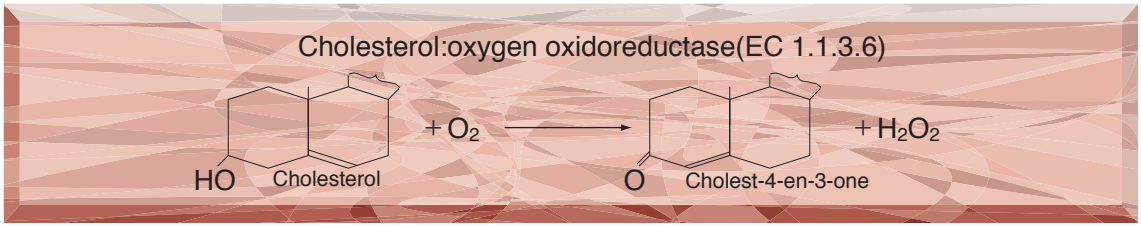
●TOYOBO ENZYMES●

(Diagnostic Reagent Grade)

CHOLESTEROL OXIDASE

from Microorganism



PREPARATION and SPECIFICATION

Appearance	: Yellowish amorphous powder, lyophilized
Activity	: Grade Ⅲ 12U/mg-solid or more
Contaminants	: Catalase ≤1.0×10^{-1}%
	Cholesterol esterase ≤1.0×10^{-2}%
Stabilizers	: BSA, amino acids

PROPERTIES

Stability	: Stable at −20℃ for at least one year	(Fig.1)
Molecular weight	: approx. 55,000 (by gel-filtration)	
Isoelectric point	: 4.6±0.1, 4.9±0.1 and 5.2±0.1	
Michaelis constant	: 2.1×10^{-5}M (Cholesterol)	
Inhibitors	: Ionic detergents, Ag^{+}, Hg^{++}	
Optimum pH	: 7.0−8.0	(Fig.4)
Optimum temperature	: 60℃	(Fig.5)
pH Stability	: pH 5.0−10.0 (25℃, 20hr)	(Fig.6)
Thermal stability	: below 55℃ (pH 7.0, 15min)	(Fig.7)
Substrate specificity	: (Table 1)	
Effect of various chemicals	: (Table 2)	

APPLICATIONS

This enzyme is useful for enzymatic determination of cholesterol in serum when coupled with cholesterol esterase (COE-301, COE-311, COE-313) in clinical analysis.

ASSAY

Principle:

$$\text{Cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{Cholest-4-en-3-one} + H_2O_2$$

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

The appearance of quinoneimine dye formed when coupled with 4-aminoantipyrine and phenol is measured at 500nm 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. 0.1M K-Phosphate buffer, pH 7.0

B. Cholesterol solution : To 5.0ml of Triton X-100 on a hot plate or in a water bath, add 500mg of cholesterol and mix with a stirring bar until cholesterol dissolves. Add 90ml of distilled water to the hot cholesterol-Triton X-100 solution by slowly pouring along a stirring bar. Stir and allow to boil for 30 to 60 seconds. The solution will be cloudy. Cool under running water with gentle agitation, the solution will turn clear. Add 4.0g of sodium cholate and dissolve. Fill up the solution to 100ml with distilled water. This solution is stable for about one week at room temperature. If it becomes cloudy, warm slightly while stirring until it clears.

C. 4-AA solution : 1.76% (1.76g 4-aminoantipyrine/100ml of H_2O)

D. Phenol solution : 6.0% (6.0g phenol/100ml of H_2O)

E. POD solution : Horseradish peroxidase 15,000 purpurogallin units/100ml of buffer (A)

F. Enzyme diluent : 20mM K-Phosphate buffer, pH 7.0 contg.0.2% bovine serum albumin

Procedure

1. Prepare the following working solution (20 tests volume), immediately before use and store on ice in a brownish bottle.

51.0ml	Buffer solution	(A)
4.0ml	Substrate solution	(B)
1.0ml	4-AA solution	(C)
2.0ml	POD solution	(E)

Concentration in assay mixture	
K-Phosphate buffer	87 mM
Cholesterol	0.89mM
4-Aminoantipyrine	1.4 mM
Phenol	21 mM
Triton X-100	0.34 %
Sodium cholate	64 mM
BSA	33 μg/ml
POD	5 U/ml

2. Pipette 2.9ml of working solution into a cuvette (d=1.0cm) and equilibrate at 37℃ for about 3 minutes. Add 0.1ml of Phenol solution (D), mix and keep at 37℃ for another 2 minutes.
3. Add 0.1ml of the enzyme solution* and mix with gentle inversion.
4. Record the increase in optical density at 500nm against water for 3 to 4 minutes in a spectrophotometer thermostated at 37℃, and calculate the ΔOD per minute from the initial 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 (F), and dilute to 0.1−0.3 U/ml with the same buffer, and store on ice.

Calculation

Activity can be calculated by using the following formula :

$$\text{Volume activity (U/ml)} = \frac{\Delta\text{OD/min} \times (\Delta\text{OD test} - \Delta\text{OD blank}) \times Vt \times df}{13.78 \times 1/2 \times 1.0 \times Vs} = \Delta\text{OD/min} \times 4.499 \times df$$

Weight activity (U/mg) = (U/ml)×1/C

Vt : Total volume (3.1ml)

Vs : Sample volume (0.1ml)

13.78 : Millimolar extinction coefficient of quinoneimine dye under the assay conditions (cm²/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) W.Richmond; *Clin.Chem., 19,* 1350 (1973).
2) H.M.Flegg; *Ann.Clin.Biochem., 10,* 79 (1973).
3) C.C Alain et al; *Clin.Chem., 20,* 470 (1974).
4) P.N.Tarbutton and C.R.Gunter; *Clin.Chem., 20,* 724 (1974).
5) S.Nomoto; *Rinsho Kensa, 20,* 688 (1976).
6) K.Kameno et al; *Jap.J.Clin.Path., 24,* 650 (1976).
7) Y.Nishiya et al; *Protein Engng,7,* 231 (1997)

Table 1. Substrate Specificity of Cholesterol oxidase

Substrate(0.1mM)	Relative activity(%)	Substrate(0.1mM)	Relative activity(%)
Cholesterol	100	Ergosterol	37
Pregnenolone	53	Lanosterol	2
β-Cholestanol	63	Testosterone	1
β-Sitosterol	130	Androsterone	2
Stigmasterol	33	Dehydroiso-androsterone	23
5β-Pregnone-3α,20α-diol	0		

Table 2. Effect of Various Chemicals on Cholesterol oxidase

[The enzyme dissolved in 10mM phosphate buffer, pH 7.0 contg. 0.2% BSA (1.0U/ml) was incubated with each chemical at 25℃ for 1hr.]

Chemical	Concn.(mM)	Residual activity(%)	Chemical	Concn.(mM)	Residual activity(%)
None	—	100	NaF	20	98
Metal salt	2.0		NaN_3	20	95
$MgCl_2$		100	EDTA	5.0	97
$CaCl_2$		94	o-Phenanthroline	2.0	100
$Ba(OAc)_2$		100	α,α′-Dipyridyl	1.0	100
$FeCl_3$		83	Borate	50	100
$CoCl_2$		100	IAA	2.0	98
$MnCl_2$		100	NEM	2.0	98
$Zn(OAc)_2$		98	Hydroxylamine	2.0	95
$Cd(OAc)_2$		100	2-Mercaptoethanol	2.0	100
$NiCl_2$		95	Triton X-100	0.10%	100
$CuSO_2$		91	Tween 20	0.10%	98
$Pb(OAc)_2$		100	Span 20	0.10%	89
$AgNO_3$		0	Na-cholate	0.10%	100
$HgCl_2$		0	SDS	0.05%	100
PCMB	2.0	100	DAC	0.05%	100
MIA	2.0	100			

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

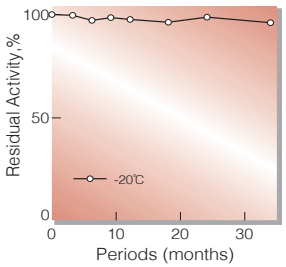


Fig.1. Stability (Powder form)
〔kept under dry conditions〕

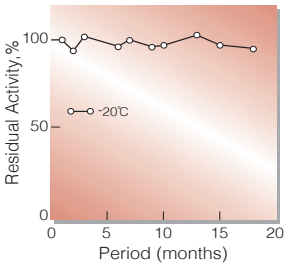


Fig.2. Stability (Powder form)
〔kept under dry conditions〕

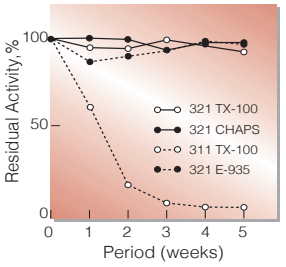


Fig.3. Stability (Liquid form)
〔40℃ in buffer solution, pH7.0〕

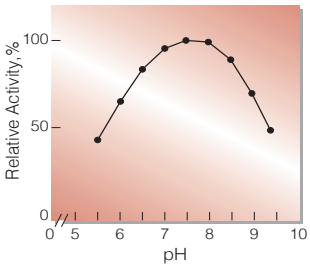


Fig.4. pH-Activity
〔37℃ in 0.1M K-phosphate buffer solution〕

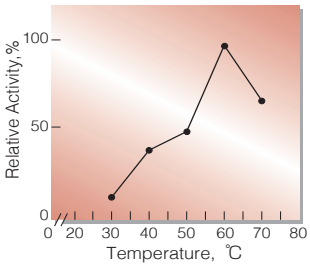


Fig.5. Temperature activity
〔in 0.1M K-phosphate buffer,pH 7.0〕

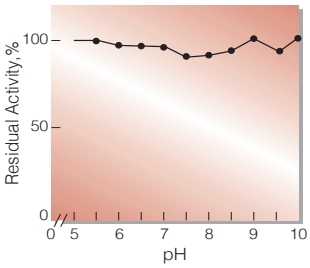


Fig.6. pH-Stability
〔25℃ 20 hr-treatment with 50mM buffer solution:pH5.0-6.0, acetate; pH 6.5-8.5, K-phosphate; pH9-10.0,K_2CO_3-$NaHCO_3$〕

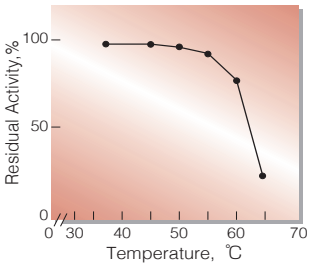


Fig.7. Thermal stability
〔15 min-treatment with 50mM K-phosphate buffer, pH7.0〕

活性測定法（Japanese）

1.原理

Cholesterol＋O_2 $\xrightarrow{\text{cholesterol oxidase}}$ Cholest-4-en-3-one＋H_2O_2
$2H_2O_2$＋4-Aminoantipyrine＋phenol $\xrightarrow{\text{peroxidase}}$
Quinoneimine dye＋$4H_2O$
4-Aminoantipyrineとフェノールの酸化縮合生成物であるQuinoneimine色素を500nm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

下記条件下で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. 0.1M K-リン酸緩衝液,pH7.0
B. コレステロール溶液〔5.0mℓのTriton X-100に500mgのコレステロールを添加し, ヒーター上で攪拌溶解する。これに90mℓの蒸留水を静かに添加し, 攪拌混合後, ヒーター上で30～60秒煮沸する (溶液は濁る)。次いでゆるやかに攪拌しながら流水中で冷却し(溶液は清澄化する), これに4.0gのコール酸ナトリウム塩(ナカライテスク製)を添加して攪拌溶解させた後, 蒸留水で最終液量を100mℓとする〕(溶液は室温で少なくとも1週間は保存可能,もし保存中に濁る場合は,攪拌しながら加温清澄化すれば良い)
C. 4-AA水溶液:1.76% (4-アミノアンチピリン1.76gを水に溶解して100mℓとする)
D. フェノール水溶液:6.0% (フェノール6.0gを水に溶解して100mℓとする)
E. POD溶液:Peroxidase 150mg(100プルプロガリン単位/mg)を100mℓの緩衝液(A)に溶解する。

酵素溶液：酵素標品を予め氷冷した0.2%のBSAを含む20mM K-リン酸緩衝液, pH7.0で溶解し, 同緩衝液で0.1～0.3 U/mℓに希釈する。

4.手順

①下記反応混液を調製する。

51.0mℓ	K-リン酸緩衝液	(A)
4.0mℓ	基質溶液	(B)
1.0mℓ	4-AA水溶液	(C)
2.0mℓ	POD溶液	(E)

(褐色瓶にて氷冷保存)

②反応混液2.9mℓをキュベット(d=1.0cm)にとり,37℃で約3分間予備加温し,0.1mℓのフェノール水溶液を加えて更に2分間加温する。
③酵素溶液0.1mℓを加え, ゆるやかに混和し,水を対照に37℃に制御された分光光度計で500nmの吸光度の増加を3～4分間記録し, その直線部分から1分間あた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20mM K-リン酸緩衝液,pH7.0)を0.1mℓ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(ΔODblank)。

5.計算式

$$U/mℓ = \frac{\Delta OD/min\ (\Delta OD\ test - \Delta OD\ blank) \times 3.1(mℓ) \times 希釈倍率}{13.78 \times 1/2 \times 1.0 \times 0.1(mℓ)}$$

$$= \Delta OD/min \times 4.499 \times 希釈倍率$$

$$U/mg = U/mℓ \times 1/C$$

13.78 : 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 : 酸素反応で生成したH_2O_2の2分子のから形成するQuinoneimine色素は1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/mℓ)