●TOYOBO ENZYMES●

(Diagnostic Reagent Grade)

L-α-GLYCEROPHOSPHATE OXIDASE

from Microorganism

sn-Glycerol-3-phosphate:oxygen 2-oxidoreductase (EC 1.1.3.21)

Glycerol-3-phosphate + O_2 ⟶ 3-Dihydroxyacetone phosphate + H_2O_2

PREPARATION and SPECIFICATION

Appearance	: Yellowish amorphous powder, lyophilized	
Activity	: Grade Ⅲ 15U/mg-solid or more	
Contaminants	: Lactate oxidase	≤2.0×10^{-4}%
	Adenosine triphosphatase	≤2.0×10^{-4}%
Stabilizers	: Amino acids , FAD	

PROPERTIES

Stability	: Stable at −20℃ for at least one year	(Fig.1)
Molecular weight	: approx. 67,000 (by SDS-PAGE)	
Isoelectric point	: 4.6±0.1	
Michaelis constant	: 1.3×10^{-3}M	
Inhibitors	: SH-reagents, ionic detergents, metal ions, etc.	
Optimum pH	: 6.0−7.0	(Fig.3)
Optimum temperature	: 45℃	(Fig.4)
pH Stability	: 4.5−8.5 (25℃, 20hr)	(Fig.5)
Thermal stability	: below 45℃ (pH6.5,15min)	(Fig.6)
Effect of various chemicals	: (Table 1)	

APPLICATIONS

This enzyme is useful for enzymatic determination of triglyceride when coupled with lipoprotein lipase (LPL-311, LPL-314) and glycerokinase (GYK-301, GYK-311) in clinical analysis.

ASSAY

Principle:

Glycerol-3-phosphate + O_2 $\xrightarrow{\text{L-}\alpha\text{-glycerophosphate oxidase}}$ Dihydroxyacetone 3-phosphate + H_2O_2

$2H_2O_2$ + 4-Aminoantipyrine + EHSPT $\xrightarrow{\text{peroxidase}}$ Quinoneimine dye + $4H_2O$

The appearance of quinoneimine dye is measured at 555nm 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. D, L-α-Glycerophosphate solution : 1.5M [Weigh 48.63g of D,L-α-Glycerophosphate(disodium salt,MW=324.17), dissolved in 60ml of H_2O and after adjusting the pH to 6.5±0.05 at 25℃ with 4.0N HCl,fill up to 100ml with H_2O]
(Stable for two weeks if stored at 0−4℃)

B. PIPES-NaOH buffer, pH 6.5 : 0.5M [Weigh 15.12g of PIPES (MW=302.36),suspend in 60ml of H_2O dissolve with 10N NaOH. After adjusting the pH to 6.5±0.05 at 25℃ with 10N NaOH, fill up to 100ml with H_2O](Stable for two weeks if stored at 0−4℃)

C. 4-AA solution : 28mM [569mg 4-aminoantipyrine(MW=203.25)/100ml of H_2O](Stable for one week if stored at 4℃ in a brownish bottle)

D. EHSPT(TOOS) solution : 20mM [591mg N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine (MW=295.3)/100ml of H_2O](Stable for one week if stored at 4℃ in a brownish bottle)

E. Peroxidase solution : 0.05% [50mg peroxidase (110 purpurogallin units/mg)/100ml of H_2O(Should be prepared fresh)

F. Enzyme diluent : 20mM PIPES-NaOH buffer, pH 6.5 contg. 0.5M NaCl

Procedure

1. Prepare the following working solution (40 tests) in a brownish bottle and store on ice.test).

40ml	Substrate solution	(A)
40ml	PIPES-NaOH buffer, pH 6.5	(B)
5ml	4-AA solution	(C)
5ml	EHSPT solution	(D)
10ml	Peroxidase solution	(E)

Concentration in assay mixture	
PIPES-NaOH buffer	193 mM
NaCl	19.2 mM
D,L-α-Glycerophosphate	577 mM
4-Aminoantipyrine	1.3 mM
EHSPT	0.96mM
Peroxidase	ca.5.3 U/ml

2. Pipette 2.5ml of working solution into a cuvetto (d=1.0cm) and equilibrate at 30℃ for about 5 minutes.
3. Add 0.1ml of the enzyme solution* and mix by gentle inversion.
4. Record the increase in optical density at 555nm against water for 3 to 4 minutes in a spectrophotometer thermostated at 30℃, and calculate the ΔOD per minute from the initial linear portion of the curve (ΔOD test).
   At the same time, measure the blank rate (ΔOD blank) by using the same method as the ΔOD test except that the enzyme diluent is added instead of enzyme solution.

* Dissolve the enzyme preparation in ice-cold enzyme diluent (F), dilute to 0.05−0.2U/ml with the same buffer and store on ice.

Calculation

Activity can be calculated by using the following formula :

$$\text{Volume activity (U/ml)} = \frac{\Delta\text{OD/min}\,(\Delta\text{OD test} - \Delta\text{OD blank}) \times \text{Vt} \times \text{df}}{29.9 \times 1/2 \times 1.0 \times \text{Vs}} = \Delta\text{OD/min} \times 1.739 \times \text{df}$$

Weight activity (U/mg) = (U/ml)×1/C

Vt : Total volume (2.6ml)

Vs : Sample volume (0.1ml)

29.9 : Millimolar extinction coefficient of quinoneimine dye under the assay condition (㎠/micromole)

1/2 : Factor based on 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)

Table 1. Effect of Various Chemicals on L-α-Glycerophosphate oxidase
[The enzyme dissolved in 0.1M PIPES-NaOH buffer, pH7.0 (10U/ml) was incubated at 25℃ for 1hr.]

Chemical	Concn.(mM)	Residual activity(%)
None	—	100
Metal salt	2.0	
$MgCl_2$		100.0
$CaCl_2$		96.5
$BaCl_2$		99.5
$FeSO_4$		67.4
$FeCl_3$		73.1
$CoCl_2$		99.5
$MnCl_2$		100.1
$ZnSO_4$		96.4
$NiCl_2$		97.9
$AgNO_3$		93.1
$CuSO_4$		84.7
MIA	2.0	98.4
NaF	2.0	99.4
NaN_3	20	100.2
EDTA	50	99.6
o-Phenanthroline	2.0	101.6
α,α′-Dipyridyl	2.0	100.0
Borate	20	101.8
IAA	2.0	98.7
NEM	2.0	99.8
Hydroxylamine	2.0	100
Triton X-100	0.10%	110.6
Brij 35	0.10%	108.9
Tween 20	0.10%	99.1
Span 20	0.10%	103.7
Na-cholate	0.10%	105.8
SDS	0.05%	3.0
DAC	0.05%	71.8

MIA, Monoiodoacetate; EDTA, Ethylenediaminetetraacetate; IAA, Iodoacetamido;
NEM, N-Ethylmaleimide; SDS, Sodium dodecyl sulfate; DAC, Dimethylbenzylalkylammonium chloride.

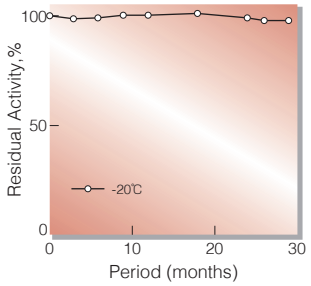

Fig.1. Stability (Powder form)

〔Kept under dry form〕

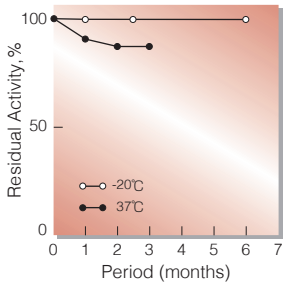

Fig.2. Stability (Powder form)

〔Kept under dry form〕

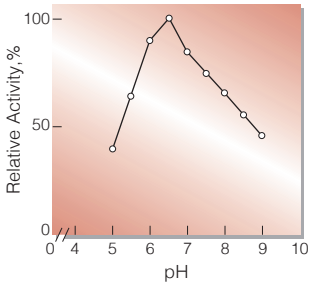

Fig.3. pH-Activity

〔0.1M buffer solution:pH5.0-6.5 ,MES;pH6.5-7.5,PIPES-NaOH ;7.5-9.0,Tris-HCl〕

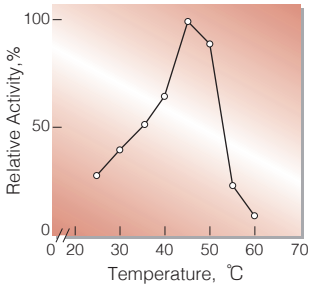

Fig.4. Temperature activity

〔in 0.2M PIPES-NaOH buffer,pH6.5〕

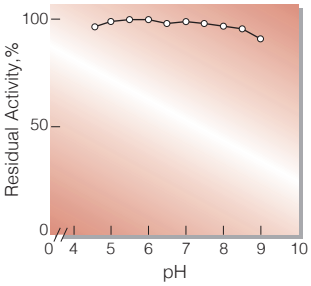

Fig.5. pH-Stability

〔25℃,20hr-treatment with 0.1M buffer solution; pH4.5-6.0,acetate;pH6.0-8.0,K-phosphate; pH8.0-9.0,Tris-HClenzyme concn.:20U/ml,〕

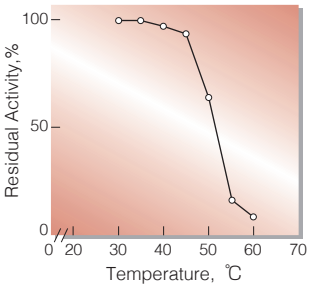

Fig.6. Thermal stability

〔15min-treatment with 0.1M PIPES-NaOH buffer,pH6.5, enzyme concn.:20U/ml〕

活性測定法（Japanese）

1.原理

Glycerol-3-phosphate＋O_2 $\xrightarrow{\text{L-}\alpha\text{-glycerophosphate oxidase}}$ Dihydroxyacetone 3-phosphate＋H_2O_2

$2H_2O_2$＋4-Aminoantipyrine＋EHSPT $\xrightarrow{\text{peroxidase}}$ Quinoneimine dye＋$4H_2O$

4-AminoantipyrineとEHSPTの酸化縮合生成物であるQuinoneimine色素を555nm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. 1.5Mグリセロリン酸溶液[48.63gのD,L-α-グリセロリン酸・2Na(MW＝324.17)を約60mℓの蒸留水で溶解後，4.0N HClでpHを6.5±0.05に調整(25℃)し，蒸留水で100mℓとする。](0～4℃保存で2週間は使用可能)

B. 0.5M PIPES-NaOH緩衝液,pH6.5[15.12gのPIPES(MW＝302.36)を約60mℓの蒸留水で溶解しながら，10N NaOHでpHを6.5±0.05に調整(25℃)し，蒸留水で100mℓとする。](0～4℃保存で2週間は使用可能)

C. 28mM 4-AA水溶液[569mgの4-アミノアンチピリン(MW＝203.25)を100mℓの蒸留水に溶解する。](褐色瓶中4℃保存で1週間は使用可能)

D. 20mM EHSPT(TOOS)水溶液[591mgのEHSPT(MW＝295.3)を100mℓの蒸留水に溶解する。(褐色瓶中4℃保存で1週間は使用可能)

E. POD溶液[50mgのペルオキシダーゼ(POD)(110プルプロガリン単位/mg)を100mℓの蒸留水に溶解する。](褐色瓶中で4℃保存)(用時調製)

酵素溶液：酵素標品を予め氷冷した0.5M NaCl含む20mM PIPES-NaOH緩衝液,pH6.5で溶解(約1mg/mℓ)し，分析直前に同緩衝液で0.05～0.20U/mℓに希釈する。

4.手順

①下記反応混液を調製する。(褐色瓶にて氷冷保存)

40mℓ	基質溶液	(A)
40mℓ	緩衝液	(B)
5mℓ	4-AA水溶液	(C)
5mℓ	EHSPT水溶液	(D)
10mℓ	POD水溶液	(E)

②反応混液2.5mℓをキュベット(d=1.0cm)にとり，30℃で約5分間予備加温する。

③酵素溶液0.1mℓを添加し，ゆるやかに混和後，水を対照に30℃に制御された分光光度計で555nm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に酵素溶液の代りに酵素希釈液(0.5M NaCl含む20mM PIPES-NaOH緩衝液，pH6.5)0.10mℓを加え，上記同様に操作を行い，1分間当りの吸光度変化を求める(ΔODblank)。

5.計算式

$$U/mℓ = \frac{\Delta OD/min\ (\Delta OD\ test - \Delta OD\ blank) \times 2.60(mℓ) \times 希釈倍率}{29.9 \times 1/2 \times 1.0 \times 0.1}$$

$$= \Delta OD/min \times 1.739 \times 希釈倍率$$

U/mg ＝U/mℓ×1/C

29.9 ：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$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)