●TOYOBO ENZYMES●
(Diagnostic Reagent Grade)

PEROXIDASE
from Horseradish

Donor:hydrogen-peroxidase oxidoreductase (EC 1.11.1.7)

$$\text{Donor} + H_2O_2 \longrightarrow \text{Oxidized donor} + 2H_2O$$

PREPARATION and SPECIFICATION

Appearance	: Reddish-brown amorphous powder, lyophilized
Activity	: GradeⅦ 60 Purpurogallin U/mg-solid or more (RZ ≥ 0.6)

PROPERTIES

Stability	: Stable at −20℃	
Molecular weight	: approx. 40,000 (by gel filtration)	
Structure	: Glycoprotein with one mole of protohaemin Ⅸ	
Inhibitors	: Cyanide, sulfide, fluoride, azide	
Optimum pH	: 8.0	(Fig.2)
pH Stability	: 5.0−10.0	(Fig.3)
Thermal stability	: below 60℃	(Fig.4)

APPLICATIONS

This enzyme is useful for enzymatic determination of H_2O_2 in clinical analysis.

ASSAY

Principle:

$2\text{Pyrogallol} + 3H_2O_2 \xrightarrow{\text{peroxidase}} \text{Purpurogallin} + 5H_2O + CO_2$

The appearance of Purpurogallin is measured at 420 nm by spectrophotometry.

Unit definition:

One Purpurogallin unit causes the formation of one milligram of purpurogallin in 20 seconds under the conditions described below.

Method:

Reagents

A. Pyrogallol solution : 5% (W/V)(Should be prepared fresh).

B. H_2O_2 solution : 0.147M [Dilute 1.67ml of 30% (W/V) H_2O_2 to 100ml with H_2O] (Should be prepared fresh)

C. Phosphate buffer, pH6.0 : 0.1M

D. H_2SO_4 solution : 2.0N

Procedure

1. Prepare the following reaction mixture in a test tube (32ϕ ×200mm) and equilibrate at 20℃ for about 5 minutes.

14.0ml	H_2O	
2.0ml	Pyrogallol solution	(A)
1.0ml	H_2O_2 solution	(B)
2.0ml	Phosphate buffer, pH6.0	(C)

Concentration in assay mixture	
Phosphate buffer	15 mM
Pyrogallol	40 mM
H_2O_2	7.4mM

2. Add 1.0 ml of the enzyme solution* and mix.
3. After exactly 20 seconds at 20℃, add 1.0 ml of 2.0N H_2SO_4 solution (D) to stop the reaction.
4. Extract the produced purogallin from the above stopped reaction mixture in five times with 15ml portions of ether and fill up the combined ether extracts to 100ml with fresh ether.
5. Measure the optical density at 420nm against water (OD test).
   At the same time, prepare the blank by first mixing the reaction with 1.0ml of 2.0N H_2SO_4 solution (D) after 20 sec-incubation at 20℃, followed by the addition of the enzyme solution and extracting with ether by the same procedure as the test (OD blank).

* Dissolve the enzyme preparation in ice-cold 0.1M phosphate buffer, pH6.0 (C), dilute to 3.0−6.0 purpurogallin U/ml with the same buffer and store on ice.

Calculation

Activity** can be calculated by using the following formula :

$$\text{Volume activity (U/ml)} = \frac{\Delta\text{OD (OD test} - \text{OD blank)} \times \text{Vt} \times \text{df}}{0.117 \times \text{Vs}} = \Delta\text{OD} \times 8.547 \times \text{df}$$

Weight activity (U/mg) = (U/ml)×1/C

Vs : Sample volume (1.0ml)

0.117 : Optical density at 420nm corresponding to 1mg% of Purpurogallin in ether

df : Dilution factor

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

**One purpurogallin unit is equivalent to 13.5 international units determined with o-dianisidine at 25℃.

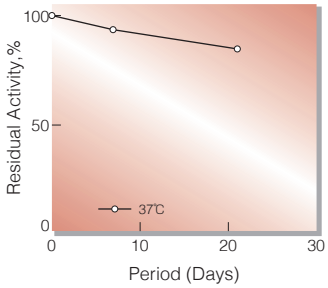

Fig.1. Stability (Powder form)

〔Kept under dry conditions〕

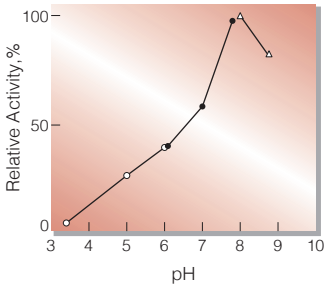

Fig.2. pH-Activity

〔37℃, in 0.1M buffer solution; pH 3.3-6, Acetate; pH 6-7.5, K-Phosphate; pH 7.5-9, Tris-HCl〕

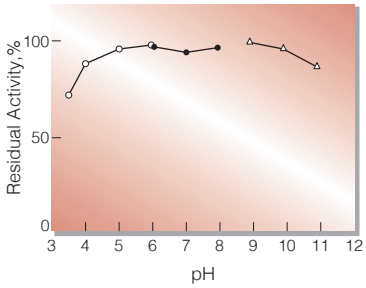

Fig.3. pH-Stability

〔25℃, 20hr-treatment with 0.1M buffer solution; pH 3-6, Acetate; pH 6-8, K-Phosphate; pH 9-11, Glycine-NaOH〕

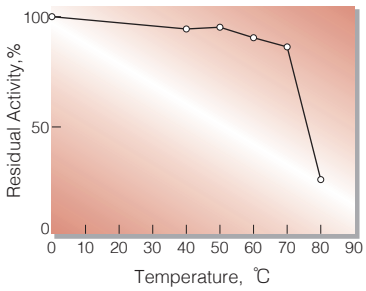

Fig.4. Thermal stability

〔10 min-treatment with 0.05M K-Phosphate buffer, pH6.0 Enzyme concentration : 2 U/ml〕

活性測定法（Japanese）

1.原理

2Pyrogallol＋3H_2O_2 $\xrightarrow{\text{peroxidase}}$ Purpurogallin＋5H_2O＋CO_2

生成するPurpurogallinをエーテル抽出し,420nmの吸光度の変化で測定する。

2.定義

下記条件下で20秒間に1.0㎎のPurpurogallinを生成する酵素量を1Purpurogallin単位(U)とする。

3.試薬

A. 5%(W/V)ピロガロール水溶液(用時調製)
B. 0.147M H_2O_2水溶液〔30%(W/V)H_2O_2溶液1.67㎖を蒸留水で希釈して100㎖とする〕(用時調製)
C. 0.1Mリン酸緩衝液,pH6.0(反応混液及び酵素希釈用)
D. 2.0N H_2SO_4溶液

酵素溶液：酵素標品を予め氷冷した0.1Mリン酸緩衝液，pH6.0で溶解し,同緩衝液で3.0～6.0Purpurogallin U/㎖に希釈して氷冷保存する。

4.手順

①試験管(32φ×200mm)に下記反応混液を調製し,20℃で約5分間予備加温する。

14.0㎖	蒸留水	
2.0㎖	ピロガロール水溶液	(A)
1.0㎖	H_2O_2水溶液	(B)
2.0㎖	リン酸緩衝液	(C)

②酵素溶液1.0㎖を加え,反応を開始する。
③20℃で正確に20秒間反応させた後，H_2SO_4溶液(D)1.0㎖を加えて反応を停止させる。反応停止後の混液から生成したPurpurogallinをエーテル15㎖で抽出する。この操作を5回繰り返し,抽出液を合わせ,更にエーテルを加えて全量を100㎖にする。この液につき420nmにおける吸光度を測定する(OD test)。
④盲検は反応混液①を20℃で20秒間放置後，H_2SO_4溶液(D)を加えて混和し,次いで酵素溶液1.0㎖を加えて調製する。この液につき上記同様にエーテル抽出を行って吸光度を測定する(ODblank)。

5.計算式

$$U/㎖ = \frac{\Delta OD\ (OD\ test - OD\ blank) \times 希釈倍率}{0.117 \times 1(㎖)}$$

$$= \Delta OD \times 8.547 \times 希釈倍率$$

U/mg ＝U/㎖×1/C

0.117 ： 1㎎% Purpurogallinエーテル溶液の420nmにおける吸光度

C ： 溶解時の酵素濃度(c ㎎/㎖)

(注)1Purpurogallin単位は13.5国際単位(o-dianisidineを基質とし, 25℃の反応条件下)に相当する。