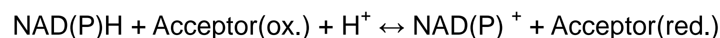
NIPRO ENZYMES

DIAPHORASE I (Di-1)

[EC 1. 6. 99. -]

from *Bacillus stearothermophilus*



SPECIFICATION

State	: Lyophilized	
Specific activity	: more than 1,000 U/mg protein	
Contaminants	: (as Diaphorase activity = 100 %)	
	Adenylate kinase	< 0.01 %
	NADH oxidase	< 0.01 %

PROPERTIES

Molecular weight	: ca. 30,000	
Optimum pH	: 8.0	(Fig. 1)
pH stability	: 7.5 - 9.5	(Fig. 2)
Isoelectric point	: 4.7	
Optimum temperature	: 70 °C	
Thermal stability	: No detectable decrease in activity up to 50 °C.	(Fig. 3, 4)
Michaelis constants	: See Table 1	
Substrate specificity	: See Table 1	
Effectors	: cations and anions	(Fig. 5, 6)

STORAGE

Stable at -20 to 5 °C for at least one year

APPLICATION

The enzyme is useful for the measurement of various dehydrogenase reactions in visible spectral range.

ASSAY

Principle

The change in absorbance is measured at 600 nm according to the following reaction.

$$\text{NAD(P)H} + \text{DCIP(ox.)} + \text{H}^+ \xrightarrow{\text{Di-1}} \text{NAD(P)}^+ + \text{DCIP(red.)}$$

Unit Definition

One unit of activity is defined as the amount of Di-1 that reduces 1 μmol of DCIP per minute at 30 °C.

Solutions

Ⅰ Buffer solution ; 500 mM Tris-HCl, pH8.5
Ⅱ NADH solution ; 13.1 mM (0.100 g NADH disodium salt·$3H_2O$/10 mL distilled water)
Ⅲ 2,6-Dichlorophenolindophenol (DCIP) solution ; 1.2 mM (2.0 mg DCIP sodium salt·$2H_2O$/5mL distilled water) (prepare freshly)

Preparation of Enzyme Solution

Dissolve the lyophilized enzyme with distilled water and dilute to 1.0 to 2.0 U/mL with 50 mM potassium phosphate buffer, pH 7.5.

Procedure

1. Prepare the following reaction mixture and pipette 2.85 mL of reaction mixture into a cuvette.

Solution Ⅰ	3.00mL
Solution Ⅱ	2.28mL
H_2O	23.22mL

2. Incubate at 30 °C for about 3 minutes.
3. Add 0.15 mL of Solution Ⅲ and 0.01 mL of enzyme solution into the cuvette and mix.
4. Read absorbance change at 600 nm per minute (ΔAbs(test)) in linear portion of curve. Repeat the Procedure 3 using distilled water in place of enzyme solution, and ΔAbs(blank) is obtained.

Calculation

$$\text{Volume activity (U/mL)} = \frac{(\Delta\text{Abs (test)} - \Delta\text{Abs (blank)}) \times (3.00 + 0.01)}{19 \times 0.01} \times \text{d.f.}$$

$$\text{Specific activity (U/mg protein)} = \frac{\text{Volume activity (U/mL)}}{\text{Protein concentration (mg/mL)*}}$$

d.f. ; dilution factor
19 ; millimolar extinction coefficient of DCIP (cm^2/μmol)
*Protein concentration ; determined by Bradford's method

REFERENCE

1. Mains, I., Power, D.M., Thomas, E.W. and Buswell J. A.; *Biochem. J.*, **191**, 457 (1980)

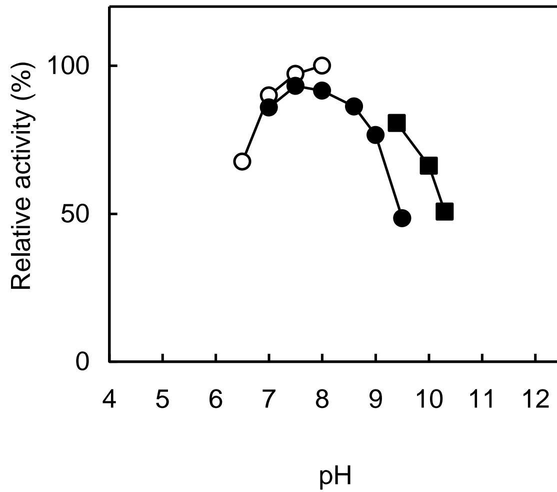


Fig. 1 pH profile

(○ phosphate, ● Tris-HCl, ■ Gly-KCl-KOH)

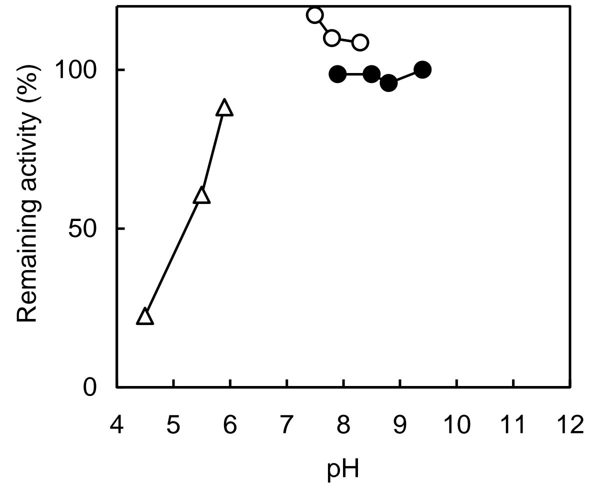


Fig. 2 pH stability

(treated for 24 hr at 4 °C in the following buffer solution (0.1 M); △ acetate, ○ phosphate, ● Tris-HCl)

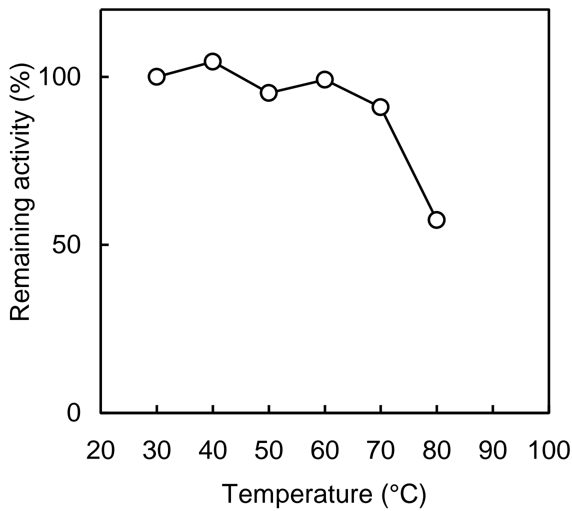


Fig. 3 Thermal stability

(treated for 15 min in 0.1 M potassium phosphate buffer, pH 7.5)

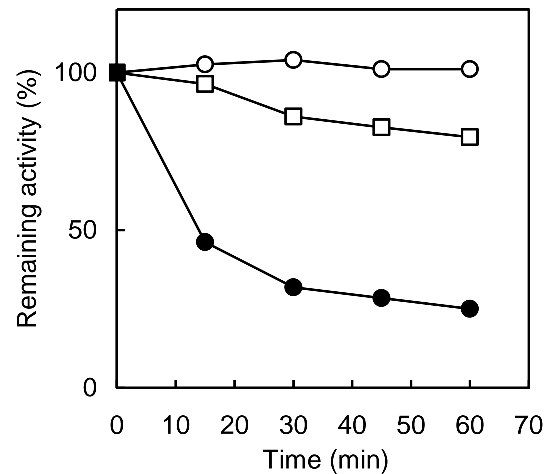


Fig. 4 Thermal stability

(treated in 0.1 M potassium phosphate buffer, pH 7.5 ○ 50 °C, □ 60 °C, ● 70 °C)

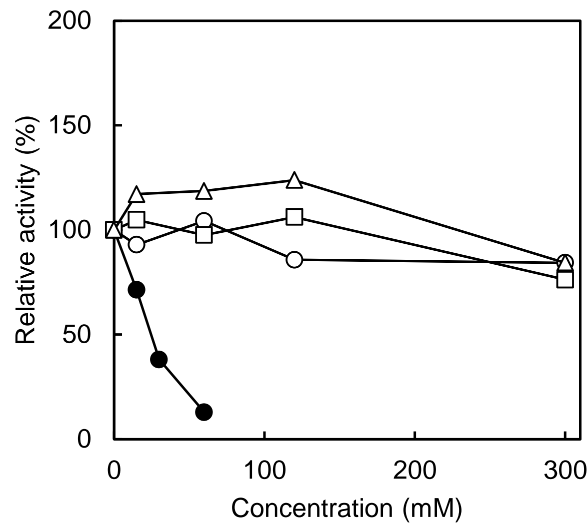


Fig. 5 Effect of various cations on the activity of DIAHORASE

Measurement : 0.30 mL of each cation solution and 3.00 mL of assay mixture were mixed, and incubated at 30° C for about 3 minutes. After incubation, 0.01mL of enzyme solution was added to the reaction mixture and the activity of DIAPHORASE was measured.
○ NaCl, △ KCl, □ $MgCl_2$, ● $CaCl_2$

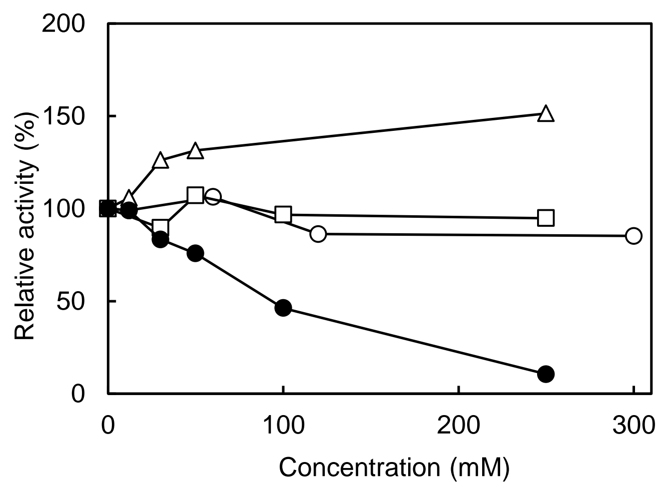


Fig. 6 Effect of various anions on the activity of

Measurement : 0.30 mL of each anion solution and 3.00 mL of assay mixture were mixed, and incubated at 30° C for about 3 minutes. After incubation, 0.01 mL of enzyme solution was added to the reaction mixture and the activity of DIAPHORASE was measured.
○ NaCl, △CH_3COONa, □ Na_2SO_4, ●$NaHCO_3$

Table 1. SUBSTRATE SPECIFICITY OF DIAPHORASE

Acceptor	DCIP*1	NBT*2	INT*3	FMN*4
$Km^{Acceptor}$ (mM)	0.015	0.15	0.40	-
Km^{NADH} (mM)	0.50	0.02	0.07	-
Km^{NADPH} (mM)	0.52	0.19	0.50	-
Optimum pH	8.0	> 10	7.5	< 6.5
$Activity^{NADH}$ (U/mg)	1,200	225	290	18
$Activity^{NADPH}$ (U/mg)	4	150	120	-
Assay Mixture	Tris-HCl (pH 8.5) 50 mM NAD(P)H 1 mM DCIP 0.06 mM	TEA (pH 7) 50 mM NAD(P)H 1 mM NBT 0.5 mM Triton X-100 0.1 %	Phosphate (pH 7.5) 96 mM NAD(P)H 1 mM INT 3 mM DMSO*6 2 % BSA*5 1 mg/mL	Phosphate (pH 7) 88 mM NADH 0.2 mM FMN 0.13 mM
Wavelength for measurement (nm)	600	550	492	340
Extinction coefficient ($cm^2/\mu mol$)	19	12.4	19.2	6.2

*1 2,6-Dichlorophenolindophenol
*2 Nitro blue tetrazolium
*3 *p*-Iodonitrotetrazolium violet
*4 Flavin mononucleotide
*5 Bovine serum albumin
*6 Added 1/40 volume of 120mM INT (0.607g/10mL 80% DMSO) into the Assay Mixture

Effect of BSA on the activity of DIAPHORASE: (See next page)

BSA stimulates the activity with INT as electron acceptor and the activation can be increased 30 fold with concentrations above 1 mg/mL BSA (Fig. 10). The extent of activation for DCIP is about 35 %, whereas the activities with NBT and FMN are not affected by BSA.

Effect of Triton X-100 on the activity of DIAPHORASE: (See next page)

The activity with NBT is little in the absence of Triton X-100, but is greatly increased by the addition of Triton X-100 (Fig. 8). On the other hand, Triton X-100 has no effect on the activities with DCIP, INT and FMN.

NBT (Nitro blue tetrazolium)

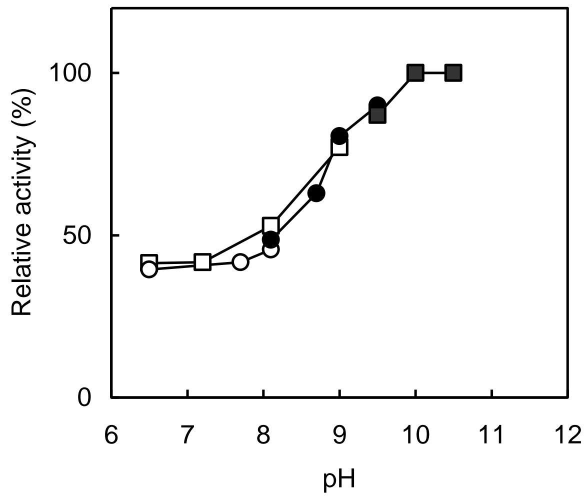
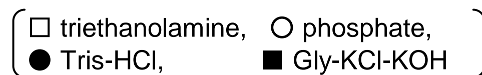


Fig. 7 pH profile



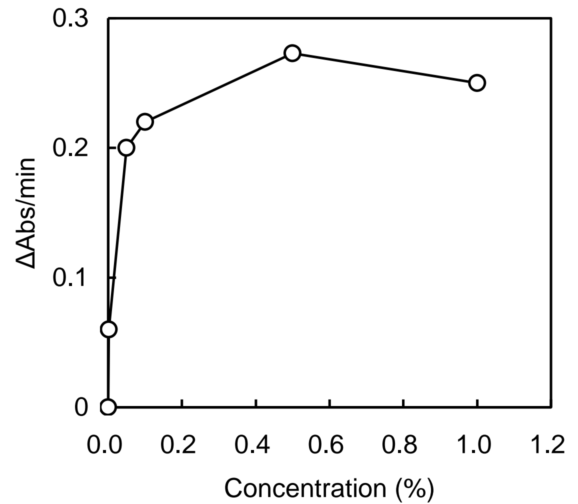


Fig. 8 Effect of Trion- X-100 on the activity of DIAPHORASE

INT (*p*-Iodonitrotetrazolium violet)

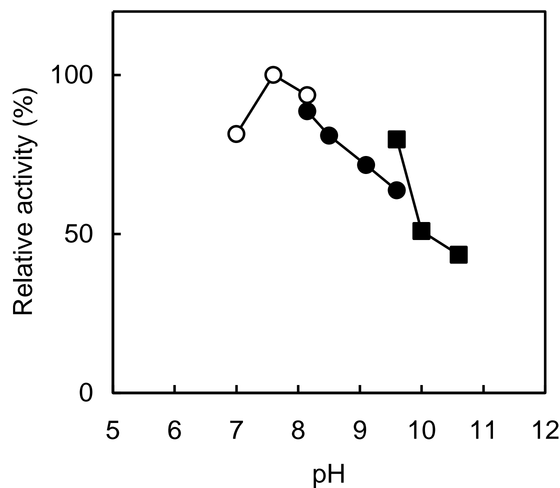
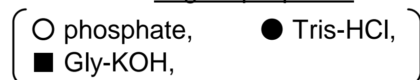


Fig. 9 pH profile



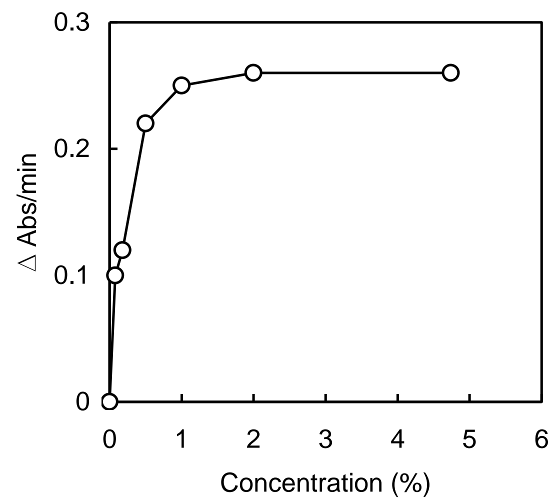


Fig. 10 Effect of BSA on the activity of DIAPHORASE