●TOYOBO ENZYMES●
(Diagnostic Reagent Grade)

DIAPHORASE
from Microorganism

NAD(P)H:(acceptor)oxidoreductase (EC 1.6.99. —)

$$NAD(P)H + H^+ + Acceptor(ox) \longrightarrow NAD(P)^+ + Acceptor(red)$$

PREPARATION and SPECIFICATION

Appearance	: Yellowish amorphous powder, lyophilized	
Activity	: Grade Ⅲ 500 U/mg-solid or more	
Contaminants	: Myokinase	≤5.0×10^{-1}%
	NADH oxidase	≤1.0×10^{-1}%

PROPERTIES

Stability	: Stable at −20℃	(Fig.1)
Molecular weight (Gel-filtration)	: approx. 48,000	
Michaelis constant	: 2.2×10^{-4}M (NADH), 2.9×10^{-2}M (NADPH)	
Inhibitors	: Fe^{3+}, Mn^{2+}, Cu^{2+}, Pb^{2+}	
Isoelectric point	: 5.0	
Optimum pH	: 8.0	(Fig.3)
Optimum temperature	: 60℃	(Fig.4)
pH Stability	: 5.0−10.0	(Fig.5)
Thermal stability	: below 70℃	(Fig.6)
Substrate specificty	: Either NADH or NADPH can be used as a reductant.	
Effect of various chemicals	: (Table 1)	

APPLICATIONS

This enzyme is useful for colorimetric determination of NAD(P)H and many dehydrogenases when coupled with various dyes which act as hydrogen acceptors from NAD(P)H.

ASSAY

Principle:

$NADH + H^+ + DCPIP \xrightarrow{diaphorase} NAD^+ + DCPIP(red)$

The reduction of DCPIP(2,6-dichlorophenol-indophenol) is measured at 600 nm by spectrophotometry.

Unit definition:

One unit causes the decrease of one micromole of DCPIP per minute under the conditions described below.

Method:

Reagents

A. Buffer solution : 0.2M Tris-HCl, pH 8.0
B. NADH solution : 36mM (Prepare freshly and store on ice)
C. DCPIP solution : 2.4mM [7.8mg DCPIP(Mw:326.11)/10ml of H_2O] (Should be prepared fresh)
D. Enzyme diluent : Buffer solution (A) containing 0.5% of Tween20

Procedure

1. Prepare the following reaction mixture in a cuvette (d=1.0cm) and equilibrate at 37℃ for about 4 minutes.

2.4ml	H_2O	
0.3ml	Buffer solution	(A)
0.1ml	NADH solution	(B)

Concentration in assay mixture	
Tris buffer	27 mM
NADH	1.2 mM
DCPIP	80 μM
Tween20	ca.167 μg/ml

2. Add 0.1 ml of the enzyme solution* and mix by gentle pipetting and equilibrate at 37℃ for another 1 min.
3. Add 0.1 ml of DCPIP solution (C) and mix by rapid inversion.
4. Record the decrease of optical density at 600 nm against water for 3 to 4 min in a spectrophotometer thermostated at 37oC, and calculate the ΔOD per minute from the initial linear portion of the curve (OD test).
   At the same time , measure the blank rate (OD blank) by the same method as test except that the enzyme diluent is added instead of the enzyme solution.

* Dissolve the enzyme preparation in ice-cold buffer solution (A) (approx. 1.0% solution), dilute to 0.10−0.25U/ml with ice-cold enzyme diluent (D) and store on ice.

Calculation

Activity can be calculated by using the following formula :

$$\text{Volume activity (U/ml)} = \frac{\Delta OD/min\ (OD\ test - OD\ blank) \times Vt \times df}{20.9 \times 1.0 \times Vs} = \Delta OD/min \times 1.43 \times df$$

Weight activity (U/mg) = (U/ml)×1/C

Vt : Total volume (3.0ml)
Vs : Sample volume (0.1ml)
20.9 : Millimolar extinction coefficient of DCPIP under the assay conditions (㎠/micromole)
1.0 : Light path length (cm)
df : Dilution factor
C : Enzyme concentration in dissolution (c mg/ml)

Table 1. Effect of Various Chemicals on Diaphorase

[The enzyme dissolved in 0.1M HEPES buffer, pH7.5 (5U/ml) was incubated with each chemical at 25℃ for 1hr.]

Chemical	Concn.(mM)	Residual activity(%)
None	—	100
Metal salt	2.0	
$MgCl_2$		102
$CaCl_2$		99
$Ba(OAc)_2$		100
$FeCl_3$		4.4
$CoCl_2$		94
$MnCl_2$		55
$ZnCl_2$		84
$Cd(OAc)_2$		101
$NiCl_2$		101
$CuSO_4$		23
$Pb(OAc)_2$		46
$AgNO_3$		94
MIA	1.0	104
NaF	2.0	105

Chemical	Concn.(mM)	Residual activity(%)
NaN_3	2.0	104
EDTA	5.0	105
o-Phenanthroline	2.0	105
α,α'-Dipyridyl	1.0	102
Borate	5.0	104
IAA	2.0	105
NEM	2.0	106
Hydroxylamine	2.0	107
TritonX-100	0.10%	109
Brij 35	0.10%	109
Tween 20	0.10%	116
Span 20	0.10%	113
Na-Cholate	0.10%	110
SDS	0.05%	91
DAC	0.05%	110

MIA, Monoiodoacetate; EDTA, ethylenediaminetetraacetate; IAA, iodoacetamide; NEM, N-Ethylmaleimide; SDS, sodium dodecyl sulfate; DAC, Dimethylbenzylalkylammonium chloride

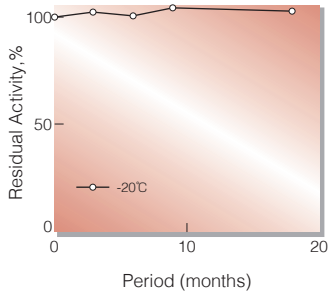

Fig.1. Stability (Powder form)

〔Kept under dry conditions〕

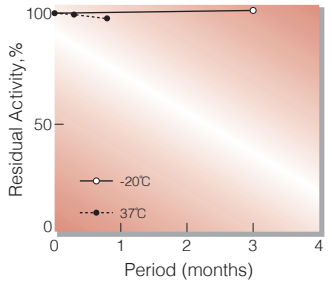

Fig.2. Stability (Powder form)

〔Kept under dry conditions〕

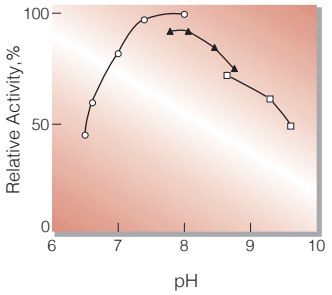

Fig.3. pH-Activity

〔37℃, in 0.1M buffer solution; ○—○, KPB; ▲—▲, Tris-HCl; □—□, Gly-NaOH〕

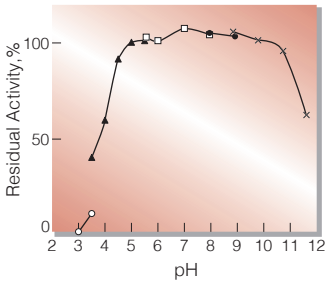

Fig.4. pH-Stability

〔25℃, 20hr-treatment with 0.1M buffer solution; ○—○, Glycine-HCl; ▲—▲, Acetate; □—□, KPB; ●—●, Tris-HCl; ×—×, Glycine-NaOH〕

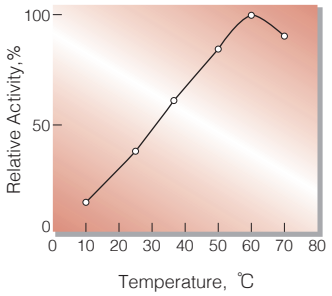

Fig.5. Temperature activity

〔in 50mM K-Phoshate buffer, pH7.5〕

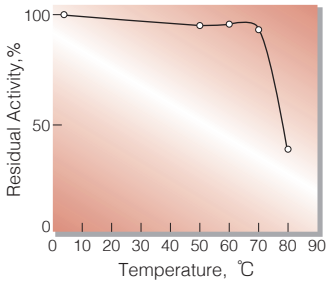

Fig.6. Thermal stability

〔15 min-treatment with 0.1M K-Phosphate buffer, pH7.5 Enzyme concentration : 10 U/ml〕

活性測定法（Japanese）

1.原理

$NADH + H^+ + DCPIP \xrightarrow{diaphorase} NAD^+ + DCPIP(red)$

DCPIP(2,6-dichlorophenol-indophenol)の還元量を600nm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600nmの吸光度を1.0減少させる酵素量を1単位(U)とする。

3.試薬

A. 0.2M Tris-HCl緩衝液, pH8.0
B. 36mM NADH水溶液(用時調製, 氷冷保存)
C. 2.4mM DCPIP水溶液〔7.8㎎のDCPIP(Mw:326.11)を10㎖の蒸留水で溶解する〕(用時調製)

酵素溶液：酵素標品を予め氷冷した蒸留水で溶解し, 分析直前に酵素希釈液(D)で0.10～0.25 U/㎖に希釈する。

4.手順

①下記反応液をキュベット(d=1.0cm)に採り,25℃で約5分間予備加温する。

2.4㎖	蒸留水	
0.3㎖	0.2M Tris-HCl緩衝液、pH 8.0	(試薬A)
0.1㎖	36mM NADH水溶液	(試薬B)

②酵素溶液0.1㎖を加えてピペッティングによる混和後,さらに約1分間予備加温する。

③DCPIP水溶液(試薬C)を0.1㎖を加えて,速やかに転倒混和した後,水を対照に37℃に制御された分光光度計で600nmの吸光度変化を3～4分間記録し,その初期直線部分より1分間当たりの吸光度変化を求める(ODtest)。

④盲検は①の反応混液に酵素希釈液(0.5%のTween20を含む試薬A),DCPIP水溶液各0.1mlを加え,上記同様に操作を行って1分間当たりの吸光度変化量を求める(ODblank)。

5.計算式

$$U/㎖ = \frac{\Delta OD/min\ (OD\ test - OD\ blank) \times 3.0(㎖) \times 希釈倍率}{20.9 \times 1.0 \times 0.10(㎖)}$$

$$= \Delta OD/min \times 1.43 \times 希釈倍率$$

$U/mg = U/㎖ \times 1/C$

20.9 ： DCPIP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 ㎎/㎖)