

Enzymatic Assay of PHOSPHOLIPASE D (EC 3.1.4.4)

PRINCIPLE:

L- α -Phosphatidylcholine + H₂O $\xrightarrow{\text{Phospholipase D}}$ Choline + Phosphatidic Acid

Choline + O₂ + H₂O $\xrightarrow{\text{Choline Oxidase}}$ Betaine Aldehyde + H₂O₂

2H₂O₂ + 4-AAP + Phenol $\xrightarrow{\text{Peroxidase}}$ 4H₂O + Quinoneimine Dye

Abbreviation used:

4-AAP = 4-Aminoantipyrine

CONDITIONS: T = 30°C, pH = 8.0, A_{500nm}, Light path = 1 cm

METHOD: Colorimetric

REAGENTS:

- A. 100 mM Tris HCl Buffer, pH 8.0 at 30°C (0.1 M Tris Buffer)
(Prepare 100 ml in deionized water using Trizma Base, Sigma Prod. No. T-1503. Adjust to pH 8.0 at 30°C with 1 M HCl.)
- B. 50 mM Sodium Lauryl Sulfate Solution (SDS)
(Prepare 10 ml in deionized water using Lauryl Sulfate, Sodium Salt, Sigma Prod. No. L-5750.)
- C. 17.9% (v/v) Ethanol Solution (EtOH)
(Prepare 1 ml in deionized water using Ethyl Alcohol Denatured, Sigma Stock No. 27,074-1.)
- D. 0.46% (w/v) L- α -Phosphatidylcholine Substrate Solution
(Prepare by transferring 2.2 ml (220 mg) of L- α -Phosphatidylcholine, Sigma Prod. No. P-5388, to a 50 ml Erlenmeyer flask. Evaporate off the hexane by bubbling nitrogen gas through the liquid. Place the Erlenmeyer flask containing the substrate into a desiccator connected to a vacuum line for 4 hours. Add 6 ml of Reagent A (Buffer), 3 ml of Reagent B (SDS), and 39 ml of deionized water. Mix, using a magnetic stirrer, until a uniform suspension is obtained. Add 0.272 ml of Reagent C (EtOH) to obtain a 0.1% (v/v) ethanol concentration in the substrate solution. **PREPARE FRESH.**)

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REAGENTS: (continued)

- E. 500 mM Calcium Chloride Solution (CaCl₂)
(Prepare 25 ml in deionized water using Calcium Chloride, Dihydrate, Sigma Prod. No. C-3881.)
- F. 10 mM Tris HCl Buffer pH 8.0, with
2 mM Ethylenediaminetetraacetic Acid and 1.0% (w/v) Potassium Chloride (Enzyme Diluent)
(Prepare 10 ml in Reagent A using Ethylenediaminetetraacetic Acid, Disodium Salt, Dihydrate, Sigma Stock No. ED2SS and Potassium Chloride, Sigma Prod. No. P-4504.)
- G. Choline Oxidase Enzyme Solution (COD)
(Prepare a solution containing 10 units/ml of Choline Oxidase, Sigma Prod. No. C-5896, in cold Reagent F.)
- H. 1.0 mM Choline Chloride Standard (Chol Std Soln)
(Prepare 50 ml in deionized water using Choline Chloride, Sigma Prod. No. C-1879.
PREPARE FRESH.)
- I. Choline Color Reagent Mixture (CCRM)
(Prepare by dissolving 39 mg of 4-Aminoantipyrine, Free Base, Sigma Prod. No. A-4382, 80 mg of Phenol, Sigma Prod. No. P-3653 and 4 mg of Peroxidase, Sigma Prod. No. P-8250 in 5.5 ml of Reagent A. Store in an amber bottle to protect from light.)
- J. 2 M Tris HCl Buffer, pH 9.0 at 25°C
(Prepare 25 ml in deionized water using Trizma Base, Sigma Prod. No. T-1503. Adjust to pH 9.0 at 25°C with 1 M HCl.)
- K. Phospholipase D Enzyme Solution (PLD)
(Immediately before use, prepare a solution containing 10 - 20 units/ml in cold deionized water.)

PROCEDURE:

Pipette (in milliliters) the following reagents into suitable containers:

	<u>Test</u>	<u>Blank</u>
Reagent D (Substrate Soln)	2.40	2.40
Reagent E (CaCl ₂)	0.30	0.30
Deionized Water	0.20	0.30

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PROCEDURE: (continued)

Mix vigorously by vortexing and equilibrate to 30°C using a thermostatted metabolic shaker. Then add:

	<u>Test</u>	<u>Blank</u>
Reagent K (PLD)	0.10	-----

Immediately mix by swirling and incubate the containers, for exactly 10 minutes at 30°C. The vials should be swirled several times during the reaction. Transfer the Test and Blank to a boiling water bath. Remove tubes from the water bath after 5 minutes and let cool to room temperature. Add 0.05 ml of Reagent J (Tris HCl Buffer), centrifuge and filter both Test and Blank through a 0.45 µm filter. Pipette (in milliliters) the following reagents into suitable containers.

Test Filtrate	2.00	-----
Blank Filtrate	-----	2.00
Reagent I (CCRM)	0.10	0.10
Reagent G (COD)	0.10	0.10

Mix by inversion and let stand between 2 - 3 hours at room temperature. Then add:

Deionized Water	2.00	2.00
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Centrifuge to clarify and then transfer the solutions to suitable cuvettes. Record the A_{500nm} for both Test and Blank using a suitable spectrophotometer.

COLORIMETRIC ASSAY:

Standard Curve:

Pipette (in milliliters) the following reagents into suitable containers:

	<u>Std 1</u>	<u>Std 2</u>	<u>Std 3</u>	<u>Std 4</u>	<u>Std Blank</u>
Reagent D (Substrate Soln)	2.40	2.40	2.40	2.40	2.40
Reagent E (CaCl ₂)	0.30	0.30	0.30	0.30	0.30
Reagent H (Chol Std Soln)	0.05	0.10	0.20	0.30	----
Deionized Water	0.25	0.20	0.10	----	0.30

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COLORIMETRIC ASSAY: (continued)

Mix vigorously by vortexing and then place Standard and Standard Blank in a boiling water bath. Remove tubes after 5 minutes from the water bath and let cool to room temperature. Add 0.05 ml of Reagent J (Tris HCl Buffer), centrifuge and filter the Standards and Standard Blank through a 0.45 μm filter.

Pipette (in milliliters) the following reagents into suitable containers:

Std 1 Filtrate	2.00	----	----	----	----	----
Std 2 Filtrate		----	2.00	----	----	----
Std 3 Filtrate	----	----	2.00	----	----	----
Std 4 Filtrate	----	----	----	2.00	----	----
Blank Filtrate		----	----	----	----	2.00
Reagent I (CCRM)		0.10	0.10	0.10	0.10	0.10
Reagent G (COD)		0.10	0.10	0.10	0.10	0.10

Mix by inversion and let stand between 2-3 hours at room temperature. Then add:

Deionized Water		2.00	2.00	2.00	2.00	2.00
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Clarify the solutions by centrifugation. Transfer the solutions to cuvettes and record the $A_{500\text{nm}}$ for both Standards and Standard Blank using a suitable spectrophotometer.

CALCULATIONS:

Standard Curve:

$$\Delta A_{500\text{nm}} \text{ Standard} = A_{500\text{nm}} \text{ Standard} - A_{500\text{nm}} \text{ Standard Blank}$$

Prepare a standard curve by plotting $\Delta A_{500\text{nm}}$ Standard versus the micromoles of Choline.

Sample Determination:

$$\Delta A_{500\text{nm}} \text{ Sample} = A_{500\text{nm}} \text{ Test} - A_{500\text{nm}} \text{ Blank}$$

Determine the total micromoles of Choline liberated using the Standard curve.

$$\text{Units/ml enzyme} = \frac{(\text{micromoles choline liberated}) (6) (df)}{(0.1)}$$

6 = Time conversion factor for one hour (as per Unit Definition)

0.1 = Volume (in milliliter) of enzyme used

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CALCULATIONS: (continued)

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

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

UNIT DEFINITION:

One unit will liberate 1.0 μ mole of choline from L- α -phosphatidylcholine (egg yolk) per hour at pH 8.0 at 30°C.

FINAL ASSAY CONCENTRATION:

In a 3.00 ml reaction mixture, the final concentrations are 0.37% (w/v) L- α -phosphatidylcholine, 0.08% (v/v) ethanol, 9.9 mM Tris, 2 mM sodium lauryl sulfate, 50 mM calcium chloride, and 1 - 2 units phospholipase D.

REFERENCES:

Artiss, J. D., Draisey, T.F., Thibert, R.J., Zak, B., and Taylor, K.E., (1980) *Microchemical Journal* **25**, 153-168

Imamura, S. and Horiuchi, Y. (1979) *Journal of Biochemistry* **85**, 79-95

Yang, S.F., Freer, S., and Benson, A.A. (1967) *Journal of Biological Chemistry* **242**, 477-484

NOTES:

1. Choline Oxidase Unit Definition: One unit will form 1.0 μ mole of H₂O₂ per minute from choline and H₂O at pH 8.0 at 37EC.
2. Peroxidase Unit Definition: One unit will form 1.0 mg purpurogallin from pyrogallol in 20 sec at pH 6.0 at 20EC.
3. This assay is based on the cited references.
4. Where Sigma Product or Stock numbers are specified, equivalent reagents may be substituted.

This procedure is for informational purposes. For a current copy of Sigma's quality control procedure contact our Technical Service Department.