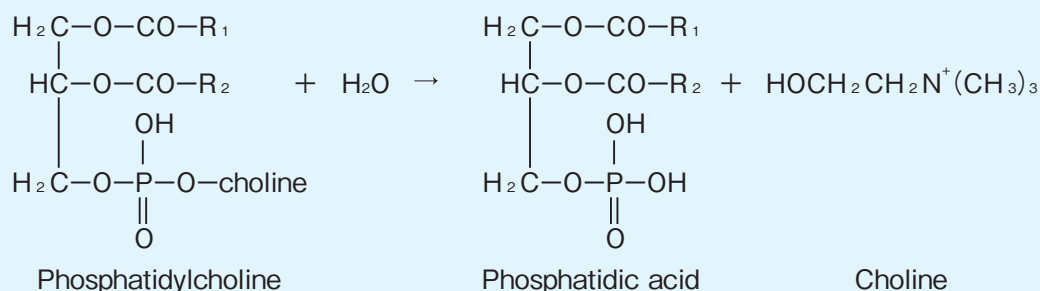


(Diagnostic Reagent Grade)

T-222

PHOSPHOLIPASE D [PLD II]

from *Streptomyces chromofuscus*
(Phosphatidylcholine phosphatidohydrolase: EC 3.1.4.4)



Preparation and Specification

Appearance : Pale grayish to grayish or brownish to light purple lyophilizate
 Specific activity : More than 30 U/mg solid
 Contaminants :
 Catalase : Less than 0.6 % (U/U)
 Glucose oxidase : Less than 0.02 % (U/U)

Properties

Substrate specificity : See Table 1
 Molecular weight : 58 kDa (SDS-PAGE)
 Isoelectric point : pH 5.9 (estimated from amino acid sequence)
 Michaelis constants : 1,2-Dioleoyl-sn-glycero-3-phosphocholine 9.3×10^{-4} M
 Optimum pH : 6.7-7.1
 pH stability : 5.3-9.7
 Thermal stability : Stable at 60°C and below (pH 8.0, 10 min)
 Storage stability : At least one year at -20°C
 Effect of metal ions : See Table 2
 Activators : Ca^{2+}

Applications for Diagnostic Test

This enzyme is useful for enzymatic determination of **phospholipids** when coupled with choline oxidase (T-05)

PLD II

Phosphatidylcholine + H₂O → Choline + Phosphatidic acid

COD

Choline + 2 O₂ + H₂O → Betaine + 2 H₂O₂

POD

2 H₂O₂ + 4-AA + Phenol → Quinoneimine dye + 4 H₂O

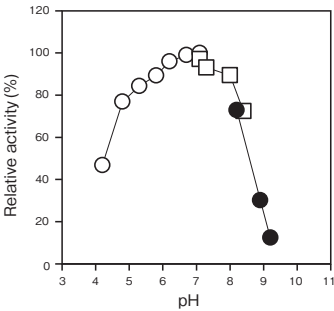
Table 1. Substrate specificity

Substrate	Specific activity (%)
1,2-Dioleoyl-sn-glycero-3-phosphocholine	100
2-Oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine	98
L- α -Phosphatidylcholine	95
L- α -Lysophosphatidylcholine	99
1-Oleoyl-sn-glycero-3-phosphocholine	99
L- α -phosphatidylethanolamine	14
Sphingomyelin	26

Table 2. Effect of metal ions (Activators)

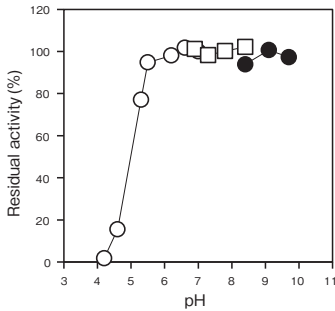
Divalent cation (1 mM)	Relative activity (%)
None	2
Ca ²⁺	100
Mg ²⁺	2
Mn ²⁺	0
Ba ²⁺	1

Fig.1 pH Optimum



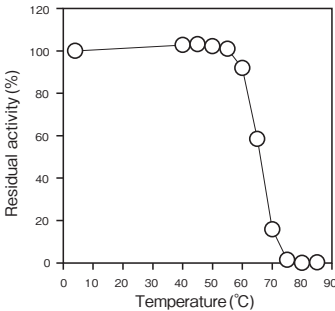
○ : 3,3-Dimethylglutarate-NaOH
buffer
□ : Tris-HCl buffer
● : Glycine-NaOH buffer

Fig.2 pH Stability



37°C, 60 min.
10 mM buffer containing 0.1%
TritonX-100 and 0.05% BSA
○ : 3,3-Dimethylglutarate
-NaOH buffer
□ : Tris-HCl buffer
● : Glycine-NaOH buffer

Fig.3 Thermal Stability

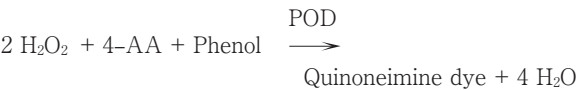
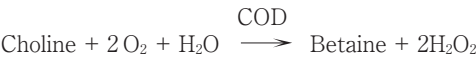


pH 8.0, 10 min.
10 mM Tris-HCl buffer containing
0.1% TritonX-100 and 0.05% BSA

Assay

■ Principle

The assay is based on the increase in absorbance at 500 nm as the formation of quinoneimine dye proceeds in the following reactions:



COD: Choline oxidase

■ Unit definition

One unit is defined as the amount of enzyme which hydrolyzes 1 μ mole of phosphatidylcholine to phosphatidic acid and choline per minute at 37°C under the conditions specified in the assay procedure.

■ Reagents

1. Reaction mixture for the first reaction
0.1 M Tris-HCl buffer pH 8.0 0.20 ml
0.1 M CaCl₂ solution 0.05 ml
25 mM substrate solution ¹⁾ 0.10 ml

Distilled water 0.15 ml

1): 25 mM substrate solution

Dissolve 88.5 mg of 1,2-Dioleoyl-sn-glycero-3-phosphocholine with 4.5 ml of 5 % (W/V) Triton X-100 solution.

2. Reaction mixture for the second reaction

- | | |
|------------------------------------|---------|
| 15 mM 4-AA solution | 0.10 ml |
| 0.2 % (W/V) Phenol solution | 0.10 ml |
| 60 mM EDTA pH 8.0 | 0.10 ml |
| 50 mM Tris-HCl buffer pH 8.0 | 2.00 ml |
| 90 U/ml POD solution ²⁾ | 0.10 ml |
| 30 U/ml COD solution ³⁾ | 0.10 ml |

EDTA: Ethylenediaminetetraacetic acid

2): 90 U/ml POD solution

Dissolve 900 U (PPU) of POD with 10 ml of distilled water.

3): 30 U/ml COD solution

Dissolve 300 U of COD with 10 ml of 10 mM Tris-HCl buffer pH 8.0.

3. Enzyme dilution buffer

10 mM Tris-HCl buffer (pH 8.0) containing 0.05% (W/V) BSA and 0.1% (W/V) Triton X-100

4. Reagents

Triton X-100: The Dow Chemical Company

1,2-Dioleoyl-sn-glycero-3-phosphocholine:

Sigma Chemical Co. #P-6354

EDTA (2 Na·2H₂O): KISHIDA CHEMICAL Co., Ltd.

#060-29133

COD: Nagase Diagnostics Co., Ltd. #T-05
 BSA: Millipore Fraction V pH 5.2 #81-053
 4-AA : NACALAI TESQUE, INC. Special grade #01907-52
 POD: Sigma Chemical Co. Type II #P-8250

Absorbance sample : As
 blank : Ab
 $\Delta A = (As - Ab) \leq 0.60 \text{ Abs}$

Enzyme solution

Accurately weigh about 20 mg of the sample and add enzyme dilution buffer to make a total of 20 ml. Dilute it with enzyme dilution buffer to adjust the concentration as required.

Procedure

- Pipette accurately 0.50 ml of reaction mixture for the first reaction into a small test tube and preincubate at 37°C.
- After 5 min, add 50 μ l of enzyme solution and mix to start the reaction at 37°C.
- At 10 min after starting the reaction, add 2.50 ml of reaction mixture to the second reaction and mix to start the second reaction.
 ※ In the case of a blank test, add 50 μ l of enzyme dilution buffer solution at this time.
- At 20 min after starting the reaction, measure the absorbance at 500 nm.

Calculation

$$\text{Activity (U/mg of powder)} = \frac{\Delta A/10}{12.2} \times \frac{3.05}{0.05} \times \frac{1}{X}$$

12.2 : millimolar extinction coefficient of quinoneimine dye
 ($\text{cm}^2 / \mu\text{mole}$)

10 : reaction time (min)

3.05 : final volume (ml)

0.05 : volume of enzyme solution (ml)

X : concentration of the sample in enzyme solution
 (mg/ml)

Storage

Storage at -20°C in the presence of a desiccant is recommended. Enzyme activity will be retained for at least one year under this condition.

References

- Imamura, S. and Horiuchi, Y. (1979) J. Biochem., **85**, 75-95.

PLDII 活性測定法 (Japanese)

I. 試薬液

1. 第一反応試薬混合液

0.1M トリス-HCl 緩衝液 pH8.0	0.20 ml
0.1M 塩化カルシウム溶液	0.05 ml
25mM 基質溶液 ¹⁾	0.10 ml
精製水	0.15 ml

1): 25mM 基質溶液

1,2-ジオレオイル sn-グリセロ-3-ホスホコリン 88.5mg を 5% (W/V) トリトン X-100 溶液 4.5ml で溶解する。

2. 第二反応試薬混合液

15mM 4-AA 溶液	0.10 ml
0.2% (W/V) フェノール液	0.10 ml
60mM EDTA 溶液 pH8.0	0.10 ml
50mM トリス-HCl 緩衝液 pH8.0	2.00 ml
90U/ml POD 溶液 ²⁾	0.10 ml
30U/ml COD 溶液 ³⁾	0.10 ml

2): 90U/ml POD 溶液

POD 900 単位 (PPU) を精製水 10ml で溶解する。

3): 30U/ml COD 溶液

COD 300 単位 (U) を 10mM トリス-HCl 緩衝液 pH8.0 10ml で溶解する。

3. 酵素溶解希釈用液

0.05% (W/V) BSA と 0.1% (W/V) トリトン X-100 を含む 10mM トリス-HCl 緩衝液 pH8.0

4. 試薬

トリトン X-100: Dow Chemical 製

1,2-ジオレオイル sn-グリセロ-3-ホスホコリン:
 シグマ製 #P-6354

EDTA (エチレンジアミン四酢酸・2Na・2H₂O):
 キシダ化学製 #060-29133

COD (コリン酸化酵素): ナガセダイアグノスティクス製 #T-05

BSA: Millipore 製 Fraction V pH5.2 #81-053

4-AA: ナカライテスク製 特級 #01907-52 POD:
 シグマ製 Type II #P-8250

II. 酵素試料液

検品約 20mg を精密に量り、酵素溶解希釈用液で全容 20ml とする。

その液を酵素溶解希釈用液で適宜希釈する。

III. 測定操作法

- 小試験管に第一反応試薬混合液 0.50ml を正確に分注し、37°C で予備加温する。
- 5 分経過後、酵素試料液 50 μ l を正確に加えて混和し、37°C で第一反応を開始する。
- 10 分経過後、第二反応試薬混合液 2.50ml を加えて混和し、37°C で第二反応を開始する。
 ※盲検はこの時点で酵素溶解希釈用液 50 μ l を加える。
- 20 分経過後、500nm における吸光度を測定する。求められた吸光度を試料液は As、盲検液は Ab とする。

$$\Delta A = (As - Ab) \leq 0.60 \text{ Abs}$$

IV. 計算

$$\text{活性 (U/mg)} = \frac{\Delta A/10}{12.2} \times \frac{3.05}{0.05} \times \frac{1}{X}$$

12.2: キノンイミン色素の 500nm におけるミリモル分子吸光係数 ($\text{cm}^2 / \mu\text{mole}$)

10 : 反応時間 (min)

3.05 : 反応総液量 (ml)

0.05 : 反応に供した酵素試料液量 (ml)

X : 酵素試料液の検品濃度 (mg/ml)