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Safety evaluation of the food enzyme phospholipase A2 from the genetically modified *Streptomyces violaceoruber* strain AS-10

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Abstract

The food enzyme phospholipase A2 (phosphatidylcholine 2-acylhydrolase EC 3.1.1.4) is produced with the genetically modified *Streptomyces violaceoruber* strain AS-10 by Nagase (Europa) GmbH. 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 four food manufacturing processes, i.e. egg processing, baking processes, degumming of fats and oils and milk processing for cheese production. Since residual amounts of total organic solids (TOS) are removed in degumming of fats and oils, dietary exposure was calculated only for the remaining three food manufacturing processes. Dietary exposure to the food enzyme–TOS was estimated to be up to 0.41 mg TOS/kg body weight (bw) per day in European populations. Genotoxicity tests did not indicate a safety concern. The systemic toxicity was assessed by means of a repeated dose 90-day oral toxicity study in rats. The Panel identified a no observed adverse effect level of 191.2 mg TOS/kg bw per day, the mid-dose tested, which, when compared with the estimated dietary exposure, results in a margin of exposure above 460. A search for similarity of the amino acid sequence of the food enzyme to known allergens was made and no matches were found. The Panel considered that, under the intended conditions of use, the risk of allergic sensitisation and elicitation reactions by dietary exposure cannot be excluded, but the likelihood for this to occur is considered to be 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 A2, phosphatidylcholine 2-acylhydrolase, EC 3.1.1.4, lecithinase A, *Streptomyces violaceoruber*, 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 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 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 companies "Nagase (Europa) GmbH" for the authorisation of the food enzyme Phospholipase A2 from a genetically modified strain of *Streptomyces violaceoruber* (strain AS-10), "Novozymes A/S" for the authorisation of the food enzymes Glucose oxidase from *Aspergillus niger* (strain NZYM-KA), "Hayashibara Co., Ltd." for the authorisation of the food enzymes 4-α-D-{(1→4)-α-D-glucano} trehalose trehalohydrolase from *Arthrobacter ramosus* and (1→4)-α-D-glucan-1-α-D-glucosylmutase from *Arthrobacter ramosus*, and the Association of Manufacturers and Formulators of Enzyme Products (AMFEP) for the authorisation of the food enzyme Alpha-amylase from *Bacillus subtilis*.

Following the requirements of Article 12.1 of Regulation (EC) No 234/2011[3] implementing Regulation (EC) No 1331/2008, 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] Regulation (EC) No. 1332/2008 of the European Parliament and of the Council of 16 December 2008 on Food Enzymes and Amending Council Directive 83/417/EEC, Council Regulation (EC) No. 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/97. OJ L 354, 31.12.2008, pp. 7–15.

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

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

1.1.2. Terms of Reference

The European Commission requests the European Food Safety Authority to carry out the safety assessments on the food enzymes Phospholipase A2 from a genetically modified strain of *Streptomyces violaceoruber* (strain AS-10), Glucose oxidase from *Aspergillus niger* (strain NZYM-KA), 4-α-D-{(1→4)-α-D-glucano} trehalose trehalohydrolase from *Arthrobacter ramosus*, (1→4)-α-D-glucan-1-α-D-glucosylmutase from *Arthrobacter ramosus* and Alpha-amylase from *Bacillus subtilis* in accordance with Article 17.3 of Regulation (EC) No 1332/2008 on food enzymes.

1.2. Interpretation of the Terms of Reference

The present scientific opinion addresses the European Commission's request to carry out the safety assessment of food enzyme Phospholipase A2 from a genetically modified strain of *S. violaceoruber* (strain AS-10).

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 A2 from a genetically modified *S. violaceoruber* (strain AS-10). The dossier was updated on 14 April 2016.

Additional information was requested from the applicant during the assessment process on 19 May 2020 and was consequently provided (see 'Documentation provided to EFSA').

Following the reception of additional data by EFSA on 7 December 2020, EFSA requested a clarification teleconference on 17 March 2021, after which the applicant provided additional data on 27 September 2021.

2.2. Methodologies

The assessment was conducted in line with the principles described in the EFSA 'Guidance on transparency in the scientific aspects of risk assessment' (EFSA, 2009b) and following the relevant guidance documents of the EFSA Scientific Committee.

The '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, 2021a).

3. Assessment

IUBMB nomenclature	Phospholipase A2
Systematic name	phosphatidylcholine 2-acylhydrolase
Synonyms	lecithinase A; phosphatidase; phosphatidolipase
IUBMB No.	3.1.1.4
CAS No.	9,001-84-7
EINECS No.	232-637-7

Phospholipases A2 catalyse the hydrolysis of ester bonds in diacylphospholipids, resulting in the liberation of free fatty acids and leaving the residual 1-acyl-2-lysophospholipid. The food enzyme is intended to be used in four food manufacturing processes, i.e. egg processing, baking processes, degumming of fats and oils and milk processing for cheese production.

3.1. Source of the food enzyme

The phospholipase A2 is produced with the genetically modified bacterium *S. violaceoruber* strain AS-10, which is deposited in the collection of the Japanese Biological Resource Center (NBRC), with deposit number [redacted].[4]

[4] Technical dossier/2nd submission /Annex A3.1-Att 1.

3.1.1. Characteristics of the parental/recipient microorganism

The recipient microorganism was originally described as [redacted] Therefore, the strain is considered sufficiently identified. *S. violaceoruber* is known to produce secondary metabolites with antimicrobial activity, and antimicrobial resistance cannot be excluded for strains of the species. No information was provided on possible antimicrobial resistances of the recipient strain.

3.1.2. Characteristics of introduced sequences

[redacted]

3.1.3. Description of the genetic modification process

[redacted]

3.1.4. Safety aspects of the genetic modification

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

The production strain *S. violaceoruber* AS-10 differs from the recipient strain [redacted]

3.2. Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004[6], with food safety procedures based on hazard analysis and critical control points, and in accordance with current good manufacturing practice.

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 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.

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.

[5] Technical dossier/2nd submission/Annex A3.1 and Annex A3.1-Att 3.

[6] 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.

3.3. Characteristics of the food enzyme

3.3.1. Properties of the food enzyme

The phospholipase A2 is a single polypeptide chain of [redacted] amino acids. The molecular mass of the mature protein, calculated from the amino acid sequence, is around [redacted] kDa. The food enzyme was analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis. A consistent protein pattern was observed across all batches. The gels showed a single major protein band corresponding to an apparent molecular mass of about [redacted] kDa, consistent with the expected mass of the enzyme. Amylase and protease activity were detected in the food enzyme. The lipase activity was below the limit of detection (LoD).[7]

The in-house determination of phospholipase A2 activity is based on hydrolysis of L-α-phosphatidylcholine and is expressed in phospholipase A2 Units/g (U/g). The enzymatic activity is determined by measuring the release of fatty acids by spectrophotometry using a commercial test kit (reaction conditions: pH 8.0, 37°C, 20 min).[8] One unit of phospholipase A2 activity (U) is defined as the amount of enzyme that generates 1 μmol of fatty acid per minute under the conditions of the assay.

The food enzyme has a temperature optimum around [redacted] ([redacted]) and a pH optimum around [redacted] ([redacted]). Thermostability was tested after a pre-incubation of the food enzyme for [redacted] at different temperatures at [redacted]. Phospholipase A2 activity decreased above [redacted], showing no residual activity above [redacted].

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme in the form of a powdered preparation, were provided for four batches used for commercialisation and one batch produced for the toxicological tests[9] (Table 1). The dried preparation contains approximately 80% w/w [redacted], which is included in the ash values shown in Table 1. The average total organic solids (TOS) of the four food enzyme preparation batches used for commercialisation is 10.6%, with an average enzyme activity/TOS ratio of 1,144 U/mg TOS.

Table 1: Compositional data of the dried food enzyme preparation

Parameters	Unit	Batches				
		1	2	3	4	5[(a)]
Phospholipase A2 activity	U/g batch[(b)]	113,400	111,600	110,810	117,600	263,200
Protein[(c)]	%	NA	NA	NA	NA	NA
Ash	%	92.7	88.0	86.2	87.0	2.6
Water	%	0.7	0.9	1.2	0.9	1.8
Total organic solids (TOS)[(d)]	%	6.6	11.1	12.6	12.1	95.6
Activity/mg TOS	U/mg TOS	1,718	1,005	879	972	275

(a): Batch used for the toxicological studies.
(b): Unit: phospholipase A2 (see Section 3.3.1).
(c): NA: not analysed.
(d): TOS calculated as 100% – % water – % ash.

3.3.3. Purity

The lead content in six commercial batches (three dried and three liquid) and in the batch used for toxicological studies was below 0.2 mg/kg, which complies with the specification for lead (≤ 5 mg/kg) as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006). In addition, the levels of arsenic, cadmium and mercury were below the LoDs of the employed methods or, when detected, found at concentrations which did not give rise to safety concerns.[10,11]

[7] Technical dossier/2nd submission /Annex A2.4; Add data December 2020/Annex 3.
[8] Technical dossier/2nd submission /Annex A2.5.
[9] Technical dossier/2nd submission /Annex A2.1 and Annex A7.1.1.
[10] LoDs: Pb = 0.1 mg/kg; As = 1 mg/kg; Cd = 0.01 mg/kg; Hg = 0.01 mg/kg.
[11] Technical dossier/2nd submission /Annex A2.1, Annex A2.2 and Annex A7.1.1.

The dried food enzyme preparation complies with the microbiological criteria as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006), which stipulate that *Escherichia coli* and *Salmonella* species are absent in 25 g of sample and total coliforms should not exceed 30 colony forming units per gram. No antimicrobial activity was detected in any of the tested batches (FAO/WHO, 2006).[12]

Streptomycetes are known to produce secondary metabolites. The presence of aflatoxins B1, B2, G1 and G2 was examined in one batch of the dried food enzyme preparation and one batch of the liquid form and were below the LoDs of the applied analytical method.[13,14] The possible presence of other secondary metabolites is addressed by the toxicological examination of the food enzyme–TOS.

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 six independent batches analysed in triplicate (three batches of the dried food enzyme and three batches of the liquid form). One mL of the liquid form was incubated in 5 mL of non-selective medium at 30°C for 5 days for resuscitation. One gram of the dried form was incubated in 20 mL of non-selective medium at 30°C for 5 days for resuscitation. From these, 3 × 2 mL were inoculated on agar plates [redacted] and incubated at 30°C for 7 days. No colonies were produced.[15]

The absence of recombinant DNA in the food enzyme was demonstrated by polymerase chain reaction (PCR) analysis of six batches in triplicate, three batches of liquid food enzyme and three batches of dried food enzyme. No DNA was detected with primers that would amplify [redacted], with a limit of detection of 1 ng spiked DNA/g food enzyme.[16]

3.4. Toxicological data

A battery of toxicological tests, including a bacterial gene mutation assay (Ames test), an *in vitro* mammalian chromosomal aberration test and a repeated dose 90-day oral toxicity study in rats, has been provided. The batch 5 (Table 1) used in these studies represents the food enzyme dried without the addition of the excipients used in the commercial batches. As a result, it has a far higher TOS content and a lower specific activity and is considered suitable as a test item.

3.4.1. Genotoxicity

3.4.1.1. Bacterial reverse mutation test

A bacterial reverse mutation assay (Ames test) was performed according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 (OECD, 1997a) and following Good Laboratory Practice (GLP).[17] Four strains of *Salmonella* Typhimurium (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* WP2 *uvrA* were used in the presence or absence of metabolic activation (S9-mix), applying the preincubation method. Two separate experiments were carried out in triplicate using five concentrations of the food enzyme (from 313 to 5,000 μg/plate, corresponding to 299, 598, 1,195, 2,390 and 4,780 μg TOS/plate). No cytotoxicity was observed at any concentration level of the test substance. There was no significant increase in revertant colony numbers above the control values in any strain at any concentration with or without S9-mix.

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

3.4.1.2. *In vitro* mammalian chromosomal aberration test

The *in vitro* mammalian chromosomal aberration test was carried out in cultured Chinese hamster lung fibroblasts (CHL/IU) according to OECD Test Guideline 473 (OECD, 1997b) and following GLP.[18] On the basis of the results of a dose-finding test, cell cultures were treated at a range of concentrations of the food enzyme inducing a maximum cell growth inhibition of 50%. In a short-term

[12] Technical dossier/1st submission/Annex A2.1, Annex A2.2 and Annex A7.1.1.
[13] Technical dossier/2nd submission /Annex A2.3.
[14] LoD: 0.005 mg/kg.
[15] Technical dossier/Add data December 2020/Annex 2.
[16] Technical dossier/Add data September 2021/Annex 1.
[17] Technical dossier/2nd submission /Annex A7.1.2.
[18] Technical dossier/2nd submission /Annex A7.1.3.

treatment (6 h followed by 18 h recovery period), the concentrations tested were 156, 313, 625 and 1,250 μg/mL (corresponding to 149.1, 299, 598 and 1,195 μg TOS/mL) in the presence of metabolic activation (S9-mix) and at 625, 1,250 and 2,500 μg/mL (corresponding to 598, 1,195 and 2,390 μg TOS/mL) without S9-mix. In continuous 24 h treatment in the absence of S9-mix, the cells were exposed to the food enzyme at 313, 625, 1,250 and 2,500 μg/mL (corresponding to 299, 598, 1,195 and 2,398 μg TOS/mL), but the analysis of chromosomal aberrations was performed only at 313 μg/mL (299 μg TOS/mL) due to low number of metaphases. In a continuous 48 h treatment in the absence of S9-mix, the cells were exposed to the food enzyme at 39.1, 78.1, 156 and 313 μg/mL (corresponding to 37.4, 74.7, 149.1 and 299 μg TOS/mL). The frequency of structural and numerical chromosomal aberrations in treated cultures was comparable to the values detected in negative controls.

The Panel concluded that food enzyme did not induce structural and numerical chromosome aberrations under the test conditions employed for this study.

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 OECD Test Guideline 408 (OECD, 1998) and following GLP.[19] Groups of 12 male and 12 female Sprague–Dawley (Crl:CD(SD)) rats received by gavage 40, 200 or 1,000 mg/kg body weight (bw) per day of the food enzyme, corresponding to 38.24, 191.2 and 956 mg TOS/kg bw per day, for 90 days. Controls received the vehicle (water for injection). No mortality was observed.

In the functional observations, a statistically significant decrease in rearing in mid-dose females in week 1, an increase in rearing in the low-dose females in week 6, a decreased grip strength of forelimb and hindlimb in the high-dose males in week 13, were observed. The Panel considered the changes as not toxicologically relevant as they were only recorded sporadically (all parameters) and they were only observed on one sex (all parameters).

The haematological investigation revealed a statistically significant increase in mean corpuscular haemoglobin concentration (MCHC) (+1.4%) in mid-dose females, an increase in lymphocyte percentage (+10% and +9%) and a decrease in segmented neutrophils percentage (−42% and −38%) in mid- and high-dose females. The Panel considered the changes as not toxicologically relevant as they were only observed in one sex (all parameters), the changes were small (MCH, lymphocyte percentage), the changes were unaccompanied by changes in total number of white blood cells (lymphocyte percentage, segmented neutrophils percentage) and there was no dose–response relationship (all parameters).

The clinical chemistry investigation revealed statistically significant decreases in sodium concentration in all treated males (−0.7% at all doses) and in chloride concentration in high-dose males (−1.9%). The Panel considered the changes as not toxicologically relevant as they were only recorded in one sex (both parameters), there was no dose–response relationship (sodium) and the changes were small (both parameters).

Statistically significant changes in organ weight included an increase in the relative liver weight (+12%) in high-dose males and a decrease in the relative kidney weight in the mid-dose females (−12%). The Panel considered these changes as not toxicologically relevant as they were only observed in one sex (both parameters), there was no dose–response relationship (the relative kidney weight), the changes were small (both parameters) and there were no histopathological changes in the liver and the kidneys.

The microscopic examination revealed several changes in the stomach of the high-dose group: minimal erosion in the glandular stomach in males (1/12 vs. 0/12), minimal or mild diffuse mucosal hyperplasia in males (4/12 vs. 0/12) and females (2/12 vs. 0/12), minimal or mild globule leukocyte cell infiltration in males (2/12 vs. 1/12) and females (5/12 vs. 0/12) and minimal to mild hyperplasia of the limiting ridge of the stomach in males (12/12 vs. 2/12 in the controls) and females (12/12 vs. 0/12). Additionally, a minimal globule leukocyte cell infiltration was seen in one mid-dose female. The Panel noted that the limiting ridge of stomach is recognised as a structure specific to rodent. However, the Panel considered the changes of the stomach mucosa possibly due to irritation from the concentration of the test compound at the high dose as test compound related.

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

[19] Technical dossier/2nd submission /Annex A7.1.4.

The Panel identified a no observed adverse effect level (NOAEL) of 191.2 mg TOS/kg bw per day, the mid-dose tested based on the microscopic changes in the stomach.

3.4.3. 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 phospholipase A2 produced with the genetically modified *S. violaceoruber* strain AS-10 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 matches were found.[20]

No information is available on oral and respiratory sensitisation or elicitation reactions of this phospholipase A2.

Phospholipase A2 is considered the major allergen of honeybee venom (Okano et al., 1999). Therefore, the applicant performed a literature search for possible adverse reactions or allergy after oral exposure to phospholipases. A study investigated the possible use of phospholipase A2 for oral immunotherapy (Guerin et al., 2002), suggesting that the oral intake of phospholipase A2 does not represent a concern even for individuals' sensitive to bee venom. In addition, no allergic reactions upon dietary exposure to any phospholipase A2 have been reported in the literature.

[redacted], a product that may cause allergies or intolerances (Regulation (EU) No 1169/2011[21]) is used as a 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 this protein source is not expected to be present.

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 is considered to be low.

3.5. Dietary exposure

3.5.1. Intended use of the food enzyme

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

Table 2: Intended uses and recommended use levels of the food enzyme preparation(c)

Food manufacturing process(a)	Raw material (RM)	Maximum recommended use level (mg TOS/kg RM)(b)
Egg processing	Egg yolk, whole liquid egg	**21.1**
Baking processes	Flour	**0.63**
Degumming of fats and oils	Crude oil or fat	0.63
Milk processing for cheese production	Milk	**2.11**

(a): The name has been harmonised by EFSA according to the 'EC working document describing the food processes in which food enzymes are intended to be used' – not yet published at the time of adoption of this opinion.
(b): Numbers in bold were used for calculation.
(c): Additional information December 2020/Annex 5, 9.

[20] Technical dossier/2nd submission /Annex A7.2.
[21] 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.

In egg processing, the food enzyme is added to egg yolk in the manufacture of mayonnaise, or to the whole liquid egg in the manufacture of other egg-based products.[22] The hydrolysis of lecithin in egg yolk with phospholipase A2 provides better emulsifying properties to foods. The food enzyme–TOS remains in the final foods.

In baking processes, the phospholipase A2 is added to flour during the preparation of the dough.[23] The hydrolysis of phospholipids in flour can replace partially or fully the need to add emulsifiers to the dough. The food enzyme–TOS remains in the bakery products.

In the degumming process, the food enzyme is added to crude vegetable oil before the centrifugation step.[24] Phospholipase A2 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 water-based 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, 2021b).

In dairy processing for cheese production, the phospholipase A2 is added to milk before pasteurisation.[25] Hydrolysis of milk phospholipids decreases the surface tension of milk, giving rise to cream with improved texture and skim milk with decreased surface tension. The treated milk can retain better fat and protein in curd, consequently increasing the yield of cheese. The food enzyme–TOS remains in cheese and whey (the by-product during cheese making) with a partition ratio of 1:9, respectively.

Based on data provided on thermostability (see Section 3.3.1), it is expected that the phospholipase A2 is inactivated during cooking, baking or pasteurisation.

3.5.2. Dietary exposure estimation

In accordance with the guidance document (EFSA CEP Panel, 2021a), a dietary exposure was calculated only for food manufacturing processes where the food enzyme–TOS remains in the final foods, namely egg processing, baking processes and milk processing for cheese production.

Chronic exposure to the food enzyme–TOS was calculated by combining the maximum recommended use level with individual consumption data (EFSA CEP Panel, 2021a). the estimation involved selection of relevant food categories and application of technical conversion factors (EFSA CEP Panel, 2021b). Exposure from all FoodEx categories was subsequently summed up, averaged over the total survey period (days) and normalised for body weight. 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 41 dietary surveys (covering infants, toddlers, children, adolescents, adults and the elderly), carried out in 22 European countries (Appendix B). The highest dietary exposure at the 95th percentile was estimated to be about 0.414 mg TOS/kg bw per day in infants.

Table 3: Summary of estimated dietary exposure to food enzyme–TOS in six population groups

Population group	Estimated exposure (mg TOS/kg body weight per day)					
	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)	0.014–0.186 (11)	0.021–0.094 (15)	0.013–0.030 (19)	0.006–0.023 (21)	0.004–0.019 (22)	0.003–0.009 (22)

[22] Additional information December 2020/Annex 5.
[23] Additional information December 2020/Annex 6.
[24] Additional information December 2020/Annex 7.
[25] Additional information December 2020/Annex 8.

Population group	Estimated exposure (mg TOS/kg body weight per day)					
	Infants	Toddlers	Children	Adolescents	Adults	The elderly
Min–max 95th percentile (number of surveys)	0.054–0.414 (9)	0.055–0.193 (13)	0.025–0.066 (19)	0.014–0.036 (20)	0.010–0.045 (22)	0.009–0.024 (21)

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 4.

Table 4: 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 for high percentiles (95th percentile)	+
Possible national differences in categorisation and classification of food	+/–
Model assumptions and factors	
FoodEx categories included in the exposure assessment were assumed to always contain the food enzyme–TOS	+
Exposure to food enzyme–TOS was always calculated based on the recommended maximum use level	+
Assuming that whey protein concentrate is used in all milk-based infant formulae and follow-on formulae	+
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	+/–
Exclusion of one process from the exposure assessment – Degumming of fats and oils	–

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

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 exclusion of one food manufacturing processes from the exposure assessment was based on > 99% of TOS removal during processing and is not expected to have an impact on the overall estimate derived.

3.6. Margin of exposure

A comparison of the NOAEL (191.2 mg TOS/kg bw per day) from the 90-day rat study with the derived exposure estimates of 0.003–0.186 mg TOS/kg bw per day at the mean and from 0.009–0.414 mg TOS/kg bw per day at the 95th percentile, resulted in a margin of exposure (MoE) of at least 462.

4. Conclusions

Based on the data provided, the removal of TOS during the degumming of fats and oils and the derived margin of exposure for the three remaining food manufacturing processes, the Panel concluded that the food enzyme phospholipase A2 produced with the genetically modified

S. violaceoruber strain AS-10 does not give rise to safety concerns under the intended conditions of use.

The production strain of the food enzyme contains multiple copies of a known antimicrobial resistance gene on a replicative plasmid. However, based on the absence of viable cells and DNA from the production organism in the food enzyme, this is not considered to be a risk.

5. Documentation as provided to EFSA

Request for the authorization of a phospholipase A2 preparation from *Streptomyces violaceoruber* AS-10 for use as a processing aid. January 2015. Submitted by Nagase (Europa) GmbH.

Additional information. December 2020, September 2021. Submitted by Nagase (Europa) GmbH.

Additional information on 'The transfer of enzymes into food for fat and oil processing'. October 2017 and February 2018. Provided by the Association of Manufacturers and Formulators of Enzyme Products.

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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
GLP	Good Laboratory Practice
GMM	genetically modified microorganism
GMO	genetically modified organism
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LoD	limit of detection
MoE	margin of exposure
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Cooperation and Development
PCR	polymerase chain reaction
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TOS	total organic solids
WHO	World Health Organization

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.2023.7458#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
Toddlers	From 12 months up to and including 35 months of age	Belgium, Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Hungary, Italy, Latvia, 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, 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, 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, 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, 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).