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Safety evaluation of the food enzyme subtilisin from the non-genetically modified *Bacillus paralicheniformis* strain DP-Dzx96

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Abstract

The food enzyme subtilisin (serine endopeptidase, EC 3.4.21.62) is produced with the non-genetically modified *Bacillus paralicheniformis* strain DP-Dzx96 by Genencor International B.V. The food enzyme was considered free from viable cells of the production organism. The food enzyme is intended to be used in five food manufacturing processes: production of protein hydrolysates from plants and fungi, production of protein hydrolysates from meat and fish proteins, production of cooked rice, production of modified meat and fish products, and yeast processing. The production strain of the food enzyme contains known antimicrobial resistance genes. Bacitracin, a medically important antimicrobial, was detected in the food enzyme. The presence of bacitracin represents a risk for the development of antimicrobial resistant bacteria. A search for the similarity of the amino acid sequence of the food enzyme to known allergens was made and three matches with respiratory and two matches with food allergens were found. The Panel considered that, under the intended conditions of use, the risk of allergic reactions upon dietary exposure to this food enzyme, particularly in individuals sensitised to muskmelon or pomegranate, cannot be excluded, but would not exceed the risk of consuming these foods. Due to the presence of bacitracin, the Panel concluded that the food enzyme subtilisin produced with the non-genetically modified *Bacillus paralicheniformis* strain DP-Dzx96 cannot be considered safe.

Keywords: food enzyme, subtilisin, serine endopeptidase, EC 3.4.21.62, alcalase, *Bacillus paralicheniformis*, non-genetically modified microorganism

Requestor: European Commission

Question number: EFSA-Q-2023-00236

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Waiver: The full opinion will be published in accordance with Article 12 of Regulation (EC) No 1331/2008 once the decision on confidentiality will be received from the European Commission.

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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 in foods, in accordance with the specifications and conditions of use provided for in Article 7(2) of Regulation (EC) No 1332/2008[3] on food enzymes.

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

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

[1] Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on Food Enzymes and Amending Council Directive 83/417/EEC, Council Regulation (EC) No 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/97. OJ L 354, 31.12.2008, pp. 7–15.

[2] Regulation (EC) No 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 354, 31.12.2008, pp. 1–6.

[3] Commission Regulation (EU) No 234/2011 of 10 March 2011 implementing Regulation (EC) No 1331/2008 of the European Parliament and of the Council establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 64, 11.3.2011, pp. 15–24.

1.1.2. Terms of Reference

The European Commission requests the European Food Safety Authority to carry out the safety assessments on the food enzymes Cyclomaltodextrin glucanotransferase from *Geobacillus stearothermophilus*, Dextranase from *Chaetomium gracile*, Subtilisin from *Bacillus licheniformis*, Mucorpepsin from *Rhizomucor miehei*, Animal rennet consisting of chymosin and pepsin from the abomasum of *Bos primigenius* (cattle), *Bubalus bubalis* (buffalo), *Capra aegagrus hircus* (goat) and *Ovis aries* (sheep), and Lipase from a genetically modified strain of *Aspergillus niger* (strain NZYM-DB) in accordance with Article 17.3 of Regulation (EC) No 1332/2008 on food enzymes.

1.2. Interpretation of the Terms of Reference

The present scientific opinion addresses the European Commission's request to carry out the safety assessment of food enzyme subtilisin from *Bacillus licheniformis* submitted by AMFEP.

The application was submitted initially as a joint dossier[4] and identified as the EFSA-Q-2015-00232. During a meeting between EFSA, the European Commission and the Association of Manufacturers and Formulators of Enzyme Products (AMFEP),[5] it was agreed that joint dossiers will be split into individual data packages.

The current opinion addresses one data package originating from the former joint dossier. This data package is identified as EFSA-Q-2023-00236 and concerns the food enzyme subtilisin produced from the *Bacillus licheniformis* strain DP-Dzx96 and submitted by Genencor International B.V. 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 a non-genetically modified *Bacillus paralicheniformis* DP-Dzx96.

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 five food processes: production of protein hydrolysates from plants and fungi, production of protein hydrolysates from meat and fish proteins, production of cooked rice, production of modified meat and fish products, and yeast processing.

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

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

3.1. Source of the food enzyme

The enzyme is produced with the non-genetically modified bacterium *Bacillus paralicheniformis* strain DP-Dzx96, which is deposited at the Westerdijk Fungal Biodiversity Institute with the deposit number [redacted].[6] The production strain was identified as *B. paralicheniformis* by [redacted].[7]

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, cytotoxic activity and the inability to synthetise bacitracin are verified for the specific strain used (EFSA BIOHAZ Panel, 2022). The production strain was not cytotoxic to [redacted].[8] The WGS analysis of the strain revealed the presence of genes coding for antimicrobial resistance, [redacted].[9]

3.2. Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004[10], with food safety procedures based on Hazard Analysis and Critical Control Points, and in accordance with current good manufacturing practice.[11]

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 then 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.[12] The process may also involve the use of [redacted] to precipitate the protein fraction. 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.[13]

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 subtilisin is a single polypeptide chain of [redacted] amino acids.[11] The molecular mass of the mature protein, calculated from the amino acid sequence, was calculated to be [redacted] kDa.[14] The food enzyme was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A consistent protein pattern was observed across all batches. The gel showed a single major protein band corresponding to an apparent molecular mass of about [redacted] kDa, consistent with the expected mass of the enzyme.[15] No other enzyme activities were reported.

The in-house determination of subtilisin activity is based on hydrolysis of N-succinyl-Ala-Ala-Ala-*p*-nitroanilide (reaction conditions: pH 8.5, 25°C, 10 min). The enzymatic activity is determined by measuring the release of p-nitroanilide spectrophotometrically at 405 nm. The enzyme activity is quantified relative to an internal enzyme standard and expressed in Delft Units/g (KDU/g).[16] The food enzyme has a temperature optimum around 60°C (pH 8.5) and a pH optimum between pH 9 and 11 (25°C). Thermostability was tested after pre-incubation of the food enzyme for 15 min at different

[6] Technical Dossier/Annex/Annex P.
[7] Technical Dossier/Annex/Annex O.
[8] Technical Dossier/Annex/Annex Q.
[9] Technical Dossier/Annex/Annex R.
[10] 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.
[11] Technical dossier/pp. 9–10, 52-53/Annex E.
[12] Technical dossier/pp. 53-60/Annex F.
[13] Technical dossier/p. 57/Annex G.
[14] Technical dossier/Annex M.
[15] Technical dossier/p. 38.
[16] Technical dossier/Annex B.

temperatures (pH 8.6). Enzyme activity decreased above 65°C, showing no residual activity above 68°C.

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches (Table 1).[17] The mean total organic solids (TOS) of the three food enzyme batches for commercialisation was 15.3% and the mean enzyme activity/TOS ratio was 6.5 KDU/mg TOS.

Table 1: Composition of the food enzyme

Parameters	Unit	Batches		
		1	2	3
Enzyme activity	KDU/g[(a)]	987	947	1,055
Protein	%	13.16	12.55	14.64
Ash	%	0.35	0.44	0.50
Water	%	84.40	85.30	83.05
Total organic solids (TOS)[(b)]	%	15.25	14.26	16.45
Activity/TOS	KDU/mg TOS	6.5	6.6	6.4

(a): KDU/g: Delft Unit/g (see Section 3.3.1).
(b): TOS calculated as 100%–% water–% ash.

3.3.3. Purity

The lead content in the three commercial batches was below 0.05 mg/kg,[18,19] which complies with the specification for lead as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006).[20]

The food enzyme preparation 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).[20] No antimicrobial activity was detected in any of the tested batches when using the test strains and procedures recommended by JECFA.[20]

Three food enzymes batches were analysed for the presence of bacitracin. In all samples, bacitracin was detected in concentrations varying from [redacted].[21]

The exposure to low concentrations of antimicrobials, including sub-inhibitory concentrations, may result in the selection of AMR bacteria (EFSA BIOHAZ Panel, 2021). For several antimicrobial agents, the lowest drug concentration that can result in enrichment of resistant bacteria, has been estimated. The predicted no effect concentration (PNEC) for bacitracin has been calculated by Bengtsson-Palme and Larsson (2016) to be 8 ng/mL. The Panel also noted that bacitracin may select for cross-resistance to colistin (Xu et al., 2018), a critically important antimicrobial.[22] 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 of the production strain

The absence of viable cells of the production strain in the food enzyme was demonstrated in [redacted]. No colonies were produced.[23]

3.4. Toxicological data

The food enzyme contains bacitracin, indicating that the production strain has the capacity to produce this antimicrobial. In addition, the production strain contains an AMR gene conferring

[17] Technical dossier/p. 37/Annex C.
[18] Technical dossier/p. 8, 40/Annex C.
[19] LoD/LoQ: Pb = 0.05 mg/kg.
[20] Technical dossier/p. 8, 40/Annex C/Annex D.
[21] Technical dossier/Annex T.
[22] Critically important antimicrobials for human medicine, 6th revision. Geneva: World Health Organization; 2019.
[23] Technical dossier/Annex/Annex U and Annex V.

resistance to bacitracin. Therefore, the production strain does not meet the requirements for the QPS approach, which in principle would trigger the need for toxicological studies. However, the presence of bacitracin in the food enzyme represents a risk to human health (see Section 3.3.3), independent from any other potential toxicological issue. For this reason, the Panel considered it not justified 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 subtilisin produced with the non-genetically modified *Bacillus paralicheniformis* strain DP-Dzx96 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, five matches were found. The matching allergens were Asp fl 13, alkaline serine protease from *Aspergillus flavus*; Asp v 13, extracellular alkaline serine protease from *Aspergillus versicolor*; Asp o 13, alkaline serine protease from *Aspergillus oryzae*, all known as respiratory allergens. In addition, two matches were identified with the food allergens Cuc m 1, a subtilisin-like protease from *Cucumis melo* (muskmelon) and Pun g 14, a chitinase III from *Punica granatum* (pomegranate).

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

Several studies have shown that adults respiratorily sensitised 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).

Allergic reaction to *Cucumis melo* and *Punica granatum* has been reported (Hassan and Venkatesh, 2015; Neeharika and Sunkar, 2021).

[redacted], a product that may cause allergies (listed in the Regulation (EU) No 1169/2011[24]), is used as raw material. However, during the fermentation process, this product 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 from these sources 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, particularly in individuals sensitised to muskmelon or pomegranate, cannot be excluded, but would not exceed the risk of consuming these foods.

3.5. Dietary exposure

3.5.1. Intended use of the food enzyme

The food enzyme is intended to be used in five food manufacturing processes at the recommended use levels summarised in Table 2.

[24] 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 and recommended use levels of the food enzyme as provided by the applicant[(b)]

Food manufacturing process[(a)]	Raw material (RM)	Recommended use level (mg TOS/kg RM)
Production of protein hydrolysates from plants and fungi	Proteins from plant or fungi	1.5–960
Production of protein hydrolysates from meat and fish proteins	Proteins from meat or fish	1.5–960
Production of cooked rice	Rice	Not provided
Production of modified meat and fish products	Meat (only)	1.5–960
Yeast processing	Yeast cells, yeast extract, yeast cell walls	13–693

(a): The name has been harmonized by EFSA according to the 'EC working document describing the food processes in which food enzymes are intended to be used' – not yet published at the time of adoption of this opinion.
(b): Technical dossier/p. 70.

For the production of protein hydrolysates, the food enzyme is added to proteins isolated from plant, microbial or animal sources during the hydrolysis step.[25] The subtilisin is used to increase the yield and to enhance the flavour of the hydrolysates. The food enzyme–TOS remains in the final processed foods.

For the production of cooked rice, the food enzyme is added to rice before cooking.[26] The hydrolysis action can improve the quality of the food products. The food enzyme–TOS remains in the cooked rice.

For the production of modified meat, the food enzyme is added to the meat before cooking or roasting.[26] The partial hydrolysis of meat proteins by the subtilisin improves the tenderness and flavour of the processed meat products. The food enzyme–TOS remains in the final meat products.

In yeast processing, the food enzyme is added to yeast biomass or after the separation of yeast cell walls and yeast extract.[27] The subtilisin hydrolyses insoluble proteins, optimising the extraction process and improving the sensory properties of the final product.

Based on the data provided on thermostability (see Section 3.3.1), it is expected that the enzyme is inactivated during all food manufacturing processes listed in Table 2.

Concerning the intended uses and the use levels, the technical dossier provided most of the information, however, some data are missing, for example, the use level in rice cooking is not provided.

As the presence of bacitracin in the food enzyme represents a risk to human health (see Section 3.3.3), the Panel did not request additional data in order to complete this section of the opinion.

3.5.2. Dietary exposure estimation

The missing details to complete Table 2 precluded an estimate of the dietary exposure.

3.6. Margin of exposure

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

4. Conclusions

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 DP-Dzx96 cannot be considered safe.

[25] Technical dossier/p. 67.
[26] Technical dossier/p. 68.
[27] Technical dossier/p. 69.

5. Documentation as provided to EFSA

Subtilisin from *Bacillus paralicheniformis* strain DP-Dzx96. January 2015. Submitted by Genencor International B.V. The dossier was updated on 28 March 2023.

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Abbreviations

AMR	antimicrobial resistance
CAS	Chemical Abstracts Service
CEP	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 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
LoQ	limit of quantification
QPS	qualified presumption of safety
TOS	total organic solids
WGS	whole genome sequencing
WHO	World Health Organization