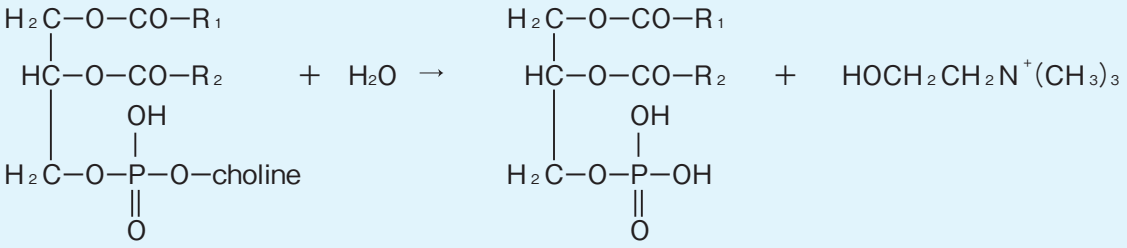
PHOSPHOLIPASE D [PLDP]

(Glycerophospholipid specific)

from *Streptomyces* sp.
(Phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4)



Phosphatidylcholine　Phosphatidic Acid　Choline

Preparation and Specification

Appearance : Light brownish amorphous powder, lyophilized
Specific activity : More than 100 U/mg solid

Properties

Substrate specificity	: See Table 1	
Molecular weight	: 46 kDa (gel filtration)	
Isoelectric point	: pH 4.2	
Optimum pH	: 5.5–6.0	Figure 1
pH stability	: 4.2–8.5 (37℃, 60 min, 0.05% BSA)	Figure 2
Storage stability	: At least one year at −20℃	Figure 3
Effect of various chemicals	: See Table 2 and Table 3	

Transphosphatidylation Catalyzed by Phospholipase D

Figure 4
Figure 5
Figure 6
Figure 7

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Table 1. Substrate specificity

Substrate	Relative activity (%)
Lecithin	100
Lysolecithin	3.4
Sphingomyelin	0.03

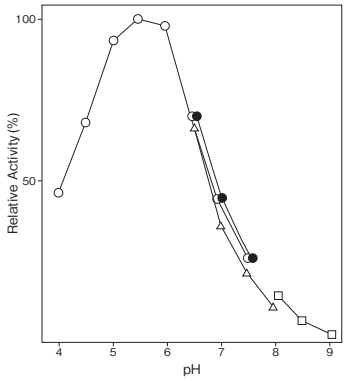
Table 2. Effect of detergents on PLDP activity

Additive	Concentration (%)	Relative activity (%)
None	0	0
Triton X-100	0.1	28
	0.5	100
	1.0	78
SDS	0.1	15
Deoxycholate	0.1	8

Table 3. Effect of metal ions on PLDP activity

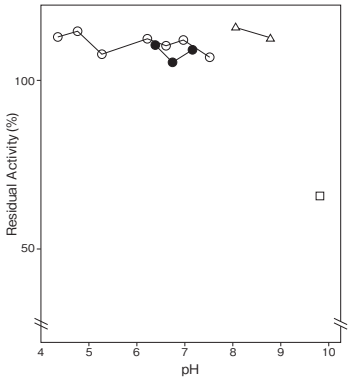
Metal ion	Concentration (mM)	Relative activity (%)
None	0	100
$CaCl_2$	10	99
$MgCl_2$	10	101
$MnCl_2$	10	132
$ZnCl_2$	1	99
$CoCl_2$	1	106
$BaCl_2$	1	101
$CuCl_2$	1	65
EDTA	1	99
EDTA	10	100

Fig.1 pH Optimum



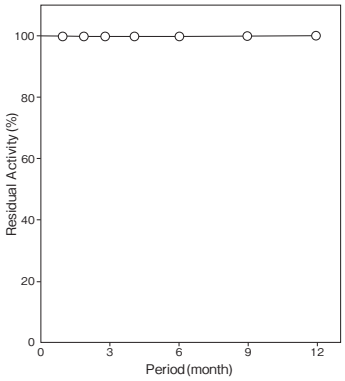
○ : 3,3-Dimethylglutarate-NaOH buffer
● : Phosphate buffer
△ : Tris-HCl buffer
□ : Glycine-NaOH buffer

Fig.2 pH Stability



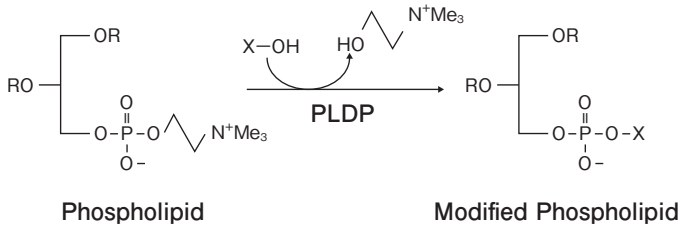
37℃, 60 min.
○ : 3,3-Dimethylglutarate-NaOH buffer
● : Phosphate buffer
△ : Tris-HCl buffer
□ : Glycine-NaOH buffer

Fig.3 Storage (lyophilized powder)



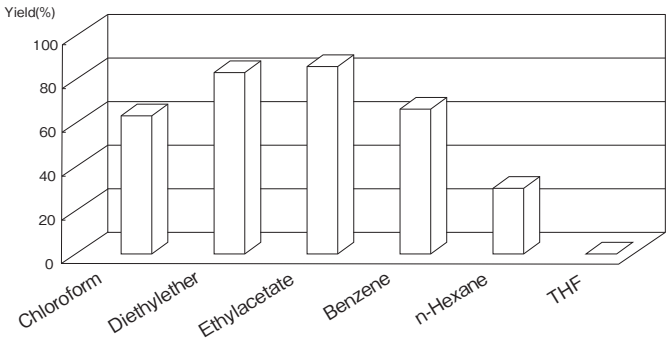
−20℃

Transphosphatidylation Catalyzed by Phospholipase D



Phospholipid　　Modified Phospholipid

Fig.4 The Influence of Organic Solvents on the Yield of 5'-Phosphatidylcytidine by PLDP-Catalyzed Transphosphatidylation



Organic phase : Solvent (0.5ml) Contained 1mg Dipalmitoylphosphatidylcholine
Aqueous phase : 0.25M Cytidine-HCl buffer pH4.5 (0.1ml) and 0.05ml of water contained 0.9 Unit of PLDP

Reaction Temp : 35℃, Reaction : 90 min.

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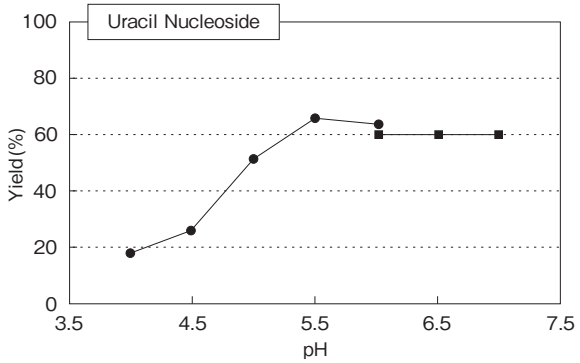
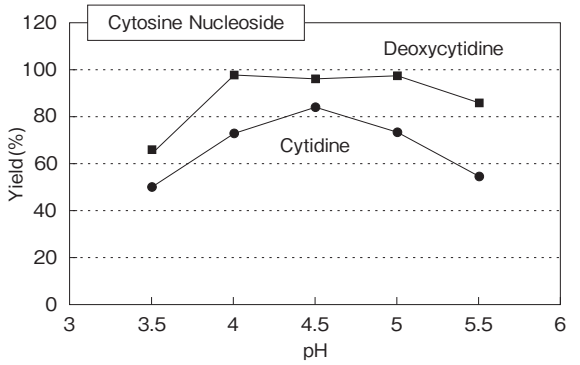
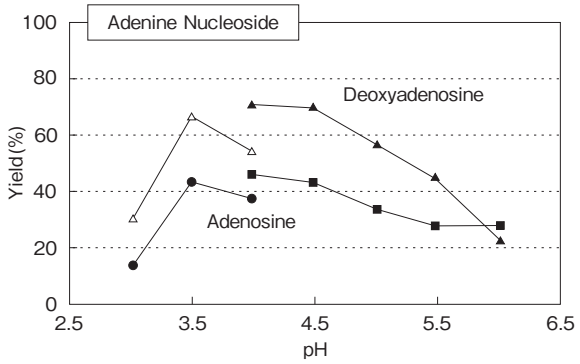
Fig.5 Optimum pH of PLDP-Catalyzed



Donor : Uridine 250 mM
pH4-6 : Acetate buffer
pH6-7 : MES-NaOH buffer



pH3.5-4 : Glycine-HCl buffer
pH4-5.5 : Acetate buffer
Cytidine, Deoxycytidine : 250mM



pH3-4 : Glycine-HCl buffer
pH4-6 : Acetate buffer
Adenosine : 50mM
Deoxyadenisine : 50mM

Phospholipid acceptor : Dipalmitoylphosphatidylcholine in Chloroform (1ml)
Buffer : 0.5ml contained 1.8 Units of PLDP

Reaction Temp : 35℃
Reaction period : 90 min.

Fig.6 The Influence of Reaction Temperature on the Yield of 5'-Phosphatidylcholine by PLDP-Catalyzed Transphosphatidylation
(Donor:dipalmitoylphosphatidylcholine, Acceptor: Cytidine, pH4.5)

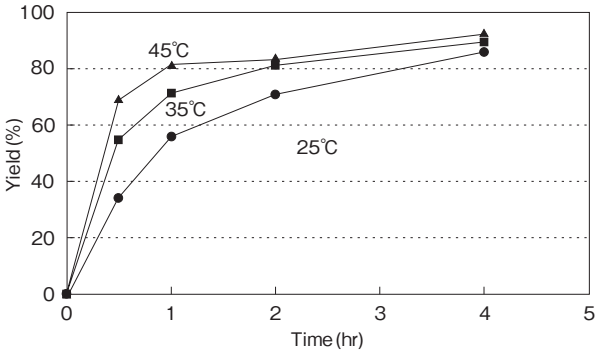
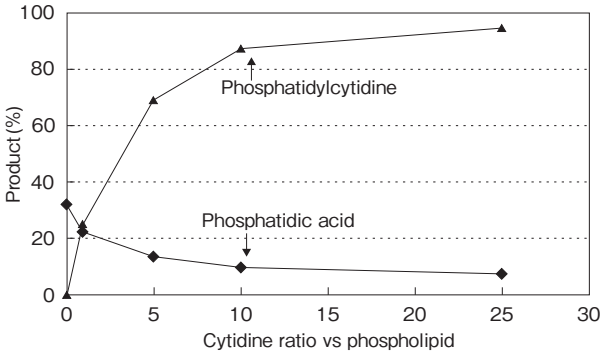


Fig.7 The Effect of Acceptor-Donor Ratio on the Yield of 5'-Posphatidylcytidine by PLDP-Catalyzed Transphosphatidylation



Organic solvent : 10 mM Dipalmitoylphosphatidylcholine in Chloroform (1ml)
Aqueous solvent : 0.1 M Glycine-HCl (pH4.5) 0.5ml containing 1.8 Units of PLDP

Reaction : at 35℃, with continuous stiiring, for 180min.

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:

$$\text{Phosphatidyl choline} + H_2O \xrightarrow{\text{PLDP}} \text{Phosphatidic acid} + \text{Choline}$$

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

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

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

0.2 M DMG–NaOH buffer pH 5.5	0.10 ml
10 mM $CaCl_2$	0.05 ml
3% (W/V) Triton X–100 solution	0.05 ml
10 mM Substrate solution [1)]	0.10 ml
Distilled water	0.15 ml

DMG: 3, 3'–Dimethylglutarate

1): 10 mM Substrate solution
Dissolve 78.6 mg of 1,2–Dioleoyl–sn–glycero–3–phosphocholine with 10 ml of 5% Triton X–100 (W/V).

2. Reaction mixture for the second reaction

15 mM 4–AA	0.05 ml
0.2% (W/V) Phenol	0.05 ml
1 M Tris–HCl buffer, pH 8.0	0.05 ml
50 U/ml POD [2)]	0.05 ml
50 U/ml COD [3)]	0.05 ml
Distilled water	0.25 ml

2) : 50 U/ml POD
Dissolve 500 U (PPU) of POD with 10 ml of distilled water.

3) : 50 U/ml COD
Dissolve 500 U of COD with 10 ml of 10 mM Tris–HCl buffer, pH 8.0.

3. Reaction stopper
1 M Tris–HCl buffer containing 10 mM EDTA and 1% (W/V) Cetyltrimetylammonium chloride
EDTA: Ethylenediamine tetraacetic acid

4. Reaction dilution solution
1% (W/V) Triton X–100

5. Enzyme dilution buffer
10 mM DMG–NaOH buffer, pH 5.5 containing 0.1% Triton X–100

6. Reagents:
DMG : Tokyo Kasei Kogyo Co., Ltd. #D1322
Triton X–100: The Dow Chemical Company
1,2–Dioleoyl–sn–glycero–3–phosphocholine: Sigma Chemical Co. #P–6354
EDTA ($2Na \cdot 2H_2O$) : KISHIDA CHEMICAL Co., Ltd. #060–29133
COD: Asahi Kasei Pharma Corporation #T–05
4–AA: NACALAI TESQUE, INC. Special grade #01907–52
Cethyltrimethylammonium chloride: FUJIFILM Wako Pure Chemical Corporation #087–06032
POD: Sigma Chemical Co. Type Ⅱ #P–8250

■ Enzyme solution

Weigh about 20 mg of test sample exactly 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 0.45 ml of reaction mixture for the first reaction into a small test tube and preincubate them at 37℃.
2. After 5 min, add exactly 50 μl of enzyme solution and mix to start the first reaction.
   ※ In the case of a test blank, add 50 μl of enzyme dilution buffer in place of enzyme solution at this point.
3. After 10 min, add 0.50 ml of reaction stopper and mix. On stopping the first reaction, add 0.50 ml of the reaction mixture for the second reaction immediately to start the second reaction at 37℃.
4. After 20 min, add 1.5 ml of reaction dilution solution and mix.
5. After 10 min, measure the absorbance at 500 nm.
   Absorbance sample:As
   blank :Ab
   $\triangle A = (As - Ab) \leqq 0.40$ Abs

■ Calculation

$$\text{Activity (U/mg)} = \frac{\triangle A/10}{12.2 \times 1/2} \times \frac{1}{2} \times \frac{3.00}{0.05} \times \frac{1}{X}$$

12.2 : millimolar extinction coefficient of quinoneimine dye at 500 nm (cm^2 / μmole)
2 : the multiplier derived from the fact that 1 mole of phosphatidyl choline produces 2 mole of H_2O_2
1/2 : the multiplier derived from the fact that 2 mole of H_2O_2 produces 1 mole of quinoneimine dye
10 : reaction time (min)
3.00 : 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 (Figure 3).

References

1. Satosi S., Shigeyuki I., Hideo S., and Jun-ichi M., (1987) Chem. Pharm. Bull., **35**. 1, 447–449
2. Satoshi S., Shigeru U., Shigeyuki I., Kiyofumi F., Akira M., and Tohru U., (1987) Tetrahedron, **28**, 2, 199–202
3. Satoshi S., Hiromichi I., Shigeru U., Shigeyuki I., Kiyofumi F., Masatoshi T., Akira M., and Tohru U., (1988) Chem. Pharm. Bull., **36**, 1, 209–217
4. Satoshi S., Shigeyuki I., Kiyofumi F., and Tohru U., (1988) Chem. Pharm. Bull., **36**, 12, 5020–5023
5. Satoshi S., Hiromichi I., Atsushi S., Keishi N., Shigeyuki I., and Akira M., (1995) Bioorg. Med. Chem., 3, 3, 235–243

PLDP活性測定法（Japanese）

Ⅰ．試薬液

1. 第一反応試薬混合液

0.2M DMG–NaOH 緩衝液 pH5.5	0.10 ml
10mM 塩化カルシウム溶液	0.05 ml
3%（W/V）トリトン X–100 溶液	0.05 ml
10mM 基質溶液 [1]	0.10 ml
精製水	0.15 ml

1):10mM 基質溶液
1, 2 - ジオレオイル sn– グリセロ –3– ホスホコリン 78.6mg を 5%（W/V）トリトン X–100 溶液 10ml で溶解する。

2. 第二反応試薬混合液

15mM 4–AA 溶液	0.05 ml
0.2%（W/V）フェノール液	0.05 ml
1M トリス –HCl 緩衝液 pH8.0	0.05 ml
50U/ml POD 溶液 [2]	0.05 ml
50U/ml COD 溶液 [3]	0.05 ml
精製水	0.25 ml

2): 50U/ml POD 溶液
POD500 単位（PPU）を精製水 10ml で溶解する。

3):50U/ml COD 溶液
COD500 単位（U）を 10mM トリス -HCl 緩衝液 pH8.0 10ml で溶解する。

3. 反応停止液
1%（W/V）塩化セチルトリメチルアンモニウムと 10mM EDTA を含む 1M トリス –HCl 緩衝液 pH8.0

4. 反応用希釈液
1%（W/V）トリトン X–100 溶液

5. 酵素溶解希釈用液
0.1% トリトン X–100 を含む 10mM DMG–NaOH 緩衝液 pH5.5

6. 試薬
DMG（3,3– ジメチルグルタール酸）:東京化成製 #D1322
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$_2$O）: 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
4–AA:ナカライテスク製 特級 #01907–52
塩化セチルトリメチルアンモニウム: 富士フイルム和光純薬製 #087–06032
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 0.45ml 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。
※盲検はこの時点で酵素試料液の代わりに酵素溶解希釈用液 50 μl を加える。
3. 10 分経過後、反応停止液 0.50ml を加えて混和し、第一反応を停止すると共に、直ちに第二反応試薬混合液 0.50ml を加えて混和し、37℃で第二反応を開始する。
4. 20 分経過後、反応用希釈液 1.50ml を加えて混和する。
5. 10 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。

$$\Delta A = (As - Ab) \leqq 0.40\ Abs$$

Ⅳ．計算

$$\text{活性 (U/mg)} = \frac{\Delta A/10}{12.2 \times 1/2} \times \frac{1}{2} \times \frac{3.00}{0.05} \times \frac{1}{X}$$

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）
2 : フォスファチジルコリン 1 モルから H_2O_2 2 モルが生成することによる係数
1/2 : H_2O_2 2 モルからキノンイミン色素 1 モルが生成することによる係数
10 : 反応時間（min）
3.00 : 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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