●TOYOBO ENZYMES●

(Diagnostic Reagent Grade)

N-ACETYLNEURAMINIC ACID ALDOLASE

from Microorganism

N-Acetylneuraminate pyruvate-lyase (EC 4.1.3.3)[1~4)]

N-Acetylneuraminate ⇄ *N*-Acetyl-D-mannosamine + Pyruvate

PREPARATION and SPECIFICATION

Appearance	: Yellowish amorphous powder, lyophilized
Activity	: Grade Ⅲ 15U/mg-solid or more (30U/mg-protein or more) (containing approx. 30% of stabilizers)
Contaminants	: Catalase ≤1.0% NADH oxidase ≤1.0×10^{-3}%
Stabilizers	: Mannitol, EDTA

PROPERTIES

Stability	: Stable at −20℃ for at least one year	(Fig.1)
Molecular weight	: approx. 98,000	
Isoelectric point	: 4.6±0.1	
Michaelis constant	: 2.5×10^{-3}M (*N*-Acetylneuraminic acid)	
Structure	: 3 subunits (approx. 35,000) per enzyme molecule	
Inhibitors	: p-Chloromercuribenzoate, SDS, Hg^{++}, Ag^{+}	
Optimum pH	: 7.5−8.0	(Fig.3)
Optimum temperature	: 70℃	(Fig.4)
pH Stability	: pH 6.0−9.0 (10℃, 25hr)	(Fig.5)
Thermal stability	: below 65℃ (pH 7.5, 30min)	(Fig.6)
Effect of various chemicals	: (Table 1)	

APPLICATIONS

This enzyme is useful for enzymatic determination of *N*-acetylneuraminic acid and sialic acid when coupled with the related enzymes in clinical analysis. [5~7)]

For industrial use, this enzyme is useful for enzymatic synthesis of sialic acid. [8~9)]

ASSAY

Principle:

N-Acetylneuraminate $\xrightarrow{\text{N-Acetylneuraminic acid aldolase}}$ Pyruvate + *N*-Acetyl-D-mannosamine

Pyruvate + NADH + H^+ $\xrightarrow{\text{lactate dehydrogenase}}$ L-Lactate + NAD^+

The disappearance of NADH is measured at 340 nm by spectrophotometry.

Unit definition:

One unit causes the oxidation of one micromole of NADH per minute under the conditions described below.

Method:

Reagents

A. NANA solution : 50mM [Dissolve 309mg of *N*-acetylneuraminic acid (MW=309) in approx. 15ml of 50mM K-phosphate buffer, pH 7.5 and, after adjusting the pH to 7.5 with 1N KOH, fill up to 20ml with the same buffer.] (Stable for at least one week if stored at 0−5℃)

B. LDH solution : approx. 50U/ml [Dilute pig heart lactate dehydrogenase (Toyobo Grade Ⅱ, ammonium sulfate suspension) to a concentration of approx. 50U/ml with ice-cold 50mM K-phosphate buffer, pH 7.5] (Should be freshly prepared)

C. NADH solution : 1.0mM [Dissolve 7.6mg of NADH・Na_2・$3H_2O$ (MW=763) in 10ml of 50mM K-phosphate buffer, pH 7.5] (Should be freshly prepared)

D. Buffer solution : 50mM K-phosphate buffer, pH 7.5

E. Enzyme diluent : 50mM K-phosphate buffer, pH 7.5 containing 0.2% BSA

Procedure

1. Prepare the following reaction mixture in a cuvette (d=1.0cm) and equilibrate at 37℃ for about 5 minutes.

1.0ml	Substrate solution	(A)
0.5ml	LDH solution	(B)
0.5ml	NADH solution	(C)
0.4ml	Buffer solution	(D)

Concentration in assay mixture	
K-Phosphate buffer	50 mM
NANA	20 mM
NADH	0.2mM
LDH	ca.10 U/ml

2. Add 0.1ml of the enzyme solution* and mix by gentle inversion.
3. Record the decrease in optical density at 340nm against water for 3 to 4 minutes in 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) using the same method the test except that the enzyme diluent (E) is added instead of the enzyme solution.

* Dissolve the enzyme preparation in ice-cold enzyme diluent (E) and dilute to 0.1−0.3U/ml with the same buffer, immediately before assay.

Calculation

Activity can be calculated by using the following formula :

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

$$= \Delta\text{OD/min} \times 4.02 \times \text{df}$$

Weight activity (U/mg) = (U/ml) × 1/C

Vt : Total volume (2.5ml)

Vs : Sample vol ume (0.1ml)

6.22 : Millimolar extinction coefficient of NADH (㎤/micromole)

1.0 : Light path length (cm)

df : Dilution factor

C : Enzyme concentration in dissolution (c mg/ml)

REFERENCES

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Table 1. Effect of Various Chemicals on *N*-Acetylneuraminic acid aldolase
[The enzyme dissolved in 0.1M Tris-HCl buffer, pH7.5 (5U/ml) was incubated at 30°C for 1hr.]

Chemical	Concn.(mM)	Residual activity(%)
None	—	100
Metal salt	2.0	
$MgCl_2$		107
$CaCl_2$		87
$Ba(OAc)_2$		95
$FeCl_3$		89
$CoCl_2$		93
$MnCl_2$		98
$ZnSO_4$		92
$NiCl_2$		99
$CuSO_4$		64
$Pb(OAc)_2$		87
$AgNO_3$		0
$HgCl_2$		0

Chemical	Concn.(mM)	Residual activity(%)
PCMB	2.0	0
NEM	2.0	103
NaF	2.0	100
NaN_3	20	100
EDTA	5.0	95
o-Phenanthroline	2.0	100
α,α'-Dipyridyl	2.0	101
Borate	50	86
Triton X-100	0.10%	109
Na-cholate	0.10%	95
SDS	0.10%	0
Tween 40	0.10%	96
Span 85	0.10%	93

Ac, CH_3CO; PCMB, p-Chloromercuribenzoate; NEM, N-Ethylmaleimide; EDTA, Ethylenediaminetetraacetate; SDS, Sodium dodecyl sulfate.

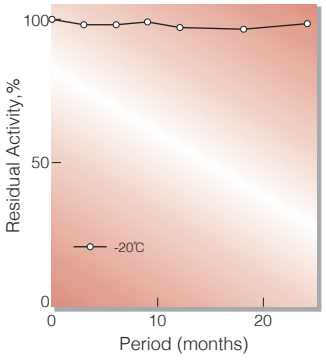

Fig.1. Stability (Powder form)

〔kept under dry conditions〕

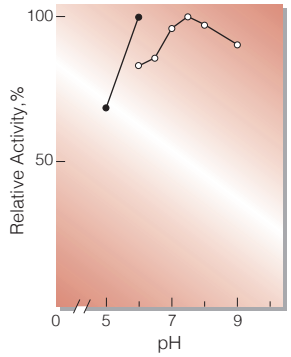

Fig.3. pH-Activity

〔37℃, 5min-reaction in 50mM buffer solution : pH5.0-6.0, acetate; pH6.0-9.0, K-phosphate;
The enzyme activity was assayed by the 2,4-dinitroplenylhydradine method.〕

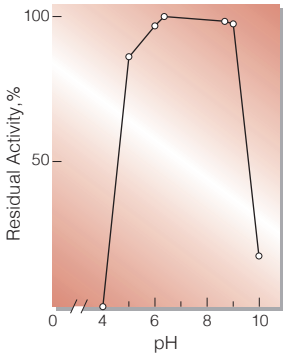

Fig.5. pH-Stability

〔10℃, 25hr-treatment with 50mM buffer solution : pH4.0-6.0, acetate; pH6.0-9.0, K-phosphate ; pH9.0-10.0, borate.〕

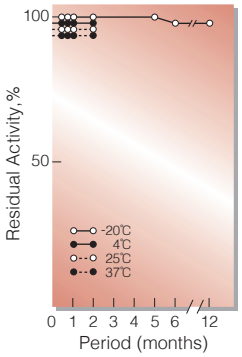

Fig.2. Stability (Powder form)

〔kept under dry conditions〕

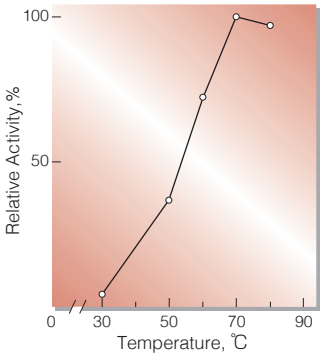

Fig.4. Temperature activity

〔5min-reaction in 50mM K-phosphate buffer pH7.5, The enzyme activity was assayed by the 2,4-dinitroplenylhydradine method.〕

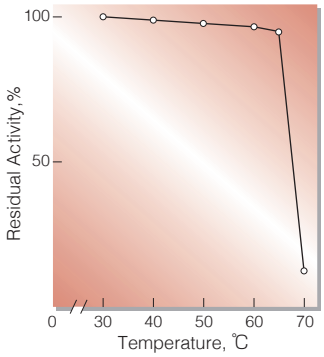

Fig.6. Thermal stability

〔30min-treatment with 50mM K-phosphate buffer, pH7.5, enzyme concentration.: 20U/ml〕

活性測定法（Japanese）

1.原理

N-acetylneuraminate $\xrightarrow{\text{*N*-acetylneuraminic acid aldolase}}$
N-Acetyl-D-mannosamine＋Pyruvate
Pyruvate＋NADH＋H^+ $\xrightarrow{\text{lactate dehydrogenase}}$
L-Lactate＋NAD^+
NADHの消失量を340nmの吸光度の変化で測定する。

2.定義

下記条件で1分間に1マイクロモルのNADHが酸化される酵素量を1単位(U)とする。

3.試薬

A. 50mM NANA溶液 〔309mgのN-アセチルノイラミン酸(MW＝309)を約15mlの50mM K-リン酸緩衝液，pH7.5に溶解し，1N KOHでpHを7.5に調整後，同緩衝液で20mlとする〕(0～5℃保存で1週間は使用可能)
B. LDH溶液〔ブタ心臓LDH(東洋紡製GradeⅡ，硫安懸濁液)を50mM K-リン酸緩衝液，pH7.5で，約50U/mlに希釈する〕(用時調製)
C. 1.0mM NADH溶液〔7.6mgのNADH・Na_2・$3H_2O$ (MW＝763)を10mlの50mM K-リン酸緩衝液，pH7.5に溶解する〕(用時調製)
D. 50mM K-リン酸緩衝液，pH7.5

酵素溶液：酵素標品を予め氷冷した0.2%のBSAを含む50mM K-リン酸緩衝液，pH7.5 (D)で溶解し，分析直前に同緩衝液で0.1～0.3U/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。

1.0ml	基質溶液	(A)
0.5ml	LDH溶液	(B)
0.5ml	NADH溶液	(C)
0.4ml	K-リン酸緩衝液	(D)

②酵素溶液0.1ml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3～4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)。
③盲検は反応混液①に酵素溶液の代りに酵素希釈液(0.2%BSAを含む50mM K-リン酸緩衝液，pH7.5)0.1ml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(ΔOD blank)。

5.計算式

$$U/ml = \frac{\Delta OD/min\ (\Delta OD\ test - \Delta OD\ blank) \times 2.5(ml) \times 希釈倍率}{6.22 \times 1.0 \times 0.1(ml)}$$
$$= \Delta OD/min \times 4.02 \times 希釈倍率$$

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)