●TOYOBO ENZYMES●

(Diagnostic Reagent Grade)

GLYCEROL DEHYDROGENASE

from Cellulomonas sp.

Glycerol:NAD^+ 2-oxidoreductase (EC 1. 1. 1. 6)

PREPARATION and SPECIFICATION

Appearance	: White amorphous powder, lyophilized
Activity	: Grade Ⅲ 50U/mg-solid or more (containing approx. 50% of stabilizers)
Contaminant	: NADH oxidase ≤1.0×10^{-3}%
Stabilizer	: BSA

PROPERTIES

Stability	: Stable at −20℃ for at least 6 months (Fig.1)
Molecular weight	: approx. 390,000
Isoelectric point	: 4.4±0.1
Michaelis constants	: 1.1×10^{-2}M (Glycerol), 8.9×10^{-5}M (NAD^+)
Structure	: 10 subunits (42,000) per enzyme molecule
Inhibitors	: p-Chloromercuribenzoate, o-phenanthroline, monoiodoacetate, heavy metal ions (Co^{++}, Ni^{++}, Cu^{++}, Zn^{++}, Cd^{++})
Optimum pH	: 10.0−10.5 (Fig.4)
Optimum temperature	: 50℃ (Fig.5)
pH Stability	: pH 7.5−10.5 (25℃, 20hr) (Fig.6)
Thermal stability	: below 55℃ (pH 7.5, 15min) (Fig.7)
Substrate specificity	: This enzyme has the highest specificity for glycerol and 1,2-propanediol, and also oxidizes glycerol-α-monochlorohydrin, ethylene glycol and 2,3-butanediol (Table 1). The oxidative reaction is stimulated by K^+, NH_4^+ and Rb^+.
Effect of various chemicals	: (Table 2)

APPLICATIONS

This enzyme is useful for enzymatic determination of glycerol and of triglyceride when coupled with lipoprotein lipase (LPL-311, LPL-314) in clinical analysis. [1,2]

ASSAY

Principle:

Glycerol + NAD^+ —(glycerol dehydrogenase)→ Dihydroxyacetone + NADH + H^+

The appearance of NADH is measured at 340nm by spectrophotometry.

Unit definition:

One unit causes the formation of one micromole of NADH per minute under the conditions described below.

Method:

Reagents

A. Carbonate-bicarbonate buffer, pH 11.0 : 0.2M (Prepare by mixing 0.2M K_2CO_3 and 0.2M $NaHCO_3$ to reach pH 11.0).

B. Glycerol solution : 0.3M

C. Ammonium sulfate solution : 1.0M

D. NAD^+ solution : 10mM [Weigh 143.5mg of NAD^+(MW=717.45) and dissolve in 18.0ml of H_2O and, after adjusting the pH to 7.0 with 0.5 N KOH, fill up to 20.0ml with H_2O] (Should be prepared fresh)

E. Enzyme diluent : 20mM K-phosphate buffer pH 7.5.

Procedure

1. Prepare the following working solution, immediately before use.

30.0ml	Carbonate-bicarbonate buffer, pH 11.0	(A)
22.0ml	Substrate solution	(B)
2.0ml	Ammonium sulfate solution	(C)
6.0ml	NAD^+ solution	(D)

Concentration in assay mixture	
Carbonate buffer	0.10 M
Glycerol	0.10 M
NAD^+	1.0mM
Ammonium sulfate	33 mM

Be sure the pH in the range (pH 10.0−10.5). If not, adjust the pH to 10.5 with 1.0 N KOH or 1.0N HCl, and store on ice in a brownish bottle.

2. Pipette 2.9ml of the working solution into a cuvette (d=1.0cm) and equilibrate at 25℃ 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 340nm against water for 3 to 4 minutes in a spectrophotometer thermostated at 25℃ 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 test except that the enzyme diluent is added instead of enzyme solution.

* Dissolve the enzyme preparation in ice-cold enzyme diluent (E), dilute to 0.10−0.25U/ml with the same buffer and store on ice.

Calculation

Activity can be calculated by using the following formula :

$$\text{Volume activity (U/ml)} = \frac{\Delta OD/min\ (\Delta OD\ test - \Delta OD\ blank) \times Vt \times df}{6.22 \times 1.0 \times Vs} = \Delta OD/min \times 4.82 \times df$$

Weight activity (U/mg) = (U/ml)×1/C

Vt : Total volume (3.0ml)

Vs : Sample volume (0.1ml)

6.22 : Millimolar extinction coefficient of NADH (㎤/micromole)

1.0 : Light path length (cm)

df : Dilution factor

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

REFERENCES

1) T.Nishina; ***Rinsho Kensa(Japanese), 22,*** 1304 (1978).

2) M.Sugiura, et al.; ***Clin, Chim.Acta, 81,*** 125 (1977).

3) R.M.Burton; ***Methods in Enzymology,*** vol.1, p.397 (S.P.Colowick & N.O.Kaplan eds.), Academic Press, New York–London (1955).

Table 1. Substrate Specificity of Glycerol dehydrogenase

Substrate	Relative activity(%)	Substrate	Relative activity(%)
Glycerol	100	2,3-Butanediol	52.6
Glycerol-α-monochlorohydrin	48.5	Xylitol	—
Ethylene glycol	7.8	D-Mannitol	—
1,2-Propanediol	132	D-Glucose	—
1,3-Propanediol	—	Methanol	—
1,3-Butanediol	—	Ethanol	—
1,4-Butanediol	—		

—, Not detected

Table 2. Effect of Various Chemicals on Glycerol dehydrogenase

Chemical	Concn.(mM)	Residual activity(%)	Chemical	Concn.(mM)	Residual activity(%)
None	—	100	PCMB	0.1	25
Metal salt	1.0		MIA	1.0	78
$MgCl_2$		100	NaN_3	1.0	110
$CaCl_2$		97	NaF	1.0	108
$SrCl_2$		98	EDTA	1.0	101
$BaCl_2$		95	KCN	1.0	94
$MnCl_2$		94	o-Phenanthroline	1.0	13
$CoCl_2$		57			
$NiCl_2$		50			
$CuSO_4$		2.4			
$ZnCl_2$		1.5			
$CdCl_2$		11			

PCMB, p-Chloromercuribenzoate; MIA, Monoiodoacetate; EDTA, Ethylenediaminetetraacetate.

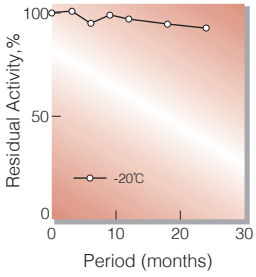

Fig.1. Stability (Powder form)
〔kept under dry conditions〕

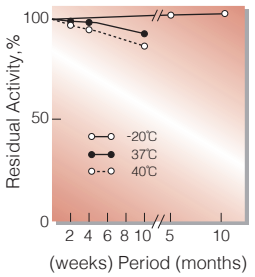

Fig.2. Stability (Powder form)
〔kept under dry conditions〕

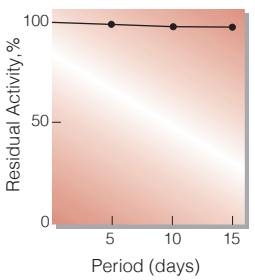

Fig.3. Stability (Liquid form at 37℃)
〔enzyme concentration: 400-500U/ml buffer composition: 50mM K-phosphate buffer contg.3.2M ammonium sulfate and 0.2% BSA, pH7.0〕

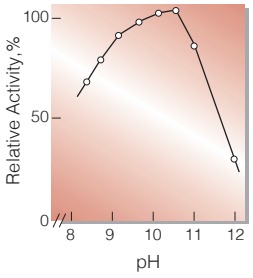

Fig.4. pH-Activity
〔25℃, in 0.1M K_2CO_3-$NaHCO_3$ buffer〕

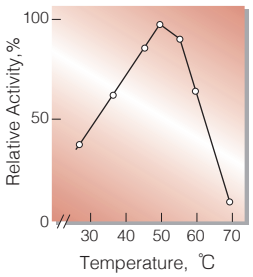

Fig.5. Temperature activity
〔in 0.1M K_2CO_3-$NaHCO_3$ buffer, pH10.5〕

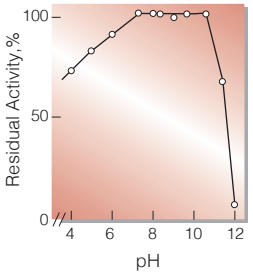

Fig.6. pH-Stability
〔25℃, 20hr-treatment with 50mM buffer solution pH4.0-6.0,acetate: pH6.0-8.5, K-phosphate;pH9.0-11.8, K_2CO_3-$NaHCO_3$〕

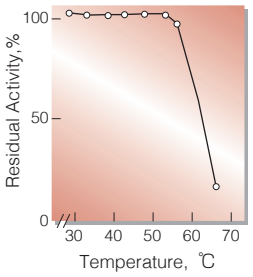

Fig.7. Thermal stability
〔15min-treatment with 50mM K-phosphate buffer, pH 7.5〕

活性測定法（Japanese）

1.原理

Glycerol＋NAD^+ —glycerol dehydrogenase→ Dihydroxy-acetone＋NADH＋H^+

NADHの生成量を340nmの吸光度の変化で測定する。

2.定義

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

3.試薬

A. 0.2M K_2CO_3-$NaHCO_3$緩衝液, pH11.0
B. 0.3Mグリセロール水溶液
C. 1.0M硫安溶液
D. 10mM NAD^+水溶液〔143.5㎎のNAD^+(MW＝717.45)を蒸留水18㎖に溶解し，0.5N KOHでpH7.0に調整後，蒸留水で20㎖とする〕(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で溶解し,同緩衝液で0.10～0.25U/㎖に希釈して氷冷保存する。

4.手順

①下記反応混液を使用直前に調製する。

30.0㎖	K_2CO_3-$NaHCO_3$緩衝液	(A)
20.0㎖	基質溶液	(B)
2.0㎖	硫安溶液	(C)
6.0㎖	NAD^+水溶液	(D)

pHが10.0～10.5の範囲にあることを確認，pHがこの範囲外の場合は1.0N KOHまたは1.0N HClで10.5に調製し,褐色瓶中で氷冷保存する。

②反応混液2.9㎖をキュベット(d＝1.0cm)に採り,25℃で約5分間予備加温する。

③酵素溶液0.1㎖を添加し,ゆるやかに混和後,水を対照に25℃に制御された分光光度計で340nm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(ΔOD test)。

④盲検は反応混液①2.9㎖に,酵素溶液の代りに酵素希釈液(20mM K-リン酸緩衝液，pH7.5)を0.1㎖加え,上記同様に操作を行って,1分間当りの吸光度変化を求める(ΔOD blank)。

5.計算式

$$U/㎖ = \frac{\Delta OD/min\ (\Delta OD\ test - \Delta OD\ blank) \times 3.0(㎖) \times 希釈倍率}{6.22 \times 1.0 \times 0.1(㎖)}$$

$$= \Delta OD/min \times 4.82 \times 希釈倍率$$

U/mg ＝U/㎖×1/C

6.22 ： NADHのミリモル分子吸光係数 (cm^2/micromole)

1.0 ： 光路長(cm)

C ： 溶解時の酵素濃度(c ㎎/㎖)