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Safety evaluation of the food enzyme phospholipase C from a genetically modified *Komagataella phaffii* (strain PRF)

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Abstract

The food enzyme phospholipase C (EC 3.1.4.3) is produced with a genetically modified *Komagataella phaffii* (formerly *Pichia pastoris*) (strain PRF) by DSM. The genetic modifications do not give rise to safety concerns. The food enzyme is free from viable cells of the production organism and recombinant DNA. This phospholipase C is intended to be used in fats and oils processing for degumming. The residual amounts of total organic solids (TOS) are removed during refinement steps applied during fats and oils processing. Consequently, no dietary exposure was calculated. 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 (NOAEL) of at least 1,672 mg TOS/kg body weight per day, the highest dose tested. Similarity of the amino acid sequence to those of known allergens was searched 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 cannot be excluded, but the likelihood is considered to be low. Based on the data provided and the removal of TOS during the fats and oils processing for degumming, the Panel concluded that this food enzyme does not give rise to safety concerns under the intended conditions of use.

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1. Introduction

Article 3 of the Regulation (EC) No 1332/2008¹ 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 1331/2008² 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, and
- 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 a food enzyme for evaluation’ (EFSA CEF Panel, 2009) 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.

Five applications have been introduced for the authorisation of the food enzymes: Alpha-amylase from a genetically modified strain of *Pseudomonas fluorescens*, Phospholipase C from a genetically modified strain of *Pichia pastoris*, Alpha-amylase from a genetically modified strain of *Bacillus licheniformis*, Xylose isomerase from a genetically modified strain of *Streptomyces rubiginosus* and Alpha-amylase from a genetically modified strain of *Bacillus amyloliquefaciens*.

Following the requirements of Article 12.1 of Commission Regulation (EU) No 234/2011³ 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 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, p. 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, p. 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, p. 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 Alpha-amylase from a genetically modified strain of *Pseudomonas fluorescens*, Phospholipase C from a genetically modified strain of *Pichia pastoris*, Alpha-amylase from a genetically modified strain of *Bacillus licheniformis*, Xylose isomerase from a genetically modified strain of *Streptomyces rubiginosus* and Alpha-amylase from a genetically modified strain of *Bacillus amyloliquefaciens* 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 the food enzyme phospholipase C from a genetically modified (GM) strain of *Komagataella phaffii* (formerly *Pichia pastoris*).

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 C from a GM strain of *K. phaffii* (strain PRF).

Additional information was requested from the applicant during the assessment process on 20 June 2018 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, 2009) as well as in the EFSA 'Scientific Opinion on Guidance on the risk assessment of GM microorganisms and their products intended for food and feed use' (EFSA GMO Panel, 2011) and following the relevant existing guidance's of EFSA Scientific Committees.

The current 'Guidance on the submission of a dossier on food enzymes for safety evaluation' (EFSA CEF Panel, 2009) has been followed for the evaluation of the application with the exception of the exposure assessment, which was carried out in accordance to the methodology described in the CEF Panel statement on the exposure assessment of food enzymes (EFSA CEF Panel, 2016).

3. Assessment

IUBMB nomenclature:	phospholipase C
Systematic name:	phosphatidylcholine cholinephosphohydrolase
Synonyms:	lipophosphodiesterase I; lecithinase C; <i>Clostridium welchii</i> α -toxin; <i>Clostridium oedematiens</i> β - and γ -toxins; lipophosphodiesterase C; phosphatidase C; heat-labile hemolysin; α -toxin
IUBMB No:	EC 3.1.4.3
CAS No:	9001-86-9
EINECS No:	232-638-2

The phospholipase C catalyses the hydrolysis of the phosphodiester bond linking glycerol and phosphate moieties at the sn-3 position of glycerophospholipids. It is intended to be used in fats and oils processing for degumming.

3.1. Source of the food enzyme

The phospholipase C is produced with a GM yeast *K. phaffii* (formerly *Pichia pastoris*) strain PRF, which is deposited at the internationally recognised public culture collection, Dutch Westerdijk Fungal Biodiversity Institute in Utrecht, the Netherlands, with deposition number [REDACTED].⁴

⁴ Technical dossier/Additional information/Annex-I.

The genomic sequence of the parental strain [REDACTED] has been published [REDACTED]. The genomic sequence of the production strain PRF has been compared with that of the strain [REDACTED]. The alignment indicates a high identity between the two genomes. [REDACTED]. This confirms that the production strain is a *K. phaffii* derivative.⁵

3.1.1. Characteristics of the parental and recipient microorganisms

The parental microorganism *K. phaffii* [REDACTED]

The recipient strain is [REDACTED]

K. phaffii has been used for many years in the biotechnology industry for production of single-cell protein and enzymes (Wegner, 1990). *K. phaffii* has been included in the list of organisms considered suitable a qualified presumption of safety (QPS) approach for safety assessment with the qualification that it only applies when the species is used for enzyme production and no viable cells are found in the final product (EFSA BIOHAZ Panel, 2018).

3.1.2. Characteristics of the introduced sequences

The phospholipase C coding gene used for the final transformation was [REDACTED]

3.1.3. Description of the genetic modification process

The recipient strain [REDACTED]

3.1.4. Safety aspects of the genetic modification

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

The recipient strain [REDACTED] differs from the parental strain [REDACTED]

The production strain [REDACTED] differs from the recipient strain [REDACTED]

⁵ Technical dossier/Annex II-2.

No genetic instability was seen by polymerase chain reaction (PCR) [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. 852/2004⁶, with food safety procedures based on Hazard Analysis and Critical Control Points, and in accordance with current Good Manufacturing Practice.⁷

The production strain is grown as a pure culture using a typical industrial medium in a submerged, fed-batch fermentation system with conventional process controls in place. After completion of the fermentation, the solid biomass is removed from the fermentation broth by filtration leaving a supernatant containing the food enzyme. 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 weight 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.

3.3. Characteristics of the food enzyme

3.3.1. Properties of the food enzyme

The phospholipase C is a single polypeptide chain of 245 amino acids. The molecular mass of the mature protein, derived from the amino acid sequence, was calculated to be 28 kDa. The secreted protein is glycosylated. The sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis showed one major protein band in all batches, migrating at about 34 kDa, and a number of other bands of minor staining intensity. No other enzyme activities were reported.

The in-house determination of phospholipase C activity is based on hydrolysis of phosphatidylcholine (reaction conditions: pH 7.3, 37°C, 6 min), releasing 1,2-diacylglycerol and phosphorylcholine. The latter lowers the pH of the reaction mixture. The enzymatic activity is determined by measuring the amount of base required to maintain the pH of the reaction mixture. The phospholipase C activity is expressed in phospholipase C units (PLCU)/g. One phospholipase C unit is defined as the amount of enzyme catalysing the hydrolysis of 1 μmol phosphatidylcholine per minute at 37°C and at pH 7.3.

The phospholipase C has been characterised with regards to its temperature and pH profiles. The phospholipase C exhibits optimal activity between pH 6.5 and 7.5 (at 37°C), and the optimum temperature is about 60°C (at pH 7.3). Thermostability was tested after a pre-incubation of the food enzyme from 2 to 60 min at different temperatures (from 50 to 100°C). Under the assay conditions used, the phospholipase C activity decreased rapidly above 70°C showing no residual activity above 90°C after 2 min.⁸

3.3.2 Chemical parameters

Data on chemical parameters of the food enzyme were provided for three batches used for commercialisation and one batch used for the toxicological testing (Table 1). The average total organic solids (TOS) content of the three commercial batches was 9.4% (w/w); the values ranged from 7.74% to 10.98% (Table 1).⁹

The average enzyme activity/TOS ratio of the commercial food enzyme batches is 577.7 PLCU/mg TOS.

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.

⁷ Technical dossier/Annex I-5 and Annex I-7.

⁸ Technical dossier/Annex I-2.

⁹ Technical dossier/Additional information August 2018.

Table 1: Compositional data provided for the food enzyme

Parameter	Unit	Batches			
		1	2	3	4 ^(c)
Phospholipase C activity	PLCU/g ^(b)	49,430	56,419	55,721	315,000
Protein	%	4.21	4.66	4.39	30
Ash	%	0.26	0.29	0.32	12.6
Water	%	92.0	90.1	88.7	3.8
Total organic solids (TOS) ^(a)	%	7.74	9.61	10.98	83.6
Phospholipase C activity/mg TOS	PLCU/mg TOS	638.6	587.1	507.5	376.8

(a): TOS calculated as 100% – % water – % ash.

(b): PLCU/g: phospholipase C unit/g.

(c): Batch used in toxicological studies.

3.3.3. Purity

The lead content in three commercial batches and in the batch used for toxicological studies was below 0.37 mg/kg which complies with the specification for lead (≤ 5 mg/kg) as laid down in the general specifications and considerations for enzymes used in food processing (FAO/WHO, 2006).¹⁰

The food enzyme complies with the microbiological criteria as laid down in the general specifications and considerations for enzymes used in food processing (FAO/WHO, 2006), which stipulate that *E. coli* and *Salmonella* species are absent in 25 g of sample and total coliforms should not exceed 30 colony forming unit per gram. No antimicrobial activity was detected in any of these batches (FAO/WHO, 2006).¹¹

The Panel considered the compositional data provided for the food enzyme as sufficient.

3.3.4. Viable cells and DNA of the production strain

The absence of the production strain in the final formulated food enzyme preparation was demonstrated in 12 independent batches analysed. 0.5 mL of product was incubated on selective agar containing ampicillin, gentamycin and streptomycin at 30°C for 72 h. No colonies were detected.

The absence of recombinant DNA in the food enzyme was demonstrated by PCR analysis of three batches in triplicate. No DNA was detected with primers that would amplify a 999-bp fragment specific for a region containing the alphaMF-PLC gene, with a limit of detection (LoD) of 1 ng spiked DNA/g food enzyme.¹²

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 used for toxicological testing (batch 4, Table 1) is a lyophilised enzyme concentrate. As shown in Table 1, the food enzyme batch 4 used for the toxicological testing has lower specific activity (enzyme activity/mg TOS), which indicates that it is less pure than the commercial batches and thus can be 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 made according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 (OECD, 1997a) and following Good Laboratory Practice (GLP).¹³ Four strains of *Salmonella* Typhimurium (TA 1535, TA 1537, TA 98 and TA 100) and *E. coli* WP2uvrA (pKM101) were used in the presence or absence of metabolic activation (S9-mix), applying the 'treat and plate' assay. Two experiments were carried out using five different concentrations of the food enzyme for the four strains of *S. Typhimurium* (154, 512, 1,540, 5,120, and

¹⁰ LoD: Pb = 0.006 mg/L.

¹¹ Technical dossier/Annex I-3.

¹² Technical dossier/Annex II-7.

¹³ Technical dossier/Annex I-16.

7,690 µg test article/mL, corresponding to 129, 428, 1,287, 4,280, and 6,429 µg TOS/mL) and seven different concentrations of the food enzyme for the *E. coli* WP2uvrA (15.4, 51.2, 154, 512, 1,540, 5,120, and 7,690 µg test article/mL, corresponding to 13, 43, 129, 428, 1,287, 4,280, and 6,429 µg TOS/ml). Cytotoxicity was observed with WP2uvrA in the presence of S9 mix as evidenced by reduced background lawns and dose-related decreases in revertant frequencies at the top three concentrations tested. Upon treatment with the food enzyme, there was no significant increase in revertant colony numbers above the control values in any strain 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 chromosome aberration test

The *in vitro* mammalian chromosome aberration test was carried out according to the OECD Test Guideline 473 (OECD, 1997b) and following GLP in cultured human peripheral blood lymphocytes.¹⁴ Two independent experiments were performed in duplicate. In the first experiment, cultures were exposed to the food enzyme at 412, 1,200, 2,450 and 5,000 µg/mL (corresponding to 344, 1,003, 2,048 and 4,180 µg TOS/mL) and 588, 1,200, 2,450 and 5,000 µg/mL (corresponding to 492, 1,003, 2,048, and 4,180 µg TOS/mL) for 3 + 19 h in the absence and in the presence of metabolic activation, respectively. In the second experiment, cultures were exposed to the food enzyme at 2,000, 3,000, 4,000 and 5,000 µg/mL (corresponding to 1,672, 2,508, 3,344, and 4,180 µg TOS/mL) for 3 + 19 h in the presence of S9-mix and for 22 + 0 h in the absence of S9-mix.

For all food enzyme concentrations tested, no statistically significant increase in cells with chromosomal aberrations, polyploidy, or endoreduplication was observed. The Panel concluded that the food enzyme did not induce structural and numerical chromosomal aberrations in cultured human peripheral blood lymphocytes when tested up to 5,000 µg/mL (corresponding to 4,180 µg TOS/mL) with and without an exogenous metabolic activation system under the experimental conditions employed.

The Panel concluded on the basis of the *in vitro* studies there is no concern for genotoxicity for the TOS enzyme tested.

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

A repeated dose 90-day oral toxicity study was performed according to OECD test guideline 408 (OECD, 1998), and following GLP.¹⁵ Groups of 20 male and 20 female Sprague–Dawley rats received the food enzyme by gavage at dose levels of 500, 1,000 and 2,000 mg/kg body weight (bw) per day, corresponding to 418, 836 and 1,672 mg TOS/kg bw per day. Controls received the vehicle (distilled water).

Three animals (1 low-dose female on day 10; 1 high-dose male on day 63; 1 high-dose female on day 72) were found dead in the study. The Panel considered this related to gavage incidents as confirmed by necropsy.¹⁶

Statistically significant intermittent differences from controls were recorded in body weights, body weight gains and food intake in the treated male and female groups. These differences were considered to be of no toxicological relevance, since they were not dose-related, not consistent between the sexes, and there was no test article related differences in body weight or overall body weight gain.

Few statistically significant changes from controls were noted in haematological parameters (decrease in the mean corpuscular haemoglobin in mid-dose males; decrease in mean corpuscular haemoglobin concentration in all treated males; decrease in percentage of basophils in mid- and high-dose males; increase in red blood cells in mid-dose females) and in clinical chemistry parameters (increase in potassium in mid-dose males and increase in triglycerides in high-dose females).¹⁷ The changes were small, not dose-related, not seen in both genders and the values were consistent with the historical control values from the laboratory. These changes were therefore considered incidental and not toxicologically relevant.

No other statistically significant effects were observed.

The Panel identified a no observed adverse effect level (NOAEL) of 1,672 mg TOS/kg bw per day, the highest dose tested.

¹⁴ Technical dossier/Annex II-17.

¹⁵ Technical dossier/Annex I-18.

¹⁶ Technical dossier/Annex I-18/p. 26.

¹⁷ Technical dossier/Annex I-18/p. 71–74, 95, 100.

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 this phospholipase C from GM *K. phaffii* (strain PRF) 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, 2017). 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 or respiratory sensitisation and elicitation reactions to this phospholipase C. Therefore, it can be concluded that an allergic reaction upon oral ingestion of phospholipase C, produced by the GM strain of *K. phaffii* (strain PRF) cannot be ruled out, but the likelihood of such reaction to occur is considered to be low.

Quantifying the risk for allergenicity is not possible in view of the individual susceptibility to food allergens. Allergenicity can be ruled out only if the proteins are fully removed. In the fats and oils processing for degumming vegetable oils, experimental data showed a significant removal (> 99%) of protein. However, traces of protein could be present in the final fats and oils.

The Panel considers that under the intended condition 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 is considered to be low.

3.5. Dietary exposure

3.5.1. Intended use of the food enzyme

Phospholipase C is intended to be used in fats and oil processing for degumming¹⁸ at an intended use level up to 133 mg TOS/kg raw material.¹⁹

When added to crude oil, phospholipase C hydrolyses the phospholipids naturally present in crude oil to form diacylglycerol and phosphate esters. This conversion results in enzymatic degumming of oil, which helps to reduce the amount of gum phospholipids.²⁰

Experimental data have been provided showing the removal (> 99%) of phospholipases A and C in the course of fats and oils processing for degumming the vegetable oils (Documentation provided to EFSA No. 4). In addition, taking into account the phase separation after degumming and purification steps applied to obtain refined oils and fats, i.e. bleaching and deodorisation, the Panel also considers that the amount of TOS in the final fats and oils will be removed to a similar degree.

3.5.2. Dietary exposure estimation

The Panel considered the evidence provided as sufficient to conclude that the presence of residual amounts of TOS after processing is negligible. Consequently, no dietary exposure was calculated.

4. Conclusions

Based on the data provided and the removal of TOS during the fats and oils processing for degumming, the Panel considers that this food enzyme phospholipase C from GM *K. phaffii* (strain PRF) does not give rise to safety concerns under the intended conditions of use.

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

Documentation provided to EFSA

- 1) Dossier 'Application for authorisation of phospholipase C from a genetically modified strain of *Pichia pastoris* in accordance with Regulation (EC) No 1331/2008'. March 2015. Submitted by DSM Food Specialties.
- 2) Additional information, August 2018. Submitted by DSM Food Specialties.

¹⁸ The description provided by the applicant 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 adoption of this opinion.

¹⁹ Technical dossier/Section 3.2.1.4.

²⁰ Technical dossier/p. 60.

- 3) Summary report on the genetically modified microorganism part. Delivered by Technical University of Denmark (Copenhagen, Denmark).
- 4) 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
EC	Enzyme Commission
EINECS	European Inventory of Existing Commercial Chemical Substances
FAO	Food and Agricultural Organization of the United Nations
GLP	Good Laboratory Practice
GM	genetically modified
GMO	genetically modified organism
IUBMB	International Union of Biochemistry and Molecular Biology
LoD	limit of detection
OECD	Organisation for Economic Cooperation and Development
PCR	polymerase chain reaction
PLCU	phospholipase C units
QPS	qualified presumption of safety
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TOS	total organic solids
WHO	World Health Organization