●TOYOBO ENZYMES●

(Diagnostic Reagent Grade)

HEXOKINASE

from Microorganism

ATP:D-Hexose 6-phosphotransferase (EC 2. 7. 1. 1)

D-Hexose + ATP $\xrightarrow{Mg^{++}}$ D-Hexose-6-phosphate + ADP

PREPARATION and SPECIFICATION

Appearance	: White amorphous powder, lyophilized	
Activity	: Grade Ⅲ 150U/mg-solid or more	
Contaminants	: Phosphoglucose isomerase	≤1.0×10^{-1}%
	6-Phosphogluconate dehydrogenase	≤1.0×10^{-2}%
	Glucose-6-phosphate dehydrogenase	≤1.0×10^{-2}%
	Myokinase	≤1.0×10^{-2}%
	Glutathione reductase	≤5.0×10^{-1}%

PROPERTIES

Stability	: Stable at −20℃ for at least one year	(Fig.1)
Molecular weight	: approx. 82,000 (by gel filtration)	
Isoelectric point	: 4.1±0.1	
Michaelis constants	: 2.3×10^{-4}M (D-Glucose), 7.7×10^{-5}M (ATP)	
Inhibitors	: Metal ions, p-chloromercuribenzoate, iodoacetamide, SDS, etc	
Optimum pH	: 8.0−9.0	(Fig.2)
Optimum temperature	: 50℃	(Fig.3)
pH Stability	: pH 4.0−9.0 (25℃, 20hr)	(Fig.4)
Thermal stability	: below 45℃ (pH 7.0, 30min)	(Fig.5)
Substrate specificity	: (Table 1)	
Effect of various chemicals	: (Table 2)	

APPLICATIONS

The enzyme is useful for enzymatic determination of glucose, adenosine-5'-triphosphate (ATP) and creatine phosphokinase when coupled with glucose-6-phosphate dehydrogenase (=G-6-PDH, G6D-311, G6D-321).

ASSAY

Principle:

D-Glucose + ATP $\xrightarrow{\text{hexokinase}}$ D-Glucose-6-phosphate + ADP

D-Glucose-6-phosphate + NAD^+ $\xrightarrow{\text{G-6-PDH}}$ Glucono-δ-lactone-6-phosphate + NADH + H^+

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

Unit definition:

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

Method:

Reagents

A. Tris-HCl buffer, pH 8.0 : 50mM, containing 13.3mM $MgCl_2$

B. Glucose solution : 0.67M in Tris-HCl buffer solution (A) (The solution Should be keep at room temperature at least for 1 hour before use)

C. ATP solution : 16.5mM in Tris-HCl buffer solution (A) (Should be prepared fresh)

D. NAD^+ solution : 6.8mM in Tris-HCl buffer solution (A) (Should be prepared fresh)

E. G-6-PDH solution : 300U/ml (Dilute with Tris-HCl buffer solution (A) and store on ice)

F. Enzyme diluent : Tris-HCl buffer solution (A) contg. 0.1% of bovine serum albumin

Procedure

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

2.30ml	Tris-HCl buffer solution	(A)
0.50ml	Glucose solution	(B)
0.10ml	ATP solution	(C)
0.10ml	NAD^+ solution	(D)
0.01ml	G-6-PDH solution	(E)

Concentration in assay mixture	
Tris-HCl buffer	50 mM
Glucose	0.11 M
ATP	0.53 mM
NAD^+	0.22 mM
$MgCl_2$	13 mM
BSA	3.2 μg/ml
G-6-PDH	ca.1.0 U/ml

2. Add 0.1ml of the enzyme solution* and mix by gentle inversion.
3. Record the increase of optical density at 340nm against water for 4 to 5 minutes in a spectrophotometer thermostated at 30℃ and calculate the ΔOD per minute from the initial portion of the curve (ΔOD test).

   At the same time, measure the blank rate (ΔOD blank) by the same method as the test except the enzyme diluent (F) is added instead of the enzyme solution.

* Dissolve the enzyme preparation on ice-cold enzyme diluent (F) 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 :

$$\text{Volume activity (U/ml)} = \frac{\Delta\text{OD/min (OD test} - \text{OD blank)} \times \text{Vt} \times \text{df}}{6.22 \times 1.0 \times \text{Vs}} = \Delta\text{OD/min} \times 5.0 \times \text{df}$$

Weight activity (U/mg) = (U/ml) × 1/C

Vt : Total volume (3.11ml)

Vs : Sample volume (0.1ml)

1.0 : Light path length (cm)

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

df : Dilution factor

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

Table 1. Substrate specificity of Hexokinase

[Pyruvate kinase-Lactate dehydrogenase system with 0.1M Tris-HCl buffer, pH 7.5]

Substrate(100mM)	Relative activity(%)	Substrate(100mM)	Relative activity(%)
D-Glucose	100	D-Galactose	0
D-Fructose	140	D-Xylose	2
D-Mannose	52	D-Glucosamine	58
2-Deoxy-D-glucose	91		

Table 2. Effect of Various Chemicals on Hexokinase

[The enzyme dissolved in 50mM K-phosphate buffer, pH 6.5 (5U/ml) contg. 0.1% bovine serum albumin was incubated with each chemical at 30℃ for 1hr]

Chemical	Concn.(mM)	Residual activity(%)	Chemical	Concn.(mM)	Residual activity(%)
None	—	100	PCMB	2.0	0
Metal salt			MIA	2.0	80
$AgNO_3$	2.0	0	IAA	2.0	7
$BaCl_2$	2.0	99	EDTA	5.0	103
$CaCl_2$	2.0	98	$(NH_4)_2SO_4$	20.0	104
$CdCl_2$	2.0	85	Borate	20.0	102
$CoCl_2$	2.0	85	o-Phenanthroline	2.0	101
$CuSO_4$	2.0	25	α,α'-Dipyridyl	2.0	102
$FeCl_3$	2.0	28	Urea	2.0	104
$FeSO_4$	2.0	80	Guanidine	2.0	103
$HgCl_2$	2.0	0	Hydroxylamine	2.0	104
$MgCl_2$	2.0	98	Na-cholate	1.0%	102
$MnCl_2$	2.0	100	Triton X-100	1.0%	105
$NiCl_2$	2.0	100	Brij 35	1.0%	0
$Pb(OAc)_2$	2.0	98	SDS	0.1%	25
$Zn(OAc)_2$	2.0	98	Tween 20	0.1%	101
$ZnSO_4$	2.0	99	Span 20	0.1%	106
NaF	20.0	101	DAC	0.1%	101
NaN_3	20.0	102			

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

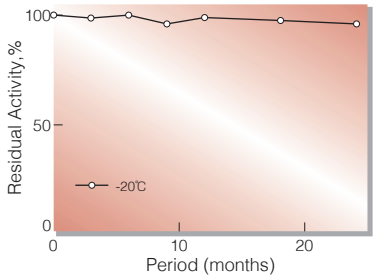

Fig.1. Stability (Powder form)

〔kept under dry conditions〕

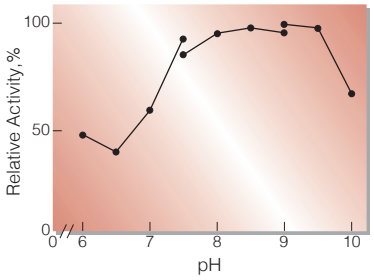

Fig.2. pH-Activity

〔30℃ in the 50mM buffer solution: pH6.2-7.5, PIPES-NaOH: pH7.5-9.0, Tris-HCl: pH9.0-10.0, Glycine-NaOH〕

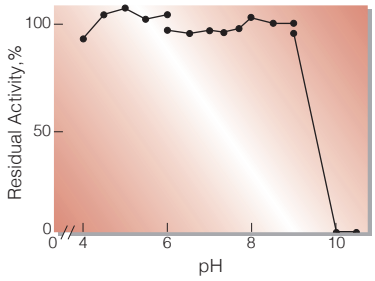

Fig.4. pH-Stability

〔25℃, 20hr-treatment in the 0.1M buffer solution: pH4.0-8.0,Acetate-NaOH;pH6.0-8.0, K-phosphate;pH7.5-9.0,Tris-HCl;pH9.0-10.5, Glycine-NaOH enzyme concn.: ca.10U/ml〕

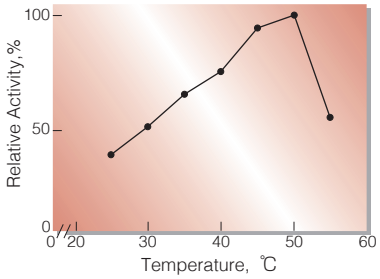

Fig.3. Temperature activity

〔in 50mM Tris-HCl buffer,pH8.0〕

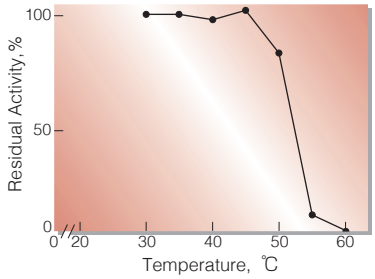

Fig.5. Thermal stability

〔30min-treatment with 50mM K-phosphate buffer, pH7.0, containing 0.1% bovine serum albumin enzyme concn.: ca.5U/ml〕

活性測定法（Japanese）

1.原理

D-Glucose＋ATP $\xrightarrow{\text{hexokinase}}$ D-Glucose-6-phosphate＋ADP

D-Glucose-6-phosphate＋NAD^+ $\xrightarrow{\text{G-6-PDH}}$ Glucono-δ-lactone-6-phosphate＋NADH＋H^+

NADHの生成量を340nmの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NADHを生成する酵素量を1単位(U)とする。

3.試薬

A. 50mM Tris-HCl緩衝液,pH8.0 (13.3mM $MgCl_2$ を含む)
B. 0.67Mグルコース緩衝液(試薬Aで溶解)(溶解後,室温に少くとも1時間保管したものを使用する)
C. 16.5mM ATP溶液(試薬Aで溶解)(用時調製)
D. 6.8mM NAD^+溶液(試薬Aで溶解)(用時調製)
E. G-6-PDH溶液(300U/mℓに試薬Aで希釈し氷冷保存する)

酵素溶液：酵素標品を予め氷冷した0.1%牛血清アルブミン(BSA)を含む試薬Aの緩衝液で溶解し，同溶液で0.1～0.3U/mℓに希釈して氷冷保存する。

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。

2.30mℓ	Tris-HCl緩衝液	(A)
0.50mℓ	グルコース溶液	(B)
0.10mℓ	ATP溶液	(C)
0.10mℓ	NAD^+水溶液	(D)
0.01mℓ	G-6-PDH溶液	(E)

②酵素溶液を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4～5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(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(0.1%BSAを含む試薬A)を0.1mℓ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。(ΔODblank)。

5.計算式

$$U/m\ell = \frac{\Delta OD/min\ (OD\ test - OD\ blank) \times 3.11(m\ell) \times 希釈倍率}{6.22 \times 1.0 \times 0.1(m\ell)}$$

$$= \Delta OD/min \times 5.0 \times 希釈倍率$$

U/mg ＝U/mℓ×1/C

6.22 ：NADHのミリモル分子吸光係数(㎠/micromole)

1.0 ：光路長(cm)

C ：溶解時の酵素濃度(c mg/mℓ)