●TOYOBO ENZYMES●

(Diagnostic Reagent Grade)

GLYCEROL KINASE

from Microorganism

ATP:glycerol 3-phosphotransferase (EC 2.7.1.30)

$$\text{Glycerol} + \text{ATP} \xrightarrow{Mg^{++}} \text{Glycerol-3-phosphate} + \text{ADP}$$

PREPARATION and SPECIFICATION

Appearance	: White amorphous powder, lyophilized	
Activity	: Grade Ⅲ 30U/mg-solid or more	
Contaminants	: Catalase	≤1.0×10^{-1}%
	NADH oxidase	≤1.0×10^{-3}%
	Adenosine triphosphatase	≤1.0×10^{-3}%

PROPERTIES

Stability	: Stable at −20℃ for at least one year	(Fig.1)
Molecular weight	: approx. 220,000 (by gel filtration)	
Structure	: Four subunits of approx. 58,000	
Isoelectric point	: 4.3	
Michaelis constants	: 9.4×10^{-5}M (Glycerol), 1.3×10^{-5}M (ATP), 2.1×10^{-3}M (Dihydroxyacetone)	
Inhibitors	: p-Chloromercuribenzoate, Hg^{++}, Ag^{+}	
Optimum pH	: 10.0	(Fig.2)
Optimum temperature	: 70℃	(Fig.3)
pH Stability	: pH 5.5−10.0 (25℃, 20hr)	(Fig.4)
Thermal stability	: below 65℃ (pH 7.5, 30min)	(Fig.5)
Substrate specificity	: (Table 1)	
Effect of various chemicals	: (Table 2)	

APPLICATIONS

This enzyme is useful for enzymatic determination of glycerol and triglyceride when coupled with glycerol-3-phosphate oxidase (=G-3-P oxidase, G3O-321) or pyruvate kinase and lactate dehydrogenase (LCD-209, LCD-211, LCD-221), lipoprotein lipase (LPL-311, LPL-314) in clinical analysis.

ASSAY

Principle:

$$\text{Glycerol} + \text{ATP} \xrightarrow[Mg^{++}]{\text{glycerol kinase}} \text{Glycerol-3-P} + \text{ADP}$$

$$\text{Glycerol-3-P} + O_2 \xrightarrow{\text{G-3-POD}} \text{Dihydroxyacetone-P} + H_2O_2$$

$$2H_2O_2 + \text{4-Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + 4H_2O$$

The appearance of quinoneimine dye is measured at 500nm by spectrophotometry.

Unit definition:

One unit causes the formation of one micromole of hydrogen peroxide (half a micromole of quinoneimine dye) per minute under the conditions described below.

Method:

Reagents

A. Glycerol solution : 0.3M (Should be prepared fresh)
B. 4-AA solution : 0.1 % (100mg of 4-aminoantipyrine / 100ml of H_2O)
C. Phenol solution : 0.1 % (100mg of phenol / 100ml of H_2O)
D. Peroxidase solution : 20mg Peroxidase (110 purpurogallin units/mg)/100ml of H_2O
E. G-3-POD solution : 20U/ml (dissolve in 200 mM HEPES buffer, pH 7.9)
F. Buffer solution : 200mM HEPES, pH 7.9 contg. 20mM $MgCl_2$ and 40mM ATP (should be prepared freshly)
G. Enzyme diluent : 20mM K-phosphate buffer, pH 7.5

Procedure

1. Prepare the following working solution in a brownish bottle and store on ice.

10ml	4-AA solution	(B)
20ml	Phenol solution	(C)
20ml	Peroxidase solution	(D)
40ml	G-3-POD solution	(E)
10ml	Buffer solution	(F)

Concentration in assay mixture	
HEPES buffer	95.2 mM
Glycerol	4.76 mM
ATP	3.81 mM
$MgCl_2$	1.90 mM
4-AA	0.469 mM
Phenol	2.02 mM
Peroxidase	ca.5.2 U/ml
G-3-POD	ca.7.6 U/ml

2. Pipette 3.0 ml of working solution in a cuvette (d=1.0cm).
3. Add 0.1ml of enzyme solution*, mix by gently inversion and equilibrate at 37℃ for about 5 minutes.
4. Add 0.05ml of glycerol solution (A) and mix by gentle inversion.
5. Record the optical density at 500nm against water for 3 to 4 minutes in a spectrophotometer thermostated at 37℃, and calculate ΔOD per minute from the initial portion of the curve (ΔOD test).

   At the same time, measure the blank rate (ΔOD blank) by the same method as test except the enzyme diluent is added instead of the enzyme solution.

* Dissolve the enzyme preparation in ice-cold enzyme diluent (G) and dilute to 0.2−0.4U/ml with the same buffer, immediately before assay.

Calculation

Activity can be calculated by using the following formula :

$$\text{Volume activity (U/ml)} = \frac{\Delta\text{OD/min (OD test} - \text{OD blank)} \times Vt \times df}{13.3 \times 1/2 \times 1.0 \times Vs} = \Delta\text{OD/min} \times 4.74 \times df$$

Weight activity (U/mg) = (U/ml)×1/C

Vt : Total volume (3.15ml)
Vs : Sample volume (0.1ml)
13.3 : Millimolar extinction coefficient of quinoneimine dye under the assay condition (cm²/micromole)
1/2 : Factor based o the fact that one mole of H_2O_2 produces half a mole of quinoneimine dye
1.0 : Light path length (cm)
df : Dilution factor
C : Enzyme concentration in dissolution (c mg/ml)

REFERENCES

1) H.-S.Huang, T.Yoshida, Y.Meng, T.Kabashima, K.Ito, Y.Nishiya, Y.Kawamura, and T.Yoshimoto; *J.Ferment.Bioeng., 83,* 328 (1997).

Table 1. Substrate Specificity of Glycerol kinase

[Pyruvate kinase-Lactate dehydrogenase system with 50mM HEPES buffer, pH 7.9]

Substrate (4.5mM)	Relative activity(%)	Substrate (4.5mM)	Relative activity(%)
Glycerol	100	2,3-Butanediol	0.2
Glycerol-α-monochlorohydrin	0.1	D-Mannitol	—
Ethylene glycol	—	D-Sorbitol	—
1,2-Propanediol	—	D-Glucose	—
1,3-Propanediol	0.2	Ribitol	—
1,3-Butanediol	—	Methanol	—
1,4-Butanediol	0.1	Ethanol	—

Table 2. Effect of Various Chemicals on Glycerol kinase

[The enzyme dissolved in 20mM K-phosphate buffer, pH 7.5 (100U/ml) was incubated with each chemical at 25℃ for 1hr.]

Chemical	Concn.(mM)	Residual activity(%)	Chemical	Concn.(mM)	Residual activity(%)
None	—	100	MIA	1.0	101
Metal salt			NaF	1.0	100
$MgCl_2$	1.0	100	NaN_3	1.0	106
$CaCl_2$		102	EDTA	5.0	100
$Ba(OAc)_2$		101	o-Phenanthroline	1.0	102
$FeSO_4$		98	α,α′-Dipyridyl	1.0	101
$FeCl_3$		89	Borate	50	103
$CoCl_2$		104	IAA	1.0	99
$MnCl_2$		99	NEM	1.0	100
$ZnCl_2$		103	Hydroxylamine	1.0	99
$Cd(OAc)_2$		101	Triton X-100	1.0%	103
$NiCl_2$		98	Brij 35	0.1%	104
$CuSO_4$		99	Tween 20	0.1%	103
$Pb(OAc)_2$		100	Span 20	0.1%	102
$AgNO_3$		10	Na-cholate	0.5%	105
$HgCl_2$		2	SDS	0.5%	1
Dithiothreitol	1.0	100	DAC	0.5%	84
PCMB	1.0	0			

Ac, CH_3CO; PCMB, p-Chloromercuribenzoate; MIA, Monoiodoacetate; EDTA, Ethylenediaminetetraacetate; IAA, Iodoacetamide; NEM, N-Ethylmaleimide; SDS, Sodium dodecyl sulfate; DAC, Dimethylbenzylalkylammonium chloride.

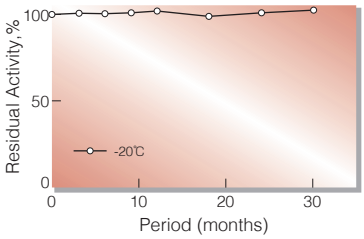

Fig.1. Stability (Powder form)

〔kept under dry conditions〕

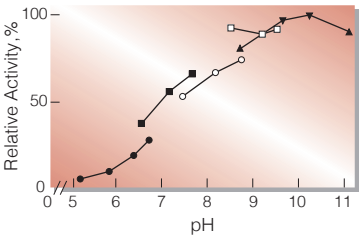

Fig.2. pH-Activity

〔37℃ 10min-reaction in 45mM buffer solution: pH5.2-6.7,MES;pH6.6-7.7,HEPES; pH7.5-8.7,TAPS;pH8.5-9.6,CHES; pH8.7-11.2,Glycine-NaOH〕

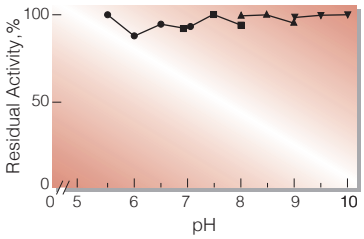

Fig.4. pH-Stability

〔enzyme concn. ca.300U/ml 25℃ 20hr-treatment in 50mM buffer solution: pH5.6-7.1,MES;pH7.1-8.0,HEPES;pH8.0-9.0, TAPS;pH9.0-10.0,CHES〕

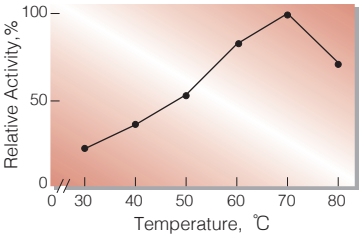

Fig.3. Temperature activity

〔10min-reaction in 45mM HEPES buffer,pH7.9〕

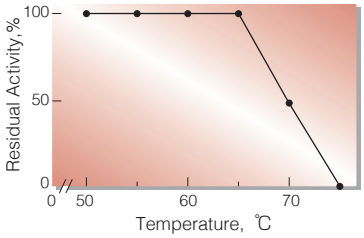

Fig.5. Thermalstability

〔enzyme concn. ca.300U/ml 30min-treatment with 20mM K-phosphate buffer, pH7.5〕

活性測定法（Japanese）

1.原理

Glycerol＋ATP $\xrightarrow[Mg^{++}]{\text{glycerol kinase}}$ Glycerol-3-P＋ADP

Glycerol-3-P＋O_2 $\xrightarrow{\text{G-3-POD}}$ Dihydroxyacetone-P＋H_2O_2

$2H_2O_2$＋4-Aminoantipyrine＋Phenol $\xrightarrow{\text{Peroxidase}}$ Qinoneimine dye＋4H_2O

4-AminoantipyrineとPhenolの酸化縮合生成物であるQuinoneimine色素を500 nmで測定し，上記反応で生成したH_2O_2量を定量する。

2.定義

下記条件下で1分間に1マイクロモルのH_2O_2を生成する酵素量を1単位(U)とする。

3.試薬

A. 0.3M グリセロール溶液(用時調製)
B. 4-AA溶液, 0.1 %(100mgの4-アミノアンチピリンを100mℓの蒸留水で溶解する。)
C. フェノール溶液,0.1%(100mgのフェノールを100mℓの蒸留水で溶解する。)
D. ペルオキシダーゼ溶液 [25mgのペルオキシダーゼ(110プルプロガリン単位/mg)を約100mℓの蒸留水で溶解する。]
E. G-3-POD溶液, 20U/mℓ(200mM HEPES緩衝液, pH7.9で溶解する。)
F. 緩衝液(20 mM $MgCl_2$と40mM ATPを含む200mM HEPES緩衝液, pH 7.9)(用時調製)

酵素溶液：酵素標品を予め氷冷した20mM Kリン酸緩衝液, pH7.5で分析直前に0.2～0.4 U/mℓに希釈する。

4.手順

①下記反応混液を褐色ビンに調製する(褐色ビンにて氷冷保存)。

10mℓ	4-AA溶液	(B)
20mℓ	フェノール溶液	(C)
20mℓ	ペルオキシダーゼ溶液	(D)
40mℓ	G-3-POD溶液	(E)
10mℓ	緩衝液	(F)

②上記反応混液をキュベット(d=1cm)に3.0mℓ採り，酵素液0.1mℓを加え，ゆるやかに混和後，37℃で約5分間予備加温する。
③グリセロール溶液(A)0.05mℓを添加し，ゆるやかに混和後，水を対照に37℃に制御された分光光度計で500 nmの吸光度変化を3～4分間記録し，その初期直線部分から1分間当たりの吸光度変化を求める(ΔODtest)。
④盲検は反応混液①に酵素溶液の代わりに酵素希釈液(20mM Kリン酸緩衝液、pH7.5)を0.1mℓ加え，上記同様に操作を行って,1分間当たりの吸光度変化を求める(ΔODblank)。

5.計算式

$$U/mℓ = \frac{\Delta OD/min\ (OD\ test - OD\ blank) \times 3.15(mℓ) \times 希釈倍率}{13.3 \times 1/2 \times 1.0 \times 0.1(mℓ)}$$

$$= \Delta OD/min \times 4.74 \times 希釈倍率$$

U/mg ＝U/mℓ×1/C

13.3 ：Quinoneimine色素の上記測定条件下でのミリモル分子吸光係数(㎠/micromole)
1/2 ：酵素反応で生成したH_2O_2の1分子から形成するQuinoneimine色素は1/2分子である事による係数
1.0 ：光路長(cm)
C ：溶解時の酵素濃度(c mg/mℓ)