## ●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*

PEO-301/302 are isolated from horseradish root in our own method. PEO-131 is chromatographically separated from the other isoenzymes using SP Sephadex C-50 by the modified method of Paul et al.[1] The peroxidase fractions having equal RZ values (ca.3.3) are combined to generate the preparation. This preparation has an RZ value of ca.3.3 and is electrophoretically homogeneous. On the other hand, GradeⅢ is partially purified preparation.

| | |
|---|---|
| Appearance | : Reddish-brown amorphous powder, lyophilized |
| Activity | : Grade Ⅰ 250 Purpurogallin U/mg-solid or more (-131) (RZ ≥ 3.0, salt free)<br>GradeⅢ 110 Purpurogallin U/mg-solid or more (-301) (RZ ≥ 2.0, containing approx. 30% of stabilizers)<br>GradeⅢ 180 Purpurogallin U/mg-solid or more (-302) (RZ ≥ 2.0, salt free) |
| Contaminant | : Phosphatase ≤$1.0\times10^{-3}$% (GradeⅢ) |

### *PROPERTIES*

| | | |
|---|---|---|
| Stability | : Stable at −20℃ for at least one year | (Fig.1,2,3) |
| Molecular weight | : approx. 40,000 | |
| Structure | : Glycoprotein with one mole of protohaeminⅨ [2] | |
| Inhibitors | : Cyanide, sulfide, fluoride, azide [3] | |
| Optimum pH | : 6.0−7.0 | (Fig.6) |
| Optimum temperature | : 45℃ | (Fig.7) |
| pH Stability | : pH 5.0−10.0 (25℃, 20hr) | (Fig.8) |
| Thermal stability | : below 50℃ (pH 6.0, 10min) | (Fig.9) |
| Effect of various chemicals | : (Table 1) | |

### *APPLICATIONS* [4~11]

This enzyme is useful for enzymatic determination of $H_2O_2$ in clinical analysis. Especially, the highly purified preparation (Grade Ⅰ) is useful as a protein tracer in histo-and cyto-chemistry and as a valuable experimental tool in hodological neurography. Also, the enzyme preparation has been used as an enzyme label in enzyme immunoassay. Grade Ⅲ(-302) is suitable for dry chemistry. On the other hand, the enzymes contribute for the reduction of phehol in waste water.

## ASSAY

**Principle:**

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

The appearance of Purpurogallin is measured at 420nm 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 (32$\phi$ ×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 1.0ml of the enzyme solution* and mix.
3. After exactly 20 seconds at 20℃, add 1.0ml of 2.0 N $H_2SO_4$ solution (D) to stop the reaction.
4. Extract the produced purpurogallin 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 mixture with 1.0ml of 2.0 N $H_2SO_4$ solution (D) after 20 a 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, pH 6.0 (C), dilute to 3.0−6.0 pur purogallin U/ml with the same buffer and store on ice.

Calculation

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

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

Weight activity (U/mg) = (U/ml)×1/C

Vs : Sample volume (1.0ml)

0.117 : Optical density at 420 nm 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℃.

## REFERENCES


1) K.G.Paul and T.Stigbrand; *Acta Chem.Scand., 24,* 3607 (1970).
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3) E.Kay et al.; *J.Biol.Chem., 242,* 2470 (1967).
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9) D.R.Colman et al.; *Brain Res., 102,* 156 (1976).
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Table 1. Effect of Various Chemicals on Peroxidase

[The enzyme dissolved in 0.1M phosphate buffer, pH 6.0 (50U/ml) was incubated with each chemical at 25℃ for 1hr.]

| Chemical | Concn.(mM) | ※Residual activity(%) |
|---|---|---|
| None | — | 100 |
| Metal salt | 2.0 | |
| $MgCl_2$ | | 102 |
| $CaCl_2$ | | 102 |
| $Ba(OAc)_2$ | | 105 |
| $FeCl_3$ | | 98 |
| $CoCl_2$ | | 97 |
| $MnCl_2$ | | 97 |
| $ZnCl_2$ | | 99 |
| $CdCl_2$ | | 99 |
| $NiCl_2$ | | 96 |
| $CuSO_4$ | | 98 |
| $Pb(OAc)_2$ | | 96 |
| $AgNO_3$ | | 91 |
| $HgCl_2$ | | 92 |
| 2-Mercaptoethanol | 2.0 | 94 |
| PCMB | 1.0 | 98 |
| MIA | 2.0 | 99 |
| NEM | 2.0 | 97 |
| IAA | 2.0 | 99 |
| Hydroxylamine | 2.0 | 98 |
| EDTA | 5.0 | 95 |
| o-Phenanthroline | 2.0 | 98 |
| $\alpha,\alpha'$-Dipyridyl | 1.0 | 96 |
| Borate | 50 | 98 |
| NaF | 2.0 | 98 |
| $NaN_3$ | 2.0 | 75 |
| Triton X-100 | 0.10% | 98 |
| Brij 35 | 0.10% | 80 |
| Tween 20 | 0.10% | 89 |
| Span 20 | 0.10% | 98 |
| Na-cholate | 0.10% | 97 |
| SDS | 0.05% | 98 |
| DAC | 0.05% | 102 |

※Residual activity was measured by 4AA-DEA method

4AA, 4-Aminoantipyrine; DEA, Diethylaniline

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

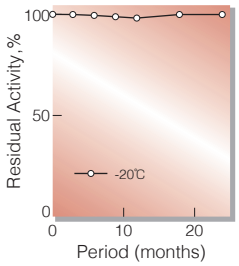


Fig.1. Stability (PEO-131) (Powder form)

〔kept under dry conditions〕

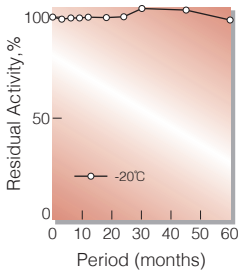


Fig.2. Stability (PEO-301) (Powder form)

〔kept under dry conditions〕

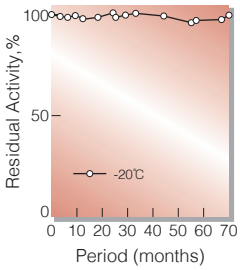


Fig.3. Stability (PEO-302) (Powder form)

〔kept under dry conditions〕

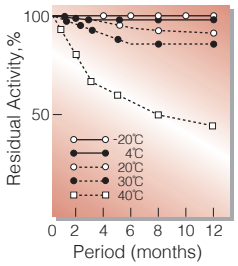


Fig.4. Stability (Powder form)

〔kept under dry conditions〕

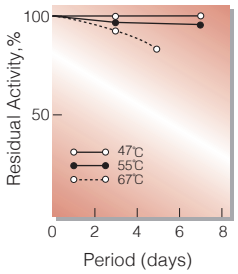


Fig.5. Stability (Powder form)

〔kept under dry conditions〕

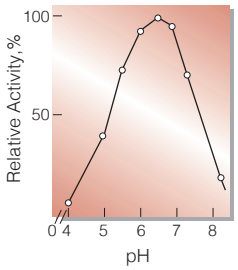


Fig.6. pH-Activity

〔20℃, 20sec-reaction in 0.1M buffer solution: pH4.0-6.0, acetate; pH6.0-8.0, phosphate〕

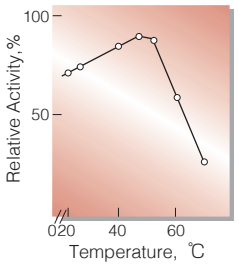


Fig.7. Temperature activity

〔20sec-reaction in 0.1M phosphate buffer, pH6.0〕

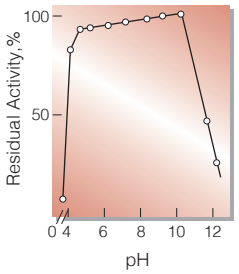


Fig.8. pH-Stability

〔25℃, 20hr-treatment with 50mM buffer solution: pH3.5-6.0, acetate; pH6.0-8.0, phosphate; pH9.0-11.0 borate〕

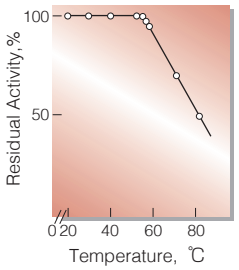


Fig.9. Thermal stability

〔10min-treatment with 50mM phosphate buffer, pH6.0〕

## 活性測定法（Japanese）

### 1.原理

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

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)1.0㎖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。