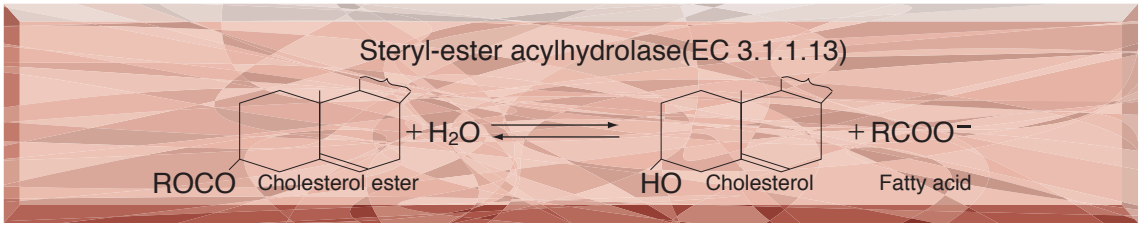
●TOYOBO ENZYMES●

(Diagnostic Reagent Grade)

CHOLESTEROL ESTERASE

from Pseudomonas sp.



PREPARATION and SPECIFICATION

Appearance	: Light brown amorphous powder,lyophilized
Activity	: Grade Ⅲ 100U/mg-solid or more (containing approx. 40% of stabilizers)
Contaminants	: Catalase ≤1.0×10^{-2}%
Stabilizers	: Mg^{++}, Na-cholate, BSA

PROPERTIES

Stability	: Stable at −20℃ for at least one year	(Fig.1)
Molecular weight	: approx. 300,000	
Isoelectric point	: 5.9±0.1	
Michaelis constants	: 5.4×10^{-5}M (Linoleate),6.6×10^{-5}M (Oleate),	
	: 3.7×10^{-5}M (Linolenate),1.5×10^{-4}M (Palmitate)	
	: 1.2×10^{-4}M (Myristate),2.3×10^{-5}M (Stearate)	
Inhibitors	: Hg^{++}, Ag^{+}, ionic detergents	
Optimum pH	: 7.0−9.0	(Fig.4)
Optimum temperature	: 40℃	(Fig.5)
pH Stability	: pH 5.0−9.0 (25℃, 24hr)	(Fig.6)
Thermal stability	: below 55℃ (pH 7.5, 10min)	(Fig.7)
Substrate specificity	: (Table 1)	
Effect of various chemicals	: (Table 2)	

APPLICATIONS

This enzyme is useful for enzymatic determination of total cholesterol when coupled with cholesterol oxidase (COO-311, COO-321, COO-331) in clinical analysis.

ASSAY

Principle:

Cholesterol ester + H_2O $\xrightarrow{\text{cholesterol esterase}}$ Cholesterol + Fatty acid

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

$2H_2O_2$ + 4-Aminoantipyrine + Phenol $\xrightarrow{\text{peroxidase}}$ Quioneimine 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. 0.2M K-Phosphate buffer, pH 7.0

B. Cholesterol linoleate solution : To 39mg of cholesterol linoleate, add 2.0ml of isopropanol and dissolve completely by heating slightly. Mix with about 80ml of 1.0% (v/v) hot Triton X-100 solution (preheated at 72−74℃) to the cholesterol linoleate solution and keep the solution in a hot water bath (72−74℃), stirring for 30 minutes. The solution will turn clear and then cloudy. Cool under running water with gentle agitation until temperature of the solution goes down to room temperature. Add 600mg of Na-cholate and dissove. Fill up the solution to 100ml with 1.0% Triton X-100 solution. This solution is stable at 4℃ for at least 5 days.

C. 4-AA solution : 1.76% (1.76g 4-aminoantipyrine/100ml of H_2O)(Store at 4℃ in a brownish bottle)

D. Phenol solution : 6.0% (6.0g phenol/100ml of H_2O)(Store at 4℃ in a brownish bottle)

E. POD solution : Horseradish peroxidase (Toyobo, GradeⅢ) 7,500 purpurogallin units/50ml of 0.1M K-phosphate buffer, pH 7.0 (150PU/ml)(Prepare freshly)

F. COD solution : Streptomyces sp. cholesterol oxidase (Toyobo, GradeⅢ) 1,500U/5.0ml of ice-cold H_2O (300 U/ml)(Should be prepared fresh)

G. Enzyme diluent : 20mM K-phosphate buffer, pH 7.5 containing 2mM $MgCl_2$, 0.5mM EDTA-Na_3 and 0.2% BSA

Procedure

1. Prepare the following working solution (50 tests) in a brownish bottle.

75 ml	Buffer solution	(A)
50 ml	Substrate solution	(B)
2.5ml	4-AA solution	(C)
5.0ml	Phenol solution	(D)
5.0ml	POD solution	(E)

(This solution is stable at 4℃ for at least 5 days.)

Concentration in assay mixture	
K-Phosphate buffer	0.11 M
Cholesterol linoleate	0.20mM
4-Aminoantipyrine	1.5 mM
Phenol	22 mM
EDTA	17 μM
Isopropanol	0.68 %
COD	ca.10 U/ml
POD	ca. 5.1 U/ml

2. Pipette 2.75ml of working solution into a cuvette (d=1.0cm) and equilibrate at 37℃ for about 5 minutes. Add 0.1ml of COD solution (F), 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 linear portion of the curve (ΔOD test).

   At the same time, measure the blank rate (ΔOD blank) using the same method as the test except that the enzyme diluent (G) is added instead of the enzyme solution.

* Dissolve the enzyme preparation in ice-cold enzyme diluent (G), and dilute to 0.08−0.22U/ml with the same buffer, immediately before assay.

Calculation

Activity can be calculated by using the following formula :

$$\text{Volume activity (U/ml)} = \frac{\Delta\text{OD/min}\ (\Delta\text{OD test} - \Delta\text{OD blank}) \times \text{Vt} \times \text{df}}{13.78 \times 1/2 \times 1.0 \times \text{Vs}} = \Delta\text{OD/min} \times 4.282 \times \text{df}$$

Weight activity (U/mg) = (U/ml) × 1/C

Vt : Total volume (2.95ml)

Vs : Sample volume (0.1ml)

13.78 : Millimolar extinction coefficient of quinoneimine dye under the assay conditions (㎤/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) C.C.Allain, L.S.Poon, C.S.G.Chan, W.Richmond, and P.C.Fu; *Clin.Chem., 20,* 470 (1974).
2) Y.Kameno, N.Nakano, and S.Baba; *Jap.J.Clin.Path., 24,* 650 (1976).

Table 1. Substrate Specificity of Cholesterol esterase
[The reaction was carried out at 37℃ in 0.1M K-phoshate buffer, pH 7.0 contg. 0.2mM cholesterol ester, 0.33% Triton X-100 and 0.2% Na-cholate.]

Cholesterol ester		Relative activity(%)	Cholesterol ester		Relative activity(%)
Linoleate	(18 :2)	100	Tridecanoate	(13 :0)	53.3
Acetate	(2 :0)	2.9	Myristate	(14 :0)	49.3
Propionate	(3 :0)	21.3	Pentadecanote	(15 :0)	46.6
Crotonate	(4 :1)	0.0	Palmitate	(16 :0)	33.5
Valerate	(5 :0)	8.0	Heptadecanoate	(17 :0)	0.0
Caproate	(6 :0)	17.3	Stearate	(18 :0)	8.0
Heptanoate	(7 :0)	18.6	Oleate	(18 :1)	105.3
Caprylate	(8 :0)	58.6	Lelaidate	(18 :3)	166.5
Nonanoate	(9 :0)	48.0	Phenyl acetate		0.0
Decylate	(10 :0)	40.0	Cinnamate		0.0
10-Undecenoate	(11 :1)	114.6	Benzoate		58.6
Laurate	(12 :0)	54.6			

Number of carbon atoms and double bonds are given in parenthesis.

Table 2. Effect of Various Chemicals on Cholesterol esterase
[The enzyme (13.5U/ml) was incubated at 25℃ for 1hr with each chemical.]

Chemical	Concn.(mM)	Residual activity(%)	Chemical	Concn.(mM)	Residual activity(%)
None	—	100	MIA	2.0	96
Metal salt	2.0		NaF	20.0	99
$CaCl_2$		96	NaN_3	20.0	100
$Ba(OAc)_2$		100	EDTA	5.0	100
$FeCl_3$		87	o-Phenanthroline	2.0	100
$CoCl_2$		97	α,α'-Dipyridyl	2.0	99
$MnCl_2$		88	Borate	20.0	100
$Zn(OAc)_2$		83	Triton X-100	1.0%	97
$NiCl_2$		99	Brij 35	1.0%	100
$Pb(OAc)_2$		95	SDS	0.1%	8
$AgNO_3$		51	Na-cholate	1.0%	100
$HgCl_2$		14	Taurocholate	0.1%	100
NEM	2.0	100			
PCMB	2.0	100			

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

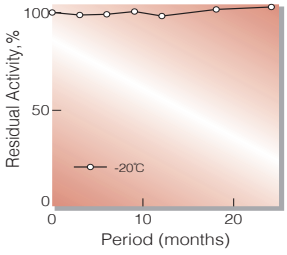


Fig.1. Stability (Powder form)
〔kept under dry conditions〕

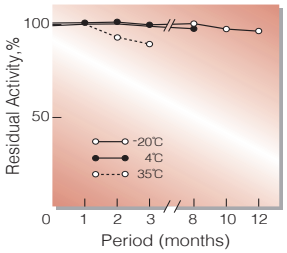


Fig.2. Stability (Powder form)
〔kept under dry conditions〕

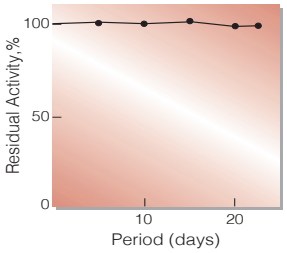


Fig.3. Stability (Liquid form at 5℃)
〔enzyme concentration:673.5U/ml
buffer composition :0.1M K-phosphate buffer,pH7.0〕

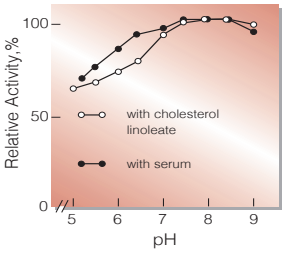


Fig.4. pH-Activity
〔37℃,in 0.2 M K-phosphate buffer〕

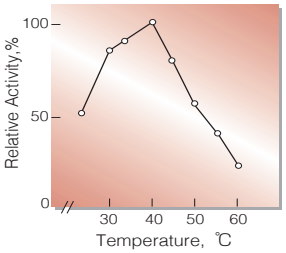


Fig.5. Temperature activity
〔in 0.1M K-phosphate buffer, pH7.0〕

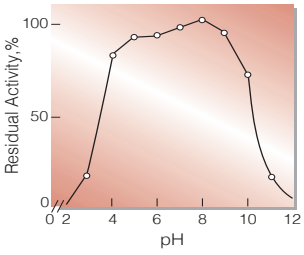


Fig.6. pH-Stability
〔25℃, 24hr-treatment with 0.2M Britton-Robinson buffer〕

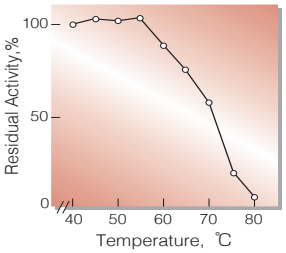


Fig.7. Thermal stability
〔10min-treatment with 20mM K-phosphate buffer,pH7.5 contg. 2mM $MgCl_2$& 0.5mM EDTA・Na_3〕

活性測定法（Japanese）

1.原理

Cholesterol ester＋H_2O $\xrightarrow{\text{cholesterol esterase}}$
Cholesterol＋Fatty acid
Cholesterol $\xrightarrow{\text{cholesterol oxidase}}$ Cholest-4-en-3-one＋H_2O_2
$2H_2O_2$＋4-Aminoantipyrine＋Phenol $\xrightarrow{\text{peroxidase}}$
Quinoneimine dye＋$4H_2O$
4-AA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(加水分解されたCholesterol esterの量)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cholesterol esterを加水分解する酵素量を1単位 (U)とする。

3.試薬

A. 0.2M K-リン酸緩衝液, pH7.0
B. コレステロールリノレート溶液〔39㎎のコレステロールリノレートを精秤し,2.0㎖のイソプロパノールを加え完全に加温溶解する。それを予め72～74℃に加温しておいた約80㎖の1.0%(V/V)トリトンX-100溶液と混和し,更に72～74℃の温湯中で攪拌しながら30分間保ったのち,流水中で室温まで冷却する。次いで600㎎のコール酸ナトリウムを加えて溶解させ,前記1.0%トリトンX-100溶液で最終液量を100㎖とする〕(4℃保存で5日間は使用可能)
C. 1.76%4-AA水溶液〔1.76gの4-アミノアンチピリンを100㎖の蒸留水に溶解し,褐色瓶中で4℃に保存する〕
D. 6.0%フェノール水溶液〔6.0gのフェノールを100㎖の蒸留水に溶解し,褐色瓶中で4℃に保存する〕
E. POD溶液〔西洋ワサビ由来ペルオキシダーゼ(東洋紡製,GradeⅢ)7,500プルプロガリン単位を50㎖の0.1M K-リン酸緩衝液, pH7.0に溶解する(150PU/㎖)〕(用時調製)
F. COD溶液〔ストレプトミセス属由来のコレステロールオキシダーゼ(東洋紡製,GradeⅢ)1,500単位を5.0㎖の氷冷蒸留水に溶解する(300 U/㎖)〕(用時調製)
G. 酵素溶液：酵素標品を予め氷冷した2mM $MgCl_2$ 0.5mM ETDA-Na_3及び0.2% BSAを含む20mM K-リン酸緩衝液, pH7.5で溶解し,分析直前に同緩衝液で0.08～0.22U/㎖に希釈する。

4.手順

①下記反応混液を(50テスト)を褐色瓶中で調製する(4℃保存で5日間は使用可能)。

75 ㎖	K-リン酸緩衝液	(A)
50 ㎖	基質溶液	(B)
2.5㎖	4-AA水溶液	(C)
5.0㎖	フェノール水溶液	(D)
5.0㎖	POD溶液	(E)

②反応混液2.75㎖を試験管に採り, 37℃で約5分間予備加温し,0.1㎖のCOD溶液を加えて更に2分間加温する。
③酵素溶液0.1㎖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500nm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分間あたりの吸光度変化を求める(ΔOD test)。
④盲検はCOD添加液②に酵素溶液の代りに酵素希釈液(2mM $MgCl_2$, 0.5mM EDTA-Na_3及び0.2% BSA)を含む20mMK-リン酸緩衝液, pH7.5を0.1㎖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

$$U/㎖ = \frac{\Delta OD/min\ (\Delta OD\ test - \Delta OD\ blank) \times 2.95(㎖) \times 希釈倍率}{13.78 \times 1/2 \times 1.0 \times 0.1(㎖)}$$
$$= \Delta OD/min \times 4.282 \times 希釈倍率$$

U/mg ＝U/㎖×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 ：H_2O_2の1分子のから形成するQuinoneimine色素は1/2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/㎖)