, yeast processing and production of flavouring preparations.

[5] 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, pp. 21–23.

[6] The full detail is available online 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 enzyme is produced with the non-genetically modified bacterium *Bacillus paralicheniformis* strain LMG S-30155, which is deposited at the Belgian Coordinated Collection of Microorganism, Laboratory of Microbiology-UGent (BCCM/LMG, Belgium) with the deposit number LMG S-30155.[7] It was identified as *B. paralicheniformis* by whole genome sequencing (WGS) analysis, which showed an average nucleotide identity (ANI) of 98.89% with *B. paralicheniformis* type strain.[8]

The species *Bacillus paralicheniformis* is included in the list of organisms for which the qualified presumption of safety (QPS) may be applied, provided that the absence of acquired antimicrobial resistance (AMR) genes, toxigenic activity and the inability to synthetise bacitracin are verified for the specific strain used (EFSA BIOHAZ Panel, 2020).[9] The production strain was not cytotoxic against Vero cells using the Lactate Dehydrogenase assay.[10] WGS analysis did not identify genes of concern involved in virulence, but identified the presence of genes involved in bacitracin biosynthesis. The broth supernatant of the production strain showed antibacterial activity against the test strain [redacted].[11]

[redacted].[12]

3.2. Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004[13], with food safety procedures based on Hazard Analysis and Critical Control Points, and in accordance with current Good Manufacturing Practice.[14]

The production strain is grown as a pure culture using a typical industrial medium in a submerged, [redacted] fermentation system with conventional process controls in place. After completion of the fermentation, the solid biomass is removed from the fermentation broth by filtration. The filtrate containing the enzyme is further purified and concentrated, including an ultrafiltration step in which enzyme protein is retained, while most of the low molecular mass material passes the filtration membrane and is discarded. A final concentration step is carried out by evaporation. Finally, the food enzyme was spray-dried prior to analysis.[15] The applicant provided information on the identity of the substances used to control the fermentation and in the subsequent downstream processing of the food enzyme.[16]

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

Subtilisin is a single polypeptide chain of [redacted] amino acids.[17] The molecular mass of the mature protein, calculated from the amino acid sequence, is [redacted] kDa.[18] The food enzyme was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A consistent protein pattern was observed across all batches. The gels showed several bands of different intensity, one corresponding to an apparent mass of about [redacted].[19] No other enzymatic activities were reported.

[7] Technical Dossier/Annex I.
[8] Technical Dossier/Annex J/pp. 6–10.
[9] https://zenodo.org/record/4917383#.ZBhvT3bMKUl
[10] Technical Dossier/Annex M.
[11] Technical Dossier/Annex L.
[12] Technical Dossier/Annex J/p. 10; APPENDIX 1/VIII.
[13] 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.
[14] Technical dossier/Dossier p. 45 & Annex N.
[15] Technical dossier/Dossier pp. 45–51 & Annex O.
[16] Technical dossier/Dossier pp. 45–51 & Annex P.
[17] Technical dossier/Annex R.
[18] Technical dossier/Dossier p. 34.
[19] Technical dossier/Annex E.

The in-house determination of subtilisin activity is based on hydrolysis of casein (reaction conditions: pH 8.5, 40°C). The enzymatic activity is determined by measuring the release of solubilised casein peptides spectrophotometrically at 275 nm. The subtilisin activity is expressed in Detergent Alkaline Protease Unit/g (DAPU/g). One DAPU is defined as the activity that liberates the equivalent of 4 μmol of tyrosine per minute under the conditions of the assay.[20]

The food enzyme has a temperature optimum around 50°C (pH 8.5) and a pH optimum around pH 10 (40°C). Thermostability was tested after a pre-incubation for 15 min at different temperatures. The enzyme activity decreased above 60°C, showing no residual activity above 80°C.[21]

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches (Table 1). The mean total organic solids (TOS) of the three food enzyme batches for commercialisation was 82% and the mean enzyme activity/TOS ratio was 4.1 DAPU/mg TOS.

Table 1: Composition of the food enzyme[(c)]

Parameters	Unit	Batches		
		1	2	3
Subtilisin activity	DAPU/g[(a)]	3,363	3,221	3,295
Protein	%	51	50	48
Ash	%	10	11	11
Water	%	7	8	8
Total organic solids (TOS)[(b)]	%	83	81	81
Activity/TOS	DAPU/mg TOS	4.1	4.0	4.1

(a): UNIT: Detergent Alkaline Protease Unit (DAPU) (see Section 3.3.1).
(b): TOS calculated as 100% – % water – % ash.
(c): Technical dossier/Annex F & dossier p. 32.

3.3.3. Purity

The lead content in the three batches was below 5 mg/kg, which complies with the specification for lead as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006).[22,23]

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).[24]

The analysis of the WGS indicated that the production strain may have a capacity to synthesise bacitracin, an agent included by the WHO in the list of medically important antimicrobials.[25] Samples were taken from three production batches at various stages in the production process [redacted] and analysed using a quantitative ELISA test for the presence of bacitracin. In all [redacted] samples, bacitracin was detected in concentrations varying from [redacted] ng/mL.[26] In addition, phenotypic analysis found that the production strain shows antibacterial activity against the test strain [redacted].[27]

As reported by EFSA (EFSA BIOHAZ Panel, 2021), the exposure to low concentrations of antimicrobials, including sub-inhibitory concentrations, may result in the selection of AMR bacteria. For several antimicrobial agents, the Minimal Selective Concentration the lowest drug concentration that can result in enrichment of resistant bacteria, has been established. The value for bacitracin has been estimated by Bengtsson-Palme and Larsson (2016) as 8 ng/mL, lower than the concentration found in

[20] Technical dossier/Annex D.
[21] Technical dossier/Dossier pp. 36–38.
[22] LoD: Pb = 0.250 mg/kg.
[23] Technical dossier/ Annex F & Annex G.
[24] Technical dossier/Annex F & Annex G.
[25] Critically important antimicrobials for human medicine, 6th revision. Geneva: World Health Organization; 2019.
[26] Technical dossier/Annex K.
[27] Technical dossier/Annex L.

the food enzyme produced by *B. paralicheniformis*. The Panel also noted that bacitracin may select for cross-resistance to Colistin (Xu et al., 2018), a critically important antimicrobial,[25] through the mcr-1 gene. The Panel considered that the presence of bacitracin in the food enzyme represents a risk for the development of resistance in bacteria.

3.3.4. Viable cells and DNA from production strain

Data on the absence of viable cells and DNA from the production strain in the food enzyme were not provided. As the presence of bacitracin in the food enzyme represents a risk to human health (see Section 3.3.3), the Panel considered it unnecessary to request the data to complete this section of the opinion.

3.4. Toxicological data

The production strain does not satisfy the requirements for the QPS approach to risk assessment. No toxicological data were provided. However, as the presence of bacitracin in the food enzyme represents a risk to human health (see Section 3.3.3), the Panel considered it unnecessary to request the toxicological data to complete this section of the opinion.

3.4.1. Allergenicity

The allergenicity assessment considered only the food enzyme and not carriers or other excipients that may be used in the final formulation.

The potential allergenicity of the enzyme produced with the non-genetically modified *Bacillus paralicheniformis* strain LMG S-30155 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, several matches were found. The matching allergens were microbial peptidases, all known as respiratory allergens.[28]

No information is available on oral and respiratory sensitisation or elicitation reactions of this subtilisin.

Several studies have shown that adults respiratorily sensitized to a food enzyme may be able to ingest the corresponding allergen without acquiring clinical symptoms of food allergy (Cullinan et al., 1997; Poulsen, 2004; Armentia et al., 2009).

[redacted] and [redacted] (listed in the Regulation (EU) No 1169/2011[29]) and [redacted] products that may cause allergies or intolerances are used as raw materials. However, during the fermentation process, these products will be degraded and utilised by the microorganisms for cell growth, cell maintenance and production of enzyme protein. In addition, the microbial biomass and fermentation solids are removed. Taking into account the fermentation process and downstream processing, the Panel considered that no potentially allergenic residues are present in the food enzyme.

The Panel considered that, under the intended conditions of use, the 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 several food manufacturing processes summarized in Table 2.

[28] Technical dossier-pp. 10–11, 63–64; Annex R.

[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.

Table 2: Intended uses of the food enzyme as provided by the applicant[(a)]

Food manufacturing process	Raw material (RM)	Recommended use level (mg TOS/kg RM)
Oil production	Algal fermentation broth	[redacted]
Hydrolysis of vegetable proteins	Almond protein, amaranth protein, barley protein, buckwheat protein, canola protein, corn protein, chia seed protein, chickpea protein, hemp protein, lentil protein, moringa protein, oats protein, peanut protein, pea protein, pumpkin protein, quinoa protein, rice protein, sacha inchi protein, soy protein, sunflower protein, wheat protein	[redacted]
Hydrolysis of microbial protein	Chlorella protein, seaweed protein, (micro)algae (Spirulina, etc.) protein	[redacted]
Hydrolysis of animal protein	Beef bones, skin and viscera Fish (salmon, redfish, mackerel, trout, cod, blue whiting, herring, fish from the Epipelagic, Mesopelagic group) bones, skin and viscera poultry bones, skin and viscera lamb bones, skin and viscera turkey bones, skin and viscera pork bones, skin and viscera whey protein	[redacted]
Yeast processing	Whole yeast cells, autolysed yeasts yeast extract, yeast cell walls	[redacted]
Production of flavouring preparations	Materials of vegetable origin Materials of animal origin	[redacted]

(a): Technical dossier/p. 57.

In the treatment of algae for edible oil production, the food enzyme is added to algal fermentation broth during the hydrolysis step.[30] The enzyme is used to increase the oil extraction yield.[31] The food enzyme–TOS is removed in the final processed foods by extraction and subsequent refinement of the extract (EFSA CEP Panel, 2021).

In the production of protein hydrolysates, the food enzyme is added to plant, microbial or animal protein during the hydrolysis step.[32] The enzyme is used to increase the yield and to enhance the flavour of the hydrolysates.[33] The food enzyme–TOS remains in the final processed foods.

In yeast processing, the food enzyme could be added to yeast biomass or after the separation between yeast cell walls and yeast extract.[34] The enzyme is used to hydrolyze insoluble proteins, which helps optimising the extraction process. In addition, it improves the sensory properties of the yeast-derived products.[35] The food enzyme–TOS remains in the final processed foods.

Several flowcharts were provided to indicate the use of food enzymes in the production of flavouring substances and/or preparations.[36] However, the information is too generic to be useful for the evaluation of the food enzyme under assessment.

Based on data provided on thermostability (see Section 3.3.1) and the downstream processing step applied in the food processes, it is expected that the subtilisin is inactivated by heat during all the food processes in which the food enzyme–TOS remains.

The information provided in the technical dossier lacks important details about certain food manufacturing processes to which the food enzyme may be applied. [redacted]

[30] Technical dossier/p. 54.
[31] Technical dossier/p. 74.
[32] Technical dossier/p. 55.
[33] Technical dossier/p. 75.
[34] Technical dossier/p. 56.
[35] Technical dossier/p. 79.
[36] Technical dossier/Annex S.

As the presence of bacitracin in the food enzyme represents a risk to human health (see Section 3.3.3), the Panel considered it unnecessary to request additional data to complete this section of the opinion.

3.5.2. Dietary exposure estimation

The missing use levels and the limited technical information about possible overlapping uses of the food enzyme in different food manufacturing processes precluded an estimate of the dietary exposure.

3.6. Margin of exposure

In the absence of appropriate data, the margin of exposure was not calculated.

4. Conclusion

Due to the presence of bacitracin, a medically important antimicrobial, the Panel concluded that the food enzyme subtilisin produced with the non-genetically modified *Bacillus paralicheniformis* strain LMG S-30155 cannot be considered safe.

5. Documentation as provided to EFSA

Technical dossier 'Application for authorization of subtilisin from the non-genetically modified *Bacillus paralicheniformis* strain LMG S-30155 in accordance with Regulation (EC) No 1331/2008'. 9 June 2022. Submitted by ENMEX SA de CV, now part of Kerry Food Ingredients (Cork) Ltd.

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Abbreviations

ANI	average nucleotide identity
AMR	antimicrobial resistance
CAS	Chemical Abstracts Service
CEP	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
EINECS	European Inventory of Existing Commercial Chemical Substances
FAO	Food and Agricultural Organisation of the United Nations
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kDa	kiloDalton
LoD	limit of detection
QPS	qualified presumption of safety
TOS	total organic solids
WGS	whole genome sequencing
WHO	World Health Organization