●TOYOBO ENZYMES●
(Diagnostic Reagent Grade)

LEUCINE DEHYDROGENASE

from Bacillus sp.[1)]

L-Leucine:NAD⁺ oxidoreductase (deaminating) (EC 1. 4. 1. 9)

L-Leucine + NAD^+ + H_2O α-Ketoisocaproate + NH_3 + NADH + H^+

PREPARATION and SPECIFICATION

Appearance	: White amorphous powder, lyophilized
Activity	: Grade Ⅱ 20U/mg-solid or more (containing approx. 70% of stabilizers)
Contaminants	: Leucylpeptide decomposing enzymes (Leu-Val) $\leq 1.0 \times 10^{-2}$% (Leu-Gly-Gly) $\leq 1.0 \times 10^{-2}$% NADH oxidase $\leq 1.0 \times 10^{-2}$%
Stabilizers	: 2-Mercaptoethanol, L-cysteine, dithiothreitol, ethylenediaminetetraacetate

PROPERTIES

Stability	: Stable at −20℃ for at least one year	(Fig.1)
Molecular weight [2)]	: 245,000	
Michaelis constants [2)]	: 1.0×10^{-3}M (L-Leucine), 3.9×10^{-4}M (NAD^+), 3.5×10^{-5}M (NADH), 3.1×10^{-4}M［α-Ketoisocaproate (α-KIC)］, 2.0×10^{-1}M (NH_3)	
Structure [2)]	: 6 subunits per enzyme molecule	
Inhibitors [2)]	: Na_2S, Hg^{++}, Cu^{++}, Co^{++}, Mg^{++}, p-chloromercuribenzoate	
Optimum pH	: 10.5−10.8 (L-Leu→α-KIC), 9.4 (α-KIC→L-Leu)	(Fig.3)
Optimum temperature	: above 70℃	(Fig.4)
pH Stability	: pH 5.5−10.5 (25℃, 20hr)	(Fig.5)
Thermal stability	: below 60℃ (pH 6.9, 10min)	(Fig.6)
Substrate specificity	: (Table 1)	

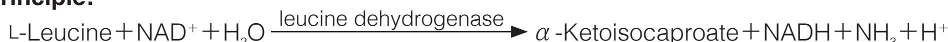
APPLICATIONS [4)]

This enzyme is useful for enzyme determination of L-leucine and the activity of leucine amino-peptidase.

ENZYMES Diagnostic Reagent Grade

ASSAY

Principle:



L-Leucine + NAD^+ + H_2O —(leucine dehydrogenase)→ α-Ketoisocaproate + NADH + NH_3 + 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. L-Leucine solution : 0mM L-leucine in 0.2M glycine-KCl-KOH buffer, pH 10.5 (Prepare freshly)
B. NAD^+ solution : 12.5mM (Should be prepared fresh)
C. Enzyme diluent : 25mM K-phosphate buffer, pH 7.2

Procedure

1. Prepare the following reaction mixture in a cuvette (d=1.0cm) and equilibrate at 37℃ for about 5 minutes.
   3.0ml Substrate solution (A)
   0.3ml NAD^+ solution (B)

Concentration in assay mixture	
Glycine buffer	0.18 M
L-Leucine	18 mM
NAD^+	1.1mM

2. Add 0.05ml 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 (C) is added instead of the enzyme solution.

* Dissolve the enzyme preparation in ice-cold enzyme diluent (C) (ca. 5mg/ml) and dilute to 0.25−0.33U/ml with the same buffer, immediately before assay.

Calculation

Activity can be calculated by using the following formula :

$$\text{Volume activity (U/ml)} = \frac{\Delta OD/min\ (\Delta OD\ test - \Delta OD\ blank) \times Vt \times df}{6.22 \times 1.0 \times Vs} = \Delta OD/min \times 10.77 \times df$$

Weight activity (U/mg) = (U/ml) × 1/C

Vt : Total volume (3.35ml)
Vs : Sample volume (0.05ml)
6.22 : Millimolar extinction coefficient of NADH (cm^2/micromole)
1.0 : Light path length (cm)
df : Dilution factor
C : Enzyme concentration in dissolution (c mg/ml)

REFERENCES

1) K.Soda et al.; *Biochem.Biophys.Res.Commun., 44,* 931 (1971).
2) T.Ohshima et al.; *J.Biol.Chem., 253,* 5719 (1978).

Table 1. Substrate Specificity of Leucine dehydrogenase[2)]

Substrate(10mM)	Relative activity(%)
L-Leucine	100
L-Valine	74
L-Isoleucine	58
L-Norvaline	41
L-Norleucine	10
L-Methionine	0.6
L-Cysteine	0.3
Inert:L-Ala, L-Glu, L-Thr, L-Ser Gly, L-Phe, L-Lys, L-Arg, D-Leu, D-Val, D-Ile	

Substrate(10mM)	Relative activity(%)
α-Ketoisocaproate	100
α-Ketoisovalerate	126
α-Ketovalerate	76
α-Ketobutyrate	57
α-Ketocaproate	46
Inert:Pyruvate, α-Ketoglutarate, Phenylpyruvate, Oxaloacetate, Glyoxylate	

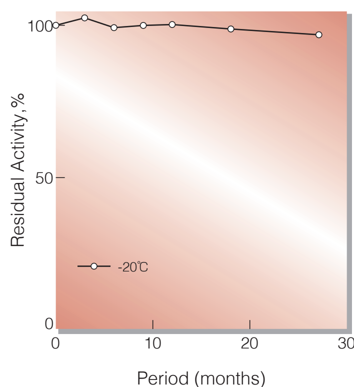


Fig.1. Stability (Powder form)
〔kept under dry conditions〕

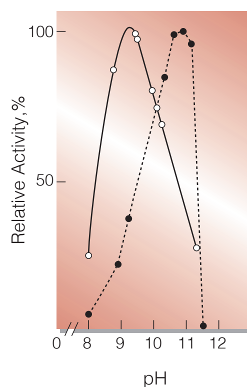


Fig.3. pH-Activity
〔○——○: α-KIC→Leu in 1M ammonium buffer
●-------●:Leu→ α-KIC in 0.2M glycine-KOH buffer〕

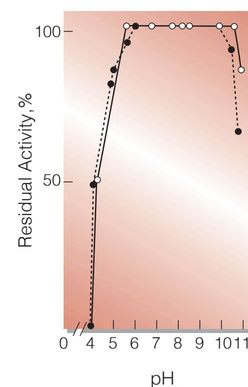


Fig.5. pH-Stability
〔25℃,20hr-treatment with 50mM buffer solution:pH4.0-6.0,acetate; pH6.0-8.5,phosphate;pH9.0-11.0, carbonate:
○——○:with 0.01% mercaptoethanol
●-------●:without mercaptoethanol〕

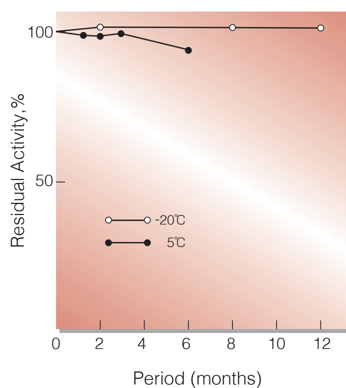


Fig.2. Stability (Powder form)
〔kept under dry conditions〕

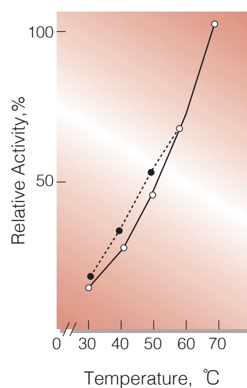


Fig.4. Temperature activity
〔○——○: α-KIC→Leu in 1.0M ammonium buffer pH9.5
●-------●:Leu→ α-KIC in 0.2M glycine-KOH buffer pH10.5〕

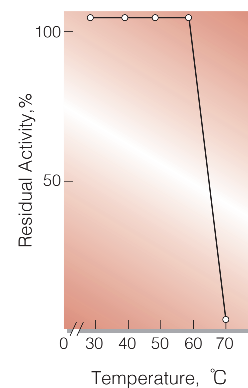


Fig.6. Thermal stability
〔10min-treatment with 50mM phosphate buffer,pH6.9〕

活性測定法（Japanese）

1.原理

L-Leucine＋NAD^+＋H_2O $\xrightarrow{\text{leucine dehydrogenase}}$
α-Ketoisocaproate＋NH_3＋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. 20mM L-ロイシン溶液〔L-ロイシンを20mMになるように0.2Mグリシン-KCl-KOH緩衝液(pH10.5)に溶解する〕(用時調製)
B. 12.5mM NAD^+水溶液(用時調製)
酵素溶液：酵素標品を予め氷冷した25mM K-リン酸緩衝液,pH7.2で溶解(約5mg/mℓ)し,分析直前に同緩衝液で0.25～0.33U/mℓに希釈する。

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。

3.0mℓ	基質溶液	(A)
0.3mℓ	NAD^+水溶液	(B)

②酵素溶液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)。
④盲検は反応混液①に酵素溶液の代りに酵素希釈液(25mM K-リン酸緩衝液,pH7.2)を0.05mℓを加え,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(ΔODblank)。

5.計算式

$$U/m\ell = \frac{\Delta OD/min\ (\Delta OD\ test - \Delta OD\ blank) \times 3.35(m\ell) \times 希釈倍率}{6.22 \times 1.0 \times 0.05(m\ell)}$$

$$= \Delta OD/min \times 10.77 \times 希釈倍率$$

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)