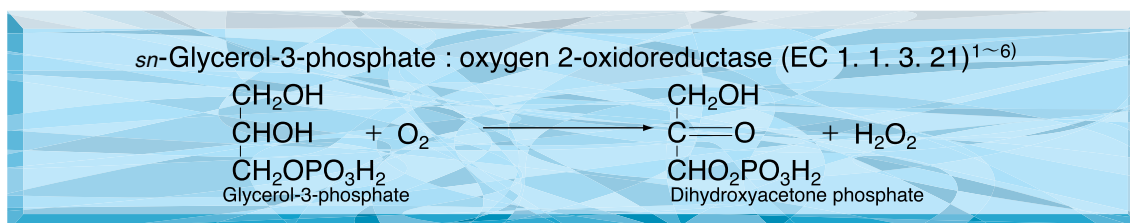


● TOYOBO ENZYMES ●

(Diagnostic Reagent Grade)

L- α -GLYCEROPHOSPHATE OXIDASE

from Microorganism



PREPARATION and SPECIFICATION

Appearance	: Yellowish amorphous powder, lyophilized
Activity	: Grade III 15U/mg-solid or more (containing approx. 60% of stabilizer)
Contaminants	: Lactate oxidase $\leq 2.0 \times 10^{-4}\%$ Phosphatase $\leq 1.0 \times 10^{-3}\%$
Stabilizers	: Amino acids, FAD

PROPERTIES

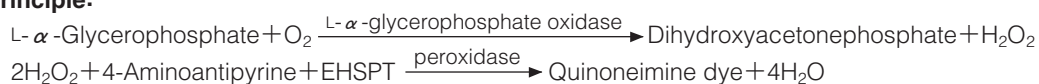
Stability	: Stable at -20°C for at least 6 months	(Fig.1)
Molecular weight	: approx. 93,000 (by gel filtration)	
Isoelectric point	: 4.6 ± 0.1	
Michaelis constant	: $2.3 \times 10^{-3}\text{M}$ (L- α -Glycerophosphate)	
Inhibitors	: SH-reagents, ionic detergents, metal ions, etc.	
Optimum pH	: 6.5–7.0	(Fig.3)
Optimum temperature	: 40°C	(Fig.4)
pH Stability	: 5.0–7.5 (25°C , 60min)	(Fig.5)
Thermal stability	: below 45°C (pH 6.5, 10min)	(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:



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), dissolve in 60ml of H₂O and after adjusting the pH to 6.5±0.05 at 25°C with 4.0 N HCl, fill up to 100ml with H₂O] (Stable for two weeks if stored at 0–4°C)
- B. PIPES-NaOH buffer, pH 6.5 : 0.5M [Weigh 15.12g of PIPES (MW=302.36), suspend in 60ml of H₂O, dissolve with 10 N NaOH. After adjusting the pH to 6.5±0.05 at 25°C with 10 N NaOH, fill up to 100ml with H₂O] (Stable for two weeks if stored at 0–4°C)
- C. 4-AA solution : 28mM [569mg 4-aminoantipyrine (MW=203.25)/100ml of H₂O] (Stable for one week if stored at 4°C in a brownish bottle)
- D. EHSPT (TOOS) solution : 20mM [591mg N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine (MW=295.3)/100ml of H₂O] (Stable for one week if stored at 4°C in a brownish bottle)
- E. Peroxidase solution : 0.05% [50mg peroxidase (110 purpurogallin units/mg)/100ml of H₂O] (Should be prepared fresh)
- F. Enzyme diluent : 20mM PIPES-NaOH buffer, pH 6.5 contg. 0.5M NaCl

Procedure

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

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
- Pipette 2.5ml of working solution into a cuvette (d=1.0cm) and equilibrate at 30°C for about 5 minutes.
- Add 0.10 ml of the enzyme solution* and mix by gentle inversion.
- Record the increase in optical density at 555nm against water for 3 to 4 minutes in a spectrophotometer thermostated at 30°C, and calculate the Δ OD per minute from the initial linear portion of the curve (Δ OD test).

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

- * Dissolve the enzyme preparation in ice-cold enzyme diluent (F), dilute to 0.05–0.20U/ml with the same buffer and store on ice.

Calculation

Activity can be calculated by using the following formula :

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

$$\text{Weight activity (U/mg)} = (\text{U/ml}) \times 1/C$$

V_t : Total volume (2.60ml)

V_s : Sample volume (0.10ml)

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

1/2 : Factor based on the fact that one mole of H₂O₂ produces half a mol of quinoneimine dye

1.0 : Light path length (cm)

df : Dilution factor

C : Enzyme concentration in dissolution (c mg/ml)

REFERENCES

L- α -Glycerophosphate oxidase from

Yeast

1) C.Gancedo, J.M.Gancedo and A.Sols; *European J.Biochem*, 5,165 (1968).

Escherichia coli

2) W.S.Kistler, C.A.Hirsch, N.R.Cozzarelli and E.C.C.Lin; *J.Bacteriology*, 100, 1133 (1969).

Lactobacillus casei

3) C.F.Strittmatter; *J.Biol.Chem.*, 234,2794 (1959).

Streptococcus faecalis

4) N.J.Jacobs and P.J.VanDemark; *Arch.Biochem. and Biophys.*, 88,250 (1960).

Streptococcus feacium

5) L.K.Koditschek and W.W.Umbreit; *J.Bacteriology*, 98,1063 (1969).

6) T.W.Esders and C.A.Michrina; *J.Biol.Chem.*, 254,2710 (1979).

Table 1. Effect of Various Chemicals on L- α -Glycerophosphate oxidase

[The enzyme dissolved in 0.1M dimethylglutaric acid-NaOH buffer, pH 7.0 (20U/ml) was incubated at 25°C for 10min.]

Chemical	Concn.(mM)	Residual activity	Chemical	Concn.(mM)	Residual activity
None	—	100%	PCMB	2.0	3.3
Metal salt	2.0		MIA	2.0	3.3
MgCl ₂		98	NaF	2.0	100
CaCl ₂		107	NaN ₃	200	96
Ba(OAc) ₂		99	EDTA	100	99
FeCl ₂		0.3	o-Phenanthroline	2.0	95
FeCl ₃		0.8	α,α' -Dipyridyl	1.0	99
CoCl ₂		103	Borate	50	10
MnCl ₂		100	IAA	2.0	2.8
ZnSO ₄		99	NEM	2.0	97
NiCl ₂		98	Hydroxylamine	2.0	9.7
CuSO ₄		7.5	Triton X-100	0.10%	106
Pb(OAc) ₂		87	Brij 35	0.10%	3.9
AgNO ₃		79	Tween 20	0.10%	3.4
HgCl ₂		96	Span 20	0.10%	94
			Na-cholate	0.10%	92
			SDS	0.05%	4.8
			DAC	0.05%	70

Ac, CH₃CO; PCMB, p-Chloromercuribenzoate; MIA, Monoiodoacetate; EDTA, Ethylenediaminetetraacetate; IAA, Iodoacetamide; NEM, N-Ethylmaleimide; SDS, Sodium dodecyl sulfate; DAC, Dimethyl-benzyl-alkyl-ammonium chloride.

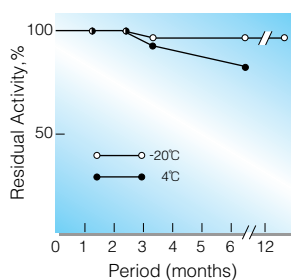


Fig. 1. Stability (Powder form)
(kept under dry conditions)

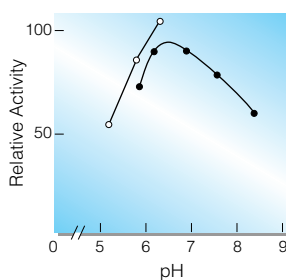


Fig. 3. pH-Activity
[0.1M buffer solution:pH5.0-5.5,] [MES;pH6.0-8.5, PIPES]

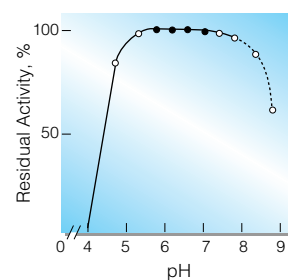


Fig. 5. pH-Stability
[25°C, 60min-treatment with 0.1M buffer solution;pH4.0-5.5, dimethylglutaric acid-NaOH;pH5.0-7.5,K-phosphate;pH7.5-9.0, Tris-HCl, enzyme concn.:20U/ml]

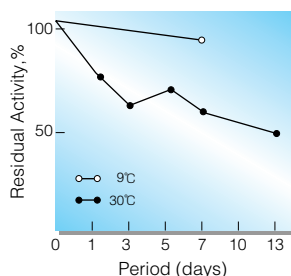


Fig. 2. Stability (Liquid form)
[in Goods buffer solution,pH6.5 (contg. 0.1% Triton X-100,0.01mM FAD,0.5mM ATP) enzyme concn.:12U/ml]

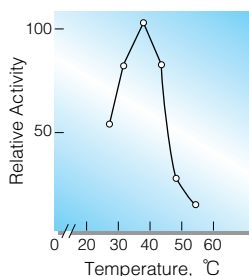


Fig. 4. Temperature activity
(in 0.2M PIPES-NaOH buffer,pH6.5)

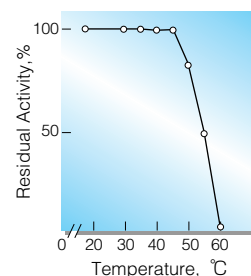
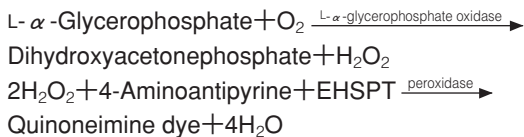


Fig. 6. Thermal stability
[10min-treatment with 0.2M K-phosphate buffer,pH6.5,enzyme concn.:20U/ml]

活性測定法 (Japanese)

1.原理



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H₂O₂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H₂O₂を生成する酵素量を1単位 (U)とする。

3.試薬

- 1.5Mグリセロリン酸溶液 [48.63gの D,L- α -グリセロリン酸 \cdot 2Na (MW=324.17)を約60mlの蒸留水で溶解後、4.0N HClでpHを6.5 \pm 0.05に調整(25 $^{\circ}$ C)し、蒸留水で100mlとする。] (0 \sim 4 $^{\circ}$ C保存で2週間は使用可能)
- 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 \pm 0.05に調整(25 $^{\circ}$ C)し、蒸留水で100mlとする。] (0 \sim 4 $^{\circ}$ C保存で2週間は使用可能)
- 28mM 4-AA水溶液 [569 mgの4-アミノアンチピリン (MW=203.25)を約100mlの蒸留水で溶解する。] (褐色瓶中4 $^{\circ}$ C保存で1週間は使用可能)
- 20mM EHSPT (TOOS)水溶液 [591mgの EHSPT (MW=295.3)を約100mlの蒸留水に溶解する。] (褐色瓶中4 $^{\circ}$ C保存で1週間は使用可能)
- 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 $^{\circ}$ C保存)(用時調製)

酵素溶液：酵素標品を予め水冷した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 \sim 0.20 U/mlに希釈する。

4.手順

- 下記反応混液を調製する。(褐色瓶にて水冷保存)

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)
- 反応混液2.5mlをキュベット(d=1.0cm)に採り、30 $^{\circ}$ Cで約5分間予備加温する。
- 酵素溶液0.1mlを添加し、ゆるやかに混和後、水を対照に30 $^{\circ}$ Cに制御された分光光度計で555nmの吸光度変化を3 \sim 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(Δ OD test)。
- 盲検は反応混液①に酵素溶液の代わりに酵素希釈液(0.5M NaClを含む20mM PIPES-NaOH緩衝液,pH 6.5) 0.10mlを加え、上記同様に操作を行ない、1分間当りの吸光度変化を求める(Δ OD blank)。

5.計算式

$$\begin{aligned} U/ml &= \frac{\Delta OD/min (\Delta OD\ test - \Delta OD\ blank) \times 2.60(ml) \times \text{希釈倍率}}{29.9 \times 1/2 \times 1.0 \times 0.10(ml)} \\ &= \Delta OD \times 1.739 \times \text{希釈倍率} \\ U/mg &= U/ml \times 1 / C \\ 29.9 &: \text{Quinoneimine色素の上記測定条件下での} \\ &\quad \text{ミリモル分子吸光係数 (cm}^2/\text{micromole)} \\ 1/2 &: \text{酸素反応で生成したH}_2\text{O}_2\text{の1分子のから形} \\ &\quad \text{成するQuinoneimine色素は1/2分子である} \\ &\quad \text{事による係数} \\ 1.0 &: \text{光路長(cm)} \\ C &: \text{溶解時の酵素濃度(c mg/ml)} \end{aligned}$$