DIAPHORASE (NADPH) [DIP]

from *Bacillus megaterium*
(NADPH: dye oxidoreductase, EC 1.6.99.1)

$$NADPH + H^+ + A \longrightarrow NADP^+ + AH_2$$

A : Hydrogen acceptor

Preparation and Specification

Appearance : Yellowish amorphous powder, lyophilized
Specific activity : More than 5 U/mg solid

Properties

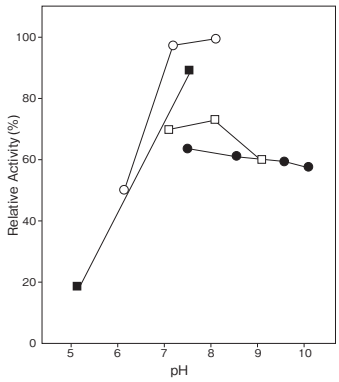
Molecular weight	: 48 kDa (gel filtration)	
Isoelectric point	: pH 3.0	
Michaelis constant	: NADPH 2.9×10^{-4}M	
Optimum pH	: 7.0–9.0	Figure 1
pH stability	: 6.5–9.0 (37℃, 60 min)	Figure 2
Thermal stability	: Stable at 60℃ and below (pH 7.5, 10 min)	Figure 3
Storage stability	: At least one year at −20℃	Figure 4
Activators	: FMN, FAD	

Applications for Diagnostic Test

This enzyme is useful for enzymatic determination of **reduced NADP.**

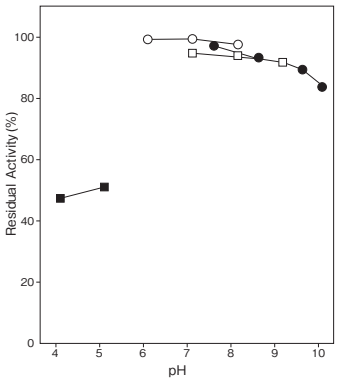
$$NADPH + H^+ + NTB \xrightarrow{DIP} \text{Formazane dye} + NADP^+$$

Fig.1 pH Optimum



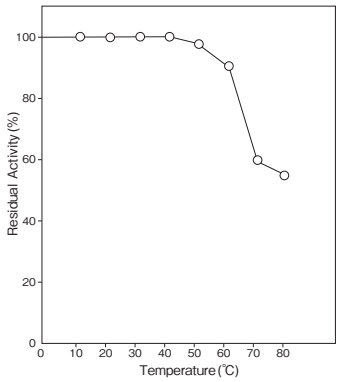
■ : 3,3-Dimethylglutarate-NaOH buffer
○ : Phosphate buffer
□ : Tris-HCl buffer
● : Glycine-NaOH buffer

Fig.2 pH Stability



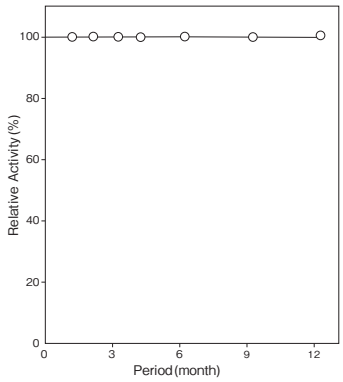
37℃, 60 min
■ : Acetate buffer
○ : Phosphate buffer
□ : Tris-HCl buffer
● : Glycine-NaOH buffer

Fig.3 Thermal Stability



pH 7.5, 10 min.
Phosphate buffer

Fig.4 Storage (lyophilized powder)



−20℃

D

Assay

■ Principle

The assay is based on the increase in absorbance at 520 nm as the formation of formazan dye proceeds in the following reaction:

$$\text{NADPH} + \text{H}^+ + \text{NTB} \xrightarrow{\text{DIP}} \text{Formazane dye} + \text{NADP}^+$$

NADPH: Nicotineamido adenine dinucleotide phosphate
NTB: Nitrotetrazolium blue

■ Unit definition

One unit is defined as the amount of enzyme which oxidizes 1 μmole of NADPH to $NADP^+$ per minute at 30℃ under the conditions specified in the assay procedure.

■ Reagents

1. Reaction mixture

0.4 M Tris-HCl buffer	pH 8.0	1.0 ml
0.5% (W/V) NTB solution		1.0 ml
5% (W/V) BSA solution	pH 8.0	0.8 ml
Distilled water		0.6 ml

2. Enzyme dilution buffer
   10mM KH_2PO_4-NaOH buffer pH 8.0 containing 0.1% (W/V) BSA
3. Reagents
   NTB: Dojindo Laboratories #344-02033
   BSA: Celliance Fraction V pH 5.2 #3220-80
   NADPH (Reduced form):
   FUJIFILM Wako Pure Chemical Corporation #305-50473
   FMN (Flavin Mononucleotide Sodium Salt):
   FUJIFILM Wako Pure Chemical Corporation Special Grade #063-00172

■ Enzyme solution

Accurately weigh about 20 mg of the sample and add enzyme dilution buffer to make a total of 20 ml. Dilute it with enzyme dilution buffer to adjust the concentration as required.

■ Procedure

1. Pipette accurately 3.4 ml of reaction mixture, 0.5 ml of 20 mM NADPH and 0.1 ml of 0.2 mM FMN into a small test tube and preincubate it at 30℃.
2. After 4 min, add 100 μl of enzyme solution and mix to start the reaction at 30℃.
   ※ In the case of a test blank, add 100 μl of enzyme dilution buffer in place of enzyme solution.
3. After starting the reaction, measure the rate of increase per minute in absorbance at 520 nm. The rate must be measured within the linear portion of the absorbance curve.

Absorbance sample : As/min
blank : Ab/min
△A/min = (As/min − Ab/min) ≦ 0.050 Abs/min

■ Calculation

$$\text{Activity (U/mg of powder)} = \frac{\triangle\text{A/min}}{16.4} \times \frac{4.10}{0.10} \times \frac{1}{\text{X}}$$

16.4 : millimolar extinction coefficient of Formazane dye at 520 nm (cm^2 / μmole)
4.10 : final volume (ml)
0.10 : volume of enzyme solution (ml)
X : concentration of the sample in enzyme solution (mg/ml)

Storage

Storage at −20℃ in the presence of a desiccant is recommended. Enzyme activity will be retained for at least one year under this condition (Figure 4).

References

1. Gerlo, E. and Charlier, J. (1975) Eur. J. Biochem., **57**, 461-467.
2. Jablonski, E. and Deluca, M. (1977) Biochemistry, **16**, 2932-2936.
3. Watanabe, H. and Hasting, J. W. (1982) Mol. Cell. Biochem., **44**, 181-187.

DIP 活性測定法（Japanese）

Ⅰ．試薬液

1. 反応試薬混合液

0.4M トリス –HCl 緩衝液　pH8.0	1.0 ml
0.5%（W/V）NTB 溶液	1.0 ml
5%（W/V）BSA 溶液　pH8.0	0.8 ml
精製水	0.6 ml

2. 酵素溶解希釈用液
   0.1%（W/V）BSA を含む 10mM KH_2PO_4–NaOH 緩衝液 pH8.0
3. 試薬
   NTB（ニトロテトラゾリウムブルー）：
   同仁化学製　#344–02033
   BSA: Celliance Fraction V pH 5.2　#3220-80
   NADPH（ニコチンアミドアデニンジヌクレオチド・リン酸還元型）：富士フイルム和光純薬製
   #305–50473
   FMN（フラビンモノヌクレオチドナトリウム）：
   富士フイルム和光純薬製　特級　#063–00172

Ⅱ．酵素試料液

検品約 20mg を精密に量り、酵素溶解希釈用液に溶解して全容 20ml とする。
その液を酵素溶解希釈用液で適宜希釈する。

Ⅲ．測定操作法

1. 小試験管に反応試薬混合液 3.4ml と 20mM NADPH 溶液 0.5ml 及び 0.2mM FMN 溶液 0.1ml を正確に分注し、30℃で予備加温する。
2. 4 分経過後、酵素試料液 100 μl を正確に加えて混和し、30℃で反応を開始する。
   ※盲検は酵素試料液の代わりに酵素溶解希釈用液 100 μl を加える。
3. 反応開始後、520nm における吸光度を測定して直線的に反応している 1 分間当たりの吸光度変化を求める。
   求められた吸光度変化を試料液は As/min、盲検液は Ab/min とする。
   $\Delta A/min = (As/min - Ab/min) \leqq 0.050$ Abs/min

Ⅳ．計算

$$\text{活性 (U/mg)} = \frac{\Delta A/min}{16.4} \times \frac{4.10}{0.10} \times \frac{1}{X}$$

16.4 : $NTBH_2$ の 520nm におけるミリモル分子吸光係数（cm^2/ μmole）
4.10 : 反応総液量（ml）
0.10 : 反応に供した酵素試料液量（ml）
X　: 酵素試料液中の検品濃度（mg/ml）