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SCIENTIFIC OPINION

Safety evaluation of the food enzyme phospholipase A_2 from the genetically modified *Aspergillus niger* strain PLA

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Abstract

The food enzyme phospholipase A_2 (phosphatidylcholine 2-acylhydrolase, EC 3.1.1.4) is produced with the genetically modified *Aspergillus niger* strain PLA by DSM Food Specialties 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 egg and egg products, in the processing of fats and oils by degumming and for the production of modified lecithins (lysolecithin). As residual total organic solids (TOS) are removed in the refined fats and oils during degumming, dietary exposure was calculated only for the remaining two food manufacturing processes. For egg processing, the dietary exposure was estimated to be up to 1.712 mg TOS/kg body weight (bw) per day in European populations. Wet gum can be used to produce lysolecithin with the highest dietary exposure of 1.61 mg TOS/kg bw per day in children at the 95th percentile when used as a food additive. Genotoxicity tests did not raise a safety concern. The systemic toxicity was assessed by a repeated dose 90-day oral toxicity study in rats. The Panel identified a no observed adverse effect level of 1350 mg TOS/kg bw per day, the highest dose tested, which, when compared with the estimated overall dietary exposure, resulted in a margin of exposure of at least 851. A search for the similarity of the amino acid sequence of the food enzyme to those of known allergens was made and no match was found. The Panel considered that the risk of allergic reactions by 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

food enzyme, phospholipase A_2, phosphatidylcholine 2-acylhydrolase, EC 3.1.1.4, phosphatidolipase, *Aspergillus niger*, genetically modified microorganism

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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 European Union 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 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.

Five applications have been introduced by the Association of Manufacturers and Formulators of Enzyme Products (AMFEP), and by the companies "DSM Food Specialties B.V" and "Novozymes A/S" for the authorisation of the food enzymes Pectinase, Poly-galacturonase, Pectin esterase, Pectin lyase and Arabanase from *Aspergillus niger*, Phospholipase A_2 from a genetically modified strain of Aspergillus niger (strain PLA), Pectinesterase from a genetically modified strain of *Aspergillus niger* (strain PME), Endo-1,4-13-xylanase from a genetically modified strain of *Aspergillus niger* (strain XEA) and Maltogenic amylase produced by a genetically modified strain of *Bacillus subtilis* (strain NZYM-SO) respectively.

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

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 Pectinase, Poly-galacturonase, Pectin esterase, Pectin lyase and Arabanase from *Aspergillus niger*, Phospholipase A_2 from a genetically modified strain of *Aspergillus niger* (strain PLA), Pectinesterase from a genetically modified strain of *Aspergillus niger* (strain PME), Endo-1,4-B-xylanase from a genetically modified strain of *Aspergillus niger* (strain XEA) and Maltogenic amylase produced by a genetically modified strain of *Bacillus subtilis* (strain NZYM-SO) in accordance with Article 17.3 of Regulation (EC) No 1332/2008 on food enzymes.

[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.

1.2 | 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 Phospholipase A_2 from a genetically modified strain of *A. niger* (strain PLA).

2 | DATA AND METHODOLOGIES

2.1 | Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme Phospholipase A_2 from a genetically modified strain of *A. niger* (strain PLA).

Additional information was requested from the applicant during the assessment process on 18 May 2020, 8 June 2021 and 5 June 2023 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) and following the relevant guidance documents of EFSA Scientific Committee.

The current 'Guidance on the submission of a dossier on food enzymes for safety evaluation' (EFSA, 2009a) as well as the 'Statement on characterisation of microorganisms used for the production of food enzymes' (EFSA CEP Panel, 2019) have 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, 2021).

3 | ASSESSMENT

IUBMB nomenclature	phospholipase A_2
Systematic name	phosphatidylcholine 2-acylhydrolase
Synonyms	lecithinase A, phosphatidase, phosphatidolipase,
IUBMB No	EC 3.1.1.4
CAS No	9001-84-7
EINECS No	232-637-7

Phospholipases A_2 catalyse the hydrolysis of the fatty acyl ester bond at the sn-2 position of the glycerol moiety, resulting in formation of 1-acyl-2-lysophospholipids and free fatty acids. The enzyme under assessment is intended to be used in the processing of egg and egg products, in the processing of fats and oils for the production of refined edible fats and oils by degumming and for the production of modified lecithin (lysolecithin).

3.1 | Source of the food enzyme

The phospholipase A_2 is produced with a genetically modified filamentous fungus *A. niger* strain PLA (██████), which is deposited at the Westerdijk Fungal Biodiversity Institute (The Netherlands) with the deposit number ████████.[4] The production strain was identified as *A. niger* by whole genome sequence (WGS) analysis██████████████████████████.[5]

3.1.1 | Characteristics of the parental and recipient microorganisms

The parental strain ██.

[4]Technical Dossier/Additional data April 2021/Annex II-1A.

[5]Technical Dossier/1st submission/Annex II-2, Additional data April 2021/Annex II-2A.

The recipient strain ▇▇.[6]

3.1.2 | Characteristics of the introduced sequences

The sequence encoding the phospholipase A_2 ▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇

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▇▇▇▇▇▇[7] ▇▇▇▇[8] ▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇.

3.1.3 | Description of the genetic modification process

The purpose of the genetic modification was to enable the production strain to synthesise phospholipase A_2.

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▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇▇[10] ▇▇▇▇▇▇▇▇▇▇▇▇▇▇[11,12]

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.

▇▇.[12]

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[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, 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 then further purified and

[6]Technical Dossier/1st submission/Annex II-3.

[7]Technical Dossier/1st submission/Annex II-5 and II-7.

[8]Technical Dossier/1st submission/Annex II-6 and II-8.

[9]Technical Dossier/1st submission/Annex II-9.

[10]Technical Dossier/1st submission/Annex II-10.

[11]Technical Dossier/1st submission/Annex II-11.

[12]Technical Dossier/Additional data April 2021/Annex II-2A.

[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/1st submission/Annex I-5.

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. Depending on the intended use of the food enzyme, a further purification by cation-exchange chromatography may be applied to remove the glucoamylase activity.[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

The phospholipase A_2 is a single polypeptide chain of 124 amino acids.[17] The molecular mass of the mature protein, derived from the amino acid sequence, was calculated to be 14 kDa. The food enzyme was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis.[18] A consistent protein pattern was observed across all batches. The gel showed a protein band migrating close to the marker protein of 14 kDa in all batches, consistent with the calculated mass of the enzyme. The protein profile also included, among bands of lower staining intensity, a major band of about 60 kDa, which corresponds to the product of ████████. Consistent with this, the food enzyme contains glucoamylase activity.[19] No other enzymatic activities were reported.

The in-house determination of phospholipase A_2 activity is based on the hydrolysis of 1,2-dithiodioctanoyl phosphatidylcholine (reaction conditions: pH 8.3, 37°C, 3 min). The enzymatic activity is determined by measuring the release of 1,2-dimercapto-1-octanoyl-phosphatidylcholine by a colourimetric reaction. The phospholipase A_2 activity is quantified relative to an internal enzyme standard and expressed in chromogenic phospholipase a_2 units (CPU).[20]

The food enzyme has a temperature optimum around 45°C (pH 8.3) and a pH optimum around pH 8.5 (37°C). Thermostability was tested after a pre-incubation of the food enzyme at different temperatures for different periods of time. The phospholipase A_2 activity decreased above 65°C, showing no residual activity after 15 min at 90°C.[18]

3.3.2 | Chemical parameters

Data on the chemical parameters of the food enzyme (before chromatographic purification) were provided for three batches intended for commercialisation (batches 1–3), one of them also used for toxicological analysis,[21] and further two batches used for toxicological studies only (batches 4–5)[22] (Table 1). The mean total organic solids (TOS) of the three food enzyme batches intended for commercialisation was 15.2% and the mean enzyme activity/TOS ratio was 20.4 CPU/mg TOS.

TABLE 1 Compositional data of the food enzyme.

		Batch				
Parameters	**Unit**	**1**	**2**	**3**[a]	**4**[b]	**5**[c]
Phospholipase A_2 activity	CPU/g[d]	4360	2900	2230	1882	5375
Protein	%	12.5	11.4	10.2	NA	16.5
Ash	%	0.4	0.3	1.2	1.1	1.2
Water	%	82.3	84.8	85.3	81.9	78.1
Total organic solids (TOS)[e]	%	17.3	14.9	13.5	17.0	20.7
Activity/TOS ratio	CPU/mg TOS	25.2	19.5	16.5	11.1	26.0

[a]Batch used for the bacterial reverse mutation test (Ames test), the *in vitro* mammalian chromosomal aberration test, the first *in vivo* mammalian erythrocyte micronucleus test and the repeated dose 90-day oral toxicity study.

[b]Batch used for the second *in vivo* mammalian erythrocyte micronucleus test.

[c]Batch used for the *in vitro* mammalian cell micronucleus test.

[d]CPU: Chromogenic Phospholipase A_2 Units (see Section 3.3.1).

[e]TOS calculated as 100% – % water – % ash.

[15]Technical Dossier/1st submission/Annex I-6 and Additional data April 2022, part I.

[16]Technical Dossier/1st submission/Annex I-7.

[17]Technical Dossier/page 49.

[18]Technical Dossier/Additional data April 2021.

[19]Technical Dossier/2nd submission.

[20]Technical Dossier/1st submission/Annex I-2.

[21]Technical Dossier/Additional data April 2021/Annex I-1A.

[22]Technical Dossier/ Additional data April 2021/Annex I-6A and Additional data April 2022/Annex III.

3.3.3 | Purity

The lead content in three commercial batches was below 1 mg/kg[23,24] which complies with the specification for lead as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006).

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). No antimicrobial activity was detected in any of the tested batches.[23]

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 B_1, B_2 and B_3 and ochratoxin A was examined in the three food enzyme batches, and all were below the limits of detection (LoD) of the applied analytical methods.[25,26] Adverse effects caused by the possible presence of other secondary metabolites were addressed by the toxicological examination of the food enzyme–TOS.

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 .[27]

The absence of recombinant DNA in the food enzyme was demonstrated .[28]

3.4 | Toxicological data

A battery of toxicological tests was provided, consisting of a bacterial reverse mutation test (Ames test), an *in vitro* mammalian chromosomal aberration test, an *in vitro* mammalian cell micronucleus test, two *in vivo* mammalian erythrocyte micronucleus tests and a repeated dose 90-day oral toxicity study in rats.

The batches 4, 5 and 6 (Table 1) used in these studies have lower or similar activity/TOS value as the batches intended for commercialisation and were considered suitable as test items.

3.4.1 | Genotoxicity

3.4.1.1 | *In vitro genotoxicity studies*

3.4.1.1.1 | *Bacterial reverse mutation test*

A bacterial reverse mutation assay (Ames test) was performed according to the Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 (OECD, 1997a) and following Good Laboratory Practice (GLP).[29]

Four strains of *S.* Typhimurium (TA98, TA100, TA1535, TA1537) and *E. coli* WP2uvrA were used with or without metabolic activation (S9-mix), applying the standard plate incorporation method. Two separate experiments were carried out in triplicate, using five concentrations of the food enzyme from 100 to 5000 μg dry matter/plate of the enzyme preparation, corresponding to 91.8, 305, 918, 3058, 4592 μg TOS/plate.

No cytotoxicity was observed at any concentration of the test substance. Upon treatment with the food enzyme, there was no biologically relevant increase in the number of revertant colonies above the control values in any strain tested, with or without S9-mix.

The Panel concluded that the food enzyme phospholipase A_2 did not induce gene mutations under the test conditions applied in this study.

[23]Technical Dossier/1st submission/Annex I-3.

[24]LOD: Pb = 0.06 mg/L.

[25]LoQ: fumonisins B_1, B_2 and B_3 = 10 μg/kg each; ochratoxin A = 0.1 μg/kg.

[26]Technical Dossier/1st submission/Annex I-3.

[27]Technical dossier/Add data April 21/Annexes part II/AnnexII-3A.

[28]Technical Dossier/Additional data June 2023/Annex 1.

[29]Technical dossier/Additional data April 2021/Annex I-3A.

3.4.1.1.2 | *In vitro mammalian chromosomal aberration test*

The *in vitro* mammalian chromosomal aberration test was carried out according to the OECD Test Guideline 473 (OECD, 1997b) and following GLP.[30]

Three experiments were performed with duplicate cultures of human peripheral whole blood lymphocytes. The cell cultures were treated with the food enzyme, either with or without metabolic activation (S9-mix). Based on the results of a dose range-finding test, an appropriate range of concentrations was selected to reach a 50% inhibition of the mitotic index. In the first experiment, the cells were exposed to the food enzyme and scored (100 metaphases per culture) for chromosomal aberrations at concentrations of 1000, 3330 and 5000 μg dry matter/mL (corresponding to 918, 3058, 4592 μg TOS/mL) in a short-term treatment (3 h exposure and 21 h recovery period) either with or without S9-mix. In the second experiment, the cells were exposed to the food enzyme and scored for chromosomal aberrations at concentrations of 1000, 1800, 3330 μg dry matter/mL (corresponding to 918, 1653, 3058 μg TOS/mL) in 24 h continuous treatment and of 1800, 4200, 5000 μg dry matter/mL (corresponding to 1653, 3857, 4592 μg TOS/mL) in a 48 h continuous treatment without S9 mix. In addition, duplicate cultures were exposed to 1000, 3330, 5000 μg dry matter/mL (corresponding to 918, 3058, 4,592 μg TOS/mL) in presence of S9 mix in a short-term treatment (3 h exposure and 45 h recovery period). In the third experiment, the cells were exposed to the enzyme preparation inactivated with EDTA and scored for chromosomal aberrations at concentrations of 560, 1000 and 1300 μg dry matter/mL (corresponding to 514, 918, 1194 μg TOS/mL) in a 48 h continuous treatment without S9-mix.

The frequency of structural and numerical aberrations was not statistically significantly different to the negative controls in the short-term treatment and in 24 h continuous treatment in the first and second experiments. The frequency of structural chromosome aberrations was statistically significantly different from the negative controls (12% and 18% vs. 1% in controls) at concentrations of 4200 and 5000 μg dry matter/mL (corresponding to 3857 and 4592 μg TOS/mL), associated with a cytotoxicity of 45% and 55%, respectively, only in the 48 h continuous treatment. The large majority of structural chromosomal aberrations recorded were chromatidic breaks. A statistically significant increase of cells with chromosomal aberration (>50% percent cells with chromatidic breaks) was also observed at all the concentrations of inactivated enzyme tested in the third experiment, associated with high level of toxicity (71% reduction of the mitotic index at 1300 μg TOS/mL).

The Panel concluded that the food enzyme phospholipase A_2 did induce an increase in the frequency of structural chromosomal aberrations in mammalian cells under the test conditions applied in this study.

3.4.1.1.3 | *In vitro mammalian cell micronucleus test*

The *in vitro* mammalian cell micronucleus test was carried out according to the OECD Test Guideline 487 (OECD, 2016) and following GLP.[31]

Four separate experiments were performed with duplicate cultures of human peripheral whole blood lymphocytes. The cell cultures were treated with the food enzyme with or without metabolic activation (S9-mix). In the first experiment, cells were exposed to the food enzyme and scored for the frequency of bi-nucleated cells with micronuclei (MNBN) at concentrations of 1633, 2857 and 5000 μg TOS/mL in a short-term treatment (3 h exposure and 25 h recovery period) without S9-mix. In the second experiment, cells were exposed to the food enzyme and scored for the frequency of MNBN at concentrations of 1633, 2857 and 5000 μg TOS/mL in a long-term treatment (48 h exposure without recovery period) without S9-mix. In the third experiment, cells were exposed to the food enzyme and scored for the frequency of MNBN at concentrations of 1633, 2857 and 5000 μg TOS/mL in a long-term treatment (28 h exposure without recovery period) without S9-mix. In the fourth experiment, cells were exposed to the food enzyme and scored for MNBN at concentrations of 21.8, 32.7 and 49 μg TOS/mL in a short-term treatment (3 h exposure and 25 h recovery period) with S9-mix.

No cytotoxicity, evaluated as reduction of proliferation index, was seen either in the short-term with and/or without S9-mix or in the long-term treatment. In the fourth experiment, precipitation was observed at 49 μg TOS/mL and above in the presence of S9 mix at end of the treatment. The frequency of MNBN was not statistically significant different to the negative controls at all concentrations tested.

The Panel concluded that the food enzyme phospholipase A_2 did not induce an increase in the frequency of MNBNs under the test conditions applied in this study.

3.4.1.2 | *In vivo genotoxicity studies*

3.4.1.2.1 | *In vivo mammalian erythrocyte micronucleus test*

First study

The *in vivo* mammalian erythrocyte micronucleus test in NMRI BR mice was carried out according to the OECD Test Guideline 474 (OECD, 1997c) and following GLP.[32]

A preliminary toxicity assay was performed on two male and two female NMRI BR mice that were dosed by oral gavage with 2,000 mg/kg body weight (bw) of food enzyme. No toxic effects were detected. In the main experiment, five male and

[30] Technical dossier/Additional data April 2021/Annex I-4A.

[31] Technical dossier/Additional data April 2022/Annex I.

[32] Technical dossier/Additional data April 2021/Annex I-5A.

five female mice per group were treated with a single oral administration by gavage of the food enzyme at doses of 500, 1000 and 2000 mg/kg bw (corresponding to 1836, 918 and 459 mg TOS/kg bw). Bone marrow was sampled 24 and 48 h after dosing.

No statistically significant increases in the frequency of micronucleated polychromatic erythrocytes (MNPCE) and no decrease in the ratio polychromatic/normochromatic erythrocytes were observed in animals treated with the food enzyme compared with the historical controls (data not shown).

The study presents important deviations from the OECD Test Guideline 474, in particular the lack of concurrent controls and the absence of data on bone marrow exposure. In addition, the maximum tolerated dose (MTD) was not reached with the highest administered dose.

Second study

The *in vivo* mammalian erythrocyte micronucleus test in NMRI BR mice was carried out according to the OECD Test Guideline 474 (OECD, 1997c) and following GLP.[33]

Groups of five male mice were treated with a single oral administration by gavage of the food enzyme at doses of 500, 1000 and 2000 mg TOS/kg bw. Bone marrow was sampled 24 and 48 h (the highest dose only) after dosing.

No treatment-related clinical signs or mortality were noted in any animal treated with the food enzyme. No statistically significant increases in the frequency of MNPCE and no decrease in the ratio polychromatic/normochromatic erythrocytes were observed in animals treated with the food enzyme compared with the concurrent controls. No data on bone marrow exposure were provided and the MTD was not reached with the highest administered dose. The study was considered inconclusive.

3.4.1.3 | *Conclusions on genotoxicity*

The food enzyme phospholipase A_2 was tested in a basic battery of *in vitro* genotoxicity studies. The test item in the presence or absence of S9 mix did not induce gene mutations in bacteria (four strains of *S.* Typhimurium, TA1535, TA1537, TA98 and TA100 and one strain of *E. coli*, WP2 *uvrA*) and did not induce chromosomal damage, evaluated as micronuclei frequency in human peripheral blood lymphocytes. The increase of structural chromosomal aberrations reported in an *in vitro* chromosomal aberration test, associated with cytotoxicity levels in the higher end of the acceptable cytotoxicity range (OECD, 1997b), was not reproduced in the *in vitro* micronucleus test carried out under the same experimental conditions.

The Panel concluded that the food enzyme phospholipase A_2 did not raise concern for genotoxicity.

3.4.2 | Repeated dose 90-day oral toxicity study in rodents

The repeated dose 90-day oral toxicity study was performed in accordance with the OECD Test Guideline 408 (OECD, 1998) and following GLP.[34] Groups of 10 male and 10 female Wistar (Crl: (WI)BR) rats received by gavage the food enzyme in doses of 500, 2000 or 10,000 mg/kg bw per day, corresponding to 67.5; 270 and 1350 mg TOS/kg bw per day. Controls received the vehicle (water).

One mid-dose male was sacrificed on day 22 due to the presence of abnormal, hunched posture, piloerection and emaciation, and one high-dose female died accidentally after blood sampling. The Panel considered these deaths as accidental, as there were no remarkable findings at necropsy.

The bw was statistically significantly increased in low- and high-dose males (+4% and +5%, respectively) on day one. The bw gain was statistically significantly decreased on days 29, 36, 57, 64, 78, 85 and 92 of administration in low-dose females (−16% on average) and on days 36, 50, 57, 64, 71, 77, 85 and 92 of administration in high-dose females (−17% on average). The Panel considered the change as not toxicologically relevant, as they were only recorded sporadically, they were only observed in one sex and the changes were without a statistically significant effect on the final bw.

The haematological investigations revealed a statistically significant increase in the white blood cell (WBC) count in mid- and high-dose males (+23, +32%), a decrease in the prothrombin time (PT) in mid-dose males (−3%), a decrease in the WBC count in mid-dose females (−22%), a decrease in haemoglobin (Hb) concentration in mid-dose females (−4%), a decrease in haematocrit (HCT) in mid-dose females (−6%), a decrease in mean corpuscular volume (MCV) in mid-dose females (−4%), and an increase in partial thromboplastin time (PTT) in mid- and high-dose females (+9, +10%). The Panel considered the changes as not toxicologically relevant, as they were only observed in one sex (PT, HB, HCT, MCV), there was no consistency between the change in males and females (WBC count), changes were small (PT, HB, HCT, MCV), there was no dose–response relationship (PT, WBC in females, Hb, HCT, MCV) and there were no changes in other relevant parameters (absence of any corroborative changes in a differential WBC count or histopathological correlates).

The clinical chemistry investigations revealed statistically significant increases in bilirubin concentration in high-dose males (+32%) and in high-dose females (+21%), glucose concentration in low-dose males (+16%), cholesterol (+23%) and triglycerides (+47%) in mid-dose males, phosphorous in mid- and high-dose males (+7%, +9%), potassium in high-dose males (+10%) and in high-dose females (+8%), calcium in low- and mid-dose males (+5%, +4%), and a decrease in calcium in high-dose females (−3%). The Panel considered changes as not toxicologically relevant, as there was no dose–response

[33]Technical dossier/Additional data April 2021/Annex I-6A.

[34]Technical dossier/Additional data April 2021/Annex I-7A.

relationship (glucose, cholesterol, triglycerides, calcium), they were only observed in one sex (glucose, cholesterol, triglycerides, phosphorous), the changes were small (phosphorus, calcium) and there were no changes in other relevant parameters (absence of corroborative changes in alkaline phosphatase activity and histopathological changes in the liver or kidneys).

Statistically significant changes in organ weights detected were an increase in relative brain weight in low- and high-dose females (+10%, +11%) and increase in relative kidney weight in high-dose females (+12%). The Panel considered the changes as not toxicologically relevant, as they were only observed in one sex (both parameters), there was no apparent dose–response relationship (relative brain weight) and there were no histopathological changes in the brain and kidneys.

No other statistically significant or biologically relevant differences to controls were reported.

The Panel identified a no observed adverse effect level (NOAEL) of 1350 mg TOS/kg bw per day, the highest dose tested.

3.4.3 | Allergenicity

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

The potential allergenicity of the phospholipase A_2 and of the truncated glucoamylase produced with the genetically modified *A. niger* strain PLA was assessed by comparing their amino acid sequences 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, 2013). Using higher than 35% identity in a sliding window of 80 amino acids as the criterion, no match was found.[35]

No information was available on oral and respiratory sensitisation or elicitation reactions of this phospholipase A_2. The applicant provided a comprehensive literature search for possible allergic reactions to phospholipases. Different publications were found identifying adverse effects caused by phospholipase A_2 from bee, wasp and snake venoms. However, no evidence was found of allergic reactions upon oral intake of phospholipase A_2.[36]

Glucoamylase from *S. commune* (Toyotome et al., 2014) is known as an occupational respiratory allergen associated with baker's asthma. However, several studies have shown that adults with occupational asthma are usually able to ingest respiratory allergens without acquiring clinical symptoms of food allergy (Armentia et al., 2009; Brisman, 2002; Poulsen, 2004). In addition, no allergic reactions upon dietary exposure to any glucoamylase have been reported in the literature.

█████████████, a known source of allergens, is used as a raw material in the media fed to the microorganisms. However, during the fermentation process, █████████████ 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 potentially allergenic residues of █████████████ are not expected to be present in the food enzyme.

The Panel considered that 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 three food manufacturing processes at the recommended use levels summarised in Table 2.

TABLE 2 Intended uses and recommended use levels of the food enzyme as provided by the applicant.[c]

Food manufacturing process[a]	Raw material (RM)	Recommended use level (mg TOS/kg RM)[b]
Processing of egg and egg products	Egg yolk	Up to 1470 (dry weight)[d]
	Liquid egg	Up to **735**[e]
Processing of fats and oils		
• Production of refined edible fats and oils by degumming	Crude oil	2.4–12.5
• Production of modified lecithin (lysolecithin)	Wet gum	147–1471[e]
	Dry lecithin	165–**2795**[f]

[35]Technical dossier/1st submission/Annex I–18.

[36]Technical dossier/Additional data April 2021.

Abbreviation: TOS, total organic solids.

[a]The name has been harmonised by EFSA according to the 'Food manufacturing processes and technical data used in the exposure assessment of food enzymes' (EFSA CEP Panel et al., 2023).

[b]Numbers in bold were used for calculations.

[c]Technical dossier/1st submission/p. 106, Additional data April 2021/Answers 12, 13, Additional data April 2022/Answer 4, Additional data June 2023/Answer 1.

[d]Technical dossier/Additional data April 2021.

[e]Technical dossier/Additional data April 2022.

[f]Technical dossier/Additional data June 2022.

In egg processing, the phospholipase A_2 is added to egg yolk or liquid egg to hydrolyse the naturally present phospholipids. The reaction products are then spray dried or pasteurised.[37] The food enzyme–TOS remains in the processed egg products. Based on the data provided on thermostability (see Section 3.3.1), it is expected that the phospholipase A_2 is heat-inactivated during egg processing.

In the degumming process, the food enzyme is added to crude vegetable oil before the centrifugation step.[38] Phospholipase A_2 hydrolyses phospholipids naturally present in crude oil to form 1-acyl-2-lysophospholipids and free fatty acids. The resulting phosphatides together with the phospholipase migrate into the aqueous phase and are subsequently removed as aqueous sludge. This process results in higher oil yields, cleaner final products, better stability and processability of the oils. The food enzyme–TOS is removed by repeated washing applied after degumming (EFSA CEP Panel, 2023). The aqueous sludge, known as wet gum, can be dried to obtain enzymatically hydrolysed lecithin (lysolecithin).[39] In this case, the food enzyme–TOS ends in the dried lysolecithin.

To obtain enzymatically hydrolysed lecithin (lysolecithin), alternatively, the wet gum is treated with the phospholipase A_2[39] at the recommended use level of 147–1471 mg TOS/kg wet gum[39] or 165–2975 mg TOS/kg dried lecithin.[40]

Enzymatically hydrolysed lecithin is a type of lecithins (E322) that is an authorised food additive in the EU according to Annex II and Annex III to Regulation (EC) No 1333/2008 on food additives.[41] In the framework of Regulation (EC) No 1333/2008 and of Commission Regulation (EU) No 257/2010 regarding the re-evaluation of approved food additives, the EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS Panel) re-evaluated lecithins used as food additive in 2017. The re-evaluation was supported by a public call for occurrence data (usage level and/or concentration data) on lecithins (E322). In response to this public call, updated information on the actual use levels of lecithins (E322) in foods was made available to EFSA by industry.

3.5.2 | Dietary exposure estimation

Two sets of estimation were calculated: one for the dietary exposure of the food enzyme–TOS via the processing of egg and egg products, the other for the use of modified lecithin (lysolecithin) as a food additive.

3.5.2.1 | *Dietary exposure via the processing of egg and egg products*

Chronic exposure to the food enzyme–TOS was calculated by combining the maximum recommended use level with individual consumption data (EFSA CEP Panel et al., 2021). the estimation involved selection of relevant food categories and application of technical conversion factors (EFSA CEP Panel, 2023). Exposure from all FoodEx categories was subsequently summed up, averaged over the total survey period (days) and normalised for bw. This was done for all individuals across all surveys, resulting in distributions of individual average exposure. Based on these distributions, the mean and 95th percentile exposures were calculated per survey for the total population and per age class. Surveys with only 1 day per subject were excluded and high-level exposure/intake was calculated for only those population groups in which the sample size was sufficiently large to allow calculation of the 95th percentile (EFSA, 2011).

Table 3 provides an overview of the derived exposure estimates across all surveys. Detailed mean and 95th percentile exposure to the food enzyme–TOS per age class, country and survey, as well as contribution from each FoodEx category to the total dietary exposure are reported in Appendix A – Tables 1 and 2. For the present assessment, food consumption data were available from 43 dietary surveys (covering infants, toddlers, children, adolescents, adults and elderly), carried out in 22 European countries (Appendix B). The highest dietary exposure was estimated to be about 1.712 mg TOS/kg bw per day in toddlers at the 95th percentile.

[37]Technical dossier/1st submission/p. 71.

[38]Technical dossier/1st submission/p. 72.

[39]Technical dossier/Additional data April 2022.

[40]Technical dossier/Additional data June 2023.

[41]Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on Food additives. OJ L 354, 16.12.2008, pp. 1–33.

TABLE 3 Summary of the estimated dietary exposure to food enzyme–TOS from egg processing in six population groups.

	Estimated exposure (mg TOS/kg body weight per day)					
Population group	**Infants**	**Toddlers**	**Children**	**Adolescents**	**Adults**	**The elderly**
Age range	3–11 months	12–35 months	3–9 years	10–17 years	18–64 years	≥65 years
Min–max mean (number of surveys)	0.001–0.128 (12)	0.047–0.566 (15)	0.167–0.583 (19)	0.051–0.297 (21)	0.039–0.160 (22)	0.016–0.154 (23)
Min–max 95th percentile (number of surveys)	0–0.847 (11)	0.324–1.712 (14)	0.492–1.554 (19)	0.221–0.842 (20)	0.132–0.487 (22)	0.126–0.463 (22)

Abbreviation: TOS, total organic solids.

3.5.2.2 | *Dietary exposure via modified lecithin as a food additive*

Since an exposure assessment to lecithins (E322) was carried out by the EFSA ANS Panel as part of the re-evaluation program and published in 2016, the so-derived exposure estimates were used in this opinion and combined with the food enzyme use levels in the assessment of exposure to food enzymes used in the production of modified lecithin. For the assessment of lecithins (E322), food consumption data were available from 33 different dietary surveys (covering infants, toddlers, children, adolescents, adults and the elderly), carried out in 19 European countries. Chronic exposure estimates were obtained. Two different exposure assessment scenarios were considered by the ANS Panel, i.e. a maximum level exposure assessment and a refined exposure assessment scenario (EFSA ANS Panel, 2016).

For the purposes of assessing exposure to the food enzyme–TOS under assessment, the CEP Panel decided to use the most conservative lecithin exposure estimates, i.e. data derived using the maximum level exposure scenario. The so-derived exposure estimates to lecithin were combined with the highest concentration of TOS in the lysolecithin (i.e. 2795 mg TOS/kg dried lysolecithin). Table 4 provides an overview of the derived exposure estimates across all surveys. The highest dietary exposure was estimated to be 1.61 mg TOS/kg bw per day in children at the 95th percentile.

TABLE 4 Summary of the estimated dietary exposure to food enzyme–TOS through lecithins (E 322) as a food additive.

Population group	Infants	Toddlers	Children	Adolescents	Adults	The elderly
Age range	3–11 months	12–35 months	3–9 years	10–17 years	18–64 years	≥65 years
Min–max mean (number of surveys)	0.140–0.498 (6)	0.193–1.020 (10)	0.198–0.878 (18)	0.089–0.495 (17)	0.196–0.330 (17)	0.201–0.324 (14)
Min–max 95th percentile (number of surveys)	0.305–1.029 (5)	0.363–1.453 (7)	0.333–1.610 (18)	0.165–0.906 (17)	0.375–0.662 (17)	0.369–0.556 (14)

3.5.2.3 | *Overall dietary exposure*

The consumption and occurrence data of these two sets of exposure estimation are different and were, therefore, reported separately.

To obtain an approximation of the overall dietary exposure, since the estimates shown in Tables 3 and 4 are in the same order of magnitude with 2-4-fold difference, the highest mean of the population age group were summed up. The highest sum was 1.586 mg TOS/kg bw per day in toddlers, followed by 1.461 in children, 0.792 in adolescents, 0.626 in infants, 0.49 in adults and 0.478 in the elderly population.

3.5.3 | Uncertainty analysis

In accordance with the guidance provided in the EFSA opinion related to uncertainties in dietary exposure assessment (EFSA, 2006), the following sources of uncertainties have been considered and are summarised in Table 5.

TABLE 5 Qualitative evaluation of the influence of uncertainties on the dietary exposure estimate.

Sources of uncertainties	Direction of impact
Model input data	
Consumption data: different methodologies/representativeness/underreporting/misreporting/no portion size standard	+/–
Use of data from food consumption surveys of a few days to estimate long-term (chronic) exposure	+
Possible national differences in categorisation and classification of food	+/–

Sources of uncertainties	Direction of impact
Model assumptions and factors	
Exposure to food enzyme–TOS was always calculated based on the recommended maximum use level	+
Selection of broad FoodEx categories for the exposure assessment	+
Use of recipe fractions in disaggregation FoodEx categories	+/–
Use of technical factors in the exposure model	+/–
Estimation of dietary exposure via modified lecithin as a food additive is based on consumption data obtained from all lecithins	+
Exclusion of other processes from the exposure assessment – Production of refined edible fats and oils by degumming	–
Overall dietary exposure	
The sum of the highest mean of each population group shown in Tables 3 and 4 is used to derive the lowest margin of exposure	+

Abbreviations: +, uncertainty with potential to cause overestimation of exposure; –, uncertainty with potential to cause underestimation of exposure.

For figures reported in Tables 3 and 4, the conservative approach applied to the exposure estimate to food enzyme–TOS, in particular assumptions made on the occurrence and use levels of this specific food enzyme, is likely to have led to an overestimation of the exposure.

The choice of using the sum of the highest mean estimates reported in Tables 3 and 4 to derive the margin of exposure (MoE) may lead to an overestimation of the overall intake. However, estimates in both tables are in the same order of magnitude, thus, the overestimation, if it occurs, would still likely be within the same magnitude.

3.6 | Margin of exposure

A comparison of the NOAEL (1350 mg TOS/kg bw per day) from the 90-day study with the approximated overall exposure of 0.478–1.586 mg TOS/kg bw per day at the maximum mean results in a MoE of at least 851.

4 | CONCLUSIONS

Based on the data provided and the derived margin of exposure, the Panel concluded that the food enzyme phospholipase A_2 produced with the genetically modified *A. niger* strain PLA 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 authorisation of phospholipase A_2 from a genetically modified strain of *Aspergillus niger*. (strain PLA). January 2015. Submitted by DSM Food Specialties B.V.

Additional data. April 2021. Submitted by DSM Food Specialties B.V.

Additional data. April 2022. Submitted by DSM Food Specialties B.V.

Additional data. June 2023. Submitted by DSM Food Specialties B.V.

Summary report on allergenicity related to phospholipase A_2 produced with a strain of *Aspergillus niger* (strain PLA) by DSM Food Specialties. March 2016. Delivered by FoBiG, (Freiburg, Germany).

ABBREVIATIONS

bw body weight
CAS Chemical Abstracts Service
CEF EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
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
GLP Good Laboratory Practice
GMO genetically modified organism
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
MNBN	bi-nucleated cells with micronuclei
MoE	margin of exposure
MTD	maximum tolerated dose
OECD	Organisation for Economic Co-operation and Development
PCR	polymerase chain reaction
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TOS	total organic solids
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.

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European Commission

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NOTE

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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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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APPENDIX A

Dietary exposure estimates to the food enzyme–TOS in details

Information provided in this appendix is shown in an excel file (downloadable https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2023.e8400#support-information-section).

The file contains two sheets, corresponding to two tables.

Table 1: Average and 95th percentile exposure to the food enzyme–TOS per age class, country and survey.

Table 2: Contribution of food categories to the dietary exposure to the food enzyme–TOS per age class, country and survey.

APPENDIX B

Population groups considered for the exposure assessment

Population	Age range	Countries with food consumption surveys covering more than 1 day
Infants	From 12 weeks on up to and including 11 months of age	Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Portugal, Slovenia, Spain
Toddlers	From 12 months up to and including 35 months of age	Belgium, Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Hungary, Italy, Latvia, the Netherlands, Portugal, Slovenia, Spain
Children	From 36 months up to and including 9 years of age	Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, the Netherlands, Portugal, Spain, Sweden
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, the Netherlands, Portugal, Romania, Slovenia, Spain, Sweden
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, the Netherlands, Portugal, Romania, Slovenia, Spain, Sweden
The elderly[a]	From 65 years of age and older	Austria, Belgium, Cyprus, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, the Netherlands, Portugal, Romania, Slovenia, Spain, Sweden

[a] The terms 'children' and 'the elderly' correspond, respectively, to 'other children' and the merge of 'elderly' and 'very elderly' in the Guidance of EFSA on the 'Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment' (EFSA, 2011).