●TOYOBO ENZYMES●

(Diagnostic Reagent Grade)

PHOSPHOENOLPYRUVATE CARBOXYLASE

from Microorganism

Orthophosphate:oxaloacetate carboxy-lyase(Phosphorylating)(EC 4.1.1.31)

$$\text{Phosphoenolpyruvate} + CO_2 + H_2O \xrightarrow[Mg^{++}]{} \text{Oxaloacetate} + Pi$$

PREPARATION and SPECIFICATION

Appearance	: White amorphous powder, lyophilized
Activity	: Grade Ⅲ 5.0U/mg-solid or more
Contaminants	: Lactate dehydrogenase ≤1.0×10^{-3}%
	Pyruvate kinase ≤0.05%
Stabilizers	: BSA, sugar alcohols

PROPERTIES

Stability	: Stable at −20℃ for at least one year	(Fig.1)
Molecular weight	: approx. 390,000 (by gel filtration)	
Isoelectric point	: 6.0±0.1	
Structure	: 4 Subunits (M.W.100,000) per enzyme molecule	
Michaelis constant	: 1.9×10^{-4}M (Phosphoenolpyruvate)	
Optimum pH	: 7.5−8.0	(Fig.2)
Optimum temperature	: 60℃	(Fig.3)
pH Stability	: pH 5.0−8.0 (25℃, 24hr)	(Fig.4)
Thermal stability	: below 40℃ (pH 7.0, 15min)	(Fig.5)

APPLICATIONS

This enzyme is useful for enzymatic determination of carbon dioxide when coupled with malate dehydrogenase (MAD-211) in clinical analysis.

ASSAY

Principle:

Phosphoenolpyruvate + CO_2 + H_2O $\xrightarrow{\text{phosphoenolpyruvate carboxylase}}$ Oxaloacetate + Pi

Oxaloacetate + NADH + H^+ $\xrightarrow{\text{malate dehydrogenase}}$ L-Malate + NAD^+

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

Unit definition:

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

Method:

Reagents

A. Buffer solution : 0.1M Tris-HCl Buffer, pH 8.0

B. Na_2CO_3 solution : 0.1M [Dissolve 1.06g of $Na2_2CO_3$(MW=105.99)/100ml of H_2O]

C. K-Phosphoenolpyruvate solution : 32mM [Dissolve 33.0mg of PEP・K(MW=206.1)/5ml of H_2O] (Should be prepared fresh)

D. $MgSO_4$ solution : 1M [Dissolve 4.93g of $MgSO_4$・$7H_2O$(MW=246.48)/20 ml of H_2O]

E. NADH solution : 1.4mM [Dissolve 5.34mg of NADH・$3H_2O$(MW=763)/5ml of H_2O]

F. MDH solution : ca.100U/ml [Dissolve malate dehydrogenase (TOYOBO GradeⅡ) to approx.100U/ml with 20mM Tris-HCl Buffer,pH 8.0] (Should be prepared fresh)

G. Enzyme diluent : 20mM K-phosphate buffer, pH 7.0

Procedure

1. Prepare the following reaction mixture in a cuvette (d=1.0cm) and equilibrate at 30℃ for about 5 minutes.

1.77ml	Buffer solution	(A)
0.3 ml	Na_2CO_3 solution	(B)
0.3 ml	K-Phosphoenolpyruvate solution	(C)
0.03ml	$MgSO_4$ solution	(D)
0.3 ml	NADH solution	(E)
0.3 ml	MDH solution	(F)

Concentration in assay mixture	
K-Phosphoenolpyruvate	3.1 mM
Tris-HCl	57 mM
Na_2CO_3	9.7 mM
$MgSO_4$	9.7 mM
NADH	0.14mM
MDH	9.7 U/ml
K-Phosphate	0.65mM

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 a spectrophotometer thermostated at 30℃, and calculate the ΔOD per minute from initial liner 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 (G) is added instead of the enzyme solution.

* Dissolve the enzyme preparation in ice-cold enzyme diluent (G) and dilute to 0.2−0.7U/ml with the same buffer and store on ice.

Calculation

Activity can be calculated by using the following formula :

$$\text{Volume activity (U/ml)} = \frac{\Delta\text{OD/min (}\Delta\text{OD test} - \Delta\text{OD blank)} \times \text{Vt} \times \text{df}}{6.22 \times 1.0 \times \text{Vs}} = \Delta\text{OD/min} \times 4.98 \times \text{df}$$

Weight activity (U/mg) = (U/ml)×1/C

Vt : Total volume (3.1ml)

Vs : Sample volume (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

1) W.Wilson, P.Jesyk, R.Rand and R.D.Bevill; *Clin.Chem.,19,* 640(1973)

2) R.L.Forrester, L.J.Wataji, D.A.Silverman and K.J.Pierre; *Clin.Chem.,22,* 243(1976)

Table 1. Effect of Various Chemicals on Phosphoenolpyruvate carboxylase
[The enzyme solution dissolved in 20mM K-phosphate buffer, pH 7.0 (20U/ml) was incubated with each chemical at 25℃ for 1hr.]

Chemical	Concn.(mM)	Residual activity(%)
None	—	100
Metal salt	2.0	
$MgCl_2$		105
$CaCl_2$		105
$Ba(OAc)_2$		103
$FeCl_3$		92
$CoCl_2$		106
$MnCl_2$		107
$ZnSO_4$		103
$Cd(OAc)_2$		104
$NiCl_2$		0
$CuSO_4$		0
$Pb(OAc)_2$		105
$AgNO_3$		0
$HgCl_2$		0
MIA	2.0	60
2-Mercaptoethanol	2.0	101

Chemical	Concn.(mM)	Residual activity(%)
PCMB	0.1	80
NEM	2.0	87
IAA	2.0	90
Hydroxylamine	2.0	95
EDTA	5.0	100
o-Phenanthroline	2.0	103
α,α'-Dipyridyl	2.0	109
Borate	5.0	103
NaF	2.0	106
NaN_3	2.0	106
Triton X-100	0.10%	111
Brij 35	0.10%	110
Tween 20	0.10%	112
Span 20	0.10%	109
Na-cholate	0.10%	108
SDS	0.05%	1
DAC	0.05%	99

Ac, CH_3CO; PCMB, p-Chloromercuribenzoate; MIA, Monoiodoacetate; EDTA, Ethylenediaminetetraacetate; IAA, Iodoacetamide; NEM, N-Ethylmaleimide; SDS, Sodium dodecyl sulfate; DAC, Dimethylbenzylallkylammonium chloride.

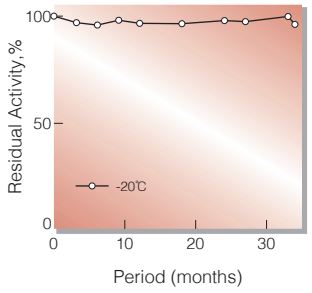

Fig.1. Stability (Powder form)
〔kept under dry conditions〕

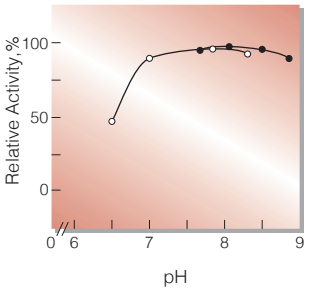

Fig.2. pH-Activity
〔30℃, in 50mM buffer solution: pH6.0-8.5, MES: pH7.5-9.0, Tris-HCl〕

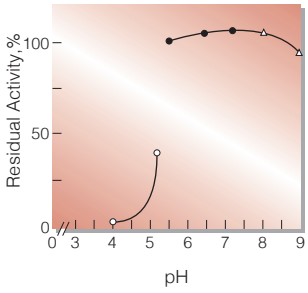

Fig.4. pH-Stability
〔25℃,24hr-treatment with 50mM buffer solution contg. 10mM $MgSO_4$: pH3.0-5.0, Acetate;pH5.0-8.0, K-phosphate; pH8.0-9.0, Tris-HCl〕

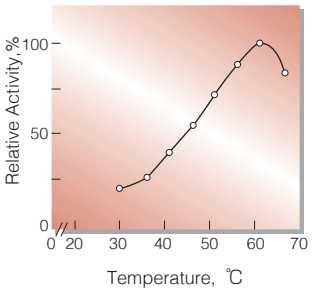

Fig.3. Temperature activity
〔in 20mM K-phosphate buffer,pH7.0〕

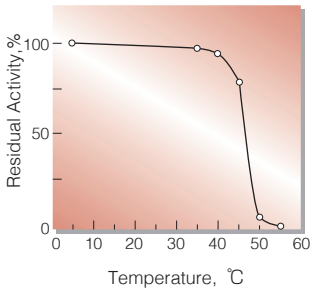

Fig.5. Thermal stability
〔15min-treatment with 20mM K-phosphate buffer,pH7.0 enzyme concn.: 2.0U/ml〕

活性測定法（Japanese）

1.原理

Phosphoenolpyruvate＋CO_2＋H_2O
$\xrightarrow{\text{Phosphoenolpyruvate carboxylase}}$ Oxaloacetate＋Pi

Oxaloacetate＋NADH＋H^+
$\xrightarrow{\text{malate dehydrogenase}}$ L-Malate＋NAD^+

NADHの減少量を340nmにおける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を酸化する酵素量を1単位(U)とする。

3.試薬

A. 0.1M Tris-HCl緩衝液,pH8.0
B. 0.1M Na_2CO_3水溶液〔1.06gの無水炭酸ナトリウム(MW＝105.99)を蒸留水100mℓに溶解する。〕
C. 32.0mM K-Phosphoenolpyruvate水溶液〔33.0mgのK-phosphoenolpyruvate(MW＝206.1)を蒸留水5.0mℓに溶解する。〕(用時調製)
D. 1.0M $MgSO_4$水溶液〔4.93gの$MgSO_4 \cdot 7H_2O$(MW＝246.48)を蒸留水20mℓに溶解する。〕
E. 1.4mM NADH水溶液〔5.34mgのNADH·Na_2(MW＝763)を蒸留水5.0mℓに溶解する。〕
F. 100U/mℓ Malate dehydrogenase溶液〔リンゴ酸脱水素酵素(東洋紡製　GradeⅡ)を20mM Tris-HCl pH8.0で溶解する。〕(用時調製)

酵素溶液：酵素標品を予め氷冷した20mM K-リン酸緩衝液,pH7.0で溶解し,同緩衝液で0.2～0.7U/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,30℃で約5分間予備加温する。

1.77mℓ	Tris-HCl緩衝液	(A)
0.30mℓ	Na_2CO_3 水溶液	(B)
0.30mℓ	K-Phosphoenolpyruvate水溶液	(C)
0.03mℓ	$MgSO_4$水溶液	(D)
0.30mℓ	NADH水溶液	(E)
0.30mℓ	Malate dehydrogenase溶液	(F)

②酵素溶液0.1mℓを添加し,ゆるやかに混和後,水を対照に30℃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(ΔODtest)。
③盲検は,酵素溶液の代わりに酵素希釈液を0.1mℓ加え,上記同様に操作を行って1分間当たりの吸光度変化を求める(ΔODblank)。

5.計算式

$$U/m\ell = \frac{\Delta OD/min\ (\Delta OD\ test - \Delta OD\ blank) \times 3.1(m\ell) \times 希釈倍率}{6.22 \times 1.0 \times 0.1(m\ell)}$$

$$= \Delta OD/min \times 4.984 \times 希釈倍率$$

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)