●TOYOBO ENZYMES●

(Diagnostic Reagent Grade)

PROTOCATECHUATE 3,4-DIOXYGENASE

from Pseudomonas sp.

Protocatechuate:oxygen 3,4-oxidoreductase (decyclizing) (EC 1.13.11.3)

Protocatechuate + O_2 ⟶ β-Carboxymuconate

PREPARATION and SPECIFICATION

Appearance	: Light brown amorphous powder, lyophilized
Activity	: Grade Ⅲ 3.0U/mg-solid or more (containing approx. 40% of stabilizers)
Contaminant	: NADPH oxidase ≤1.0×10^{-1}%
Stabilizers	: Sugars

PROPERTIES

Stability	: Store at −20℃ (A decrease in activity of ca. 20% may occur within one year)	(Fig.1)
Molecular weight	: approx. 700,000	
Michaelis constant	: 1.85×10^{-5}M (Protocatechuate)	
Structure	: Protein with nonheme iron	
Inhibitors	: Ag^{+}, Hg^{++}, PCMB	
Optimum pH	: 9.0	(Fig.3)
Optimum temperature	: 60−65℃	(Fig.4)
pH Stability	: pH 7.0−9.0 (25℃, 72hr)	(Fig.5)
Thermal stability	: below 50℃ (pH 6.0, 1hr)	(Fig.6)
Effect of various chemicals	: (Table 1)	

APPLICATIONS

This enzyme is useful for enzymatic determination of choline esterase when coupled with p-hydroxybenzoate hydroxylase.

ASSAY

Principle:

Protocatechuate + O_2 —(protocatechuate 3,4-dioxygenase)→ β-Carboxymuconate

The disappearance of protocatechuate is measured at 290nm by spectrophotometry.

Unit definition:

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

Method:

Reagents

A. Tris-acetate buffer, pH 7.5 : 50mM [Dissolve 6.1g of Tris (MW=121.14) in ca.800ml of H_2O and, after adjusting pH to 7.5 at 25℃ with 0.2M acetic acid, fill up to 1,000ml with H_2O.]

B. Protocatechuate acid solution : 0.4mM [Dissolve 6.16mg of protocatechuate in ca.80ml of buffer (A) and, after adjusting pH to 7.5 at 25℃ with 1.0N KOH, fill up to 100ml with buffer (A).] (Should be prepared fresh)

Procedure

1. Pipette 3.0ml of protocatechuate solution (B) into a cuvette (d=1.0cm) and equilibrate at 37℃ for about 5 minutes.
2. Add 0.05ml of the enzyme solution* and mix by gentle inversion.
3. Record the decrease in optical density at 290nm 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 (A) is added instead of the enzyme solution.

Concentration in assay mixture	
Tris-acetate buffer	50 mM
Protocatechuate	0.39mM

* Dissolve the enzyme preparation in ice-cold diluent (A) (1.0mg/ml or more) and dilute to 0.2−0.8U/ml with the same buffer, immediately before assay.

Calculation

Activity can be calculated by using the following formula :

$$\text{Volume activity (U/ml)} = \frac{\Delta OD/min\ (\Delta OD\ test - \Delta OD\ blank) \times Vt \times df}{3.8 \times 1.0 \times Vs} = \Delta OD/min \times 16.1 \times df$$

Weight activity (U/mg) = (U/ml) × 1/C

Vt : Total volume (3.05ml)

Vs : Sample volume (0.05ml)

3.8 : Millimolar extinction coefficient of protocatechuate (㎠/micromole)

1.0 : Light path length (cm)

df : Dilution factor

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

REFERENCES

1) H.Fujisawa and O.Hayashi; *J.Biol.Chem., 243,* 2673 (1968)

Table 1. Effect of Various Chemicals on Protocatechuate 3,4-dioxygenase
[The enzyme was incubated with each chemical at 30℃ for 1hr.]

Chemical	Concn.(mM)	Residual activity
None	—	100%
Metal salt	1.0	
$ZnCl_2$		91
$CuSO_4$		90
$AgNO_3$		0
$MgSO_4$		95
$BaCl_2$		100
$MnCl_2$		107
$NiCl_2$		98
$CaCl_2$		109
$HgCl_2$		3.4
$CrCl_2$		100
$CdCl_2$		100
$CoCl_2$		95
$FeSO_4$		88

Chemical	Concn.(mM)	Residual activity
PCMB	1.0	0
NaF	1.0	98
NaN_3	1.0	79
EDTA	5.0	100
Borate	50	90
Tween 20	0.1%	95
Brij 35	0.1%	112
SDS	0.05%	85
Na-cholate	0.1%	93

PCMB, p-Chloromercuribenzoate; EDTA, Ethylenediaminetetraacetate; SDS, Sodium dodecyl sulfate

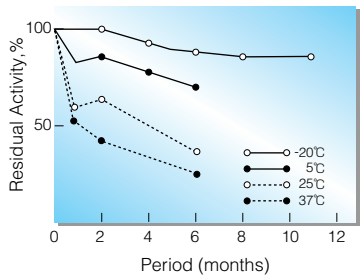

Fig.1. Stability (Powder form)
〔kept under dry conditions〕

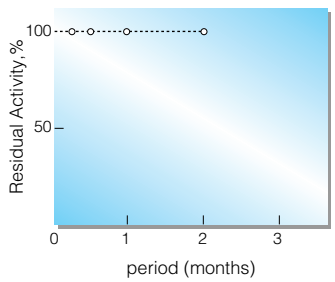

Fig.2. Stability (Liquid form at 25℃)
〔enyzme concentraton:35U/ml
buffer composition:50mM Tris-HCl buffer, pH7.8
contg. 0.1%NaN_3〕

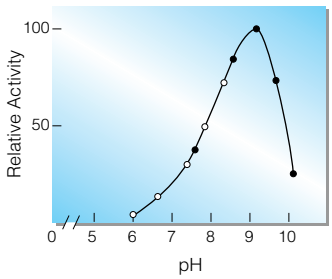

Fig.3. pH-Activity
〔37℃ in 50mM buffer solution:
pH6.0-8.0,Tris-acetate;pH7.5-10.5,
glycine-NaOH〕

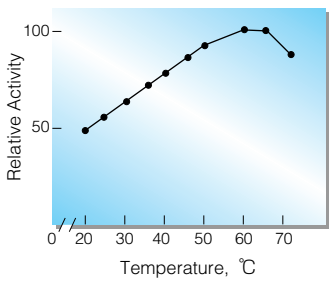

Fig.4. Temperature activity
〔in 50mM K-phosphate buffer .pH7.5〕

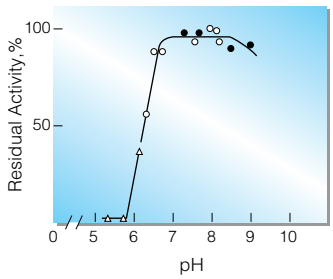

Fig.5. pH-Stability
〔25℃, 72hr-treatment with 50mM
buffer solution:pH3.3-5.5,
acetate:pH5.5-8.0 K-phosphate;
pH6.5-9.0, Tris-acetate〕

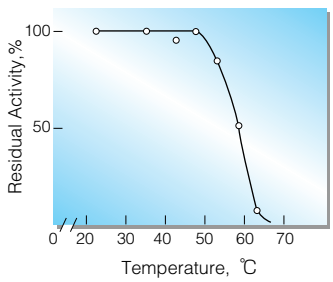

Fig.6. Thermal stability
〔1hr-treatment with 50mM K-phosphate
buffer,pH6.0〕

活性測定法（Japanese）

1.原理

Protocatechuate＋O_2 $\xrightarrow{\text{protocatechuate 3,4-dioxygenase}}$ β-Carboxymuconate

プロトカテキュ酸の消失量を290nm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マイクロモルのプロトカテキュ酸が酸化される酵素量を1単位(U)とする。

3.試薬

A. 50mM Tris-酢酸緩衝液，pH7.5〔6.1gのトリス(MW＝121.14)を約800mℓの蒸留水で溶解し，0.2M酢酸でpH7.5(25℃)に調整後，蒸留水で1,000mℓとする〕

B. 0.4mMプロトカテキュ酸溶液〔6.16mgのプロトカテキュ酸を緩衝液(A)で溶解し，1N KOHでpH7.5(25℃)に調整後，緩衝液(A)で100mℓとする〕(用時調製)

酵素溶液：酵素標品を予め氷冷した緩衝液Aで溶解(1.0mg/mℓ以上)し，分析直前に同緩衝液で0.2～0.8U/mℓに希釈する。

4.手順

①基質溶液(B)3.0mℓをキュベット(d＝1.0cm)に採り，37℃で約5分予備加温する。

②酵素溶液0.05mℓ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290nm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盲検は基質溶液(B)に，酵素溶液の代わりに酵素希釈液(A)を0.05mℓ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(ΔOD blank)。

5.計算式

$$U/m\ell = \frac{\Delta OD/min\ (\Delta OD\ test - \Delta OD\ blank) \times 3.05(m\ell) \times 希釈倍率}{3.8 \times 1.0 \times 0.05(m\ell)}$$

$$= \Delta OD/min \times 16.1 \times 希釈倍率$$

U/mg ＝ U/mℓ×1 / C

3.8 ： プロトカテキュ酸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)