



PHOSPHOLIPASE A2 (PLA2) ENZYME PREPARATION FROM *STREPTOMYCES VIOLACEORUBER* EXPRESSING A GENE ENCODING PLA2 FROM *S. VIOLACEORUBER*

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1. Summary

This Chemical and Technical Assessment summarizes data and information on the Phospholipase A2 (PLA2) enzyme preparation from *Streptomyces violaceoruber* expressing a PLA2 gene from the same species (PLA2 enzyme preparation) that was submitted to JECFA. This document also discusses published information relevant to the safety of PLA2 enzyme, including the *S. violaceoruber* production organism and details related to the manufacturing, specifications, use and use levels of the enzyme in food. This document uses the expression “PLA2” to refer to the modified enzyme and its amino acid sequence, and the expression “PLA2 enzyme preparation” to refer to the product formulated for commercial use.

PLA2 catalyses the hydrolysis of the sn-2 ester bonds of diacylphospholipids to form 1-acyl-2-lysophospholipids and free fatty acids. The PLA2 enzyme preparation is used as a processing aid in the manufacture egg yolks and lecithin to improve emulsification properties; in flour to optimise gas cell stability to improve crumb structure and volume; in vegetable oil to provide a degumming effect; and in dairy products to improve fat and protein retention. It is intended for use at levels up to 457 milligrams of Total Organic Solids per kilogram of raw material (mg TOS/kg) as a liquid preparation, and 105 mg TOS/kg raw material as a powder preparation.

The *S. violaceoruber* production organism is also referred to as *S. lividans* or *S. coelicolor*. It has been shown to be non-pathogenic and non-toxigenic, with a history of safe use in industrial applications. (Korn-Wendish and Kutzner 1992; Bergey's Manual, 1994). It occurs in nature as a component of soil (Duangmal et al., 2005), and has a history in the production of enzymes intended for use in food processing (Pariza and Johnson, 2001).

S. violaceoruber strains deposited at public type culture collections have been designated as Safety Level 1. The production strain, *S. violaceoruber* AS-10, was obtained by transforming the host organism *S. violaceoruber* 1326 with a plasmid containing the phospholipase A2 gene from *S. violaceoruber* ATCC 35287.

The PLA2 enzyme preparation is manufactured by submerged controlled fermentation of *S. violaceoruber* AS-10 in accordance with Good Manufacturing Practices (GMP). The PLA2 enzyme is released into the fermentation medium and subsequently recovered and concentrated using multiple filtration techniques. The enzyme is formulated into a liquid preparation with water and sorbitol; potassium sorbate and sodium chloride are added as preservation agents. Alternately, the liquid filtrate can be filtered and freeze dried, followed by standardization and formulation with sodium chloride to produce a powdered enzyme preparation. The PLA2 enzyme preparation complies with the General Specifications and Considerations for Enzyme Preparations Used in Food Processing (FAO/WHO, 2006).

PLA2 is not known to be allergenic when used in food processing. The sponsor examined the potential for this enzyme to be a food allergen by comparing its amino acid sequence to sequences of known allergens contained within the AllergenOnline and Allermatch databases using internationally accepted search criteria. No meaningful identity with known allergens was observed. Based on the results obtained, oral intake of PLA2 is not anticipated to pose any allergenicity concern.

2. Description

Brown powder or brown liquid.

3. Method of manufacture

3.1 *S. violaceoruber*

S. violaceoruber belongs to the genus *Streptomyces*, which is the type genus of the family *Streptomycetaceae*. Bacteria belonging to this genus are mainly found in soil but are also occasionally isolated from manure and other sources. *Streptomyces* show a Gram-positive reaction. *S. violaceoruber* occurs in nature as a component of soil (Duangmal et al., 2005). It is also referred to as *S. lividans* or *S. coelicolor*.

The taxonomic classification of this microorganism is as follows:

Kingdom:	Bacteria
Phylum:	Actinobacteria
Class:	Actinobacteria
Order:	Streptomycetales
Family:	Streptomycetaceae
Genus:	<i>Streptomyces</i>
Species:	<i>Streptomyces violaceoruber</i>

S. violaceoruber is a known source organism for production of enzymes intended for use in food processing (Pariza and Johnson, 2001). Strains of *S. violaceoruber* that have been deposited at public type culture collections have been designated as Safety Level 1. In Europe, *S. violaceoruber* is not included on the list of pathogens Annex III of Directive 2000/54/EC on the protection of workers from risks related to exposure to biological agents at work or on the list of pathogens in Belgium (EC, 2000; Belgian Biosafety Server, 2008). The host organism, *S. violaceoruber* 1326, was confirmed to be non-toxicogenic and non-pathogenic by the French Haut Conseil des Biotechnologies (HCB), and was classified as self-cloned and in Class 1. Strains of *S. violaceoruber* that have been deposited in public type culture collections, such as American Type Culture Collection (ATCC) and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), have been designated as Safety Level 1. *S. violaceoruber* is globally regarded as a safe microorganism (EC, 2000; Belgian Biosafety Server, 2008). A search of the scientific literature identified publications reporting other *Streptomyces* species, such as *S. somaliensis*, *S. madurae*, and *S. sudanensis*, as pathogenic (Boiron et al., 1998; Khatri et al., 2002; Dieng et al., 2003, 2005; Quintana et al., 2008), but no such report of pathogenicity of *S. violaceoruber* were identified in the scientific literature.

S. violaceoruber is a recognised source for food enzyme preparations, such as phospholipase A2 and beta-glucanase, that have Generally Recognized as Safe (GRAS) status for use in food in the U.S. (U.S. FDA, 2004, 2007, 2014).

3.2 *S. violaceoruber* production strain

The *S. violaceoruber* AS-10 production strain was obtained by transforming a plasmid containing an expression cassette with the phospholipase A2 encoding gene from the *S. violaceoruber* NBRC 15146

donor, a suitable promoter and terminator encoding phospholipase D from *S. cinnamomeum* and a selectable marker, ligated with a plasmid obtained from *S. violaceoruber* ATCC 35287. The resulting plasmid was incorporated into the host organism, *S. violaceoruber* 1326, by electroporation. The stability of the introduced sequences was confirmed by cultivating the production strain over three generations and by measuring phospholipase A₂ activity each time. The final enzyme preparations were tested for the absence of an antibiotic resistance gene by PCR. The production strain has been deposited at the National Institute of Technology and Evaluation in Japan.

3.3 Fermentation, recovery, and formulation

PLA₂ is produced by controlled submerged fermentation of a pure culture of *S. violaceoruber* AS-10. The manufacture of the PLA₂ enzyme preparation consists of three steps: fermentation (pre, seed and main fermentation), recovery, and formulation. Control measures are in place for physical and chemical quality control during fermentation. Samples are tested for identity, viability, and microbial purity. All raw materials used in the manufacture of PLA₂ enzyme preparation are food-grade.

Following fermentation, the culture broth containing the enzyme is separated from the biomass that consists of the production organism, other microbials, and spent fermentation medium, by sedimentation with ammonium sulphate, followed by several filtration steps (vacuum drum and germ filtration). The liquid filtrate containing the enzyme is formulated with water and sorbitol, and potassium sorbate and sodium chloride are added as preservation agents. The liquid filtrate is formulated with water, sorbitol, potassium sorbate, and sodium chloride to obtain the liquid PLA₂ enzyme preparation. Alternatively, the liquid filtrate is freeze dried and formulated with sodium chloride to a powdered PLA₂ enzyme preparation. The entire process is performed in accordance with current Good Manufacturing Practices using raw materials of food grade quality. The final powdered enzyme preparation was tested for absence of any major food allergens from the fermentation medium, and these results were applicable to the liquid preparation as well. The enzyme concentrate was also tested to be free from the production organism.

4. Identity and Characterization

4.1 PLA₂

PLA₂ catalyses the hydrolysis of the sn-2 ester bonds of diacylphospholipids to form 1-acyl-2-lysophospholipid and free fatty acid. It is classified by the Enzyme Commission of the International Union of Biochemistry and Molecular Biology (IUBMB) as follows:

Accepted name:	Phospholipase A ₂
Other name(s):	lecithinase A; phosphatidase; phosphatidolipase; phospholipase A
Reaction:	phosphatidylcholine + H ₂ O = 1-acylglycerophosphocholine + a carboxylate Also acts on phosphatidylethanolamine, choline plasmalogen and phosphatides, removing the fatty acid attached to the 2 position. Requires Ca ²⁺
Systematic name:	phosphatidylcholine 2-acylhydrolase
EC No.:	3.1.1.4
CAS No.	9001-84-7

PLA₂ produced by *S. violaceoruber* is not known to have any subsidiary or secondary enzymatic activities. The primary sequence of PLA₂ has been determined to consist of 151 amino acids; its molecular weight by calculation from the determined amino acid sequence is 16.4 kDa.

PLA₂ activity is determined spectrophotometrically by measuring the hydrolysis of phosphatidylcholine substrate by PLA₂ at 550 nm; 1 unit of activity is defined as the quantity of enzyme required to liberate 1 μmol of fatty acid from L- α -phosphatidylcholine per minute under the conditions

of the assay. The mean activities of phospholipase A2 from three batches of the liquid and the powder enzyme concentrates are 10 400 U/g and 114 200 U/g, respectively.

4.2 PLA2 Enzyme Preparation

The PLA2 enzyme preparation consists of the enzyme, PLA2, 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,

W is the % water and

D is the % diluents and/or other formulation ingredients.

The PLA2 enzyme preparation is marketed as a powder or liquid formulation under the trade names PLA2 Nagase 10P or PLA2 Nagase, respectively. A representative composition of a commercial liquid formulation of the PLA2 enzyme preparation is provided below:

Enzyme TOS:	5.6 %
Ash:	9.5 %
Water:	59.9 %
Excipients*:	25 %
*= Sorbitol, Sodium chloride and Potassium sorbate	

A representative composition of the powdered PLA2 enzyme preparation is provided below:

Enzyme TOS:	11.1 %
Ash:	88 %
Water:	0.9 %
Sodium chloride:	80%

The specifications for the liquid PLA2 preparation include activity (8500-11000 U/g), appearance, lead (≤ 5 mg/kg), arsenic ≤ 3 ppm, mercury ≤ 0.5 ppm, cadmium ≤ 0.5 ppm, coliforms (≤ 30 CFU/g), total viable aerobic count (≤ 50000 CFU/g), *Salmonella* (negative in 25 g), *E. coli* (negative in 25 g), *Staphylococcus aureus* (negative in 1g), sulphur-reducing anaerobe (<30 CFU/g), antimicrobial activity (absent by test), and loss on drying.

The specifications for the powdered PLA2 preparation include activity (100,000-120,000 U/g), appearance, lead (≤ 5 mg/kg), arsenic ≤ 4 ppm, mercury ≤ 0.5 ppm, cadmium ≤ 0.5 ppm, coliforms (≤ 30 CFU/g), total viable aerobic count (≤ 50000 CFU/g), *Salmonella* (negative in 25 g), *E. coli* (negative in 25 g), *S. aureus* (negative in 1g), sulphur-reducing anaerobe (<30 CFU/g), antimicrobial activity (absent by test), and loss on drying (0.7%).

PLA2 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 PLA2 enzyme preparation is intended to be used as a processing aid in the production of enzyme-modified egg yolk and lecithin, to hydrolyze triglycerides and phospholipids in cereal flour, dairy products, and vegetable oil. The PLA2 in all the applications will be inactivated by heat treatment prior to use of the final foods. The PLA2 enzyme preparation is used at a maximum level of 105 mg TOS/kg raw material (as a powder), and 459 mg TOS/kg raw material (as a liquid).

6. Fate in food

PLA2 is a naturally occurring substance in microorganisms, plants and animal tissues that are commonly ingested by humans. In addition to PLA2, the enzyme preparation will contain proteins, peptides, carbohydrates and salts from the fermentation process that are common to the human diet.

PLA2 enzyme preparation is intended to be used in the manufacture of food ingredients that may be added to chocolate, cocoa-based products, sauces such as mayonnaise, cheese, yogurt, milk, and ice cream, flour, and vegetable oil, that are intended to be consumed by the general population. While it is assumed that the PLA2 is carried over to final foods, the enzyme is inactivated and denatured during processing by treatment at high temperatures and is not expected to have any technical effect on the final food. If present, PLA2 will be digested, as would any other protein occurring in food. Therefore, use of PLA2 in the processing of food categories described will not have a significant effect on the human body.

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