PHOSPHOLIPASE C EXPRESSED IN *PICHIA PASTORIS*
Chemical and Technical Assessment (CTA)

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1. Summary

This Chemical and Technical Assessment summarizes data and information on the phospholipase C enzyme preparation submitted to JECFA by Verenium Corporation[1] in a dossier dated December 12, 2007 (Verenium, 2007). It also discusses published information relevant to the production organism, *Pichia pastoris*.

Phospholipase C catalyzes the hydrolysis of the *sn*-3 phosphate head group in phospholipids including phosphatidylcholine, phosphatidyletanolamine, and phosphatidylserine with the formation of 1,2-diacylglycerol and water soluble phosphate esters phosphorylcholine, phosphorylethanolamine, and phosphorylserine. Phospholipase C from *P. pastoris* is intended for use in the refining of soybean oil and other vegetable oils used in food (Ciofalo et al., 2006). The enzyme would be added to the crude oil at levels sufficient to hydrolyse phospholipids. The resulting phosphate esters would be removed from the oil, while 1,2-diacylglycerol would remain in the oil. The residues of phospholipase C would also be removed from the oil during subsequent purification steps.

Certain microbial phospholipases are known to possess haemolytic activity. Phospholipase C from *P. pastoris* was shown to be devoid of the amino acid sequence associated with haemolytic activity. The absence of haemolytic activity was also confirmed using an appropriate haemolysis assay. To assess potential allergenicity, the amino acid sequence of phospholipase C was compared to the sequences of known allergens. No amino acid homology that would suggest that phospholipase C is an allergen was detected.

Phospholipase C is manufactured by pure culture fermentation of a genetically modified strain of *P. pastoris*, which expresses the phospholipase C gene derived from a soil environmental DNA library. *P. pastoris* is a methylotrophic yeast that can utilize methanol for growth. It is not known to be either pathogenic or toxigenic and has not been associated with human or animal disease. *P. pastoris* has been used in the production of recombinant proteins for therapeutic and research applications.

The phospholipase C production strain was constructed by transformation of the *P. pastoris* host strain SMD1168 with a purified DNA fragment containing 6 copies of the expression cassette. Each expression cassette consisted of the phospholipase C gene, regulatory DNA sequences promoter and terminator from the *P. pastoris AOX1* (alcohol oxidase) gene, and the α-mating factor sequence from *Saccharomyces cerevisiae* (baker's yeast), which directs secretion of phospholipase C to the fermentation broth. The DNA fragment used in transformation also contained the *P. pastoris HIS4* gene and the *P. pastoris AOX1 3'* gene fragment. The *HIS4* gene encodes histidinol dehydrogenase and serves as a selectable marker to identify the transformed cells. The *AOX1 3'* gene fragment facilitates DNA insertion at the *AOX1* locus by homologous recombination.

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The transformed strain was shown to contain 12 expression cassettes, all integrated at the *AOX1* locus. The strain was designated DVSA-PLC-004 and used as the phospholipase C production strain. The strain complies with the criteria suggested by the Organisation for Economic Co-operation and Development for r-DNA GILSP (Good Industrial Large Scale Practice) microorganisms and cell cultures (OECD, 1992).

Phospholipase C is produced during fermentation of the production strain and is secreted to the fermentation broth. The cells are initially grown in a fermentation medium containing glycerol as a primary carbon source. After the cellular mass has reached the desired density, the expression of phospholipase C is induced by the addition of methanol. The residual methanol is subsequently removed from the fermentation broth during the purification and concentration process. The purified enzyme concentrate is formulated with appropriate food-grade ingredients. The formulated enzyme preparation complies with the General Specifications and Considerations for Enzyme Preparations Used in Food Processing (JECFA, 2006). It is marketed under the trade name Purifine™ for use as a processing aid in the refining of soybean oil and other vegetable oils intended for use in food.

2. Description

Yellow to brown liquid.

3. Method of manufacture

3.1. Pichia pastoris

P. pastoris is a methylotrophic yeast that can utilize methanol as a sole source of carbon. During the 1980s, *P. pastoris* has been developed for heterologous protein expression (Cregg et al., 1985; Cereghino and Cregg, 2000; Cereghino et al., 2001; Cereghino et al., 2002). Since then, *P. pastoris* has been successfully used for expression of numerous recombinant proteins mainly for research and pharmaceutical applications (Cereghino and Cregg, 2000; Herbst et al., 2002; Hussain et al., 2005; Malkin et al., 2005). *P. pastoris* is not known to be either pathogenic or toxigenic and has not been associated with human or animal illness. In 1993, the U.S. Food and Drug Administration approved *P. pastoris* as a source of protein in broiler feed (FDA, 1993).

3.2. Phospholipase C production strain

The phospholipase C production strain, DVSA-PLC-004, is a genetically modified derivative of strain SMD1168, which was used as a host for the phospholipase C gene. Strain SMD1168 contains two mutations, *his4* and *pep4*. The *his4* mutation allows the selection of transformed cells without the use of antibiotic resistance markers, while the *pep4* mutation prevents the production of proteinase A, which could potentially degrade phospholipase C.

The SMD1168 host strain is a descendant of a wild-type strain discovered in the 1970s by Philips Petroleum Co. and identified as *Komagataella pastoris*. The strain was deposited in the National Regional Research Laboratory Culture Collection (Peoria, IL, USA; now Agricultural Research Service Culture Collection, National Center for Agricultural Utilization and Research) as strain NRRL Y-11430. The strain was subsequently transferred to the American Type Culture Collection (Gaithersburg, MD, USA) as strain ATCC-76273. The mutant derived from strain NRRL Y-11430, known as *P. pastoris* strain GS115, was deposited in ATCC as strain ATCC-20864. Strain SMD1168 is a mutant derived from the ATCC-20864 strain. Strain SMD1168 and

its predecessors can utilize methanol for growth due to the presence of two genes, *AOX1* and *AOX2*, both encoding alcohol oxidase. One of these genes, *AOX1*, was used as a target for insertion of a DNA fragment carrying the phospholipase C gene.

The phospholipase C production strain was constructed by transformation of the host strain SMD1168 with a purified DNA fragment isolated from the expression vector pAO815-6xBD16449. The expression vector was constructed in *Escherichia coli* on the basis of a commercially-available plasmid vector pBK-CMV. The DNA fragment used in transformation contained six expression cassettes multimerized in a head to tail fashion. Each cassette consisted of the phospholipase C gene and the following regulatory sequences: 1) the *P. pastoris AOX1* promoter for methanol-inducible expression of phospholipase C; 2) the *S. cerevisiae* α-factor secretion signal for directing phospholipase C to the fermentation broth; and 3) the *P. pastoris AOX1* gene transcriptional termination sequence. The DNA fragment also contained: 1) the *P. pastoris HIS* gene for selection of transformed cells by complementing the *his4* mutation in the host strain; 2) the *P. pastoris AOX1 3'* gene fragment for the integration of the DNA fragment into the *AOX1* locus by homologous recombination; and 3) short vector sequences with no function.

After transformation, a single colony transformant was selected and designated DVSA-PLC-004. This clone, which is referred to as the phospholipase C production strain, was used to create the "Master Cell Bank" for phospholipase C production. The genomic DNA isolated from the production strain was subjected to Southern blot analysis. The analysis showed that the strain contains 12 expression cassettes, all integrated by homologous recombination into the *AOX1* locus. The vector sequences necessary for vector replication and selection in *E. coli* (including the origin of replication and an antibiotic resistance marker) were separated from the DNA fragment intended for transformation and were not introduced into the phosholipase C production strain. The strain complies with the criteria suggested by the Organisation for Economic Co-operation and Development for r-DNA GILSP (Good Industrial Large Scale Practice) microorganisms and cell cultures (OECD, 1992).

The phospholipase C gene was derived from an environmental DNA library constructed in *E. coli* using DNA purified from a soil sample. The DNA fragment initially isolated from the soil environmental library consisted of sequences encoding a secretion signal and a pro-sequence in addition to the sequence encoding the mature phospholipase C. The DNA encoding the secretion signal and pro-sequence was deleted. The deletion resulted in a phospholipase coding sequence of 735 base pairs that encodes only the mature enzyme. It should be noted that the coding sequence of the mature enzyme lacks the carboxy-terminal domain responsible for the haemolytic activity characteristic of certain microbial phospholipases. In order to improve phospholipase C activity, the coding sequence was modified by site-specific mutagenesis. These changes resulted in three amino acid substitutions in the mature enzyme. The modified gene was designated BD16449 PLC gene.

3.3. Fermentation, recovery, and formulation

Phospholipase C is manufactured by fermentation of the production strain using the fermentation medium composed of food-grade materials such as glycerol, salts of ammonium, potassium or magnesium, trace minerals, and vitamins. The cells are initially grown on glycerol as a primary carbon source. After the cellular mass has reached the desired density, the expression of phospholipase C is induced by the addition of methanol. Methanol also serves as a carbon source to maintain the cell growth. The fermentation process is monitored and controlled by a computerized control system to maintain appropriate pH, dissolved oxygen content, temperature,

airflow, pressure, and agitation. During fermentation, phospholipase C is secreted to the fermentation broth. After 80-200 hours of cultivation, the process is terminated and the fermentation broth is chilled. Phospholipase C is subsequently recovered from the broth. The recovery process includes five major steps: (1) cell separation; (2) clarification; (3) ultrafiltration; (4) heat treatment; (5) diafiltration; and (6) polish filtration. The enzyme concentrate is subsequently formulated to a desired activity. Stabilizers and preservatives are added to stabilize the final product. The formulated enzyme preparation is stored at 4-8°.

At the termination of the fermentation process, the residual methanol in the fermentation broth is within the range of 0.01-0.05 g/l. The preponderance of methanol would be removed during ultrafiltration. Methanol has a molecular weight of 32.04 and it would not be retained by the ultrafiltration membrane, which has a molecular mass cutoff of 10 kDa. It is estimated that the level of residual methanol after ultrafiltration would range from 0.09 to 0.5 mg/l. Methanol residues would be further reduced (or eliminated) during heat treatment. The heat treatment is preformed at a temperature above the boiling point of methanol. The phospholipase C enzyme preparation was tested for the presence of residual methanol using gas chromatography. Although methanol was detected, its level was below the quantification limit of the GC method of 9 mg/l. For comparison, methanol levels in fruit and vegetable juices range from 12 to 640 mg/l.

4. Characterization

4.1. Phospholipase C

Phospholipase C catalyzes the hydrolysis of phospholipids. It acts on the ester bond linking glycerol and the phosphate head group at the *sn*-3 position in phospholipids including phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phospahtidylserine (PS). The reaction products include 1,2-diacylglycerol and the corresponding phosphate esters, phosphorylcholine, phosphorylethanolamine, and phosphorylserine. The Chemical Abstract Service Registry Number (CAS No.) of phospholipase C is 9001-86-9. Phospholipase C is classified by the Enzyme Commission of the International Union of Biochemistry and Molecular Biology (IUBMB, online edition) as follows:

Accepted name:	phospholipase C
Other name(s):	lipophosphodiesterase I; lecithinase C; lipophosphodiesterase C; phosphatidase C[2]
Reaction:	phosphatidylcholine + H_2O → 1,2-diacylglycerol + choline phosphate
Systematic name:	phosphatidylcholine cholinephosphohydrolase
EC number:	3.1.4.3

Phospholipase C activity is determined by measuring the rate of hydrolysis of phosphatidylcholine to phosphorylcholine and 1,2-diacylglycerol. Phosphorylcholine is subsequently titrated with potassium hydroxide. Phospholipase C activity is expressed in phospholipase C units (PLCUs). One PLCU is defined as the quantity of the enzyme that hydrolyses 1 μmol phosphatidylcholine per minute under standard conditions (pH=7.3; 37°).

[2] IUBMB also lists several microbial phospholipases, including *Clostridium welchii* α-toxin, *Clostridium oedematiens* β- and γ-toxins, heat-labile hemolysin, and α-toxin, which have haemolytic activity. Phospholipase C described in the subject dossier does not have the C-terminal domain responsible for haemolytic activity.

Phospholipase C expressed in *P. pastoris* consists of 245 amino acids. Its amino acid sequence is 82% identical to the sequence of phospholipase C from *Bacillus cereus*. *B. cereus* phospholipase C also consists of 245 amino acids and is similar to mammalian phospholipases. Neither the *P. pastoris* nor the *B. cereus* enzyme contains the carboxy-terminal domain responsible for haemolytic activity characteristic of certain microbial phospholipases. To confirm the absence of haemolytic activity, the haemolysis assay was performed on *P. pastoris* phospholipase C using *Clostridium perfringens* phospholipase C as a positive control. No haemolytic activity was detected for *P. pastoris* phospholipase C.

Phospholipase C has also been evaluated for potential allergenicity. According to the Report of the Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology (FAO/WHO, 2001), one of the tools for assessing potential allergenicity is the comparison of the amino acid sequence of the protein in question to the sequences of known allergens. Cross-reactivity should be considered if the protein has either more that 35% sequence identity in any amino acid window of 80 amino acids, or shows identity of short stretches of amino acids. The amino acid sequence of phospholipase C was compared to the sequences of known allergens in the Food Allergy Research and Resource Program (FARRP) allergen database (http://www.allergenonline.com). No sequence homology that would suggest cross-reactivity of phospholipase C with known allergens was detected.

4.2. Phospholipase C enzyme preparation

The *P. pastoris* phospholipase C enzyme preparation is marketed under the trade name Purifine™. The activity of the product is not less than 27,500 PLCU/g. The typical composition of the enzyme preparation is (approximately):

Total Organic Solids (TOS)	7%
Glycerol	35%
Sodium acetate	0.15%
Zinc sulfate	0.03%
Methyl paraben	0.1%
Potassium sorbate	0.15%
Water	Balance of the formulation

TOS is expressed in percent and is defined as follows:

TOS (%) = 100 – (A + W + D)

where:
A = % ash, W = % water and D = % diluents and/or other formulation ingredients (NAS/NRC, 1981; JECFA, 2006). TOS consists of the enzyme of interest and residues of organic materials, such as proteins, peptides, and carbohydrates, derived from the production organism and the manufacturing process.

The phospholipase C enzyme preparation complies with the General Specifications and Considerations for Enzyme Preparations Used in Food Processing (JECFA, 2006). It does not contain coding sequences of recombinant DNA. This has been established using Southern dot blot analysis with the detection limit of 0.01 picograms of recombinant DNA.

5. Functional uses

Phospholipase C will be used in degumming of vegetable oils intended for human consumption (Ciofalo, et al., 2006). Degumming is the first step in the refining of crude oil. The subsequent steps include bleaching and deodorization. In the standard degumming procedure, water and acid are used to remove phospholipids. In enzymatic degumming, phospholipase C will be added to the crude oil under conditions commonly used in the refining of edible oils, i.e., heating at 60°C in the presence of water (2-4%). Under these conditions, phospholipase C will hydrolyse the major oil phospholipids, phosphatidylcholine and phosphatidylethanolamine. The resulting esters, phosphorylcholine and phosphorylethanolamine, will be solubilised in water and removed from the oil by centrifugation. 1,2-Diacylglycerol, which is also formed during the hydrolysis, will remain in the oil.

The phospholipase C enzyme preparation will be added to crude vegetable oils, such as soybean, corn, canola, rape, and sunflower, at levels no higher than necessary to achieve an intended effect, generally in the range of 100 to 1000 grams of the preparation per metric ton of oil, depending on the oil to be treated and the reaction conditions.

6. Reactions and fate in food

After the degumming reaction has been completed, the aqueous phase containing phospholipase C, is separated from the oil by centrifugation. The subsequent steps used in refining, i.e., repeated washing of the oil with hot water, bleaching, and deodorization, will remove the residual enzyme. An ELISA sandwich assay was developed to detect and quantify the residual phospholipase C in the refined oil. The enzyme was not detected in the oil at the detection limit of the ELISA sandwich assay, which is one microgram of the enzyme per one kilogram of the oil.

7. References

Cereghino, G.P.L., Cereghino, J.L., Ilgen, C., and Cregg, J.M., 2002. Production of recombinant proteins in fermenter cultures of the yeast, *Pichia pastoris*. Curr. Opinion Biotechnol. 13, 329-332.

Cereghino, J.L. and Cregg, J.M., 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris.* FEMS Microbiol. Rev. 24,45-66.

Cereghino, G.P.L., Sunga, A.J., Lin, C.J., and Cregg, J.M., 2001. Expression of foreign genes in the yeast, *Pichia pastoris*. In *Genetic Engineering: Principles and Methods*. Edited by J.K. Setlow, Kluver Academic/Plenum Publishers, London. Vol. 23, 157-169.

Ciofalo, V., Barton, N., Kreps, J., Coats, I., and Shanahan, D., 2006. Safety evaluation of a lipase enzyme preparation, expressed in *Pichia pastoris*, intended for use in the degumming of edible vegetable oil. Regul. Toxicol. Pharmacol. 45, 1-8.

Cregg, J.M., Barringer, K.J., Hessler, A.Y., and Madden, K.R., 1985. *Pichia pastoris* as a host system for transformations. Mol. Cell. Biol. 5, 3376-3385.

FAO/WHO, 2001. Evaluation of Allergenicity of Genetically Modified Foods. Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology, 22-25 January 2001, Food and Agricultural Organization of the United Nations (FAO), Rome, Italy. (http://www.who.int/foodsafety/publications/biotech/ec_jan2001/en/print.html)

FDA, 1993. 21 CFR Part 573, Food additives permitted in feed and drinking water of animals. Sec. 573.750. *Pichia pastoris* dried yeast. [58 FR 59170, Nov. 8,1993].

Herbst, R.S., Hess, K.R., Tran, H.T., et al., 2002. Phase 1 study of recombinant human endostatin in patiens with advanced solid tumors. J. Clin. Oncol. 20, 3792-3803.

Hussain, Z., Ali, S.S., Hussain, S.A., Raish, M., Sharma, D.R., and Kar, P., 2005. Evaluation of immunogenicity and reactogenicity of recombinant DNA hepatitis vaccine produced in India. World J. Gastroenterol. 11, 7165-7168.

IUBMB, online edition. International Union of Biochemistry and Molecular Biology. (http://www.chem.qmul.ac.uk/iubmb/enzyme/search.html)

JECFA, 2006. Joint FAO/WHO Expert Committee on Food Additives. "General Specifications and Considerations for Enzyme Preparations Used in Food Processing." Prepared at the 67th meeting (Rome, Italy, 20-29 June 2006). FAO JECFA Monographs 1, Vol. 4. Online edition: http://www.fao.org/ag/agn/jecfa-additives/search.html?lang=en

Malkin, E.M., Diemert, D.J., McArthur, J.H., et al., 2005. Phase 1 clinical trial of apical membrane antigen 1: an asexual blood-stage vaccine for *Plasmodium falciparium* malaria. Infect. Immun. 73, 3677-3685.

NRC/NAS, 1981. The 1978 Enzyme Survey Summarized Data. National Research Council/National Academy of Sciences. Washington, D.C., USA. U.S. Department of Commerce. National Technical Information Service.

OECD, 1992. Safety Considerations for Biotechnology 1992. Organisation for Economic Co-operation and Development. Part One. Elaboration of Criteria and Principles for Good Industrial Large-Scale Practice (GILSP). (http://www.oecd.org/dataoecd/8/3/2375496.pdf)

Verenium, 2007. "Phospholipase C enzyme preparation from *Pichia pastoris* expressing a heterologous phospholipase C gene." December 2007. A dossier submitted to JECFA for consideration at the 69th meeting. Rome, Italy, 17-26 June 2008.