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SIGMA QUALITY CONTROL TEST PROCEDURE

Enzymatic Assay of PHOSPHOLIPASE C (EC 3.1.4.3) from C. perfringens

PRINCIPLE:

L-α-Lecithin + H_2O $\xrightarrow{\text{Phospholipase C}}$ 1,2-Diglyceride + Choline Phosphate

Choline phosphate + H_2O $\xrightarrow{\text{Alkaline Phosphatase}}$ Choline + P_i

Abbreviations used:
L-α-Lecithin = L-α-Phosphatidylcholine
P_i = Inorganic Phosphate

CONDITIONS: T = 37°C, pH 7.3, A_{660nm}, Light path = 1 cm

METHOD: Colorimetric

REAGENTS:

A. 50 mM Tris Maleate Buffer, pH 7.3 at 37°C
(Prepare 100 ml in deionized water using Trizma Maleate, Sigma Prod. No. T-3128. Adjust to pH 7.3 at 37°C with 10 M NaOH.)

B. 50 mM Calcium Chloride Solution ($CaCl_2$)
(Prepare 25 ml in Reagent A using Calcium Chloride, Dihydrate, Sigma Prod. No. C-3881.)

C. 2.0% (w/v) L-α-Phosphatidylcholine (Lecithin)
(Prepare 25 ml in deionized water using L-α-Phosphatidylcholine, from Fresh Frozen Egg Yolk, Sigma Prod. No. P-9671.[1])

D. 50 mM Tris Maleate Buffer with 1.0% (w/v) Bovine Serum Albumin, pH 7.3 at 37°C (Enzyme Dil)
(Prepare 25 ml in Reagent A using Albumin, Bovine, Sigma Prod. No. A-4503.)

REAGENTS: (continued)

E. 270 mM Ethylenediaminetetraacetic Acid Solution, pH 7.3 at 37°C (EDTA)
(Prepare 25 ml in deionized water using Ethylenediaminetetraacetic Acid, Tetrasodium Salt, Hydrate, Sigma Prod. No. ED4S. Adjust to pH 7.3 at 37°C with 5 M HCl.)

F. Alkaline Phosphatase Enzyme Solution (Alk Phos)
(Immediately before use, prepare a solution containing 40 units/ml in deionized water using Phosphatase, Alkaline, Sigma Prod. No. P-4377.)

G. 20% (w/v) Lauryl Sulfate Solution (SDS)
(Prepare 25 ml in deionized water using Lauryl Sulfate, Sodium Salt, Sigma Prod. No. L-4509.)

H. Phosphorus Std (P Std)
(Use Phosphorus Standard Solution, Sigma Stock No. 661-9. The Phosphorus concentration is 20 µg/ml, 0.645 µ/mole.)

I. 10% (w/v) Ascorbic Acid Solution (Ascorbic Acid)
(Prepare 25 ml in deionized water using L-Ascorbic Acid, Sodium Salt, Sigma Prod. No. A-7631.)

J. 4.2% (w/v) Molybdic Acid Solution (Molyb Acid)
(Prepare 25 ml in 10 N H_2SO_4 using Molybdic Acid, Ammonium Salt, Tetrahydrate, Sigma Prod. No. M-0878 and Sulfuric Acid, Sigma Prod. No. S-1526.)

K. Ames Color Reagent (Clr Rgt)
(Prepare by combining 10 ml of Reagent I (Ascorbic Acid), 6 ml Reagent J (Molyb Acid) and 54 ml of deionized water. Mix by swirling and store in the dark at room temperature. This reagent should be prepared 30 minutes before use.)

L. Phospholipase C Enzyme Solution (Phospholipase C)
(Immediately before use, prepare a solution containing 0.1-1.0 unit/ml in cold Reagent D.)

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PROCEDURE:

Pipette (in milliliters) the following reagents into 4 dram vials:

	Test	Test Blank	Std 1	Std 2	Std 3	Std 4	Std Blank
Reagent A (Buffer)	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Reagent B ($CaCl_2$)	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Reagent C (Lecithin)	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Reagent D (Enzyme Dil)	0.90	0.90	---	---	---	---	---
Reagent H (P Std)	---	---	0.25	0.50	0.75	1.00	---
Deionized Water	---	---	0.75	0.50	0.25	---	1.00

Mix by swirling and equilibrate at 37°C. Then add:

Reagent L (Phospholipase C)	0.10	---	---	---	---	---	---

Mix by swirling and incubate at 37°C for exactly 15 minutes. Then add:

Reagent E (EDTA)	0.90	0.90	0.90	0.90	0.90	0.90	0.90

Mix by swirling. Then add:

Reagent L (Phospholipase C)	---	0.10	---	---	---	---	---
Reagent F (Alk Phos)	0.10	0.10	0.10	0.10	0.10	0.10	0.10

Mix by swirling and incubate at 37°C for 120 minutes.

Pipette (in milliliters) the following reagents into 4 dram vials:

Test Solution	1.00	---	---	---	---	---	---
Test Blank Solution	---	1.00	---	---	---	---	---
Standard 1	---	---	1.00	---	---	---	---
Standard 2	---	---	---	1.00	---	---	---
Standard 3	---	---	---	---	1.00	---	---
Standard 4	---	---	---	---	---	1.00	---
Standard Blank	---	---	---	---	---	---	1.00

Mix by swirling. Then add:

Reagent G (SDS)	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Reagent K (Clr Rgt)	3.00	3.00	3.00	3.00	3.00	3.00	3.00

Mix by swirling and incubate at 37°C for 60 minutes. Centrifuge and transfer the Test, Test Blank, Standards, and Standard Blank to suitable cuvettes. Read the A_{660nm} for each of the samples and blanks using a suitable spectrophotometer.

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CALCULATIONS:

Standard Curve:

ΔA_{660nm} Standard = A_{660nm} Standard - A_{660nm} Standard Blank

Prepare a Standard curve by plotting the ΔA_{660nm} Standard versus micromoles of phosphorus.

Sample Determination:

ΔA_{660nm} Test = A_{660nm} Test - A_{660nm} Test Blank

Determine the micromoles of Phosphorus liberated using the Standard curve.

$$\text{Units/ml enzyme} = \frac{(\mu\text{moles of phosphate released})(df)}{(15)(0.1)}$$

df = Dilution factor
15 = Time of reaction (in minutes) as per the Unit Definition
0.1 = Volume (in milliliter) of enzyme used

$$\text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

$$\text{Units/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

UNIT DEFINITION:

One unit will liberate 1.0 μmole of water soluble organic phosphorus from L-α-phosphatidylcholine per min at pH 7.3 at 37°C.

FINAL ASSAY CONCENTRATION:

In a 5.00 ml reaction mix, the final concentrations are 35 mM Tris maleate, 5 mM calcium chloride, 0.6% (w/v) L-α-phosphatidylcholine, 0.2% (w/v) bovine serum albumin, and 0.01 - 0.10 unit phospholipase c.

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NOTES:

1. Break up any large lumps of L-α-phosphatidylcholine and dissolve in deionized water. Sonicate at 30 second intervals for approximately 5 minutes or until the product is completely dissolved. The resulting suspension should be homogeneous.

2. Alkaline Phosphatase Unit Definition: One unit will hydrolyze 1.0 µmole of p-nitrophenyl phosphate per minute at pH 9.8 at 37°C.

3. Where Sigma Product or Stock numbers are specified, equivalent reagents may be substituted.