Food and Agriculture Organization of the United Nations

89th JECFA - Chemical and Technical Assessment (CTA), 2020

PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C EXPRESSED IN *PSEUDOMONAS FLUORESCENS* (PI-PLC)

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1. ***Summary***

This Chemical and Technical Assessment summarizes data and information on the phosphatidylinositol-specific phospholipase C enzyme preparation from a genetically modified strain of *Pseudomonas fluorescens* (phosphatidylinositol-specific phospholipase C enzyme preparation) submitted to JECFA. This document also discusses published information relevant to the safety of phosphatidylinositol-specific phospholipase C enzyme, including the *P. fluorescens* production organism and details related to the manufacturing, specifications, use and use levels of the enzyme in food. This document uses the expression "phosphatidylinositol-specific phospholipase C" (PI-PLC) to refer to the modified PI-PLC enzyme and its amino acid sequence, and the expression "PI-PLC enzyme preparation" to refer to the product formulated for commercial use.

Phosphatidylinositol-specific phospholipase C catalyses the hydrolysis of phosphatidylinositol to inositol monophosphate and diacylglycerol. The PI-PLC enzyme preparation is used as a processing aid in the degumming (i. e. removal pf phospholipids or phosphatides) of edible vegetable oils, such as soybean, canola, corn and sunflower seed oils; it is intended for use at levels up to 15 milligrams of Total Organic Solids per gram of oil raw material (mg TOS/kg).

P. fluorescens is a common saprophyte and potential plant pathogen that inhabits plant rhizosphere and phyllosphere environments (Chew et al, 2005). It is considered a non-pathogenic and non-toxigenic species to man (OECD, 1997), with a history of safe use in industrial applications.

The production strain, *P. fluorescens* BD27719, was obtained from the parental *P. fluorescens* Biovar I strain, MB101. *P. fluorescens* Biovar MB101 was isolated from the surface of a lettuce leaf. It was identified as *P. fluorescens* Biotype A which is equivalent to *P. fluorescens* Biovar I (Sasser, 1990; Mycogen, 1991) and has been deposited in the American Type Culture Collection (ATCC) as ATCC PTA-7841.

The PI-PLC enzyme preparation is manufactured by a controlled submerged fed-batch fermentation of a pure culture of *P. fluorescens* carrying the PI-PLC gene. The PI-PLC is released from the microbial cells by a heat lysis step. It is subsequently recovered from the fermentation broth and concentrated using multiple filtration techniques. The final enzyme preparation is standardized with glycerol to the desired activity. The PI-PLC enzyme preparation complies with the General Specifications and Considerations for Enzyme Preparations Used in Food Processing (FAO/WHO, 2006).

PI-PLC is not known to be allergenic when used in food processing. Examination of the potential for this enzyme to be a food allergen was made by comparing its amino acid sequence to sequences of known allergens contained within the AllergenOnline allergen database using internationally accepted search criteria. No meaningful identity with known allergens was observed. Based on the results obtained, oral intake of PI-PLC is not anticipated to pose any allergenicity concern.

2. Description

Yellow to brown liquid.

3. Method of manufacture

3.1 P. fluorescens

P. fluorescens is a common gram negative, aerobic, saprophytic, rod-shaped bacterium, widely distributed in the environment (Bradbury, 1986). It can be found in soils, water, plants, animals, the hospital environment, and human clinical specimens; it is a normal inhabitant of the plant rhizosphere or phyllosphere environment (OECD, 1997), and it can grow on a range of organic substrates, remaining viable for long periods of time in a wide variety of habitats. *P. fluorescens* has been taxonomically identified to be from the genus *Pseudomonas.* Strains of *Pseudomonas* are ubiquitous saprophytes, with low virulence (Palleroni, 1990). The genus *Pseudomonas* underwent taxonomic reorganization and many of the strains originally described as species of this genus were reclassified (Kersters et al., 1996). It is generally recognized that the genus *Pseudomonas* (sensu stricto) should be limited to those organisms clustering with *P. aeruginosa* and *P. fluorescens* in the DNA-RNA homology group I, γ-subclass of the *Proteobacteria* (Moore et al., 1996; Kersters et al., 1996; Anzai et al., 2000; US FDA GRAS Notice No. 574).

The taxonomy of *P. fluorescens* is as follows:

Kingdom:	Bacteria
Phylum:	Proteobacteria
Class:	Gammaproteobacteria
Order:	Pseudomonadales
Family:	Pseudomonadaceae
Genus:	*Pseudomonas*
Species:	*P. fluorescens*

P. fluorescens is a non-pathogenic microbe with a long history of safe use in food. It is listed as risk group 1 in the microorganism classification of the German Federal Institute for Occupational Safety and Health (BauA) (Germany, 2015), the German Central Commission for Biological Safety (ZKBS) (Germany, 2018), and the Dutch Commission on Genetic Modification (Netherlands, 2014). The ATCC designated strains of *P. fluorescens* under Biosafety Level 1. *P. fluorescens* does not appear on the list of pathogens in Annex III of Directive 2000/54/EC (EU, 2000) on the protection of workers from risks related to exposure to biological agents at work. *P. fluorescens* has been known to act as an opportunistic pathogen in immune compromised patients. However, *P. fluorescens* is unlikely to be an opportunistic pathogen in warm-blooded animals since it cannot grow at elevated temperatures (OECD, 1997).

3.2 P. fluorescens production strain

Recipient: The parental strain, *P. fluorescens* Biovar I, MB101, isolated from nature, was used to generate the recipient strain DC454. This was accomplished by deletion of the orotidine-5'-phosphate decarboxylase gene (*pyrF*) followed by introduction of one copy of the *lacI* gene from *Escherichia coli* K12. The *lacI* gene was introduced near the levansucrase (*lsc*) locus of *P. fluorescens* Biovar I, MB101, under the control of a $lacI^{Q1}$ promoter.

Donor: The donor organism to the parent PI-PLC gene is not known as the DNA was isolated from a pasture soil sample in Texas, USA. However, the PI-PLC amino acid sequence is known, and it shows >95% similarity to phospholipases from *Bacillus* species.

Expression Plasmid: The PI-PLC gene was modified to improve thermal stability of the encoded enzyme. The gene was codon-optimized to improve expression, synthesized, and cloned into the vector, pDOW1169. The plasmid containing the synthetic codon-optimized PI-PLC gene and the pDOW1169 vector were digested and purified, and the expression plasmid, pDOW1196_BD27719, was generated by ligation of the PI-PLC gene. The insertion of the PI-PLC gene was confirmed by DNA sequencing, followed by sequencing of the pDOW1169_BD27719 plasmid itself. The predicted Open Reading Frames were confirmed to not encode for a protein that is homologous to known toxins or other harmful proteins.

Construction of the Production Strain: The BD27719 production strain was generated by transformation of *P. fluorescens* DC454 with the expression plasmid, pDOW1169_BD27719, carrying the modified PI-PLC gene and the *pyrF* gene as a selective marker. The modified gene was codon-optimized for expression in *P. fluorescens*, inserted into the plasmid pDOW1169_BD27719, and placed under regulation of the *tac* promoter. Expression of the PI-PLC is induced by the addition of isopropylthio-beta-D-galactopyranoside (IPTG) during fermentation. Insertion of the PI-PLC gene and the absence of antibiotic resistance genes were confirmed by DNA sequence analysis.

The stability of the *lacI*^{Q1} gene in the production strain BD27719 was confirmed using colony PCR at the end of the fermentation process. The stability of the expression plasmid pDOW1169_BD2719 was confirmed by quantitative PCR and restriction digestion during the fermentation process. Additionally, there was no significant variation in plasmid copy number throughout the fermentation process.

The PI-PLC enzyme is free of any transformable DNA based on sequence analysis. Like its precursor, pMYC5088, the plasmid pDOW1169_BD27719 is defective in plasmid mobility. The plasmid's mobilization potential and ability to self-transmit were characterized and confirmed to be poor. Additionally, the plasmid demonstrated poor transferability into other bacterial strains.

3.3 Fermentation, recovery, and formulation

PI-PLC is produced by a controlled submerged aerobic fed-batch fermentation of a pure culture of *P. fluorescens* BD27719. The manufacture of PI-PLC enzyme preparation consists of three steps: pre-culture fermentation, seed fermentation, and main fermentation. The fermenter is a contained system that is cleaned, rinsed and sterilized to prevent contamination prior to inoculation. Control measures are in place for physical and chemical quality control during fermentation. Samples are tested for identity, viability and microbial purity at the completion of each primary seed lot.

The fermentation is stopped by incubation with sodium benzoate and the cells are lysed using heat to promote release of the enzyme. The PI-PLC is separated from the cell debris by filtration. The supernatant containing the enzyme is polish- and germ-filtered, followed by concentration. The enzyme concentrate, free of the production strain, is formulated as a liquid PI-PLC enzyme preparation to the desired activity by the addition of glycerol. The entire process is performed in accordance with current Good Manufacturing Practices using raw materials of food grade quality. The final enzyme preparation contains no major food allergens from the fermentation medium.

The PI-PLC enzyme preparation conforms to the General Specifications for Enzyme Preparations used in Food Processing (JECFA, 2006), and the enzyme preparation is tested to be free from the production organism.

4. Identity and Characterization

4.1 PI-PLC

PI-PLC belongs to the subclass of phosphoric di-ester hydrolases. It catalyzes the hydrolysis of the phosphodiester bond linking the glycerol and phosphate moieties at the sn-3 position of the

glycerophospholipid. It is classified by the Enzyme Commission of the International Union of Biochemistry and Molecular Biology (IUBMB) as follows:

Accepted name:	Phosphatidylinositol-specific phospholipase C
Other names(s):	Triphosphoinositide phosphodiesterase; phosphoinositidase C; 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase; monophosphatidylinositol phosphodiesterase; phosphatidylinositol phospholipase C; PI-PLC; 1- phosphatidyl-D-*myo*-inositol-4,5-bisphosphate inositoltrisphosphohydrolase
Reaction:	hydrolysis of the phosphodiester bond linking the glycerol and phosphate moieties at the sn-3 position of glycerophospholipids to produce diacylglycerol and inositol monophosphate
Systematic name:	1-phosphatidyl-1D-*myo*-inositol-4,5-bisphosphate inositoltrisphosphohydrolase
EC No.:	3.1.4.11
CAS No.:	63551-76-8

PI-PLC produced by *P. fluorescens* does not possess any other enzymatic activities. The primary sequence of PI-PLC has been determined to consist of 298 amino acids; its molecular weight after secretion, is ~34 kDa based on SDS-PAGE,

PI-PLC activity is determined spectrophotometrically by measuring the hydrolysis of 4-methyl-umbelliferyl *myo*-inositol-1-phosphate substrate by PI-PLC and the liberation of 4-methyl-umbelliferol, at 380 nm for 300 s; activity is expressed in IPRU (Inositol Phosphate Releasing Unit). One IPRU unit is the quantity of enzyme that will liberate 1 µmole of 4-methylumbelliferone from 18.7mM 4-methylumbelliferyl myo-inositol-1-phosphate per minute under standard conditions at pH 7.5 and 37°C.

4.2 PI-PLC Enzyme Preparation

The PI-PLC enzyme preparation is marketed as a liquid formulation under the trade name Purifine® PI-PLC. A representative composition of a commercial liquid formulation of the PI-PLC enzyme preparation is provided below:

Enzyme concentrate:	57 %
Glycerol:	40 %
Sodium chloride:	2 %
Potassium sorbate:	1 %

A typical composition of the enzyme concentrate is provided below:

Enzyme activity:	≥ 10000 IPRU/g
Water:	84 - 90%
Ash:	0.2 - 1.0%
Proteins:	7 - 14%
TOS:	10 - 17%

The PI-PLC enzyme preparation consists of the enzyme, PI-PLC, and substances from the fermentation process; these constitute proteins, peptides, amino acids, carbohydrates, lipids and salt. The components of fermentation are referred to as Total Organic Solids (TOS).

The TOS content of an enzyme preparation is calculated according to the following equation (NAS/NRC, 1981; FAO/WHO, 2006):

$$TOS\ (\%) = 100 - (A + W + D)$$

where

A is the % ash,
Wis the % water and
D is the % diluents and/or other formulation ingredients.

The specifications for PI-PLC include activity (>10000 IPRU/g), pH, lead (≤ 5 mg/kg), arsenic ≤ 3 ppm), mercury ≤ 0.5 ppm, cadmium ≤ 0.5 ppm, coliforms (≤ 30 CFU/g), total plate count (≤ 50000 CFU/ml), *Salmonella* (absent in 25 g), *E. coli* (absent in 25 g), antimicrobial activity (absent by test), and mycotoxins (absent by test). PI-PLC enzyme preparation complies with the General Specifications for Enzyme Preparations used in Food Processing as established by the 67th meeting of the Joint Expert Committee on Food Additives (FAO/WHO, 2006).

5. Functional Uses

The PI-PLC enzyme preparation is intended to be used as a processing aid to hydrolyse phospholipids in the refining of vegetable oils, such as soybean oil. Refining is a process of purifying crude vegetable oil by the removal non-triacylgylcerol components that affect taste, colour and stability. During refining, the PI-PLC hydrolyses the phosphodiester bond linking the glycerol and phosphate moieties at the sn-3 position of glycerophospholipid (Stryer, 1988) to produce diacylglycerol and inositol monophosphate (Clausen, 2001).

$$\text{Phosphatidylinositol} + H_2O \rightarrow \text{diacylglycerol} + \text{inositol monophosphate}$$

The PI-PLC enzyme preparation is used at a maximum level of 15 μg TOS per gram vegetable oil.

6. Fate in food

PI-PLC is a naturally occurring substance in microorganisms, plants and animal tissues that are commonly ingested by humans. In addition to PI-PLC, the enzyme preparation will contain proteins, peptides, carbohydrates and salts from the fermentation process that are common to the human diet.

PI-PLC is intended for use in degumming edible vegetable oils. After the degumming step, the aqueous phase is separated from the oil by centrifugal separation, effectively removing the enzyme from the oil. Subsequent refining steps involve repeated washing of the oil with water, bleaching and deodorization. These steps will remove any remaining enzyme residues from the final oil. Any PI-PLC in the final refined vegetable oil, if present, will be inactivated and denatured, and at levels close to or below the limit of detection. If present, PI-PLC will be digested, as would any other protein occurring in food. Therefore, use of PI-PLC in oil processing will not have a significant effect on the human body.

7. References

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