INQUIRY NEEDED

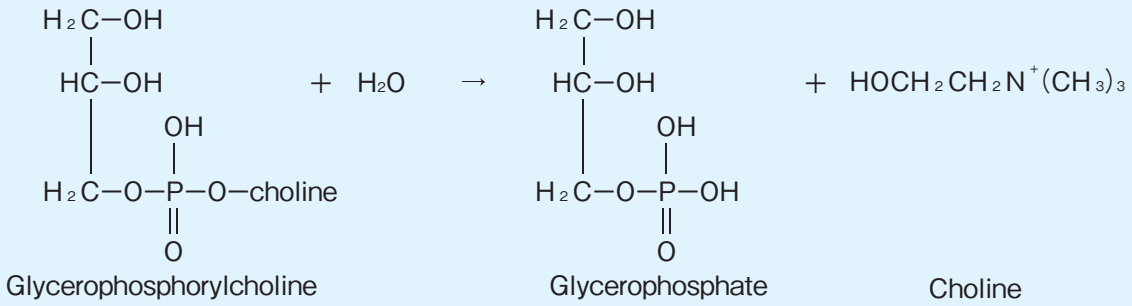
(Diagnostic Reagent Grade)

ASAHI KASEI ENZYMES T-33

GLYCEROPHOSPHORYLCHOLINE PHOSPHODIESTERASE [GPCP]

from *Gliocladium roseum*

(*sn*-Glycero-3-phosphocholine glycerophosphohydrolase, EC 3.1.4.2)



Preparation and Specification

Appearance : White to brownish amorphous powder, lyophilized
Specific activity : More than 13 U/mg solid

Properties

Substrate specificity	: See Table 1	
Isoelectric point	: pH 3.75	
Michaelis constant	: Glycerophosphorylcholine 1.0×10^{-4}M	
Optimum pH	: 8.5–9.0	Figure 1
pH stability	: 7.0–9.0 (75℃, 60 min)	Figure 2
Thermal stability	: Stable at 50℃ and below (pH 8.0, 10 min)	Figure 3
Effect of various chemicals	: See Table 2 and Table 3	
Activator	: Ca^{2+}	
Inhibitors	: Zn^{2+}, EDTA	

Applications for Diagnostic Test

This enzyme is useful for enzymatic determination of **phospholipid** when coupled with lysophospholipase (T-32) and choline oxidase (T-05).

$$\text{Lysolecithin} + H_2O \xrightarrow{\text{LYPL}} \text{Glycerophosphorylcholine} + \text{Fatty acid}$$

$$\text{Glycerophosphorylcholine} + H_2O \xrightarrow{\text{GPCP}} \text{Glycerophosphate} + \text{Choline}$$

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

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

Table 1. Substrate specificity of GPCP

Substrate	Relative activity (%)
Phosphatidylcholine	0
Lysophosphatidylcholine	0
Glycerophosphorylcholine	100
L- α -Glycerophophate	0

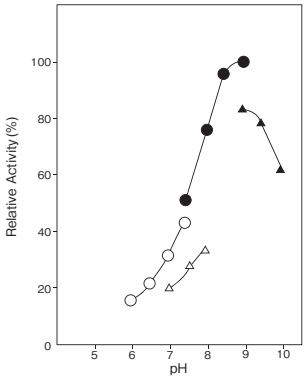
Table 2. Effect of detergents on GPCP activity

Detergent	Concentration (%)	Relative activity (%)
None	0	100
Triton X-100	0.5	104
Adekatol SO-145	0.5	108
Adekatol PC-8	0.5	101
Adekatol NP-700	0.5	103
Pluronic L-61	0.5	102
Sodium lauryl benzene sulfonate	0.5	0
Sodium lauryl sulfate	0.5	0
Cethyl pyridinium chloride	0.5	77
Cethyl trimethylammonium chloride	0.5	61

Table 3. Effect of metal ions on GPCP activity

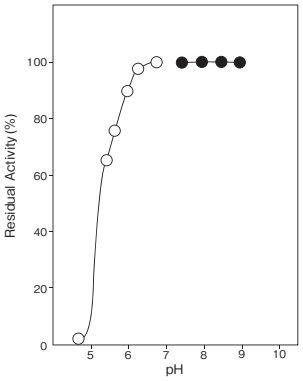
Metal ion	Concentration (mM)	Relative activity (%)
None	0	100
$CaCl_2$	1	117
$MgCl_2$	1	103
$MnCl_2$	1	16
$BaCl_2$	1	41
$CoCl_2$	1	6
$CuCl_2$	1	6
$NiCl_2$	1	0
NH_4Cl	100	115
LiCl	100	112
NaCl	100	109
KCl	100	112
EDTA	1	0

Fig.1 pH Optimum



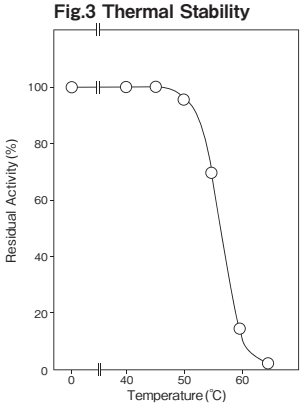
○ : 3,3-Dimethylglutarate-NaOH buffer
△ : Phosphate buffer
● : Tris-HCl buffer
▲ : Glycine-NaOH buffer

Fig.2 pH Stability



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

Fig.3 Thermal Stability



pH 8.0, 10 min.
10 mM Tris-HCl buffer

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:

$$GPC + H_2O \xrightarrow{GPCP} Glycerophosphate + Choline$$

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

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

GPCP: Glycerophosphorylcholine
COD: Choline oxidase

■ Unit definition

One unit is defined as the amount of enzyme which hydrolyzes 1 μmole of glycerophosphorylcholine per minute at 37℃ under the conditions specified in the assay procedure.

■ Reagents

1. Reaction mixture for the first reaction

0.2 M Tris-HCl buffer pH 8.0	0.10 ml
10 mM GPC solution [1)]	0.10 ml
10 mM $CaCl_2$ solution	0.05 ml
Distilled water	0.25 ml

1): 10 mM GPC solution pH 8.0
Accurately weigh 100 mg of GPC into a brown test tube and add 4 ml of distilled water and 80 μl of 5 N

NaOH. After suspending it, centrifugate at 3,000 rpm for 10 min. Remove and store supernatant. Add 4 ml of distilled water to the precipitate and suspend again. Remove and store supernatant after centrifugation.
Combine the supernatant portions and adjust pH to 8.0 (25℃) with diluted HCl and add distilled water to make a total of 20 ml.

2. Reaction mixture for the second reaction

0.2 M Tris-HCl buffer pH 8.0	0.10 ml
0.1 M EDTA solution pH 8.0	0.20 ml
0.3% 4-AA solution	0.10 ml
0.2 % (W/V) Phenol solution	0.10 ml
60 U/ml COD solution [2)]	0.10 ml
100 U/ml POD solution [3)]	0.05 ml
Distilled water	0.35 ml

EDTA: Ethylenediaminetetraacetic acid

2): 60 U/ml COD solution
Dissolve 600 U of COD with 10 ml of 10 mM Tris-HCl buffer pH 8.0.

3): 100 U/ml POD solution
1,000 U (PPU) of POD with 10 ml of distilled water.

3. Enzyme dilution buffer
10 mM Tris-HCl buffer pH 8.0 containing 0.05% (W/V) BSA.

4. Reagents
GPC: Sigma Chemical Co. #G-8005
EDTA ($2Na \cdot 2H_2O$):
KISHIDA CHEMICAL Co., Ltd. #060-29133
4-AA: NACALAI TESQUE, INC. Special grade #01907-52
COD: Asahi Kasei Pharma Corporation #T-05
POD: Sigma Chemical Co. Type Ⅱ #P-8250
BSA: Millipore Fraction V pH 5.2 #81-053

■ 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 at 37℃.
2. After 5 min, add exactly 50 μl of enzyme solution and mix to start the first reaction at 37℃.
※ In the case of a test blank, add 50 μl of enzyme dilution buffer in place of enzyme solution.
3. After 10 min, add 1.0 ml of reaction mixture for the second reaction and mix to start the second reaction at 37℃.
4. After 20 min, add 1.5 ml of distilled water to stop the reaction.
5. Measure the absorbance at 500 nm.

Absorbance sample : As
blank : Ab
△A = As − Ab

■ Calculation

$$\text{Activity (U/mg)} = \frac{\triangle A/10}{12.0 \times 1/2} \times \frac{1}{2} \times \frac{3.00}{0.05} \times \frac{1}{X}$$

12.0 : millimolar extinction coefficient of quinoneimine dye at 500 nm (cm^2 / μmole)
2 : a multiplier derived from the fact that 1 mole of glycerophosphorylcholine produces 2 mole of H_2O
1/2 : a 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.

References

1. Abra. R. M. and Quinn. P. J. (1975) Biochim. Biophys. Acta, **380**, 436-441.

GPCP 活性測定法 (Japanese)

Ⅰ. 試薬液

1. 反応試薬混合液 I

0.2M トリス－HCl 緩衝液 pH8.0	0.10 ml
10mM GPC 溶液 [1)]	0.05 ml
10mM 塩化カルシウム溶液	0.05 ml
精製水	0.25 ml

1): 10mM GPC 溶液 pH8.0
GPC100mg を褐色試験管に量り、精製水 4ml と 5N NaOH 80 μl を加えて攪拌懸濁した後、遠心分離器で分離 (3,000rpm 10min.) させる。この上清液を回収して残った沈殿物に再度、精製水 4ml を加えて攪拌懸濁し、遠心分離した後、上清液を回収する。先の上清液と混合して希薄な HCl で pH8.0 (25℃) に調整し、精製水で全容 20ml とする。

2. 反応試薬混合液Ⅱ

0.2M トリス－HCl 緩衝液 pH8.0	0.10 ml
0.1M EDTA 溶液 pH8.0	0.20 ml
0.3% 4-AA 溶液	0.10 ml
0.2% (W/V) フェノール溶液	0.10 ml
60U/ml COD 溶液 [2)]	0.10 ml
100U/ml POD 溶液 [3)]	0.05 ml
精製水	0.35 ml

2):60U/ml COD 溶液
COD 600 単位 (U) を 10mM トリス－HCl 緩衝液 pH8.0 10ml で溶解する。

3):100U/ml POD 溶液
POD 1,000 単位 (PPU) を精製水 10ml で溶解する。

3. 酵素溶解希釈用液
0.05%（W/V）BSA を含む 10mM トリス－HCl 緩衝液 pH8.0
4. 試薬
GPC（グリセロリン酸コリン）：シグマ社製 #G–8005
EDTA（エチレンジアミン四酢酸･2Na･2H$_2$O）：キシダ化学社製 #060–29133
4–AA：ナカライテスク社製　特級　#01907–52
COD（コリン酸化酵素）：旭化成ファーマ製　#T–05
POD：シグマ社製　Type Ⅱ #P–8250
BSA: Millipore 社製　Fraction V pH5.2 #81–053

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溶解して全容 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. 小試験管に反応試薬混合液Ⅰ 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、反応試薬混合液Ⅱ 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、精製水 1.50ml を加えて混和し、反応を停止する。
5. 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。

$$\Delta A = As - Ab$$

Ⅳ．計算

$$\text{活性 (U/mg)} = \frac{\Delta A/10}{12.0 \times 1/2} \times \frac{1}{2} \times \frac{3.00}{0.05} \times \frac{1}{X}$$

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/ μmole）
1/2 : H$_2$O$_2$ 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
2 : グリセロリン酸コリン 1 モルから H$_2$O$_2$ 2 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 : 酵素試料液中の検品濃度（mg/ml）