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SCIENTIFIC OPINION

Safety evaluation of the food enzyme lysophospholipase from the genetically modified *Trichoderma reesei* strain DP-Nyc81

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Abstract

The food enzyme lysophospholipase (2-lysophosphatidylcholine acylhydrolase, EC 3.1.1.5) is produced with the genetically modified *Trichoderma reesei* strain DP-Nyc81 by Genencor International B.V. 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 in the processing of cereals and other grains for the production of glucose syrups and other starch hydrolysates. Since residual amounts of food enzyme–total organic solids are removed during these food manufacturing processes, dietary exposure was not calculated and toxicological studies were considered unnecessary. A search for the identity of the amino acid sequence of the food enzyme to known allergens was made and no match was 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

EC 3.1.1.5, food enzyme, genetically modified microorganism, lecithinase B, lysophospholipase, *Trichoderma reesei*

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 European Union (EU) Community list may be placed on the market as such and used in foods, in accordance with the specifications and conditions of use provided for in Article 7(2) of Regulation (EC) No 1332/2008 on food enzymes.

On 17 May 2023, a new application has been introduced by applicant "Genencor International B.V." for the authorization of the food enzyme lysophospholipase from a genetically modified *Trichoderma reesei* (strain DP-Nyc81).

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: lysophospholipase from a genetically modified *Trichoderma reesei* (strain DP-Nyc81), in accordance with Regulation (EC) No 1331/2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings.

2 | DATA AND METHODOLOGIES

2.1 | Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme lysophospholipase from a genetically modified *Trichoderma reesei* strain DP-Nyc81.

Additional information was requested from the applicant during the assessment process on 22 January 2024 and received on 12 April 2024 and 18 April 2024 (see 'Documentation provided to EFSA').

Following the request for additional data sent by EFSA on 22 January 2024, the applicant requested a clarification teleconference on 09 February 2024, after which the applicant provided additional data on 12 April 2024 and 18 April 2024.

[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.

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[3] 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 11 June to 02 July 2024.[4] No comments were received.

3 | ASSESSMENT

IUBMB nomenclature	Lysophospholipase
Systematic name	2-Lysophosphatidylcholine acylhydrolase
Synonyms	Lecithinase B; lysolecithinase; phospholipase B
IUBMB no	EC 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 glycerophosphatides. The food enzyme under assessment is intended to be used in the processing of cereals and other grains for the production of glucose syrups and other starch hydrolysates.

3.1 | Source of the food enzyme

The lysophospholipase is produced with the genetically modified filamentous fungus *Trichoderma reesei* strain DP-Nyc81 [redacted] which is deposited at the Westerdijk Fungal Biodiversity Institute (the Netherlands) with the deposit number [redacted][5] The production strain was identified as *T. reesei* by phylogenomic analysis.[6]

3.1.1 | Characteristics of the parental and recipient microorganisms

The parental strain *T. reesei* [redacted][7]

[3]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.

[4]https://open.efsa.europa.eu/consultations/a0cTk0000034gzpIAA?search=EFSA-Q-2023-00449.

[5]Technical dossier/Additional data April 2024/Annex J.

[6]Technical dossier/Identity and specifications of the Food Enzyme/Annex C.

[7]Technical dossier/Risk assessment/Source of the food enzyme/Annexes H and I.

3.1.2 | Characteristics of introduced sequences

[redacted][8]

3.1.3 | Description of the genetic modification process

The purpose of the genetic modification was to enable the production strain to synthetise the lysophospholipase [redacted][9]

[redacted][10]

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 *T. reesei* strain DP-Nyc81 differs from the recipient strain [redacted][11]

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,[12] with food safety procedures based on Hazard Analysis and Critical Control Points, and in accordance with current good manufacturing practice.[13]

The production strain is grown as a pure culture using a typical industrial medium in a submerged, batch or 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. 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.[14]

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 lysophospholipase is a single polypeptide chain of [redacted] amino acids. The molecular mass of the mature protein, calculated from the amino acid sequence, is [redacted] kDa.[15] 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 protein

[8]Technical dossier/Risk assessment/Source of the food enzyme/p.12 and Annex H.
[9]Technical dossier/Risk assessment/Source of the food enzyme/Annex H.
[10]Technical dossier/Additional data April 2024/Annex X.
[11]Technical dossier/Additional data April 2024/Annex X.
[12]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.
[13]Technical dossier/Risk assessment/Manufacturing process of the food enzyme/Annex N.
[14]Technical dossier/Risk assessment/Manufacturing process of the food enzyme/Annex P.
[15]Technical dossier/Risk assessment/Chemical composition, properties and purity of the food enzyme/Annex T.

band migrating between the marker proteins of [redacted] and [redacted] kDa in all batches, consistent with the calculated mass of the food enzyme.[16] No other enzyme activities were reported.

The applicant's in-house determination of lysophospholipase activity is based on the hydrolysis of L-α-lysophosphatidycholine resulting in generation of free fatty acids and glycerophosphocholine (reaction conditions: pH 4.5, 55°C, 10 min). The free fatty acids, in the presence of acyl-CoA synthetase, are converted to acyl-CoA which is then oxidised in the presence of acyl-CoA oxidase, releasing hydrogen peroxide as a side product, which is used as a cosubstrate by peroxidase to allow the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline (MEHA) with 4-aminoantipyrine. The enzymatic activity is determined by measuring the amount of formed product spectrophotometrically at 550 nm.[17] The enzyme activity is expressed in lysophospholipase unit (PL)/g. One PL is defined as the amount of enzyme that releases 1 μmol of free fatty acid per minute from a 10-mM concentrated substrate at 55°C and pH 4.5.[18]

The food enzyme has a temperature optimum between 60°C and 70°C (pH 4.5) and a pH optimum between pH 3.5 and pH 4.5 (37°C). Thermostability was tested after a pre-incubation of the food enzyme for 15 min at different temperatures (pH 4.5). Enzyme activity decreased above 60°C showing no residual activity above 75°C.[19]

3.3.2 | Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches used for commercialisation (Table 1).[20] The mean total organic solids (TOS) of the three food enzyme batches was 24.2% and the mean enzyme activity/TOS ratio was 5937 PL/mg TOS.

TABLE 1 Composition of the food enzyme.

		Batches		
Parameters	**Unit**	**1**	**2**	**3**
Enzyme activity	PL/g[a]	952,340	1,720,990	1,702,467
Protein	%	13.01	18.23	18.57
Ash	%	0.21	0.17	0.28
Water	%	79.75	73.84	73.27
Total organic solids (TOS)[b]	%	20.04	25.99	26.45
Activity/TOS ratio	PL/mg TOS	4752	6622	6437

[a]PL: lysophospholipase unit (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 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).[21,22] The food enzyme complies with the microbiological criteria for total coliforms, *Escherichia coli* and *Salmonella*, and no antimicrobial activity was detected in any of the tested batches[23] as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006).

Strains of *Trichoderma*, in common with most filamentous fungi, have the capacity to produce a range of secondary metabolites (Frisvad et al., 2018). The presence of aflatoxins, fumonisins, ochratoxin A, sterigmatocystin, T-2 toxin and zearalenone was examined in all food enzyme batches and all were below the limit of detection (LoD) of the applied methods.[24,25]

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

[16]Technical dossier/Risk assessment/Chemical composition, properties and purity of the food enzyme/p. 2.
[17]Technical dossier/Risk assessment/Methods of analysis/Annex R.
[18]Technical dossier/Additional data April 2024.
[19]Technical dossier/Risk assessment/Chemical composition, properties and purity of the food enzyme/Annexes U1, U2 and U3.
[20]Technical dossier/Identity, characterisation and specifications of the enzyme/Annex E.
[21]LoD: Pb = 0.01 mg/kg.
[22]Technical dossier/Identity, characterisation and specifications of the enzyme/Annex E and additional data April 2024/Annex F.
[23]Technical dossier/Identity, characterisation and specifications of the enzyme/Annex E.
[24]LoD: aflatoxins B1, B2, G1 and G2 = 2 μg/kg each; fumonisin B1 + B2 = 200 μg/kg; ochratoxin A = 2 μg/kg; sterigmatocystin = 10 μg/kg; T-2 toxin = 10 μg/kg; zearalenone = 5 μg/kg.
[25]Technical dossier/Additional data April 2024/Annex F.

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 spread on agar plates at 30°C for 5 days. No colonies were produced. A positive control was included.[26]

The absence of recombinant DNA in the food enzyme was demonstrated by polymerase chain reaction (PCR) analysis of three batches in triplicate. No DNA was detected with primers that would amplify a 785-bp fragment [redacted] with a limit of detection of 10 ng spiked DNA/g food enzyme.[27]

3.4 | Toxicological data

The food enzyme is intended to be used only in a food manufacturing process in which the food enzyme–TOS are 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 lysophospholipase produced with the *Trichoderma reesei* strain DP-Nyc81 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.[28]

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

In addition, no allergic reactions upon dietary exposure to any lysophospholipase have been reported in the literature.

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 cereals and other grains for the production of glucose syrups and other starch hydrolysates at a recommended use level of 0.01–24.16 mg TOS/kg cereal.[29]

The food enzyme is added to starch during liquefaction and saccharification steps.[30] The food enzyme–TOS are removed from the glucose syrups by treatment with activated charcoal and with ion-exchange resins (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 glucose syrups and other starch hydrolysates are negligible (EFSA CEP Panel, 2021). Consequently, a dietary exposure was not calculated.

3.6 | Margin of exposure

Since toxicological assessment was considered unnecessary by the Panel, the margin of exposure was not calculated.

[26]Technical dossier/Additional data April 2024/Annex V.

[27]Technical dossier/Risk assessment/Source of the food enzyme/Annex M.

[28]Technical dossier/Risk assessment/Allergenicity/11.RA_Allergenicity_DP-Nyc81/Annex W.

[29]Technical dossier/Risk assessment/Intended uses in food and use levels/14.pdf.

[30]Technical dossier/Risk assessment/Intended uses in food and use levels/14.pdf.

4 | CONCLUSIONS

Based on the data provided and the removal of TOS during the food manufacturing process, the Panel concluded that the food enzyme lysophospholipase produced with the genetically modified *Trichoderma reesei* strain DP-Nyc81 does not give rise to safety concerns under the intended conditions of use.

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

5 | DOCUMENTATION AS PROVIDED TO EFSA

Lysophospholipase from a genetically modified strain of Trichoderma reesei (DP-Nyc81). June 2023. Submitted by Genencor International B.V.

Additional information. April 2024. Submitted by Genencor International B.V.

ABBREVIATIONS

bw	body weight
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 Organization of the United Nations
GMM	genetically modified microorganism
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kDa	kiloDalton
LOD	limit of detection
OECD	Organisation for Economic Cooperation and Development
PCR	polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TOS	total organic solids
WGS	whole genome sequencing
WHO	World Health Organization

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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

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