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SCIENTIFIC OPINION

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Safety evaluation of a food enzyme with phospholipase A_1 and lysophospholipase activities from the genetically modified *Aspergillus niger* strain PLN

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Abstract

The food enzyme with phospholipase A_1 (phosphatidycholine 1-acylhydrolase, EC 3.1.1.32) and lysophospholipase (2-lysophosphatidylcholine acylhydrolase, EC 3.1.1.5) activities is produced with the genetically modified *Aspergillus niger* strain PLN by DSM. The genetic modifications do not give rise to safety concerns. The food enzyme is free from viable cells of the production organism and its DNA. It is intended to be used for the production of refined edible fats and oils by degumming. Since residual amounts of total organic solids are removed during this process, dietary exposure was not calculated and toxicological studies were considered unnecessary for the assessment of this food enzyme. A search for the similarity of the amino acid sequence of the food enzyme to known allergens was made and no matches were found. The Panel considered that the 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.

KEYWORDS

Aspergillus niger, EC 3.1.1.32, EC 3.1.1.5, EFSA-Q-2023-00460, food enzyme, genetically modified microorganism, lysophospholipase, phospholipase A_1, phospholipase B

CONTENTS

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.

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 on food enzymes.

On 20 June 2023, a new application has been introduced by the applicant "DSM Food Specialties B.V." for the authorisation of the food enzyme Phospholipase Al and Lysophospholipase from a genetically modified strain of Aspergillus niger (strain PLN).

1.1.2 | Terms of Reference

The European Commission requests the European Food Safety Authority to carry out the safety assessment and the assessment of possible confidentiality requests of the following food enzyme: Phospholipase A_1 and Lysophospholipase from a genetically modified strain of *Aspergillus niger* (strain PLN), in accordance with Regulation (EC) No 1331/2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings.[3]

2 | DATA AND METHODOLOGIES

2.1 | Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme containing phospholipase A_1 and lysophospholipase from a genetically modified *Aspergillus niger* PLN.

[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]OJ L 354, 31.12.2008, p. 1.

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 guidance documents of the EFSA Scientific Committee.

The 'Scientific Guidance for the submission of dossiers on food enzymes' (EFSA CEP Panel, 2021) and the 'Food manufacturing processes and technical data used in the exposure assessment of food enzymes' (EFSA CEP Panel, 2023) have been followed for the evaluation of the application.

2.3 | Public consultation

According to Article 32c(2) of Regulation (EC) No 178/2002[4] and to the Decision of EFSA's Executive Director laying down the practical arrangements on pre-submission phase and public consultations, EFSA carried out a public consultation on the non-confidential version of the technical dossier from 26 February to 18 March 2024.[5] The comments received have been carefully evaluated and considered during the current assessment.

3 | ASSESSMENT

The food enzyme under application is a phospholipase that is encoded by a single gene and exhibits two declared enzymatic activities:

IUBMB nomenclature	Phospholipase A_1
Systematic name	Phosphatidylcholine 1-acylhydrolase
Synonyms	
IUBMB no	3.1.1.32
CAS no	9043-29-2
EINECS no	618-552-1

Phospholipases A_1 catalyse the hydrolysis of the fatty acyl ester bond at the sn-1 position of the glycerol moiety of phospholipids, resulting in the formation of 2-acyl-1-lysophospholipids and free fatty acids.

IUBMB nomenclature	Lysophospholipase
Systematic name	2-Lysophosphatidylcholine acylhydrolase
Synonyms	Phospholipase B
IUBMB no	3.1.1.5
CAS no	9001-85-8
EINECS no	618-333-0

Lysophospholipases catalyse the hydrolysis of ester bonds between a fatty acid and glycerol in lysophospholipids, resulting in the formation of free fatty acids and glycerophosphatide.

The food enzyme under assessment is intended to be used in the processing of fats and oils for the production of refined edible fats and oils by degumming.

3.1 | Source of the food enzyme

The enzyme is produced with the genetically modified filamentous fungus *Aspergillus niger* strain PLN (DS56314), which is deposited at the Westerdijk Fungal Biodiversity Institute culture collection (the Netherlands), with the deposit number CBS [redacted].[6] The production strain was identified as *A. niger* by whole genome sequence (WGS) analysis, showing an average nucleotide identity > 99% with the reference strain *A. niger* CBS 513.88.[7]

[4]Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 1.2.2002, p. 1–24.

[5]Accessible at [insert the link].

[6]Technical dossier/Risk assessment/Source of the food enzyme/Annex 5 Certificate of deposit.

[7]Technical dossier/Risk assessment/Source of the food enzyme/Annex 3 WGS analysis.

3.1.1 | Characteristics of the parental and recipient microorganisms

[redacted].

3.1.2 | Characteristics of introduced sequences

The sequence encoding the phospholipase [redacted] gene) is from *A. niger*. [redacted] gene was placed under the control of the [redacted] promoter and terminator sequences and was flanked at both sides by the downstream regions of the [redacted] gene.[9]

[redacted][10]

[redacted].[11,12]

3.1.3 | Description of the genetic modification process

The purpose of the genetic modification was to enable the production strain to overproduce phospholipase from *A. niger*.

[redacted].[14]

3.1.4 | Safety aspects of the genetic modification

The technical dossier contains all necessary information on the recipient microorganism, the donor organism and the genetic modification process.

The production strain *A. niger* strain PLN differs from the recipient strain in its ability to overproduce phospholipase.[15]

The absence of vector backbone sequences including the antimicrobial resistance gene *bla* used during the genetic modification was shown by WGS analysis.[16]

No issues of concern arising from the genetic modifications 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,[17] with food safety procedures based on Hazard Analysis and Critical Control Points, and in accordance with current Good Manufacturing Practice.[18]

[8]Technical dossier/Risk assessment/Source of the food enzyme.
[9]Technical dossier/Risk assessment/Source of the food enzyme/Annex 17.
[10]Technical dossier/Risk assessment/Source of the food enzyme/Annex 3/p. 6.
[11]Technical dossier/Risk assessment/Source of the food enzyme/p. 6 and Annex 17.
[12]Technical dossier/Risk assessment/Source of the food enzyme/pp. 7–8 and Annex 17.
[13]Technical dossier/Risk assessment/Source of the food enzyme.
[14]Technical dossier/Risk assessment/Source of the food enzyme/Annex 3.
[15]Technical dossier/Risk assessment/Source of the food enzyme/p. 9 and Annex 3/p. 7.
[16]Technical dossier/Risk assessment/Source of the food enzyme/Annex 3/p. 10.
[17]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.
[18]Technical dossier/Risk assessment/ Manufacturing process of the food enzyme/07. Manufacturing process/Annex 7.

The production strain is grown as a pure culture using a typical industrial medium in a submerged, fed-batch 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 stabilised and 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.[19] The applicant provided information on the identity of the substances used to control the fermentation and in the subsequent downstream processing of the food.[20]

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 phospholipase is a single polypeptide chain of 298 amino acids.[21] The molecular mass of the mature protein, calculated from the amino acid sequence, is around 32 kDa.[22] The food enzyme was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A consistent protein pattern was observed across all batches. The gels showed a major protein band corresponding to an apparent molecular mass of about 36 kDa, consistent with the expected molecular mass of the enzyme.[23] No other enzyme activities were reported.

The in-house determination of phospholipase A1 activity is based on the hydrolysis of L-*α*-phosphatidylcholine from egg yolk (reaction conditions: pH 4, 37°C), quantifying the release of free fatty acids using a colorimetric assay at 540 nm. The enzyme activity is expressed in phospholipase A_1 units/g (PLAU/g). One PLAU is defined as the amount of enzyme which liberates 1 μmol of free fatty acids per minute under the defined assay conditions.[24]

The in-house determination of lysophospholipase activity is based on the hydrolysis of 1-palmitoyl-2-hydroxy-sn-glycero-3 phosphocholine (reaction conditions: pH 4, 37°C), quantifying the release of free fatty acids by means of a colorimetric assay at 540 nm. It is expressed in lysophospholipase units (LPU). One LPU is defined as the amount of enzyme which liberates 1 μmol of free fatty acids per minute under the defined assay conditions.[25]

The food enzyme has a temperature optimum around 40–45°C (pH 4) and a pH optimum around pH 3.5–4 (37°C). Thermostability was tested after a pre-incubation of the food enzyme for up to 30 min at different temperatures (pH 4). The enzyme activity decreased above 60°C, showing no residual activity at 70°C after 5 min of pre-incubation.[26]

3.3.2 | Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches used for commercialization (Table 1).[27] The mean total organic solids (TOS) of the three batches was 11.0% and the mean enzyme activity/TOS ratio was 3771 PLAU/mg TOS and 2947 LPU/mgTOS.

TABLE 1 Composition of the food enzyme.

Parameters	Units	Batches		
		1	2	3
Phospholipase A_1 activity	PLAU/g[a]	447,700	287,500	506,500
Lysophospholipase activity	LPU/g[b]	349,800	224,600	395,700
Protein	%	12.28	6.03	11.21
Ash	%	0.62	0.31	0.57
Water	%	87.8	92.2	85.4

[19]Technical dossier/Risk assessment/Manufacturing process of the food enzyme/07. Manufacturing process/p.2 and Annex 8.
[20]Technical dossier/Risk assessment/Manufacturing process of the food enzyme/07. Manufacturing process/ Annex 9.
[21]Technical dossier/Risk assessment/Chemical composition, properties and purity of the food enzyme/08.Chemical composition/p. 3 and Annex 3.
[22]Technical dossier/Chemical composition, properties and purity of the food enzyme/08.Chemical composition/p. 3.
[23]Technical dossier/Risk assessment/Chemical composition, properties and purity of the food enzyme/08.Chemical composition/p. 3. and Annex 12.
[24]Technical dossier/Risk assessment/Methods of analysis/ Annex 11.
[25]Technical dossier/Risk assessment/Methods of analysis/ Annex 11.
[26]Technical dossier/Risk assessment/Chemical composition, properties and purity of the food enzyme/08.Chemical composition/Annex 14.
[27]Technical dossier/Risk assessment/Chemical composition, properties and purity of the food enzyme/08.Chemical composition/p. 1. and Annex 2.

TABLE 1 (Continued)

Parameters	Units	Batches		
		1	2	3
Total organic solids (TOS)[c]	%	11.58	7.49	14.03
Phospholipase A_1 activity/TOS ratio	PLAU/mg TOS	3866	3838	3610
Lysophospholipase activity/TOS ratio	LPU/mg TOS	3021	2999	2820

[a]PLAU: phospholipase A1 units (see Section 3.3.1).
[b]LPU: lysophospholipase units (see Section 3.3.1).
[c]TOS calculated as 100% – % water – % ash.

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).[28,29]

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).[30] No antimicrobial activity was detected in any of the tested batches.[31]

Strains of *Aspergillus*, in common with most filamentous fungi, have the capacity to produce a range of secondary metabolites (Frisvad et al., 2018). The presence of fumonisins and ochratoxin A was investigated in the three food enzyme batches, and the concentrations were below the limit of detection (LoD) of the applied analytical methods.[32,33]

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

3.3.4 | Viable cells and DNA of the production strain

The absence of viable cells of the production strain in the food enzyme was demonstrated in three independent batches analysed in triplicate. One millilitre of product was inoculated in 100 mL of medium and incubated at 30°C for 6 days for resuscitation. From this, 10 µL was plated on selective agar plates and incubated at 25°C for 5 days. No colonies were produced. A positive control was included.[34]

The absence of recombinant DNA in the food enzyme was demonstrated by polymerase chain reaction analysis of three batches in triplicate. [redacted].[35]

3.4 | Toxicological data

The food enzyme is intended to be used only in a food manufacturing process in which the food enzyme-TOS is removed from the end products by the refining steps (see Section 3.5). Consequently, toxicological studies other than the assessment of allergenicity were not considered necessary for the assessment of this food enzyme (EFSA CEP Panel, 2021).

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 exhibiting phospholipase activities produced with the *A. niger* strain PLM 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.[36]

[28]Limit of Detection/ Limit of Quantification (LoD/LoQ): Pb = 0.001/0.003 mg/kg.
[29]Technical dossier/Risk assessment/Chemical composition, properties and purity of the food enzyme/Annex 2 and Methods of analysis/Annex 1.
[30]Technical dossier/Risk assessment/Chemical composition, properties and purity of the food enzyme/Annex2.
[31]Technical dossier/Risk assessment/Chemical composition, properties and purity of the food enzyme/Annex 2.
[32]LoDs: fumonisins = 10 µg/kg each; ochratoxin A = 1 µg/kg.
[33]Technical dossier/Risk assessment/Chemical composition, properties and purity of the food enzyme/Annex 2 and Methods of analysis/Annex 1.
[34]Technical dossier/Risk assessment/Source of the food enzyme/Annex 6.
[35]Technical dossier/Risk assessment/Source of the food enzyme/Annex 4.
[36]Technical dossier/Risk assessment/Allergenicity/10.Allergenicity and Annex 13.

No information was available on oral and respiratory sensitisation or elicitation reactions of this phospholipase.

Phospholipases are implicated in allergic reactions due to insect bites.[37] No cross-reactivity between three different microbial phospholipases A_1 and the wasp allergen was found (Callesen et al., 2017). In addition, no sequence homology of the food enzyme with wasp phospholipase has been found and no allergic reactions upon ingestion of any phospholipase have been reported.

Aspergillus is a known source of respiratory allergens. However, several studies have shown that adults sensitised to respiratory allergens can ingest the allergens without acquiring clinical symptoms of food allergy (Armentia et al., 2009; Cullinan et al., 1997; Poulsen, 2004).

Yeast extract, a known source of allergens, is present in the media fed to the microorganisms. 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 fungal biomass and fermentation solids are removed. Taking into account the fermentation process and downstream processing, the Panel considered that no potentially allergenic residues from this source are present in the food enzyme.

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 processing of fats and oils for the production of refined edible fats and oils by degumming[38] at a recommended use level of 0.4–1.4 mg TOS/kg crude fat/oil.[39]

To produce refined edible fats and oils, the food enzyme is added to crude fat/oil during enzymatic degumming.[40] The enzymatic treatment helps to reduce the amount of gum phospholipids, resulting in higher oil yields and cleaner final products. The food enzyme–TOS is removed from the final foods by the repeated washing applied during the degumming process (EFSA CEP Panel, 2023).

3.5.2 | Dietary exposure estimation

The Panel accepted the evidence provided as sufficient to conclude that the residual amounts of food enzyme–TOS in the refined vegetable oils are negligible (EFSA CEP Panel, 2021). Consequently, the dietary exposure was not calculated.

3.6 | Margin of exposure

Since no toxicological or dietary exposure assessment was considered necessary by the Panel, a margin of exposure was not calculated.

4 | CONCLUSIONS

Based on the data provided and the removal of TOS during the intended food manufacturing process, the Panel concluded that the food enzyme containing phospholipase A_1 and lysophospholipase activities produced with the genetically modified *Aspergillus niger* strain PLN does not give rise to safety concerns under the intended conditions of use.

The CEP Panel considered the food enzyme free from viable cells of the production organism and recombinant DNA.

5 | DOCUMENTATION AS PROVIDED TO EFSA

Application for the authorisation of phospholipase A1/lysophospholipase from a genetically modified *Aspergillus niger* strain PLN. September 2023. Submitted by DSM Food Specialties B.V.

ABBREVIATIONS

bw body weight
LoD limit of detection

[37]Technical dossier/Risk assessment/Allergenicity/Annex 15.
[38]Technical dossier/Risk assessment/Intended use(s) in food and use level(s) /12. Intended use in food.
[39]Technical dossier/Risk assessment/Intended use(s) in food and use level(s) /17. Use levels.
[40]Technical dossier/Risk assessment/Intended use(s) in food and use level(s) /12. Intended use in food.

LoQ limit of quantification
LPU lysophospholipase units
PLAU phospholipase A_1 units
TOS total organic solids
WGS whole genome sequence

CONFLICT OF INTEREST

If you wish to access the declaration of interests of any expert contributing to an EFSA scientific assessment, please contact interestmanagement@efsa.europa.eu.

REQUESTOR

European Commission

QUESTION NUMBER

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