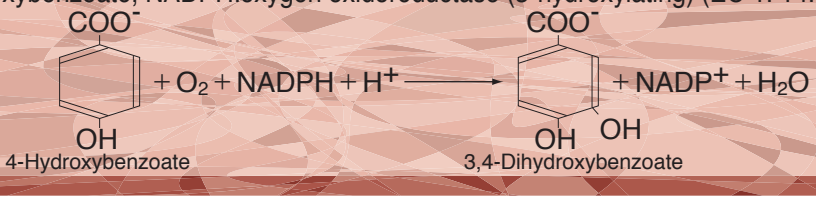
●TOYOBO ENZYMES●

(Diagnostic Reagent Grade)

p-HYDROXYBENZOATE HYDROXYLASE

from Microorganism

4-Hydroxybenzoate, NADPH:oxygen oxidoreductase (3-hydroxylating) (EC 1. 14. 13. 2)



PREPARATION and SPECIFICATION

Appearance	: Yellowish amorphous powder, lyophilized
Activity	: Grade Ⅲ 20U/mg-solid or more (containing approx. 40% of stabilizers)
Contaminant	: NADPH oxidase ≤1.0×10^{-1}%
Stabilizers	: Sugars, FAD

PROPERTIES

Stability	: Stable at −20℃ for at least one year	(Fig.1)
Molecular weight	: 55,000～60,000	
Michaelis constants	: 2.0×10^{-5}M (p-Hydroxybenzoate), 4.0×10^{-5}M (NADPH)	
Structure	: One mol of FAD per mol of enzyme	
Inhibitors	: Ag^{+}, Hg^{++}, PCMB, SDS	
Optimum pH	: 7.7−7.9	(Fig.3)
Optimum temperature	: 35℃	(Fig.4)
pH Stability	: pH 5.0−7.5 (25℃, 72hr)	(Fig.5)
Thermal stability	: below 40℃ (pH 6.0, 15min)	(Fig.6)
Substrate specificity	: (Table 1)	
Effect of various chemicals	: (Table 2)	

APPLICATIONS

This enzyme is useful for enzymatic determination of choline esterase when coupled with protocatechuate 3, 4-dioxygenase (PCO-302).

ASSAY

Principle:

p-Hydroxybenzoate + NADPH + H^+ + O_2 $\xrightarrow{\text{p-hydroxybenzoate hydroxylase}}$ Protocatechuate + $NADP^+$ + H_2O

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

Unit definition:

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

Method:

Reagents

A. Tris-malate buffer, pH 8.2 : 50mM [Dissolve 3.03g of Tris (M.W=121.14) in ca.300ml of H_2O and, after adjusting the pH to 8.2 at 25℃ with 1.0M maleic acid, fill up to 500ml with H_2O.]

B. p-hydroxybenzoate solution : 5.0mM [80mg p-hydroxybenzoate (Na salt)/100ml of buffer solution (A)] (Should be prepared fresh)

C. FAD solution : 0.2mM [19mg FAD · Na_2/100ml or buffer solution (A)] (Should be prepared fresh)

D. NADPH solution : 3.0mM [272mg NADPH · Na_4 · $4H_2O$/100ml of buffer solution (A)] (Should be prepared fresh)

E. Enzyme diluent : 50mM K-phosphate buffer, pH 6.0 containing 0.2% BSA

Procedure

1. Prepare the following working solution (10 tests) in a brownish bottle and store on ice.

21.0ml	Buffer solution	(A)
3.0ml	Substrate solution	(B)
3.0ml	FAD solution	(C)
3.0ml	NADPH solution	(D)

Concentration in assay mixture	
Tris-malate buffer	49 mM
p-Hydroxybenzoate	0.49mM
FAD	20 μM
NADPH	0.30mM

2. Pipette 3.0ml of working solution into a cuvette (d=1.0cm) and equilibrate at 37℃ for about 5 minutes.
3. Add 0.05ml of the enzyme solution* and mix by gentle inversion.
4. Record the decrease in optical density at 340nm against water for 3 to 4 minutes in a spectrophotometer thermostated at 37℃ and calculate the ΔOD per minute from 1.5 to 3 minutes 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 (E) is added instead of enzyme solution.

* Dissolve the enzyme preparation in ice-cold enzyme diluent (E) (1.0mg/ml or more) and dilute to 0.2−0.6 U/ml with the same buffer, immediately before assay.

Calculation

Activity can be calculated by using the following formula :

$$\text{Volume activity (U/ml)} = \frac{\Delta\text{OD/min (}\Delta\text{OD test} - \Delta\text{OD blank)} \times \text{Vt} \times \text{df}}{6.22 \times 1.0 \times \text{Vs}} = \Delta\text{OD/min} \times 9.8 \times \text{df}$$

Weight activity (U/mg) = (U/ml) × 1/C

Vt : Total volume (3.05ml)

Vs : Sample volume (0.05ml)

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

1.0 : Light path length (cm)

df : Dilution factor

C : Enzyme concentration (c mg/ml)

REFERENCES

1) H.Shoun and K.Arima; *Protein, Nucleic acid and Enzyme, 25,* 820, (1980).

2) K.Yano and K.Arima; *Agric.Biol.Chem., 33,* 689 (1969).

3) K.Hosokawa and R.Y.Stanier; *J.Biol.Chem., 241,* 2453 (1966).

Table 1. Substrate Specificity of p-Hydroxybenzoate hydroxylase

Substrate(0.5mM)	Relative activity(%)	Substrate(0.5mM)	Relative activity(%)
p-Hydroxybenzoic acid	100	Protocatechuic acid	3.3
Methyl-p-hydroxybenzoic acid	＜0.05	β-Resorcylic acid	4.5
Ethyl-p-hydroxybenzoic acid	＜0.05	Gentisic acid	＜0.05
n-Propyl-p-hydroxybenzoic acid	＜0.05	p-Chlorobenzoic acid	＜0.05
m-Hydroxybenzoic acid	＜0.05	p-Aminobenzoic acid	0.12
o-Hydroxybenzoic acid			

Table 2. Effect of Various Chemicals on p-Hydroxybenzoate hydroxylase (Residual activity after 1 hr-treatment at 30℃)

Chemical	Concn.(mM)	Residual activity(%)	Chemical	Concn.(mM)	Residual activity(%)
None	—	100	MIA	1.0	91
Metal salt	1.0		PCMB	1.0	3.7
$CoCl_2$		106	NaN_3	1.0	97
$ZnCl_2$		94	NaF	1.0	96
$CuSO_4$		103	o-Phenanthroline	1.0	95
$AgNO_3$		0	α,α′-Dipyridyl	1.0	90
$MgSO_4$		107	EDTA	5.0	96
$BaCl_2$		107	Borate	50	104
$FeCl_3$		106	Tween 20	0.1%	91
$MnCl_2$		90	Brij 35	0.1%	101
$NiCl_2$		104	Span 20	0.1%	94
$CaCl_2$		97	Triton X-100	0.1%	97
$SnCl_2$		102	Na-cholate	0.1%	88
$HgCl_2$		1.1	SDS	0.05%	34
$CrCl_2$		93			
$CdCl_2$		104			
$FeSO_4$		90			

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

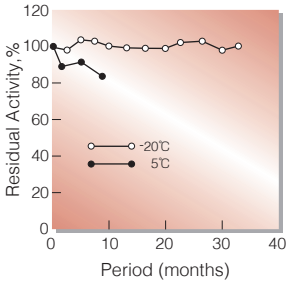


Fig.1. Stability (Powder form)
〔kept under dry conditions〕

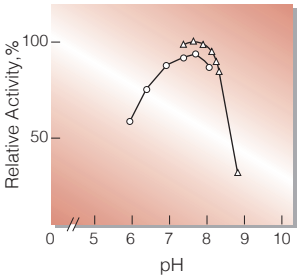


Fig.3. pH-Activity
〔37℃ in 50mM buffer solution: ○——○,K-phosphate; △——△,Tris-malate〕

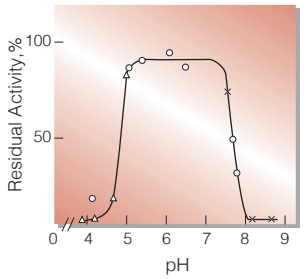


Fig.5. pH-Stability
〔25℃ in 72hr-treatment with 50mM buffer solution:△——△,acetate; ○——○,K-phosphate;×——×,glycine-NaOH〕

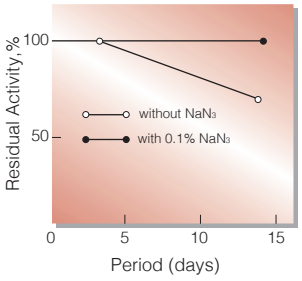


Fig.2. Stability (Liquid form at 25℃)
〔enzyme concentration:500U/ml buffer compostion:50mM K-phosphate buffer,pH6.0〕

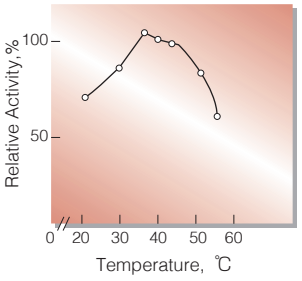


Fig.4. Temperature activity
〔in 50mM Tris-malate buffer, pH8.2〕

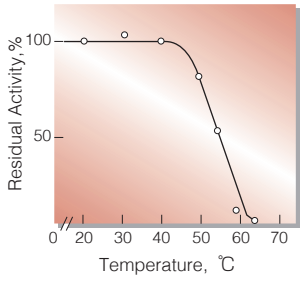


Fig.6. Thermal stability
〔15min-treatment with 50mM K-phosphate buffer,pH6.0〕

活性測定法（Japanese）

1.原理

p-Hydroxybenzoate＋NADPH＋H^+＋O_2
$\xrightarrow{\text{p-hydroxybenzoate hydroxylase}}$ Protocatechuate＋$NADP^+$
＋H_2O
NADPHの消失量を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NADPHが酸化される酵素量を1単位(U)とする。

3.試薬

A. 50mM Tris-malate緩衝液pH8.2〔3.03gのTris(MW＝121.14)を約300mℓの蒸留水で溶解し,1.0M マレイン酸でpHを8.2(25℃)に調整後,蒸留水で500mℓにする〕
B. 5.0mM p-ヒドロキシ安息香酸溶液〔80mgのp-ヒドロキシ安息香酸ナトリウムを100mℓの緩衝液(A)で溶解する〕(用時調製)
C. 0.2mM FAD溶液〔19mgのFAD・Na_2を約100mℓの緩衝液(A)で溶解する〕(用時調製)
D. 3.0mM NADPH溶液〔272mgのNADPH・Na_4-$4H_2O$を約100mℓの緩衝液(A)で溶解する〕(用時調製)

酵素溶液：酵素標品を予め氷冷した0.2% BSAを含む50mM K-リン酸緩衝液pH6.0で溶解(1.0mg/mℓ以上)し，分析直前に同緩衝液で0.2～0.6U/mℓに希釈する。

4.手順

①下記反応混液を調製する(用時調製し,褐色瓶で氷冷保存)。

21.0mℓ	Tris-malate緩衝液	(A)
3.0mℓ	基質溶液	(B)
3.0mℓ	FAD溶液	(C)
3.0mℓ	NADPH溶液	(D)

②反応混液3.0mℓをキュベット(d＝1.0cm)に採り,37℃で約5分間予備加温する。
③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340nmの吸光度変化を1.5～3.0分間記録し,その1.5～3分間の吸光度から1分間当りの吸光度変化を求める(ΔOD test)。
④盲検は反応混液に酵素溶液の代りに酵素希釈液(0.2% BSAを含む50mM K-リン酸緩衝液pH6.0)を0.05mℓ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(ΔOD blank)。

5.計算式

$$U/m\ell = \frac{\Delta OD/min\ (\Delta OD\ test - \Delta OD\ blank) \times 3.05(m\ell) \times 希釈倍率}{6.22 \times 1.0 \times 0.05(m\ell)}$$
$$= \Delta OD/min \times 9.8 \times 希釈倍率$$

U/mg ＝U/mℓ×1/C
6.22 ：NADPH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)