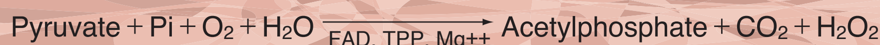
●TOYOBO ENZYMES●

(Diagnostic Reagent Grade)

PYRUVATE OXIDASE

from Microorganism

Pyruvate:oxygen 2-oxidoreductase (phosphorylating)(EC 1.2.3.3)



PREPARATION and SPECIFICATION

Appearance	: Yellowish amorphous powder, lyophilized
Activity	: Grade Ⅲ 1.5U/mg-solid or more
Contaminants	: ATPase ≤5.0×10^{-2}%
	GOT, GPT ≤5.0×10^{-2}%
Stabilizers	: Sugars, FAD

PROPERTIES

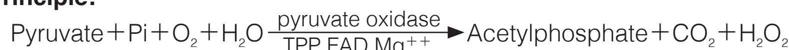
Stability	: Stable at −20℃ for at least one year	(Fig.1)
Molecular weight	: approx. 260,000	
Isoelectric point	: 4.3	
Michaelis constant	: 3.4×10^{-4}M (Pyruvate)	
Inhibitors	: Fe^{++},Zn^{++},Cu^{++},Ag^{+},Hg^{++}	
Optimum pH	: 5.7	(Fig.2)
Optimum temperature	: 65℃	(Fig.3)
pH Stability	: pH 5.7−6.5 (25℃, 20hr)	(Fig.4)
Thermal stability	: below 45℃ (pH 6.0, 15min)	(Fig.5)
Substrate specificity	: (Table 1)	
Effect of various chemicals	: (Table 2)	

APPLICATIONS

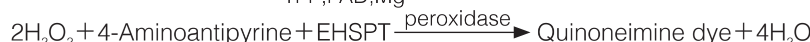
This enzyme is useful for enzymatic determination of pyruvate, GOT, GPT in clinical analysis.

ASSAY

Principle:



$Pyruvate + Pi + O_2 + H_2O \xrightarrow[TPP,FAD,Mg^{++}]{pyruvate\ oxidase} Acetylphosphate + CO_2 + H_2O_2$



$2H_2O_2 + 4\text{-}Aminoantipyrine + EHSPT \xrightarrow{peroxidase} Quinoneimine\ dye + 4H_2O$

The appearance of quinoneimine dye is measured at 550nm by spectrophotometry.

Unit definition:

One unit causes the formation of one micromole of hydrogen peroxide (half a micromole of quinoneimine dye) per minute under the conditions described below.

Method:

Reagents

A. Pyruvate solution	: 0.3M [378mg of Pyruvate・K salt (MW=126.15)/10ml of H_2O]
B. K-phosphate buffer, pH 5.9	: 0.15M
C. 4-Aminoantipyrine solution	: 0.15%(150mg of 4-Aminoantipyrine/100ml of H_2O)
D. EHSPT (TOOS) solution	: 0.3% [300mg of EHSPT (N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine) /100ml of H_2O]
E. TPP solution	: 3mM [13.8mg of TPP (Thiamine pyrophosphate)(MW=460.77)/10ml of H_2O]
F. FAD solution	: 0.15mM [1.3mg of FAD・2Na salt (MW=865.55)/10ml of H_2O]
G. EDTA solution	: 15mM [590mg of EDTA・2Na salt (MW=394.22)/100ml of H_2O]
H. $MgSO_4$ solution	: 0.15M [3.4g of $MgSO_4$・$7H_2O$(246.48)/100ml of H_2O]
I. Peroxidase solution	: 50U/ml [45mg of peroxidase (110purpurogallin units/mg)/100ml of H_2O]
J. Enzyme diluent	: 50mM K-phosphate buffer, pH 5.7

Procedure

1. Prepare the following working solution in a brownish bottle and store on ice.

10ml	K-phosphate buffer, pH 5.9	(B)
2ml	4-Aminoantipyrine solution	(C)
2ml	EHSPT solution	(D)
2ml	TPP solutionn	(E)
2ml	FAD solution	(F)
2ml	EDTA solution	(G)
2ml	$MgSO_4$ solution	(H)
3ml	Peroxidase	(I)

Concentration in assay mixture	
Pyruvate	48 mM
K-phosphate buffer	50 mM
4-Aminoantipyrine	0.48mM
EHSPT	0.58mM
TPP	0.19mM
FAD	0.01mM
EDTA	0.97mM
$MgSO_4$	9.7 mM
Peroxidase	ca.4.8 U/ml

2. Pipette 2.5ml of working solution into a cuvette (d=1.0cm), add 0.5ml of pyruvate solution (A), and equilibrate at 37℃ for about 5minutes.
3. Add 0.1ml of the enzyme solution* and mix by gentle inversion.
4. Record the increase in optical density at 550nm against water for 3 to 4 minutes in a 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) by using the same method as the test except that the enzyme diluent is added instead of the enzyme solution.

* Dissolve the enzyme preparation in ice-cold enzyme diluent (J), dilute to 0.1−0.5U/ml with the same buffer and store on ice.

Calculation

Activity can be calculated by using the following formula :

$$\text{Volume activity (U/ml)} = \frac{\Delta OD/min\ (\Delta OD\ test - \Delta OD\ blank) \times Vt \times df}{36.88 \times 1/2 \times 1.0 \times Vs} = \Delta OD/min \times 1.68 \times df$$

Weight activity (U/mg) = (U/ml)×1/C

Vt	: Total volume (3.10ml)
Vs	: Sample volume (0.10ml)
36.88	: Millimolar extinction coefficient of quinoneimine dye under the assay condition (㎠/micromole)
1/2	: Factor based on the fact that one mole of H_2O_2 produces half a mole of quinoneimine dye.
1.0	: Light path length (cm)
df	: Dilution factor
C	: Enzyme concentration in dissolution (c mg/ml)

REFERENCES

1) L.P.Hager, D.M.Geller and F.Lipman; Fed.Proc.,13, 734 (1954).
2) B.Sedewitz, K.H.Schleifer and F.Gotz; J.Bacteriol,160, 273 (1984).
3) B.Sedewitz, K.H.Schleifer and F.Gotz; J.Bacteriol,160, 462 (1984).

Table 1. Substrate Specificity of Pyruvate oxidase

Substrate(50mM)	Relative activity(%)	Substrate(50mM)	Relative activity(%)
Pyruvate	100	Acetate	0
α-Ketobutyrate	5.8	Acetoacetate	0
α-Ketoglutarate	0	L-Alanine	0
Oxaloacetate	0	L-Aspartate	0
DL-Lactate	0		

Table 2. Effect of Various Chemicals on Pyruvate oxidase
[The enzyme dissolved in 50mM K-phosphate, pH 6.0 (10U/ml) was incubated with each chemical at 25℃ for 1hr]

Chemical	Concn.(mM)	Residual activity(%)
None	—	100
Metal salt	2.0	
$MgCl_2$		96
$CaCl_2$		93
$Ba(OAc)_2$		97
$FeCl_3$		8.4
$CoCl_2$		84
$MnCl_2$		76
$ZnSO_4$		48
$Cd(OAc)_2$		86
$NiCl_2$		119
$CuSO_4$		0.9
$Pb(OAc)_2$		33
$AgNO_3$		0
$HgCl_2$		0
PCMB	1.0	66
MIA	2.0	96

Chemical	Concn.(mM)	Residual activity(%)
NaF	2.0	100
NaN_3	20	94
EDTA	5.0	107
o-Phenanthroline	2.0	97
α,α′-Dipyridyl	1.0	95
Borate	50	102
IAA	2.0	102
NEM	2.0	104
Hydroxylamin	2.0	98
Triton X-100	0.10%	143
Brij 35	0.10%	133
Tween 20	0.10%	146
Span 20	0.10%	121
Na-cholate	0.10%	116
SDS	0.05%	85
DAC	0.05%	53

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

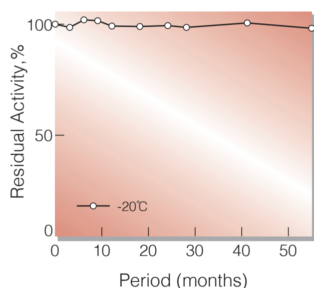


Fig.1. Stability (Power form)
〔kept under dry conditions〕

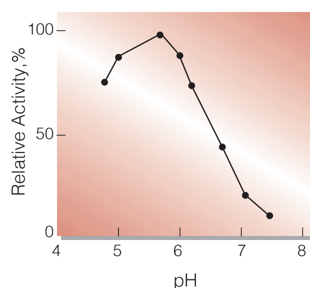


Fig.2. pH-Activity
〔37℃ in 50mM K-phosphate buffer〕

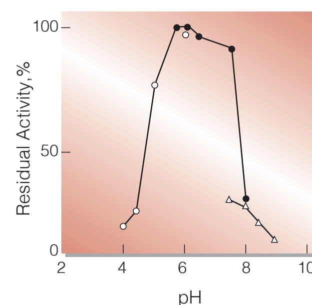


Fig.4. pH-Stability
〔25℃,20hr-treatment with 50mM buffer solution (contg. 10mM $MgSO_4$. 10μM FAD, 0.2mM TPP):pH4.0-6.0, acetate;pH5.7-8.0 K-phosphate; pH7.5-9.0,Tris-HCl〕

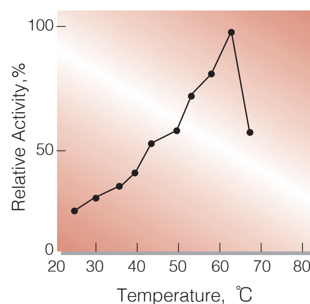


Fig.3. Temperature activity
〔in 50mM K-phosphate buffer, pH5.7〕

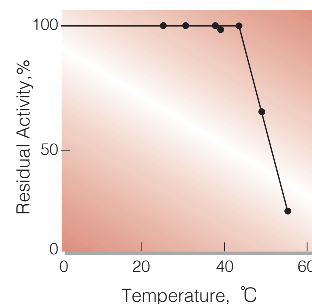


Fig.5. Thermal stability
〔15min-treatment with 50mM K-phosphate buffer(cong. 10mM $MgSO_4$. 10μM FAD, 0.2mM TPP), pH6.0. enzyme concn.:10U/ml〕

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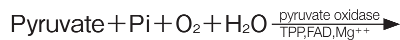
活性測定法（Japanese）

1.原理



Pyruvate＋Pi＋O_2＋H_2O $\xrightarrow[\text{TPP,FAD,Mg}^{++}]{\text{pyruvate oxidase}}$

Acetylphosphate＋CO_2＋H_2O_2

2H_2O_2＋4-Aminoantipyrine＋EHSPT

$\xrightarrow{\text{peroxidase}}$ Quinoneimine dye＋4H_2O

4-AminoantipyrineとEHSPTの酸化縮合生成物であるQuinoneimine色素を550nmで測定し,上記反応で生成したH_2O_2量を定量する。

2.定義

下記条件で1分間に1マイクロモルのH_2O_2を生成する酵素量を1単位(U)とする。

3.試薬

A. 0.3Mピルビン酸水溶液〔378㎎のピルビン酸・K塩(MW＝126.15)を10㎖の蒸留水に溶解する。〕
B. 0.15Mリン酸カリウム緩衝液，pH5.9
C. 0.15%4-AA水溶液(150㎎の4-アミノアンチピリンを100㎖の蒸留水に溶解する。)
D. 0.3%EHSPT(TOOS)水溶液〔300㎎のEHSPTを100㎖の蒸留水に溶解する〕
E. 3.0mM TPP水溶液〔13.8㎎のTPP(MW＝460.77)を10㎖の蒸留水に溶解する。〕
F. 0.15mM FAD水溶液〔1.3㎎のFAD・2Na塩(MW＝865.55)を10㎖の蒸留水に溶解する。〕
G. 15mM EDTA水溶液〔590㎎のEDTA・2Na塩(MW＝394.22)を100㎖の蒸留水に溶解する。〕
H. 0.15M $MgSO_4$水溶液〔3.4gの$MgSO_4$・7H_2O(MW＝246.48)を100㎖の蒸留水に溶解する。〕
I. 50U/㎖ POD水溶液〔45㎎ペルオキシダーゼ(POD)(110プルプロガリン単位/mg)を100㎖の蒸留水に溶解する。〕

酵素溶液：酵素標品を予め氷冷した50mM K-リン酸緩衝液，pH5.7で溶解し,分析直前に同緩衝液で0.1～0.5U/㎖に希釈する。

4.手順

①下記反応混液を調製する(褐色瓶にて氷冷保存)。

10㎖	K-リン酸緩衝液	(B)
2㎖	4-AA水溶液	(C)
2㎖	EHSPT水溶液	(D)
2㎖	TPP水溶液	(E)
2㎖	FAD水溶液	(F)
2㎖	EDTA水溶液	(G)
2㎖	$MgSO_4$水溶液	(H)
3㎖	POD水溶液	(I)

②反応混液2.5㎖をキュベット(d＝1.0cm)に採り,ピルビン酸水溶液(A)0.5㎖を添加し,37℃で約5分間予備加温する。

③酵素溶液0.1㎖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550nm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)。

④盲検は反応混液①に酵素溶液の代わりに酵素希釈液(50mM K-リン酸緩衝液,pH5.7)を0.1㎖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

$$\text{U/m}\ell = \frac{\Delta\text{OD/min }(\Delta\text{OD test}-\Delta\text{OD blank})\times 3.1(\text{m}\ell)\times \text{希釈倍率}}{36.88\times 1/2\times 1.0\times 0.1(\text{m}\ell)}$$

$$= \Delta\text{OD/min}\times 1.68\times \text{希釈倍率}$$

$$\text{U/mg} = \text{U/m}\ell\times 1/\text{C}$$

36.88 ：Quinoneimine色素の上記測定条件下でのミリモル分子吸光係数(㎠/micromole)
1/2 ：酵素反応で生成したH_2O_2の1分子から形成するQuinoneimine色素は1/2分子である事による係数。
1.0 ：光路長(cm)
C ：溶解時の酵素濃度(c mg/㎖)