●TOYOBO ENZYMES●

(Diagnostic Reagent Grade)

D-3-HYDROXYBUTYRATE DEHYDROGENASE

from Pseudomonas sp.

(R)-3-Hydroxybutanoate:NAD^+oxidoreductase (EC 1. 1. 1. 30)[1~5)]

D-3-Hydroxybutyrate + NAD^+ ⟷ Acetoacetate + NADH + H^+

PREPARATION and SPECIFICATION

Appearance	: White amorphous powder, lyophilized
Activity	: Grade Ⅲ 100U/mg-solid or more
Contaminants	: Malate dehydrogenase ≤2.0×10^{-3}%
	Lactate dehydrogenase ≤2.0×10^{-3}%
	NADH oxidase ≤2.0×10^{-3}%
Stabilizers	: Sucrose, mannitol, BSA

PROPERTIES

Stability	: Stable at −20℃ for at least one year (Fig.1)
Molecular weight	: approx. 130,000 (by gel filtration)
Isoelectric point	: 5.6±0.1
Michaelis constants	: 4.2×10^{-4}M (25℃, pH8.3), 7.0×10^{-4}M (37℃, pH8.3) (D-3-Hydroxybutyrate)
	4.9×10^{-5}M (25℃, pH8.3), 7.2×10^{-5}M (37℃, pH8.3)(NAD^+)
	8.1×10^{-5}M (25℃, pH7.1), 2.4×10^{-4}M (37℃, pH7.1)(Acetoacetate)
	8.4×10^{-6}M (25℃, pH7.1), 1.5×10^{-5}M (37℃, pH7.1)(NADH)
Inhibitors	: PCMB, MIA, IAA, Ag^+, Hg^{++}, SDS, DAC
Optimum pH	: 8.3 (Fig.3)
Optimum temperature	: 55℃ (Fig.4)
pH Stability	: pH 5.0−8.5 (25℃, 20hr) (Fig.5)
Thermal stability	: below 40℃ (pH 6.5, 15min) (Fig.6)
Substrate specificity	: (Table 1)
Effect of various chemicals	: (Table 2)

APPLICATIONS

This enzyme is useful for enzymatic determination of ketone bodies (D-3-hydroxybutyrate and acetoacetate) in clinical analysis.

ASSAY

Principle:

D-3-Hydroxybutyrate + NAD^+ $\xrightarrow{\text{D-3-hydroxybutyrate dehydrogenase}}$ Acetoacetate + 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. Tris-HCl buffer, pH 8.5 (25°C) : 0.1M

B. 3-Hydroxybutyrate solution : 158mM [200mg D,L-3-Hydroxybutyrate Na salt (MW=126.09)/10ml of Tris-HCl buffer (A)] (Stable at least 5 days if stored at 4℃)

C. NAD^+ solution : 27.9mM [80mg $NAD^+ \cdot 3H_2O$ (MW=717.45)/4.0ml of Tris-HCl buffer (A)] (Stable for at least 5 days if stored at 4℃)

D. Enzyme diluent : 0.1M Tris-HCl buffer, pH 8.5 contg. 0.1% BSA

Procedure

1. Prepare the following reaction mixture in a cuvette (d=1.0cm) and equilibrate at 37℃ for about 5 minutes.

2.3ml	Tris-HCl buffer, pH 8.5	(A)
0.5ml	Substrate solution	(B)
0.2ml	NAD^+ solution	(C)

Concentration in assay mixture	
Tris-HCl buffer	0.1 M
3-Hydroxybutyrate	25 mM
NAD^+	1.8mM

2. Add 0.1ml of the enzyme solution* and mix by gentle inversion.
3. Record the increase in optical density at 340nm against water for 2 to 3 minutes in a spectrophotometer thermostated at 37℃ 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 (D), dilute to 0.1−0.5U/ml with the same buffer and store on ice.

Calculation

Activity can be calculated by using the following formula :

$$\text{Volume activity (U/ml)} = \frac{\Delta\text{OD/min (}\Delta\text{OD test} - \Delta\text{OD blank)} \times \text{Vt} \times \text{df}}{6.22 \times 1.0 \times \text{Vs}} = \Delta\text{OD/min} \times 4.98 \times \text{df}$$

Weight activity (U/mg) = (U/ml)×1/C

Vt : Total volume (3.1ml)

Vs : Sample volume (0.1ml)

6.22 : Millimolar extinction coefficient of NADH at 340nm (㎤/micromole)

1.0 : Light path length (cm)

df : Dilution factor

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

REFERENCES

1) H.U.Bergmeyer, K.Gawehn, H.Klotzsh, H.A.Krebs and D.H.Williamson; ***Biochem.J., 102,*** 423 (1967).

2) F.P.Delafield, K.E.Cooksey and M.Doudoroff; ***J.Biol.Chem., 240,*** 4023 (1965).

3) C.W.Shuster and M.Doudoroff; ***J.Biol.Chem., 237***, 603 (1962).

4) I.Sekuzu, P.Jurtshuk and D.E.Green; ***J.Biol.Chem., 238,*** 975 (1963).

5) J.D.Smiley and G.Ashwell; ***J.Biol.Chem., 236,*** 357 (1961).

Table 1. Substrate Specificity of D-3-Hydroxybutyrate dehydrogenase

Substrate	Relative activity(%)	Substrate	Relative activity(%)
3-Hydroxybutyrate	100	sec-Butyl alcohol	0
3-Hydroxypropionate	0.14	Gluconate	0
Lactate	0	Glycolate	0.04
Glycerate	0		
2-Hydroxybutyrate	0	NAD^+	100
L-Malate	0	$NADP^+$	4.74
D,L-Malate	0		

Table 2. Effect of Various Chemicals on D-3-Hydroxybutyrate dehydrogenase

[The enzyme dissolved in 50 mM K-phosphate buffer, pH 6.5(10U/ml) was incubated at 25℃ for 1hr.]

Chemical	Concn.(mM)	Residual activity(%)	Chemical	Concn.(mM)	Residual activity(%)
None	—	100	NaF	2.0	100
Metal salt	2.0		NaN_3	20	104
$MgCl_2$		105	EDTA	5.0	97
$CaCl_2$		101	o-Phenanthroline	2.0	96
$Ba(OAc)_2$		98	α,α'-Dipyridyl	1.0	97
$FeCl_3$		101	Borate	50	103
$CoCl_2$		102	IAA	2.0	4
$MnCl_2$		103	NEM	2.0	59
$ZnSO_4$		100	Hydroxylamine	2.0	101
$Cd(OAc)_2$		100	Triton X-100	0.10%	113
$NiCl_2$		103	Brij 35	0.10%	37
$CuSO_4$		83	Tween 20	0.10%	68
$Pb(OAc)_2$		96	Span 20	0.10%	104
$AgNO_3$		2.5	Na-cholate	0.10%	107
$HgCl_2$		0	SDS	0.05%	5
PCMB	2.0	0	DAC	0.05%	4
MIA	2.0	1			

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

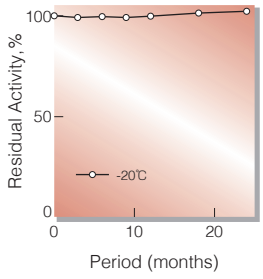

Fig.1. Stability (Powder form)

〔kept under dry conditions〕

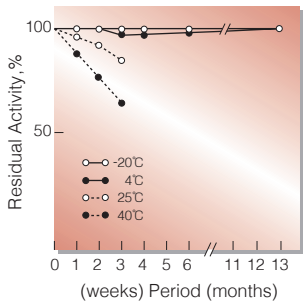

Fig.2. Stability (Powder form)

〔kept under dry conditions〕

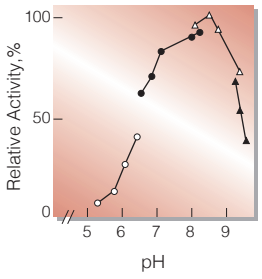

Fig.3. pH-Activity

〔37℃,5min-reaction in 0.1M buffer solution: pH5.3-6.5, dimethylglutaric acid-NaOH;pH5.9-8.3,K-phosphate;pH7.9-9.1,Tris-HCl: pH8.9-9.2,K_2CO_3-$NaHCO_3$〕

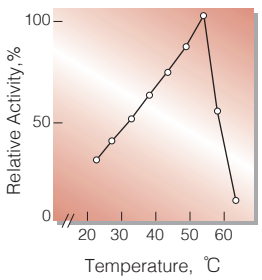

Fig.4. Temperature activity

〔in 0.1M Tris-HCl buffer, pH8.3〕

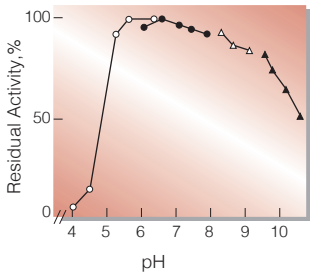

Fig.5.pH-Stability

〔25℃,20hr-treatment with 50mM buffer solution:pH4.0-6.0, dimethylglutaric acid-NaOH;pH6.0-8.0, K-phosphate;pH8.0-9.0, Tris-HCl;pH9.0-10.5, K_2CO_3-$NaHCO_3$〕

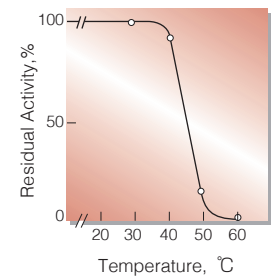

Fig.6. Thermal stability

〔15min-treatment with 50mM K-phosphate buffer,pH6.5. enzyme concn.:50U/ml〕

活性測定法（Japanese）

1.原理

D-3-Hydroxybutyrate＋NAD^+
$\xrightarrow[\text{D-3-hydroxybutyrate dehydrogenase}]{}$ Acetoacetate＋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. 0.1M Tris-HCl緩衝液，pH 8.5 (25℃)
B. 158mM 3-ヒドロキシ酪酸溶液〔200mgのD,L-3-ヒドロキシ酪酸ナトリウム塩 (MW＝126.09)を10mℓのTris-HCl緩衝液(A)に溶解する〕(4℃保存で，少なくとも5日間は使用可能)
C. 27.9mM NAD^+溶液〔80mgの$NAD^+ \cdot 3H_2O$(MW＝717.45)を4.0mℓのTris-HCl緩衝液(A)に溶解する〕(4℃保存で，少なくとも5日間は使用可能)

酵素溶液：酵素標品を予め氷冷した0.1%牛血清アルブミンを含む0.1M Tris-HCl緩衝液,pH8.5で溶解し，同緩衝液で0.1～0.5 U/mℓに希釈して氷冷保存する。

4.手順

①下記反応混液をキュベット(d＝1.0cm)に調製し,37℃で約5分間予備加温する。

2.3mℓ	0.1MTris-HCl緩衝液	(A)
0.5mℓ	基質溶液	(B)
0.2mℓ	NAD^+溶液	(C)

②酵素溶液0.1mℓ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340nmの吸光度変化を2～3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)。
③盲検は反応混液①に酵素溶液の代りに酵素希釈液(0.1%牛血清アルブミンを含む0.1MTris-HCl緩衝液，pH 8.5)を0.1mℓ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

$$U/m\ell = \frac{\Delta OD/min\ (\Delta OD\ test - \Delta OD\ blank) \times 3.1(m\ell) \times 希釈倍率}{6.22 \times 1.0 \times 0.1(m\ell)}$$
$$= \Delta OD/min \times 4.98 \times 希釈倍率$$

U/mg ＝U/mℓ×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 mg/mℓ)