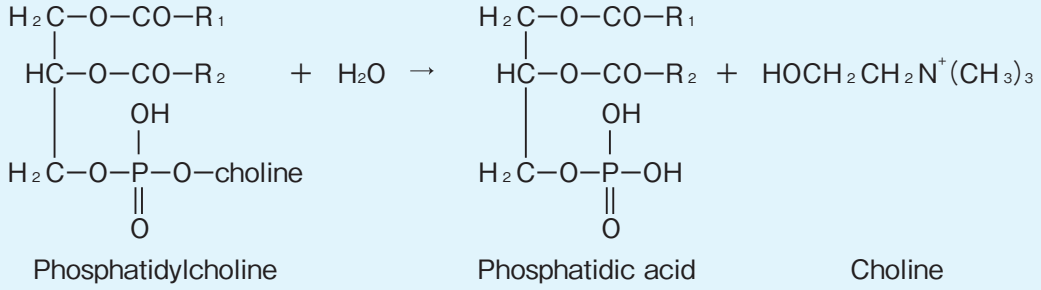
PHOSPHOLIPASE D [PLD Ⅱ]

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	Figure 1
pH stability	: 5.3–9.7	Figure 2
Thermal stability	: Stable at 60℃ and below (pH 8.0, 10 min)	Figure 3
Storage stability	: At least one year at −20℃	
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)

$$\text{Phosphatidylcholine} + H_2O \xrightarrow{\text{PLD Ⅱ}} \text{Choline} + \text{Phosphatidic acid}$$

$$\text{Choline} + 2\,O_2 + H_2O \xrightarrow{\text{COD}} \text{Betaine} + 2\,H_2O_2$$

$$2\,H_2O_2 + \text{4-AA} + \text{Phenol} \xrightarrow{\text{POD}} \text{Quinoneimine dye} + 4\,H_2O$$

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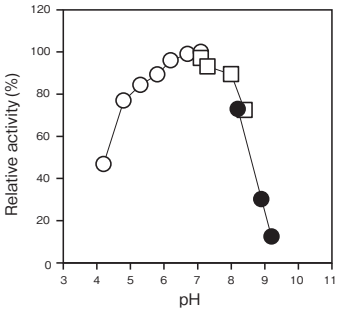
Table 1. Substrate specificity

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)

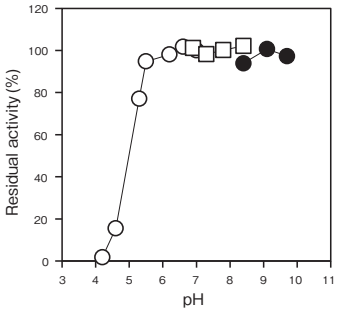
Divalent cation (1 mM)	Relative activity (%)
None	2
Ca^{2+}	100
Mg^{2+}	2
Mn^{2+}	0
Ba^{2+}	1

Fig.1 pH Optimum



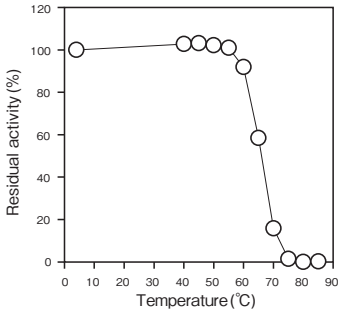
○ : 3,3-Dimethylglutarate-NaOH buffer
□ : Tris-HCl buffer
● : Glycine-NaOH buffer

Fig.2 pH Stability



37℃, 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:

$$\text{Lecithin} + H_2O \xrightarrow{\text{PLD II}} \text{Phosphatidic acid} + \text{Choline}$$

$$\text{Choline} + 2\,O_2 + H_2O \xrightarrow{\text{COD}} \text{Betaine} + 2H_2O_2$$

$$2\,H_2O_2 + \text{4-AA} + \text{Phenol} \xrightarrow{\text{POD}} \text{Quinoneimine dye} + 4\,H_2O$$

COD: Choline oxidase

■ Unit definition

One unit is defined as the amount of enzyme which hydrolyzes 1 μmole of phosphatidylcholine to phosphatidic acid and choline per minute at 37℃ 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 $CaCl_2$ 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·2H_2O): KISHIDA CHEMICAL Co., Ltd. #060-29133

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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

■ 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

1. Pipette accurately 0.50 ml of reaction mixture for the first reaction into a small test tube and preincubate at 37℃.
2. After 5 min, add 50 μl of enzyme solution and mix to start the reaction at 37℃.
3. 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 μl of enzyme dilution buffer solution at this time.
4. At 20 min after starting the reaction, measure the absorbance at 500 nm.

Absorbance sample : As
blank : Ab
△ A = (As − Ab) ≦ 0.60 Abs

■ Calculation

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

12.2 : millimolar extinction coefficient of quinoneimine dye (cm^2 / μmole)
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℃ in the presence of a desiccant is recommended. Enzyme activity will be retained for at least one year under this condition.

References

1. 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・$2H_2O$): キシダ化学製 #060-29133
COD (コリン酸化酵素): 旭化成ファーマ製 #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

1. 小試験管に第一反応試薬混合液 0.50ml を正確に分注し、37℃で予備加温する。
2. 5 分経過後、酵素試料液 50 μl を正確に加えて混和し、37℃で第一反応を開始する。
3. 10 分経過後、第二反応試薬混合液 2.50ml を加えて混和し、37℃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 μl を加える。
4. 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。

$$\Delta A = (As - Ab) \leqq 0.60 Abs$$

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 (cm^2/ μmole)
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)

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