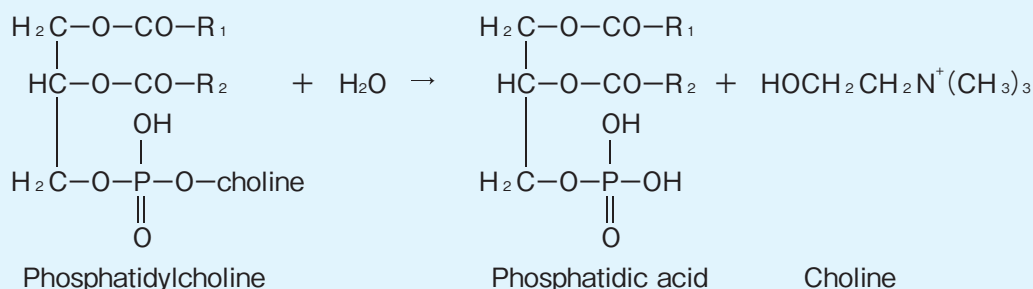


# PHOSPHOLIPASE D [PLD]

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



## Preparation and Specification

Appearance : Brownish amorphous powder, lyophilized

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 : 50 kDa (Sephadex G-100)  
57 kDa (SDS-PAGE)

Isoelectric point : pH 5.1

Michaelis constants : Phosphatidylcholine  $1.4 \times 10^{-3}$  M

Optimum pH : 7.5-8.5

pH stability : 6.0-10.0 (in 0.1% BSA)

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<sup>2+</sup>, Triton X-100, Adekatol SO-120, Deoxycholate

Inhibitor : EDTA

Figure 1

Figure 2

Figure 3

Figure 4

## Applications for Diagnostic Test

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

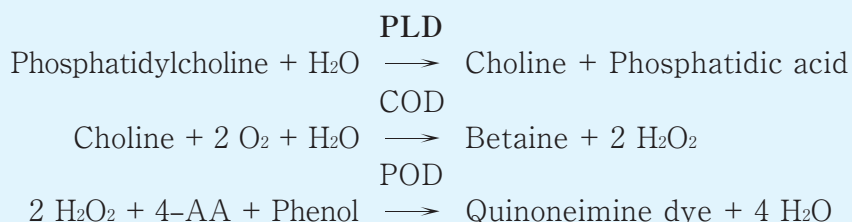


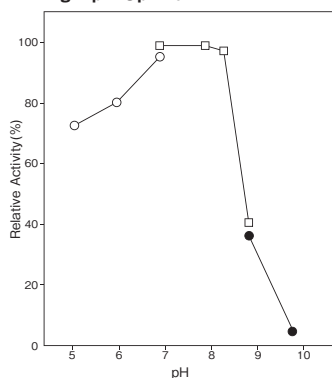
Table 1. Substrate specificity

Substrate	Specific activity (%)
Lysophosphatidylcholine	100
Phosphatidylcholine	87
Sphingomyelin	22
Phosphatidylethanolamine	27
Lysophosphatidyl- ethanolamine	52
Phosphatidylinositol	11

Table 2. Effect of metal ions on PLD activity

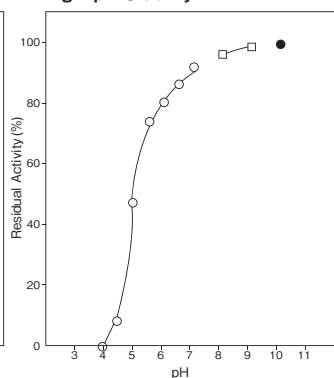
Divalent cation (1 mM)	Relative activity (%)	
	None	+ Triton X-100
None	11	100
Ca <sup>2+</sup>	28	192
Mg <sup>2+</sup>	24	92
Mn <sup>2+</sup>	7	13
Ba <sup>2+</sup>	1	2
Co <sup>2+</sup>	0	0
Zn <sup>2+</sup>	0	0

Fig.1 pH Optimum



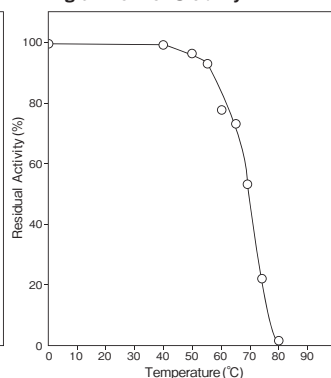
○ : 3,3-Dimethylglutarate-NaOH  
buffer  
□ : Tris-HCl buffer  
● : Glycine-NaOH buffer

Fig.2 pH Stability



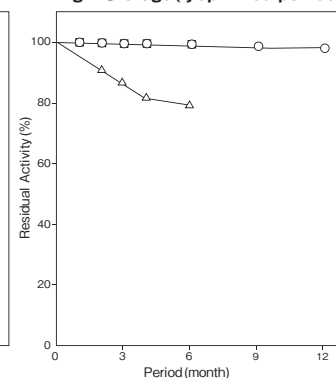
37°C, 60 min  
○ : 3,3-Dimethylglutarate  
-NaOH buffer  
□ : Tris-HCl buffer  
● : Glycine-NaOH buffer

Fig.3 Thermal Stability



pH 8.0, 10 min.  
Tris-HCl buffer

Fig.4 Storage (lyophilized powder)

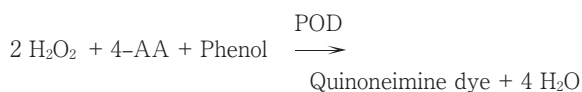
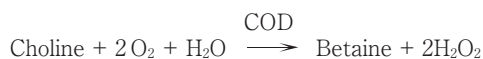


○ : -20°C  
□ : 5°C  
△ : 30°C

## Assay

### Principle

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



COD: Choline oxidase

### Unit definition

One unit is defined as the amount of enzyme which hydrolyzes 1  $\mu$ 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 CaCl <sub>2</sub> solution	0.05 ml
25 mM substrate solution <sup>1)</sup>	0.10 ml

Distilled water 0.15 ml  
1): 25 mM substrate solution

Dissolve 88.5 mg of phosphatidylcholine, dioleoyl 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 <sup>2)</sup>	0.10 ml
30 U/ml COD solution <sup>3)</sup>	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

L- $\alpha$ -Phosphatidylcholine, dioleoyl (C18:1,[Cis]-9):

Sigma Chemical Co. #P-6354

EDTA (2 Na·2H<sub>2</sub>O): KISHIDA CHEMICAL Co., Ltd.

#060-29133

COD: Asahi Kasei Pharma Corporation #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/min  
 blank : Ab/min  
 $\Delta A/\text{min} = (\text{As}/\text{min} - \text{Ab}/\text{min}) \leq 0.60 \text{ Abs}/\text{min}$

### ■ 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  $\mu\text{l}$  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  $\mu\text{l}$  of enzyme dilution buffer solution at this time.
- At 20 min after starting the reaction, measure the absorbance at 500 nm. The rate must be measured within the linear portion of the absorbance curve.

### ■ 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  $-20^\circ\text{C}$  in the presence of a desiccant is recommended. Enzyme activity will be retained for at least one year under this condition (Figure 4).

### References

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

## PLD 活性測定法 (Japanese)

### I. 試薬液

- 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 <sup>1)</sup>	0.10 ml
精製水	0.15 ml

1): 25mM 基質溶液  
 ジオレオイルフォスファチジルコリン 88.5mg  
 を 5% (W/V) トリトン X-100 溶液 4.5ml で溶解する。

- 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 液 <sup>2)</sup>	0.10 ml
30U/ml COD 溶液 <sup>3)</sup>	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  
 COD300 単位 (U) を 10mM トリス-HCl 緩衝液 pH8.0 10ml で溶解する。

- 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

- 試薬  
 トリトン X-100: Dow Chemical 社製  
 L- $\alpha$ - フォスファチジルコリン, ジオレオイル (C18:1, [Cis9]): シグマ社製 #P-6354

EDTA (エチレンジアミン四酢酸 $\cdot 2\text{Na}\cdot 2\text{H}_2\text{O}$ ):  
 キシダ化学社製 #060-29133

COD (コリン酸化酵素): 旭化成ファーマ製 #T-05  
 BSA: Millipore 社製 Fraction V pH5.2 #81-053  
 4-AA: ナカライテスク社製 特級 #01907-52  
 POD: シグマ社製 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  $\mu\text{l}$  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  $\mu\text{l}$  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。

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

### 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)