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Safety evaluation of the food enzyme phosphoinositide phospholipase C from the genetically modified *Bacillus licheniformis* strain NZYM-DI

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Abstract

The food enzyme phosphoinositide phospholipase C (1-phosphatidyl-1D-myo-inositol-4,5-bisphosphate inositoltrisphosphohydrolase, EC 3.1.4.11) is produced with the genetically modified *Bacillus licheniformis* strain NZYM-DI by Novozymes A/S. The genetic modifications did not give rise to safety concerns. The production strain has been shown to qualify for the qualified presumption of safety (QPS) status. The food enzyme was co nsidered free from viable cells of the production organism and its DNA. It is intended to be used for degumming of fats and oils. Since residual amounts of total organic solids are removed during washing and purification steps applied during degumming, dietary exposure was not estimated. As the production strain B. *licheniformis* NZYM-DI qualifies for the QPS approach to safety assessment and no issue of concern arose from the production process, no toxicological data were required. A search for similarity of the amino acid sequence of the food enzyme to known allergens was made and no match was found. The Panel considered that, under the intended conditions of use, the risk of allergic sensitisation and elicitation reactions by dietary exposure could not be excluded, but the likelihood for this to occur was considered 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: food enzyme, phosphoinositide phospholipase C, EC 3.1.4.11, triphosphoinositide phosphodiesterase, *Bacillus licheniformis*, genetically modified microorganism

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Table of 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 micro-organisms or products thereof including a product obtained by a fermentation process using micro-organisms: (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 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.

An application has been introduced by the applicant "Novozymes A/S" for the authorisation of the food enzyme Phosphoinositide phospholipase C from a genetically modified strain of *Bacillus licheniformis* (strain NZYM-DI).

Following the requirements of Article 12.1 of Commission Regulation (EC) No 234/2011[3] implementing Regulation (EC) No 1331/2008, the Commission has verified that the application falls within the scope of the food enzyme Regulation and contains all the elements required under Chapter II of that Regulation.

1.1.2. Terms of Reference

In accordance with Article 29(1)(a) of Regulation (EC) No 178/2002, the European Commission requests the European Food Safety Authority to carry out the safety assessment on the following food enzyme: Phosphoinositide phospholipase C from a genetically modified strain of *Bacillus licheniformis*

[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.

(strain NZYM-DI) in accordance with Regulation 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 phosphoinositide phospholipase C from a genetically modified *B. licheniformis* strain NZYM-DI.

Additional information was requested from the applicant during the assessment process on 27 October 2021 and was consequently provided (see 'Documentation provided to EFSA').

2.2. Methodologies

The assessment was conducted in line with the principles described in the EFSA 'Guidance on transparency in the scientific aspects of risk assessment' (EFSA, 2009b) as well as in the 'Statement on characterisation of microorganisms used for the production of food enzymes' (EFSA CEP Panel, 2019) and following the relevant existing guidance of EFSA Scientific Committees.

The current 'Guidance on the submission of a dossier on food enzymes for safety evaluation' (EFSA, 2009a) has been followed for the evaluation of the application with the exception of the exposure assessment, which was carried out in accordance with the updated 'Scientific Guidance for the submission of dossiers on food enzymes' (EFSA CEP Panel, 2021a).

3. Assessment

IUBMB nomenclature	Phosphoinositide phospholipase C
Systematic name	1-Phosphatidyl-1D-myo-inositol-4,5-bisphosphate inositoltrisphosphohydrolase
Synonyms	Triphosphoinositide phosphodiesterase, Phosphoinositidase C, 1-Phosphatidylinositol-4,5-bisphosphate phosphodiesterase, monophosphatidylinositol phosphodiesterase, phosphatidylinositol phospholipase C
IUBMB No	EC 3.1.4.11
CAS No	63551–76-8
EINECS No	849–293-4

Phosphoinositide phospholipases C catalyse the hydrolysis of phosphatidylinositol at the *sn-3* position in phospholipids, resulting in the formation of 1,2-diacylglycerol and inositol phosphate. The food enzyme under this assessment is intended to be used in the degumming of fats and oils.

3.1. Source of the food enzyme

The phosphoinositide phospholipase C is produced with the genetically modified *B. licheniformis* strain NZYM-DI, which is deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany) with deposit number [redacted][4] The production strain was identified as *B. licheniformis* [redacted][5]

The species *B. licheniformis* 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 and toxigenic activity are verified for the specific strain used (EFSA, 2007; EFSA BIOHAZ Panel, 2020). The absence of cytotoxic activity was confirmed using the lactate dehydrogenase release assay in VERO cells.[6] [redacted] did not identify known genes encoding AMR.[7] Therefore, the production strain was considered to qualify for the QPS status.

[4] Technical dossier/Annex 4 – Confidential - GMM dossier/Annex A2.
[5] Technical dossier/Annex 4 – Confidential - GMM dossier/Annex A1.
[6] Technical dossier/Annex 4 – Confidential - GMM dossier/Annex A4.
[7] Technical dossier/Additional information May 2022/Annexes 5.1–5.2.

3.1.1. Characteristics of the parental microorganism

The parental strain is [redacted]

3.1.2. Characteristics of introduced sequences

The sequence encoding the phosphoinositide phospholipase C [redacted][8]

3.1.3. Description of the genetic modification process

The purpose of the genetic modification was to enable the production strain to synthesise phosphoinositide phospholipase C [redacted][9][10]

3.1.4. Safety aspects of the genetic modification

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

The production strain *B. licheniformis* NZYM-DI differs from the parental strain in the capability to produce phosphoinositide phospholipase C [redacted]

[8] Technical dossier/ Annex 4 – Confidential - GMM dossier/GMM dossier Version 2.
[9] Technical dossier/ Annex 4 – Confidential - GMM dossier/Annexes C1-16 and Additional information/May 2022/Annex 6.
[10] Technical dossier/ Annex 4 – Confidential - GMM dossier/GMM dossier Version 2 p.6 and Annex A3.

[redacted] The absence of AMR genes was demonstrated [redacted]

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[11], with food safety procedures based on hazard analysis and critical control points, and in accordance with current good manufacturing practice.[12]

The production strain is grown as a pure culture using a typical industrial medium in a [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, leaving a supernatant containing the food enzyme. The filtrate containing the food enzyme is then further purified and concentrated, including an ultrafiltration step in which food enzyme protein is retained, while most of the low molecular mass material passes the filtration membrane and is discarded.[13] The applicant provided information on the identity of the substances used to control the fermentation and 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 phosphoinositide phospholipase C is a single polypeptide chain of [redacted] amino acids.[15] 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 (SDS–PAGE). A consistent protein pattern was observed across all batches. The gels showed a single major protein band migrating between the marker proteins of 20.1 and 30 kDa in all batches, consistent with the expected molecular mass of the enzyme. The protein profile also included bands of lesser staining intensity.[16] The food enzyme was tested for α-amylase, glucoamylase, lipase and protease activities, and none were detected. No other enzymatic activities were reported.[17]

The in-house determination of phosphoinositide phospholipase C activity is based on hydrolysis of the substrate *p*-nitrophenyl-phosphoinositide (reaction conditions: pH 7.8, 37°C, 200 s). The enzymatic activity is determined by measuring the release of *p*-nitrophenol spectrophotometrically at 405 nm. The phosphoinositide phospholipase C activity is quantified relative to an internal enzyme standard and expressed in phospholipase C Units/g (PLC(E)/g).[18]

The food enzyme has a temperature optimum around 50°C (pH 7) and a pH optimum around pH 7 (30°C).[19] Thermostability was tested after a pre-incubation of the food enzyme for 30 min at different temperatures (pH 7). Phosphoinositide phospholipase C activity was stable up to 50°C, but decreased sharply at higher temperatures. No residual activity was detected above 60°C.[20]

[11] 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.
[12] Technical dossier/p. 13.
[13] Technical dossier/p. 44–52.
[14] Technical dossier/p. 46 and Annex 6.
[15] Technical dossier/p. 31 and Annex 1.
[16] Technical dossier/p. 33.
[17] Technical dossier/p. 38 and Annexes 3.02–3.05.
[18] Technical dossier/p. 35,36 and Annex 3.01.
[19] Technical dossier/p. 37,38 and Annex 8.
[20] Technical dossier/Annex 8.

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches used for commercialisation (Table 1). The mean total organic solids (TOS) was 10.2% and the mean enzyme activity/TOS ratio was 58.4 PLC(E)/mg TOS.[21]

Table 1: Composition of the food enzyme[21]

Parameters	Unit	Batches		
		1	2	3
Phosphoinositidephospholipase C activity	PLC(E)/g batch[(a)]	6,990	4,490	6,160
Protein	%	8.9	6.8	7.5
Ash	%	0.9	1.4	0.7
Water	%	88.3	87.5	90.5
Total organic solids (TOS)[(b)]	%	10.8	11.1	8.8
Activity/mg TOS	PLC(E)/mg TOS	64.7	40.5	70.0

(a): PLC(E): phosphoinositide phospholipase C activity units (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 0.5 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] In addition, the levels of arsenic, cadmium and mercury were below the limits of detection (LOD) of the employed methods.[23,24]

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).[25] No antimicrobial activity was detected in any of the tested batches (FAO/WHO, 2006).[26]

The Panel considered that the information provided on the purity of the food enzyme is 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. [redacted] No colonies were produced. A positive control was included.[27]

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 [redacted][28]

3.4. Toxicological data

As the production strain qualifies for the QPS approach of safety assessment and no issue of concern arising from the production process of the food enzyme was identified (see Sections 3.1, 3.2 and 3.3), the Panel considered that no toxicological studies other than assessment of allergenicity were necessary.

[21] Technical dossier/p. 32 and Annex 9.
[22] Technical dossier/p. 12, 33 and Annex 9.
[23] LOD: Pb = 0.5 mg/kg; As = 0.3 mg/kg; Cd = 0.05 mg/kg; Hg = 0.05 mg/kg.
[24] Technical dossier/p. 33 and Annex 9.
[25] Technical dossier/p. 12, 35 and Annex 9.
[26] Technical dossier/ /p. 12, 33 and Annex 9.
[27] Annex 4 – Confidential - GMM dossier/Annex D1.
[28] Annex 4 – Confidential - GMM dossier/Annex D2.

3.4.1. Allergenicity

The allergenicity assessment considers only the food enzyme and not any carrier or other excipient, which may be used in the final formulation.

The potential allergenicity of the phosphoinositide phospholipase C produced with the genetically modified *B. licheniformis* strain NZYM-DI 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.

No information is available on oral and respiratory sensitisation or elicitation reactions of this phosphoinositide phospholipase C. Allergic reactions to phospholipases from insect bites have been reported, as well as food allergy to the phospholipase A1 from a red food colouring pigment (Ohgiya et al., 2009). However, the phosphoinositide phospholipase C does not possess sequence homologies with this phospholipase A1, while in addition, no allergic reactions upon dietary exposure to phosphoinositide phospholipase have been reported in the literature.[29]

[redacted] a product that may cause allergies or intolerances (listed in the Regulation (EU) No 1169/2011[30]) is used as raw material 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 microbial biomass and fermentation solids are removed. Taking into account the fermentation process and downstream processing, the Panel considered that potentially allergenic residues of these materials employed as protein sources are not expected to be present in the food enzyme.

The Panel considered that, under the intended conditions of use, the risk of allergic sensitisation and elicitation reactions upon dietary exposure to this food enzyme cannot be excluded, but the likelihood of such reactions to occur was considered low.

3.5. Dietary exposure

3.5.1. Intended use of the food enzyme

This phosphoinositide phospholipase C is intended to be used in degumming of fats and oils at an intended use level of 50–75 PLC(E)/kg oil, equivalent to 0.86–1.28 mg TOS/kg oil.[31]

When added to crude oil, phosphoinositide phospholipase C hydrolyses the ester linkage of phospholipids that are naturally present in crude oil to form 1,2-diacylglycerol and inositol phosphate. This conversion helps to reduce the amount of gum phospholipids. The resulting products together with the phospholipase migrate into the aqueous phase and are subsequently removed as water-based sludge by repeated washing (EFSA CEP Panel, 2021b). This treatment results in higher oil yields, cleaner final products, and better stability and processability of the oils.[32]

3.5.2. Dietary exposure estimation

In accordance with the guidance document (EFSA CEP Panel, 2021a), a dietary exposure was not calculated.

3.6. Margin of exposure

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

[29] Technical dossier/Additional information May 2022/Annex 7.1.

[30] 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.

[31] Technical dossier/p. 54.

[32] Technical dossier/pp. 66–69.

4. Conclusions

Based on the data provided, the outcome of the QPS assessment of the production strain and removal of TOS during the intended food production process, the Panel concluded that the food enzyme phosphoinositide phospholipase C produced with the genetically modified *Bacillus licheniformis* NZYM-DI does not give rise to safety concerns under the intended conditions of use.

Based on the data provided, the CEP Panel considered the food enzyme free from viable cells of the production organism and recombinant DNA.

5. Documentation as provided to EFSA

1) Dossier "Phosphoinositide phospholipase C produced by a genetically modified strain of Bacillus licheniformis (strain NZYM DI)". April 2021. Submitted by Novozymes A/S.
2) Additional information. May 2022. Submitted by Novozymes A/S.

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Abbreviations

ANI	average nucleotide identity
AMR	antimicrobial resistance
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
GMO	genetically modified organism
IUBMB	International Union of Biochemistry and Molecular Biology
kDa	kiloDalton
LOD	limit of detection
PCR	polymerase chain reaction
PLC(E)	phosphoinositide phospholipase C activity units
QPS	qualified presumption of safety
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TOS	total organic solids
WGS	whole genome sequencing
WHO	World Health Organization