PHOSPHOLIPASE A1 from *FUSARIUM VENENATUM* expressed in *ASPERGILLUS ORYZAE*

New specifications prepared at the 65th JECFA (2005) and published in FNP 52 Add 13 (2005). No ADI was allocated at the 65th JECFA (2005)

SYNONYMS

Phospholipase A1

SOURCES

Produced by submerged fed-batch pure culture fermentation of a genetically modified strain of *Aspergillus oryzae* containing the phospholipase A1 gene derived from *Fusarium venenatum*. The enzyme is isolated from the fermentation broth by filtration to remove the biomass and concentrated by ultrafiltration and/or evaporation. Residual production microorganisms are removed from the enzyme concentrate by germ filtration. The final product is formulated using food-grade stabilizing and preserving agents.

Active principles

Phospholipase A1

Systematic names and numbers

Phosphatidylcholine 1-acylhydrolase; EC 3.1.1.32; CAS No. 9043-29-2

Reactions catalysed

Hydrolysis of the sn-1 ester bond of diacylphospholipids to form 2-acyl-1-lysophospholipids and free fatty acids

DESCRIPTION

Brown liquid.

FUNCTIONAL USES

Enzyme preparation.
Used in cheese production to reduce the loss of fat and milk solids and increase cheese yield.

GENERAL SPECIFICATIONS

Must conform to the latest edition of the JECFA General Specifications and Considerations for Enzyme Preparations used in Food Processing (see Volume Introduction)

CHARACTERISTICS

IDENTIFICATION

Phospholipase A1 activity

The sample shows phospholipase A1 activity
See description under TESTS

TESTS

Phospholipase A1 activity

Principle

Phospholipase A1 activity is measured relative to a phospholipase standard using lecithin as a substrate. Phospholipase A1 catalyses the hydrolysis of lecithin to lyso-lecithin and a free fatty acid. The liberated fatty acid is titrated with 0.1 N sodium hydroxide under standard conditions (pH=8.0; 40^0 ±0.5). The activity of phospholipase A1 is determined as the rate of sodium hydroxide consumption during neutralization of the fatty acid and is expressed in Lecitase units (LEU) relative to a Lecitase (phospholipase) standard.

1 LEU is defined as the amount of enzyme that under standard conditions (pH=8.0; 40^0 ±0.5) results in the same rate of sodium hydroxide consumption (in microeq/min) as the Lecitase standard diluted to a nominal activity of 1

LEU/g. The quantification limit of the method is approximately 1.5 LEU/ml. (Note: The method can be carried out using either an automated system or standard laboratory equipment for carrying out titration experiments. Procedures and calculations for both the automated and manual versions are described.)

Automated method

Apparatus
Printer
Computer
pH-Stat Titration Manager (analytical robot; Novo Nordisk Engineering A/S), consisting of the following elements:

- PHM290 pH-Stat Controller (Radiometer)
- ABU901 Autoburette (Radiometer)
- Liquid Handler (Gilson)
- Temperature Regulator (Gilson)
- Syringe Pump (Gilson)
- Silverson Homogenizer L4R
- pH electrode (Radiometer)

Reagents and solutions
(Note: use only deionized water)

Titrant, sodium hydroxide 0.1 N: Use NaOH solution standardized for preparation of 1000 ml of 0.1 N NaOH, for example, one ampoule of Merck Titrisol No. 1.09959 or equivalent. Transfer quantitatively the NaOH solution into a 1000-ml volumetric flask containing approximately 500 ml of water. Add water to volume and mix. The solution is stable for up to 2 months at room temperature.

Calcium chloride, 0.32 M: Weigh 4.70 g calcium chloride ($CaCl_2\,2H_2O$, Merck 2382 or equivalent). Dissolve in water in a 100 ml volumetric flask. Add water to volume and mix. The solution is stable for up to one week at room temperature.

Sodium deoxycholate, 0.016 M: Weigh 6.7 g sodium deoxycholate ($C_{24}H_{39}NaO_4$). Dissolve in water in a 1000-ml volumetric flask. Add water to volume and mix. The solution is stable for up to one week at room temperature.

Lecithin substrate: Use lecithin (L-α-phosphatidylcholine) Sigma P-5638 or equivalent. Mix lecithin with a spoon and weigh exactly 20.0 g. Transfer to a 1000-ml beaker, add 400 ml of water and stir until the lecithin is dissolved. Add 20 ml of 0.32 M calcium chloride and stir for 1-2 min until calcium chloride is dissolved. Add 200 ml of 0.016 M sodium deoxycholate and 400 ml of water. Stir for about 0.5 hour and homogenize for 10 min on a Silverson L4R homogenizer. The solution is stable for up to one day at room temperature.

Hydrochloric acid, 1 N: Use HCl solution standardized for preparation of 1000 ml of 1 N HCl, for example, one ampoule of Merck Titrisol No. 1.09970 or equivalent. Transfer the HCl solution quantitatively to a 1000-ml volumetric flask. Add water to volume. The solution is stable for up to 6 months at room temperature.

Hydrochloric acid, 0.001 N: Transfer 1 ml of 1 N HCl to a 1000-ml volumetric flask. Add water to volume. The solution is stable for up to one week at room temperature.
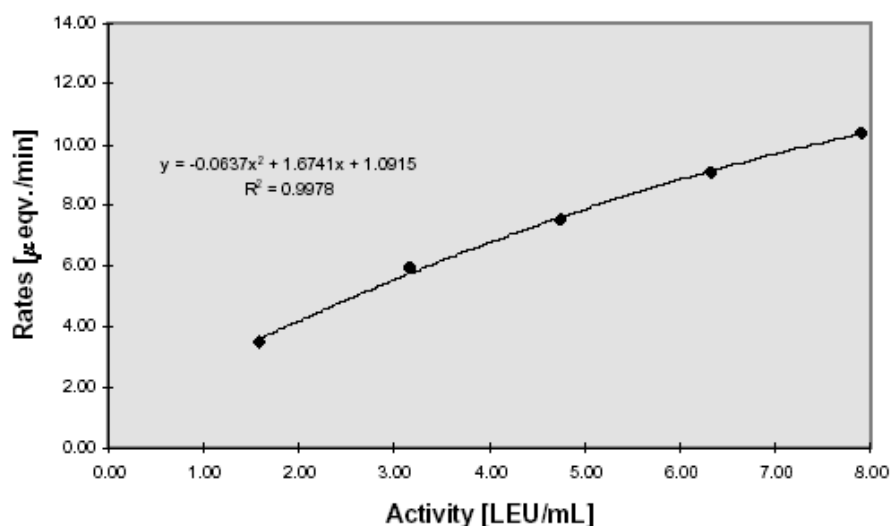
Standard and sample solutions

Standard stock solution: Use a phospholipase standard (Lecitase standard from Novozymes A/S or equivalent) with known activity, for example, 103160 LEU/g. Accurately weigh (to 4 decimal places) approximately 0.73 g of the standard. Transfer to a 250-ml volumetric flask and add 0.001 N HCl to volume. Stir for approximately 30 min. The solution can be stored for 3 weeks at 5^0.

Standard working solutions: Transfer quantitatively 5 ml of the standard stock solution to a 200-ml volumetric flask. Add 0.001 N HCl to volume. This solution contains approximately 7.5 LEU/ml and is referred to as 100% standard. Using the 100% standard, prepare additional standard solutions in 25-ml volumetric flasks according to the following table:

Standard solution	Approximate strength (LEU/ml)	Volume of 100% standard (ml)	Volume of 0.001 N HCl
80%	6.0	20	5
60%	4.5	15	10
40%	3.0	10	15
20%	1.5	5	20

The standard solutions are used by the automated system to calculate a 2nd degree polynomial standard curve. An example of the standard (calibration) curve is shown below.

Calibration Curve

$y = -0.0637x^2 + 1.6741x + 1.0915$
$R^2 = 0.9978$

Control sample: A phospholipase product with known activity, for example, 10500 LEU/ml, is used to prepare the control sample. Transfer 1 ml of the control solution to a 50-ml volumetric flask and add 0.001 HCl to volume to obtain a stock solution. Transfer 1 ml of the stock solution to a 50-ml volumetric flask and add 0.001 N HCl to volume. Analyse the control sample in each run. A result within 10 percent of the nominal activity is acceptable.

Blank sample: Use 0.001 N HCl as the blank sample.

Test samples: Remove the test sample from a refrigerator or freezer and keep it at room temperature for approximately 2 h before analysis. Accurately weigh the sample into a measuring flask and add 0.001 N HCl to volume. Repeat dilution if necessary to obtain the activity of approximately 6 LEU/ml. The dilution should be performed within one hour of the analysis. Enter the sample weight (in grams) and the dilution volume (in milliliters) into the calculation program for the automated method or into the calculation formula for the manual method.

Procedure

The method uses a pH-stat Titration Manager at pH=8.0 and $40^0 \pm 0.5$. The pH-stat Titration Manager automatically runs a two-point calibration of the pH electrode and then analyses the blank, the standard solutions, the control sample and the test samples, each in duplicate.

Start the reaction when 800 µl of the sample is added to 20 µl of the lecithin substrate. Titrate the liberated fatty acid with 0.1 N sodium hydroxide under standard conditions (pH=8.0; $40^0 \pm 0.5$). Record the rate of sodium hydroxide consumption (microeq/min) over 2 min, starting 90 sec and ending 210 sec from the start of the reaction and use to calculate the mean slope of the titration curve.

The mean slopes of the titration curves for the control sample, standard solutions, and test samples are automatically transferred to the calculation program.

Calculations

All results are calculated automatically by the calculation program. A 2nd degree polynomial standard curve is calculated based on the mean slopes (microeq/min) for the standard solutions. Based on the standard curve, the calculation program calculates the results for the control and test samples in Lecitase activity units per 1 ml (LEU/ml). Subsequently, the program calculates the activity of the test samples in Lecitase activity units per one gram of the phospholipase A1 preparation (LEU/g).

Manual method

Procedure

The method is carried out using a titrator that measures the titrant consumption rate as a function of time (e.g., TitraLab 854 from Radiometer). The titrator must be programmed to maintain pH=8 and measure the NaOH consumption rate in microequivalents per minute (microeq/min). The following procedure is followed:
Calibrate the pH electrode at pH 7 and 10. Transfer 20 ml of the lecithin substrate to a beaker and place in a water bath at $40^0 \pm 0.5$. Adjust the substrate to pH 8.0 using 0.1 N NaOH and start the titration by the addition of either 0.8 ml of the standard solution, the test sample, or the control sample. Measure the NaOH consumption rate for 4 min. Determine the NaOH consumption rate between 90 and 120 sec from the reaction start and use this information to construct a standard curve and for activity calculations.

Calculation

Construct a standard curve by plotting the NaOH consumption rate (in microeq/min) against the enzyme activity (in LEU/ml). The activity of the control sample and test samples are read from the calibration curve (in LEU/ml).

Note: a 2nd degree polynomial standard curve can be plotted using suitable software, and the activity of the test sample and control sample can be calculated from the standard curve using the same software.

The activity of the test samples in LEU/g is calculated according to the following equation:

$$\text{Activity(LEU/g)} = \frac{\text{Activity(LEU/ml)} \times \text{V(ml)}}{\text{W(g)}}$$

where: W(g) is the sample weight and
V(ml) is the volume of the volumetric flask in which the sample was diluted. For example, if the sample is weighed into a 50 ml volumetric flask and diluted to volume, V=50 ml.