●TOYOBO ENZYMES●
(Diagnostic Reagent Grade)

DIAPHORASE

from Clostridium sp.

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 30U/mg-solid or more (containing approx. 15% of stabilizers)
Contaminants	: Myokinase ≤5.0×10^{-1}%
	NAD(P)H oxidase ≤5.0×10^{-1}%
Stabilizers	: FMN, NAD(P)H

PROPERTIES

Stability	: Stable at −20℃ for at least one year	(Fig.1)
Molecular weight	: 24,000 [1)]	
Michaelis constants	: 2.0×10^{-5}M (NADH), 6.0×10^{-6}M (NADPH)	
Structure	: One mol of FMN per mol of enzyme [1)]	
Inhibitor	: N-Ethylmaleimide	
Optimum pH	: 8.5	(Fig.3)
Optimum temperature	: 50℃	(Fig.4)
pH Stability	: pH 7.5 (30℃, 3hr)	(Fig.5)
Thermal stability	: below 30℃ (pH 7.5, 30min)	(Fig.6)
Substrate specificty	: Either NADH or NADPH can be used as a reductant. The catalytic ratio (NADPH/NADH) is 0.6 in the assay method. Neither oxygen nor cytochrome C can be utilized as a hydrogen acceptor.	

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:

$NADH + H^+ + DCPIP \xrightarrow{diaphorase} NAD^+ + Leucodye$

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

Unit definition:

One unit causes the decrease of one unit absorbance (1.0) of DCPIP per minute under the condeitions described below.

Method:

Reagents

A. Buffer solution : 0.2M Tris-HCl, pH 7.5
B. NADH solution : 6.0mM (Prepare freshly and store on ice)
C. DCPIP solution : 1.2mM [3.9mg DCPIP・$2H_2O$/10ml of H_2O] (Should be prepared fresh)
D. Enzyme diluent : Buffer solution(A) containing 0.1% of bovine serum albumin.

Procedure

1. Prepare the following reaction mixture in a cuvette (d＝1.0cm) and equilibrate at 25℃ for about 5 minutes.

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

Concentration in assay mixture	
Tris buffer	27 mM
NADH	0.20mM
DCPIP	40 μM
BSA	ca.33 μg/ml

2. Add 0.1ml each of the enzyme solution* and DCPIP solution (C) in this order and mix by rapid inversion.
3. Record the decrease of optical density at 600nm against water for 2 to 3 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 the enzyme solution.

* Dissolve the enzyme preparation in ice-cold buffer solution (A) (approx.1.0% solution), dilute to 0.4－0.8U/ml with ice-cold enzyme diluent (D) and store on ice.

Calculation

Activity can be calculated by using the following formula :

$$\text{Volume activity (U/ml)} = \frac{\Delta OD/min\ (\Delta OD\ test - \Delta OD\ blank) \times df}{1.0 \times Vs} = \Delta OD/min \times 10 \times df$$

Weight activity (U/mg) ＝ (U/ml)×1/C

Vs : Sample volume (0.1ml)
1.0 : Unit absorbance at 600nm due to unit definition
df : Dilution factor
C : Enzyme concentration in dissolution (c mg/ml)

REFERENCES

1) F.Kaplan, P.Setlow and N.O.Kaplan; *Arch,Biochem.Biophys., 132,* 91 (1969).

Table 1. Effect of Various Chemicals on Diaphorase

[The enzyme dissolved in 0.2M Tris-HCl buffer, pH 7.5 (40U/ml) was incubated with each chemical at 25℃ for 1hr.]

Chemical	Concn.(mM)	Residual activity(%)
None	—	100
Metal salt	2.0	
$MgCl_2$		99
$CaCl_2$		102
$Ba(OAc)_2$		100
$FeCl_2$		90
$CoCl_2$		101
$MnCl_2$		96
$ZnCl_2$		100
$Cd(OAc)_2$		100
$NiCl_2$		99
$CuSO_4$		87
$Pb(OAc)_2$		88
$AgNO_3$		103
$HgCl_2$		103
PCMB	2.0	90
MIA	1.0	100
NaF	2.0	102
NaN_3	2.0	100
EDTA	5.0	99
o-Phenanthroline	2.0	99
α,α'-Dipyridyl	1.0	101
Borate	5.0	100
IAA	2.0	99
NEM	2.0	100
Hydroxylamine	2.0	101
TritonX-100	0.10%	106
Brij 35	0.10%	104
Tween 20	0.10%	107
Span 20	0.10%	101
Na-Cholate	0.10%	99
SDS	0.05%	32
DAC	0.05%	32

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

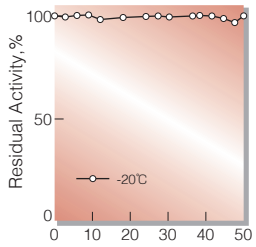

Fig.1. Stability (Powder form)

〔kept under dry conditions〕

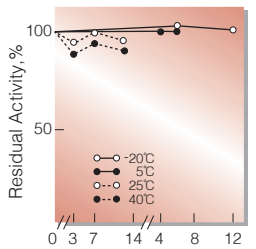

Fig.2. Stability (Powder form)

〔kept under dry conditions〕

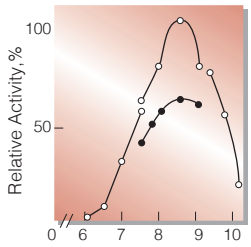

Fig.3. pH-Activity

〔The enzyme reaction was carried out with NADH(○)or NADPH(●).buffer used: pH6.0-7.5, 10mM Veronal-acetate; pH7.5-9.0, 33mM Tris-HCl; pH9.2-10.2, 33mM NH_4OH-NH_4OH〕

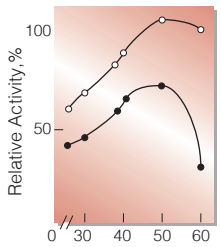

Fig.4. Temperature activity

〔The enzme reaction was carried out with NADH(○)or NADPH(●). 20mM Tris-HCl buffer,pH8.5〕

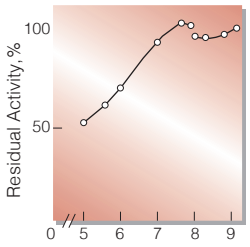

Fig.5. pH-Stability

〔30℃,3hr-treatment with the following buffer solution: pH6.0-7.5, 10mM Veronal-acetate; pH7.5-9.0, 33mM Tris-HCl〕

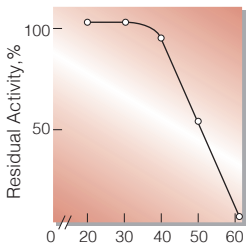

Fig.6. Thermal stability

〔30min-treatment with 0.2M Tris-HCl buffer,pH7.5 enzyme concentration:40U/ml〕

活性測定法（Japanese）

1.原理

NADH＋H^+＋DCPIP $\xrightarrow{\text{diaphorase}}$ NAD^+＋Leucodye

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, pH7.5
B. 6.0mM NADH水溶液(用時調製, 氷冷保存)
C. 1.2mM DCPIP水溶液〔3.9㎎のDCPIP・$2H_2O$ (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試薬Aに溶解し(約1.0%溶液),予め氷冷した0.1%牛血清アルブミン(BSA)を含む試薬Aで0.4～0.8U/㎖に希釈し,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)に採り,25℃で約5分間予備加温する。

2.4㎖	蒸留水	(A)
0.3㎖	Tris-HCl	(B)
0.1㎖	NADH水溶液	(C)

②酵素溶液, DCPIP水溶液(C)各0.1㎖をこの順序で添加し,ゆるやかにかつ速やかに混和後,水を対照に25℃に制御された分光光度計で600nmの吸光度変化を2～3分間記録し, その初期直線部分より1分間当りの吸光度変化を求める(ΔODtest)。

③盲検は反応混液①に酵素希釈液(0.1%BSAを含む試薬A),DCPIP水溶液(C)各0.1㎖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

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

$$= \Delta OD/min \times 10 \times 希釈倍率$$

U/mg ＝U/㎖×1/C

1.0 ： 活性定義に基いて定められた600nmにおける単位吸光度

C ： 溶解時の酵素濃度(c ㎎/㎖)