# PHOSPHOLIPASE C EXPRESSED IN *PICHIA PASTORIS*

*New specifications prepared at the 69th JECFA (2008), published in FAO JECFA Monographs 5 (2008). An ADI "not specified" was established at the 69th JECFA (2008).*

## SYNONYMS

Phospholipase C; lecithinase C; lipophosphodiesterase C; phosphatidase C

## SOURCES

Phospholipase C is produced by submerged fed-batch fermentation of a genetically modified strain of *Pichia pastoris* which contains the phospholipase C gene derived from a soil sample. The enzyme is recovered from the fermentation broth. The recovery process includes the separation of cellular biomass, clarification, ultrafiltration, diafiltration, and polish filtration. The final product is formulated using food-grade stabilizing and preserving agents and is standardized to the desired activity.

Active principles: Phospholipase C

Systematic names and numbers: Phosphatidylcholine cholinephosphohydrolase; EC 3.1.4.3; CAS No. 9001-86-9

Reactions catalysed: Hydrolysis of phosphodiester bonds at the *sn*-3 position in glycerophospholipids including phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine to yield 1,2-diacylglycerol and the corresponding phosphate esters

Secondary enzyme activities: No significant levels of secondary enzyme activities.

## DESCRIPTION

Yellow to brown liquid

## FUNCTIONAL USES

Enzyme preparation.
Used in refining vegetable oils intended for human consumption.

## GENERAL SPECIFICATIONS

Must conform to the latest edition of the JECFA General Specifications and Considerations for Enzyme Preparations Used in Food Processing.

## CHARACTERISTICS

IDENTIFICATION

Phospholipase C activity: The sample shows phospholipase C activity.
See description under TESTS.

## TESTS

Enzyme activity

**Principle**

Phospholipase C catalyses the hydrolysis of phosphatidylcholine to 1,2-diacylglycerol and phosphorylcholine. Phosphorylcholine is subsequently titrated with potassium hydroxide. The activity of phospholipase C is determined by measuring the rate of consumption of potassium hydroxide required to maintain pH 7.3 at 37°.

The enzyme activity is expressed in phospholipase C units (PLCU). One phospholipase C unit is defined as the quantity of the enzyme that will hydrolyse 1 µmol phosphatidylcholine per minute under standard conditions (pH=7.3; 37°).

**Apparatus**

Auto-titrator (Brinkmann Instruments, Titrandos® 835 or equivalent)
pH meter (Beckman Coulter, model F350 or equivalent)
Homogenizer (M133/1281-0, 2-speed, BioSpec Products, catalog # 1281, or equivalent)
Circulating water bath

**Reagents and solutions**

(NOTE: use deionized water)

*Potassium hydroxide (0.01 N):* 0.01 N KOH certified titration reagent (Brinkmann Instruments 019091104 or equivalent). Use for titration of phosphorylcholine in the phospholipase C activity assay.

*Zinc sulfate solution (100 mM)*: Weigh 2.88 g of zinc sulfate heptahydrate (crystalline, certified ACS) and dissolve in water in a 100-ml volumetric flask. Add water to volume. The solution is stable for up to 30 days at room temperature.

*Calcium chloride solution (100 mM):* Weigh 1.47 g of calcium chloride dihydrate (certified ACS) and dissolve in water in a 100-ml volumetric flask. Add water to volume. The solution is stable for up to 30 days at room temperature.

*Triton X-100 solution (approximately 10%):* Weigh 10 g of Triton X-100 (Sigma-Aldrich T9284 or equivalent) into a 200-ml beaker. Add 100 ml of water and mix for at least 1 hr on a rotating table. The solution is stable in a closed container for up to 30 days at room temperature.

*Substrate solution (20 mM phosphatidylcholine, approximately 2.5% Triton X-100, 5 mM calcium chloride):* Weigh 3.24 g of phosphatidylcholine (Phospholipon 90G (containing at least 94% phosphatidylcholine), American Lecithin Company or equivalent) into a 500 ml beaker. Add 50 ml of 10% Triton X-100 solution and 10.0 ml of 100 mM calcium chloride solution. Adjust volume to 200 ml with water and mix. Homogenize the solution using a hand-held homogenizer at low setting (7,000 rpm) for approx. 45 sec or until a uniform dispersion is obtained. Check the pH and, if necessary,

adjust to the range of 6.5-7.0 using 0.2 N sodium hydroxide solution (certified, Fisher Scientific SS274-1 or equivalent). The solution should be prepared on the day of testing.

*Dilution buffer (0.1% Triton X-100, 1 mM zinc sulfate, 1% gum arabic):* Weigh 0.5 g of Triton X-100 and 5.0 g of gum arabic (Sigma-Aldrich G9752 or equivalent). Dissolve with stirring in 450 ml of water in a 1000 ml beaker. Add 5 ml of 100 mM zinc sulfate solution and adjust the pH to the range 7.0-7.2 using 0.2 N sodium hydroxide solution. Transfer to a 500 ml volumetric flask and add water to volume. The solution is stable for up to 30 days at 4°.

*Sample solution:* Weigh to ±0.1 mg approximately 1 g of the phospholipase C enzyme preparation into a 50 ml volumetric flask. Add the dilution buffer to volume and mix. Dilute with the dilution buffer to obtain a solution with an activity of approximately 12 PLCU/ml. The solution should be prepared on the day of testing.

**Procedure**

1. Program the titrator to maintain the pH 7.3 and measure the consumption of 0.01 N KOH in milliliters per minute.
2. Set the temperature of the recirculating water bath at 37°.
3. Calibrate the pH electrode at pH 4, 7, and 10.
4. Transfer 20 ml of the substrate solution into the water-jacketed titration vessel of 50 ml capacity connected to the recirculating water bath, cover with the lid and stir.
5. Allow the substrate solution to equilibrate to 37°.
6. Start the titration program.
7. The titrator will adjust the pH of the substrate solution to 7.3 using 0.01 N KOH.
8. Add 50 µl of the sample solution.
9. Allow the titration to proceed automatically. The titrator will record the titration curve and calculate the slope. The slope between 2 and 6 minutes is used by the titrator to calculate the phospholipase C activity. Alternatively, the calculation can be performed manually.

NOTE: The slope must be within 0.02-0.1 ml/min. If the slope is outside this range or if the titration has not started within the first two minutes, adjust the activity of the sample solution.

**Calculation**

Use the following formula for manual calculation of phospholipase C activity:

$$\text{Activity (PLCU/g)} = \frac{V \times DF \times S \times N \times 1000}{V_s \times W}$$

Where:

V is the initial volume of the sample solution (50 ml)

DF is the dilution factor

S is the slope of the titration curve (ml/min)

N is the normality of potassium hydroxide (0.01 mmol/ml)

1000 is the conversion factor from millimoles to micromoles

$V_s$ is the volume of the sample solution used in the assay (0.05 ml)

W is the sample weight (g)