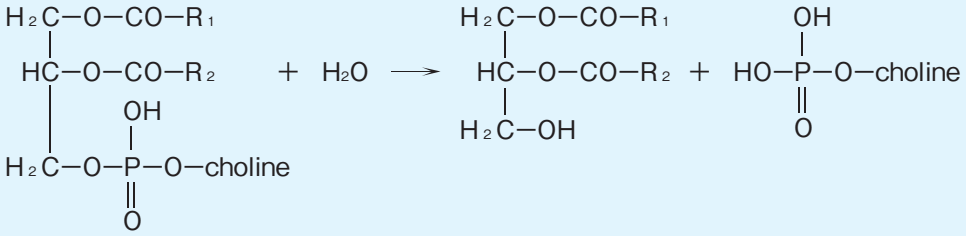
PHOSPHOLIPASE C [PLC]

from *Bacillus cereus*
(Phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3)



Phosphatidylcholine　　Diglyceride　　Phosphoryl choline

Preparation and Specification

Appearance : White to brownish amorphous powder, lyophilized
Specific activity : More than 30 U/mg solid

Properties

Molecular weight	: 20 kDa (Sephadex G-100)	
Isoelectric point	: pH 7.0	
Michaelis constant	: Phosphatidylcholine 2.0 × 10^{-2}M	
Optimum pH	: 7.0–9.0	Figure 1
pH stability	: 6.5–9.0	Figure 2
Thermal stability	: Stable at 60℃ and below (pH 7.5, 10 min)	Figure 3
Storage stability	: At least one year at −20℃	Figure 4
Effect of various chemicals	: See Table 1 and Table 2	
Activators	: Ca^{2+}, Sodium deoxycholate	
Inhibitors	: Tris-HCl buffer	

Applications for Diagnostic Test

This enzyme is useful for enzymatic determination of **lecithin** when coupled with alkaline phosphatase (T-08) and choline oxidase (T-05).

$$\text{Lecithin} + H_2O \xrightarrow{\text{PLC}} \text{Phosphorylcholine} + \text{Diglyceride}$$

$$\text{Phosphorylcholine} + H_2O \xrightarrow{\text{ALP}} \text{Choline} + Pi$$

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

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

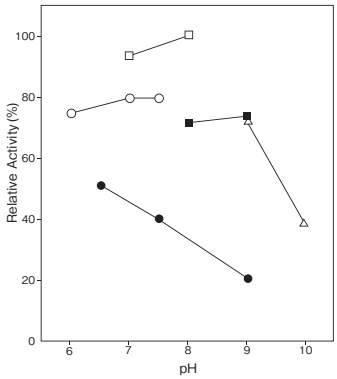
Table 1. Effect of detergents on PLC activity

Detergent	Concentration (%)	Relative activity (%)
None	0	100
Triton X-100	0.1	43
Adekatol SO-120	0.1	63
Deoxycholate	0.1	248
Tween 20	0.1	16

Table 2. Effect of metal ions on PLC activity

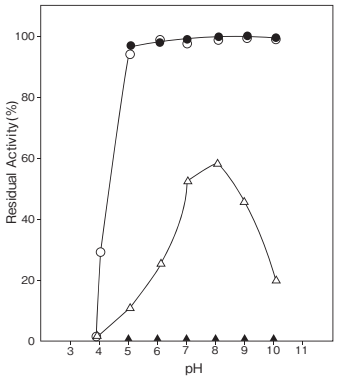
Metal ion	Concentration (mM)	Relative activity (%)
None	0	100
NaCl	100	103
KCl	100	102
$CaCl_2$	10	128
$MgCl_2$	10	123
$CoCl_2$	10	33
$MnCl_2$	10	54
$ZnCl_2$	10	79
$BaCl_2$	10	91
EDTA	10	26

Fig.1 pH Optimum



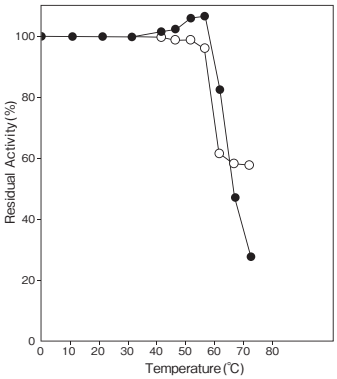
○ : 3,3-Dimethylglutarate-NaOH buffer
● : Tris-HCl buffer
□ : Collidine buffer
■ : Diethanolamine-HCl buffer
△ : Glycine-NaOH buffer

Fig.2 pH Stability



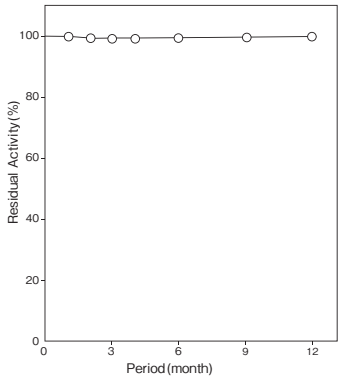
△ : -BSA, 37℃, 60 min.
▲ : -BSA, 37℃, 20 hr
○ : +BSA, 37℃, 60 min
● : +BSA, 37℃, 20 hr

Fig.3 Thermal Stability



○ : None
● : +BSA,10 min

Fig.4 Storage (lyophilized powder)



−20℃

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:

$$\text{Phosphatidyl choline} + H_2O \xrightarrow{\text{PLC}} \text{1, 2-Diglyceride} + \text{Phosphoryl choline}$$

$$\text{Phosphoryl choline} + H_2O \xrightarrow{\text{ALP}} \text{Choline} + \text{Orthophosphate}$$

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

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

ALP: Alkaline phosphatase
COD: Choline oxidase

■ Unit definition

One unit is defined as the amount of enzyme which liberates 1 μmole of phosphoryl 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 7.5	0.20 ml
10 mM Egg lecithin solution [1)]	0.20 ml
Distilled water	0.50 ml

DMG: 3, 3-Dimethylglutarate

1): 10 mM Egg lecithin solution

Pour the entire contents of one ampoule of egg lecithin into a test tube and remove ether. Then dry contents under reduced air pressure with rotary evaporator. Accurately weigh 1 g of egg lecithin into a beaker and add 135 ml of 0.5% deoxycholate (DOC) [※)] solution to dried product and suspend in homogenizer.

※): 0.5% DOC solution

Suspend 250 mg of deoxycholic acid with 40 ml of distilled water, adjust pH to 8.0 with 4 N NaOH, and add distilled water to make a total of 50 ml.

2. Reagent mixture for the second reaction

15 mM 4–AA solution	0.10 ml
0.2% (W/V) Phenol	0.10 ml
50 mM Tris–HCl buffer, pH 8.0	0.50 ml
90 U/ml POD [2]	0.10 ml
30 U/ml COD [3]	0.10 ml
80 U/ml ALP [4]	0.10 ml

2): 90 U/ml POD
Dissolve 900 U (PPU) of POD with 10 ml of distilled water.
3): 30 U/ml COD
Dissolve 300 U of COD with 10 ml of 10 mM Tris–HCl buffer (containing 1% KCl) , pH 8.0.
4): 80 U/ml ALP
Dissolve 800 U of ALP with 10 ml of 10 mM Tris–HCl buffer pH 9.0.

3. Reaction stopper
1 M Tris–HCl buffer pH 8.0 containing 0.2% (W/V) sodium dodecyl sulfate (SDS) .

4. Enzyme dilution buffer
10 mM DMG–NaOH buffer, pH 7.5 containing 0.1% (W/V) BSA

5. Reagents:
DMG: Tokyo Kasei Kogyo Co. Ltd. #D1322
Egg lecithin: Sigma Chemical Co. Type X VI –E #P–3556–1G
DOC: NACALAI TESQUE, INC. # 107–11
COD: Asahi Kasei Pharma Corporation # T–05
ALP: Asahi Kasei Pharma Corporation # T–08
4–AA: NACALAI TESQUE, INC. Special grade # 01907–52
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 as required.

■ **Procedure**

1. Pipette 0.90 ml of reagent mixture for the first reaction into a small test tube and preincubate at 37℃.
2. After 5 min, add exactly 100 μl of enzyme sample and mix to start the first reaction at 37℃.
※ In the case of a test blank, add 100 μl of enzyme dilution buffer in place of enzyme solution.
3. After 10 min, add 1.0 ml of reaction stopper and mix. On stopping the first reaction, add 1.0 ml of the reaction mixture for the second reaction immediately to start the second reaction at 37℃.
4. After 20 min, measure the absorbance at 500 nm.

Absorbance sample : As/min
blank : Ab/min

$\triangle A/min = (As/min - Ab/min) \leqq 0.30$ Abs/min

■ **Calculation**

$$\text{Activity (U/mg)} = \frac{\triangle A/10}{12.0} \times \frac{3.00}{0.10} \times \frac{1}{X}$$

12.0 : millimolar extinction coefficient of quinoneimine dye at 500 nm ($cm^2/\mu mole$)
10 : reaction time (min)
3.0 : final volume (ml)
0.10 : 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 4).

References

1. Zwaal, R. F., Roelofsen, B., Comfurius, P.S. and Van Deenen , L. L. M. (1971) Biochim. Biophys. Acta, **233**, 474.
2. Otnaess, A. B., Prydz, H., Bjoerklid, E. and Berre, A. (1972) Eur. J. Biochem., **27**, 238.

PLC 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 pH7.5	0.20 ml
10mM 卵黄レシチン溶液 [1]	0.20 ml
精製水	0.50 ml

1): 10mM 卵黄レシチン溶液
卵黄レシチン 1g をビーカーに量りとり、0.5%DOC 溶液※)135ml を加えてホモジナイザーにより分散懸濁する。
※): 0.5%DOC 溶液
デオキシコール酸 250mg を精製水 40ml に懸濁して 4N NaOH を加え pH8.0 位にして溶解した後、精製水で全容 50ml とする。

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
30U/ml COD 溶液 [2]	0.10 ml
90U/ml POD 液 [3]	0.10 ml
80U/ml ALP 溶液 [4]	0.10 ml
50mM トリス –HCl 緩衝液 pH8.0	0.50 ml

2): 30U/ml COD 溶液
COD 300 単位 (U) を 1% KCl を含む 10mM トリス –HCl 緩衝液 pH8.0 10ml で溶解する。
3): 90U/ml POD 液
POD 900 単位 (PPU) を精製水 10ml 溶解する。
4): 80U/ml ALP 溶液
ALP 800 単位 (U) を 10mM トリス –HCl 緩衝液 pH9.0 10ml で溶解する。

3. 反応停止液
   0.2%（W/V）ドデシル硫酸ナトリウム（SDS）を含む 1M トリス –HCl 緩衝液 pH8.0
4. 酵素溶解希釈用液
   0.1%（W/V）BSA を含む 10mM　DMG–NaOH 緩衝液 pH7.5
5. 試薬
   DMG（3,3–ジメチルグルタール酸）:
   東京化成製特級　#D1322
   卵黄レシチン（L–α–フォスファチジルコリン）:
   シグマ社製　Type X VI–E#P–3556–1G
   DOC（デオキシコール酸）: ナカライテスク社製
   #107–11
   COD（コリン酸化酵素）: 旭化成ファーマ製　#T–05
   ALP（アルカリフォスファターゼ）:
   旭化成ファーマ製　#T–08
   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

1. 小試験管に第一反応試薬混合液 0.90ml 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 100 μl を正確に加えて混和し、37℃で第一反応を開始する。
   ※盲検は酵素試料液の代わりに酵素溶解希釈用液 100 μl を加える。
3. 10 分経過後、反応停止液 1.0ml を加えて混和後、直ちに第二反応試薬混合液 1.0ml を加えて混和し、37℃で第二反応を開始する。
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。

$$\Delta A = (As - Ab) \leqq 0.30\ Abs$$

Ⅳ．計算

$$\text{活性 (U/mg)} = \frac{\Delta A/10}{12.0} \times \frac{3.00}{0.10} \times \frac{1}{X}$$

12.0 : キノンイミン色素の 500nm におけるミリモル分子吸光数（$cm^2/\ \mu mole$）
10　: 反応時間（min）
3.00 : 反応総液量（ml）
0.10 : 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）