●TOYOBO ENZYMES●
(Biochemical Reagent Grade)

ALKALINE PHOSPHATASE

from Calf intestine

Orthophosphoric-monoester phosphohydrolase (alkaline optimum)(EC 3.1.3.1)

Orthophosphoric monoester + H_2O ⟶ Alcohol + Orthophosphate

PREPARATION and SPECIFICATION

Appearance	: 50% glycerol solution	
Activity	: Grade Ⅱ 30,000U/ml or more	
Contaminants	: Adenosine deaminase	≤1.0×10^{-4}%
	Phosphodiesterase	≤3.0×10^{-3}%
	DNase	No degradation of the fragments is observed by agarose gel electrophoresis, after incubation of 1μg of λ-DNA with 2 units of alkaline phosphatase for 16 hr at 37°C in a 50μl reaction volume.
	RNase	No degradation of the fragments is observed by polyacrylamide gel electrophoresis, after incubation of 2μg of tRNA with 2 units of alkaline phosphatase for 16 hr 37°C in a 50μl reaction volume.

PROPERTIES [1,2]

Stability	: Stable at −20℃ for at least one year	(Fig.1)
Molecular weight	: approx. 100,000	
Isoelectric point	: 5.7	
Michaelis constant	: 1.7×10^{-3}M (p-Nitrophenyl phosphate)	
Inhibitors	: Cu^{++}, Ag^{+}, Hg^{++}, EDTA	
Optimum pH	: 10.0−10.3	(Fig.3)
Optimum temperature	: 40℃	(Fig.4)
pH Stability	: pH 8.5−10.3 (25℃, 20hr)	(Fig.5)
Thermal stability	: below 40℃ (pH 9.5, 30min)	(Fig.6)
Effect of various chemicals	: (Table 1)	

APPLICATIONS [3,4]

This enzyme is useful for molecular biology.

ASSAY

Principle:

p-Nitrophenylphosphate(pNPP)＋H_2O $\xrightarrow{\text{alkaline phosphatase}}$ p-Nitrophenol＋Pi

The appearance of p-Nitrophenol is measured at 405nm by spectrophotometry.

Unit definition:

One unit causes the formation of one micromole of p-Nitrophenol per minute under the conditions described below.

Method:

Reagents

A. Diethanolamine buffer, pH10.25 : 1M ［Dilute 9.66ml of diethanolamine (MW＝105.14) in 60ml of H_2O, add 0.25ml of 0.1M $MgCl_2$ and, after adjusting the pH to 10.25 with 2N HCl, fill up to 100ml with H_2O］(Prepare freshly)

B. pNPP solution : 0.1M ［Dissolve 371mg of p-nitrophenylphosphate disodium salt (MW＝371.16) in 10ml buffer solution A］(Prepare freshly)

C. Enzyme diluent : 0.1M Diethanolamine buffer pH 9.5 contg. 0.25mM $MgCl_2$

Procedure

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

2.6ml	Buffer solution	(A)
0.3ml	Substrate solution	(B)

Concentration in assay mixture	
Diethanolamine buffer	0.97 M
p-Nitrophenylphoshate	10 mM
$MgCl_2$	0.25mM

2. Add 0.1ml of the enzyme solution* and mix by gentle inversion.
3. Record the increase in optical density at 405nm against water for 3 to 5 minutes in a spectrophotometer thermostated at 37℃, and calculate ΔOD per minute from the intial linear portion of the curve (ΔOD test).
   At the same time, measure the blank rate (ΔOD blank) using the same method as the test except that the enzyme diluent is added instead of the enzyme solution.

* Dilute to 0.1－0.3U/ml with ice cold enzyme diluent (C),immediately before assay.

Calculation

Activity can be calculated by using the following formula :

$$\text{Volume activity (U/ml)} = \frac{\Delta\text{OD/min (}\Delta\text{OD test} - \Delta\text{OD blank)} \times \text{Vt} \times \text{df}}{18.5 \times 1.0 \times \text{Vs}} = \Delta\text{OD/min} \times 1.62 \times \text{df}$$

Vt : Total volume (3.0ml)

Vs : Sample volume (0.1ml)

18.5 : Millimolar extinction coefficient of p-nitrophenol under the assay condition (㎠/micromole)

1.0 : Light path length (cm)

df : Dilution factor

REFERENCES

1) H.N.Ferunley; ***The Enzymes, Vol.4***, (3rd ed.) 417 (1971).

2) R.K.Morton; ***Biochem.J, 61***, 232 (1955).

3) F.Dray, E.Dith and C.Rougeot; ***Methods of Enzymatic Analysis, Vol.9***, 348 (1986).

4) P.Rathman and B.B.Saxena; ***Methods of Enzymatic Analysis, Vol.9***, 396 (1986).

Table 1. Effect of Various Chemicals on Alkaline phosphatase

[The enzyme dissolved in 40mM CAPS buffer, pH9.5 (20U/ml) was incubated with each chemical at 25℃ for 1 hr.]

Chemical	Concn.(mM)	Residual activity(%)
None	—	100
Metal salt	2.0	
$MgCl_2$		108
$CaCl_2$		99
$FeCl_2$		96
$MnCl_2$		76
$CoCl_2$		86
$ZnCl_2$		73
$NiCl_2$		91
$CuSO_4$		56
$Pb(OAc)_2$		96
$AgNO_3$		0.1
$HgCl_2$		17

Chemical	Concn.(mM)	Residual activity(%)
PCMB	2.0	110
MIA	2.0	102
NaF	2.0	104
NaN_3	20	97
EDTA	5.0	19
o-Phenanthroline	2.0	80
Triton X-100	0.1%	101
Brij 35	0.1%	111
Tween 20	0.1%	95
Span 20	0.1%	82
Na-cholate	0.1%	94
SDS	0.1%	107

Ac, CH_3CO; PCMB, p-Chloromercuribenzoate; MIA, Monoiodoacetate; EDTA, Ethylenediaminetetraacetate; SDS, Sodium dodecyl sulfate.

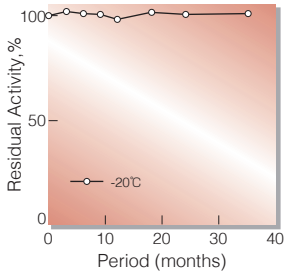

Fig.1. Stability (Liquid form)

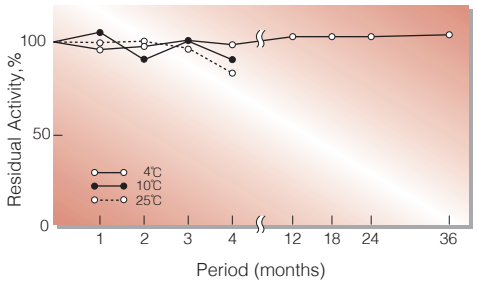

Fig.2. Stability (Liquid form)

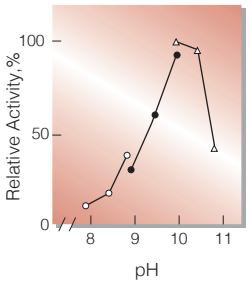

Fig.3. pH-Activity

〔37℃,5min-reaction in 0.1M buffer solution:pH8.0-9.0, Bicine;pH9.0-10.0,CHES; pH10.0-11.0,CAPS.〕

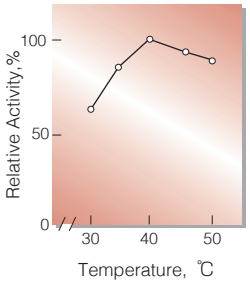

Fig.4. Temperature activity

〔5 min-reaction in 0.1M Diethanolamine buffer,pH10.25.〕

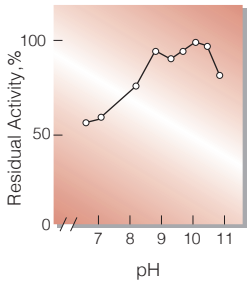

Fig.5. pH-Stability

〔25℃, 25hr-treatment with 0.1M buffer solution;pH5.5-7.0,MES;pH8.5-10.0, Diethanolamine;pH10.5-11.0,CAPS. enzyme concentration:20U/ml〕

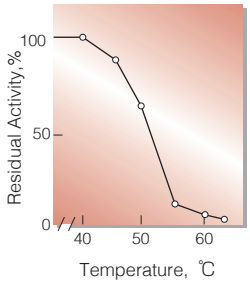

Fig.6. Thermal stability

〔30min-treatment with 0.1M Diethanolamine buffer,pH9.5.enzyme concentration:20U/ml〕

活性測定法（Japanese）

1.原理

p-Nitrophenylphosphate＋H_2O $\xrightarrow{\text{alkaline phosphatase}}$ p-Nitrophenol＋Pi

p-Nitrophenolの生成量を405nmにおける吸光度の変化で測定する。

2.定義

下記条件下で1分間に1マイクロモルのp-Nitrophenolを生成する酵素量を1単位(U)とする。

3.試薬

A. 1Mジエタノールアミン緩衝液, pH 10.25〔9.66mℓのジエタノールアミン（MW＝105.14)を蒸留水60mℓで希釈後, 0.1M $MgCl_2$ 0.25mℓを添加する。さらに, 2.0N HClでpHを10.25に調整し, 最終液量を100mℓとする〕(用時調製)

B. 0.1M pNPP溶液 〔371mgのp-ニトロフェニルリン酸二ナトリウム塩（MW＝371.16)を10mℓの緩衝液Aに溶解する〕(用時調製)

酵素溶液：酵素溶液を予め氷冷した0.25mM $MgCl_2$を含む0.1Mジエタノールアミン緩衝液,pH 9.5で分析直前に0.1～0.3/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。

2.6mℓ	ジエタノールアミン緩衝液	(A)
0.3mℓ	基質溶液	(B)

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405nmの吸光度変化を3～5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(ΔODtest)。

③盲検は反応混液①に酵素溶液の代りに酵素希釈液(0.25mM $MgCl_2$を含む0.1Mジエタノールアミン緩衝液, pH9.5)を加え, 上記同様に操作を行って1分間当りの吸光度を求める(ΔODblank)。

5.計算式

$$U/mℓ = \frac{\Delta OD/min\ (\Delta OD\ test - \Delta OD\ blank) \times 3.00(mℓ) \times 希釈倍率}{18.5 \times 1.0 \times 0.1(mℓ)}$$

$$= \Delta OD/min \times 1.62 \times 希釈倍率$$

18.5 ：p-Nitrophenolのミリモル分子吸光係数

1.0 ：光路長(cm)