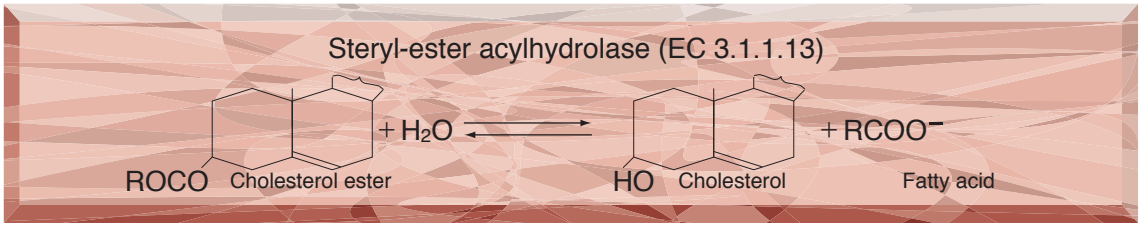
●TOYOBO ENZYMES●
(Diagnostic Reagent Grade)

CHOLESTEROL ESTERASE

from Schizophyllum commune



PREPARATION and SPECIFICATION

Appearance	: Light brown amorphous powder, lyophilized
Activity	: Grade Ⅲ 2.0 U/mg-solid or more (containing approx. 20% of stabilizers)
Stabilizer	: Na-Cholate

PROPERTIES

Stability	: Stable at −20℃ for at least one year	(Fig.1,2)
Molecular weight	: approx. 130,000	
Isoelectric point	: 4.1±0.1	
Michaelis constants	: 3.9×10^{-5}M (Linoleate),9.2×10^{-5}M (Palmitate), : 6.3×10^{-5}M (Decylate),8.8×10^{-5}M (Propionate)	
Inhibitors	: Heavy metal ions (Hg^{++}, Ag^{+}, Fe^{+++})	
Optimum pH	: 4.8−8.0 (Cholesterol linoleate), 5.0 (serum)	(Fig.5)
Optimum temperature	: 55−60℃	(Fig.6)
pH Stability	: pH 2.5−7.5 (25℃, 20hr)	(Fig.7)
Thermal stability	: below 55℃ (pH 5.5, 10min)	(Fig.8)
Substrate specificity	: (Table 1)	

APPLICATIONS

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

ASSAY

Principle:

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

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

3-Methyl-2-benzo-thiazolinone-hydrazone (MBTH)

Dimethylaniline (DMA)

Indamine dye

The appearance of indamine is measured at 590nm by spectrophotometry.

Unit definition:

One unit causes the hydrolysis of one micromole of cholesterol ester per minute under the conditions described below.

Method:

Reagents

A. COD-POD solution : 14.4g $Na_2HPO_4 \cdot 12H_2O$, 31g boric acid, 15,000 U COD (Grade Ⅲ), 25,000 PU POD, 290mg sodium cholate/1,000ml of H_2O (adjusting the pH to 6.5) (stable for a month if stored at 0−5℃)

B. DMA-MBTH solution : 400mg EDTA-Na_2, 0.7ml DMA, 150mg MBTH/1,000ml of 0.5M acetate buffer, pH 4.7 (stable for a week if stored at 0−5℃ in a brownish bottle)

C. Cholesterol linoleate solution : 0.6mM [Dissolve 40mg of cholesterol linoleate in 8.0ml of absolute ethanol on a hot plate, mix 90ml of 1.0% (V/V) of Triton X-100 solution, keep at 70℃ in a hot water bath for 15 minutes and, after cooling down to room temperature in running water, fill up to 100ml with 1.0% (V/V) Triton X-100 (Should be prepared fresh)]

D. HCl solution : 1.0 N

E. Enzyme diluent : 10mM Phosphate buffer, pH 7.0.

Procedure

1. Prepare the following reaction mixture in a test tube and equilibrate at 37℃ for about 5 minutes.

1.0ml	COD-POD solution	(A)
1.0ml	DMA-MBTH solution	(B)
0.1ml	enzyme solution*	

2. Add 1.0ml of the substrate solution (C) and mix.
3. After exactly 10 minutes at 37℃, add 1.0ml of HCl solution (D) to stop the reaction and measure the optical density at 590 nm against water (OD test).

   At the same time, prepare the blank by first mixing the reaction mixture (1.0ml of substrate solution is used instead of the enzyme solution) with 1.0ml of HCl solution (D) after 10 min-incubation at 37℃, followed by the addition of the enzyme of the enzyme solution (OD blank).

* Dissolve the enzyme preparation in ice-cold enzyme diluent and dilute to 0.02～0.05U/ml with the same buffer.

Calculation

Activity can be calculated by using the following formula :

$$\text{Volume activity (U/ml)} = \frac{\Delta\text{OD (OD test} - \text{OD blank)} \times \text{Vt} \times \text{df}}{39.0 \times \text{t} \times \text{Vs}} = \Delta\text{OD} \times 0.105 \times \text{df}$$

Weight activity (U/mg) = (U/ml) × 1/C

Vt : Total volume (4.1ml)

Vs : Sample volume (0.1ml)

39.0 : Millimolar extinction coefficient of indamine dye under the assay conditions (㎠/micromole)

t : Reaction time (10 minutes)

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.Allain 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) Y.Kameno et al.; *Jap.J.Clin.Path., 24,* 650 (1976).

Table 1. Substrate Specificity of Cholesterol esterase

[The reaction was carried out at 37℃ for 10min in 0.1M acetate buffer, pH 5.0, contg. 0.2mM each cholesterol ester and 0.33% Triton X-100.]

Cholesterol ester		Relative activity(%)	Cholesterol ester		Relative activity(%)
Linoleate	(18 :2)	100	Laurate	(12 :0)	108
Acetate	(2 :0)	9	Tridecanoate	(13 :0)	59
Propionate	(3 :0)	40	Myristate	(14 :0)	57
Butyrate	(4 :0)	38	Pentadecanote	(15 :0)	76
Crotonate	(4 :1)	0	Palmitate	(16 :0)	111
Valerate	(5 :0)	18	Heptadecanoate	(17 :0)	99
Caproate	(6 :0)	63	Stearate	(18 :0)	39
Heptanoate	(7 :0)	32	Oleate	(18 :1)	59
Caprylate	(8 :0)	94	Lelaidate	(18 :3)	43
Nonanoate	(9 :0)	120	Linolenate	(18 :3)	91
Decylate	(10 :0)	143	Arachidonate	(20 :4)	3
10-Undecenoate	(11 :1)	141			

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

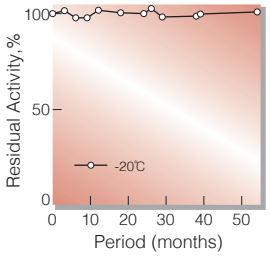


Fig.1. Stability (COE-301) (Powder form)

〔kept under dry conditions〕

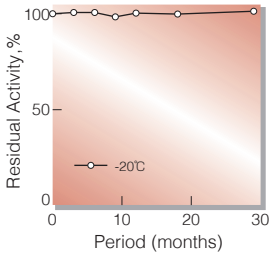


Fig.2. Stability (COE-302) (Powder form)

〔kept under dry conditions〕

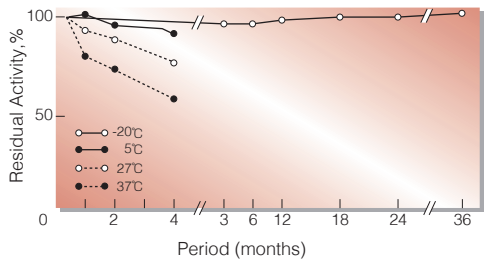


Fig.3.Stability (Powder form)

〔kept under dry conditions〕

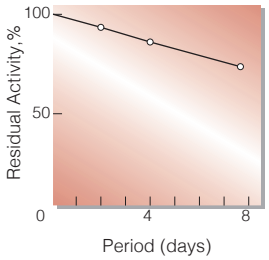


Fig.4. Stability (Liquid form at 40℃)

〔Enzyme concentration: 20mg/ml Buffer composition :0.2M boric acid-borax contg. 0.1% sodium cholate and 0.6% Trinton X-100, pH5.5.〕

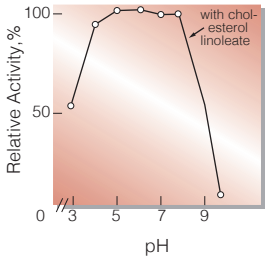


Fig.5.pH-Activity

〔37℃, 10 min-reaction in 0.1M buffer solution: pH3.0-6.0,acetate; pH6.0-8.0,phosphate; pH8.0-10.0,boric acid-KCl-sodium carbonate〕

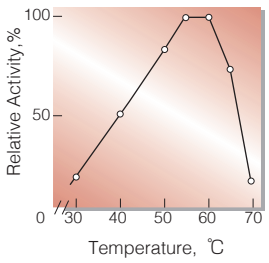


Fig.6. Temperature activity

〔10min-reaction in 0.1M acetate buffer, pH5.5.〕

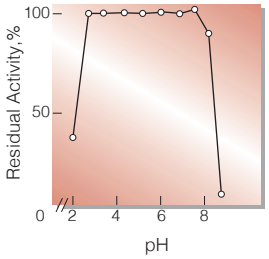


Fig.7. pH-Stability

〔25℃,20hr-treatment with 50mM buffer solution:pH2.0-4.0,citrate; pH4.0-6.0, acetate;pH6.0-8.0, phosphate;pH8.0-9.0,boric acid -KCl-sodium carbonate〕

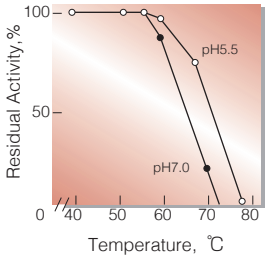


Fig.8. Thermal stability

〔10min-treatment with 50mM acetate buffer,pH5.5 and 50mM phosphate buffer, pH7.0〕

活性測定法（Japanese）

1.原理

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

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

H_2O_2＋3-Methyl-2-benzothiazolinonehydrazone (MBTH)＋Dimethylaniline (DMA) $\xrightarrow{\text{peroxidase (POD)}}$ Indamine dye＋H_2O

MBTHとDMAの酸化縮合生成物であるIndamine色素を590nm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活性を1単位 (U)とする。

3.試薬

A. COD-POD溶液〔$Na_2HPO_4 \cdot 12H_2O$ 14.4g,ホウ酸31g,cholesterol oxidase (GradeⅢ)15,000単位, POD 25,000プリプロガリン単位及びsodium cholate 290mgを1,000mℓの蒸留水に溶解し,pH6.5に調整する。(0～5℃保存で1ヶ月使用可能)〕

B. DMA-MBTH溶液 〔EDTA-Na_2 400mg, DMA 0.7mℓ及びMBTH 150mgを1,000mℓの0.5M酢酸緩衝液, pH4.7に溶解する。(0～5℃遮光保存で1週間使用可能)〕

C. 0.6mMコレステロールリノレート溶液〔40mgのコレステロールリノレートを精秤し,8.0mℓの無水エタノールを加え,ヒーター上で加温溶解する。それを90mℓの1%(V/V) トリトン X-100と混和して, ウォーターバス中で攪拌しながら70℃まで加温する。更に該温度で15分間保温した後, 室温まで流水冷却する。次いで前記1%トリトンX-100で最終液量を100mℓとする。(用時調製)〕

D. 1.0N HCl溶液

酵素溶液：酵素標品を予め氷冷した10mMリン酸緩衝液, pH7.0で溶解し,同緩衝液で0.02～0.05U/mℓに希釈する。

4.手順

①試験管に次の反応混液を調製し37℃で約5分間予備加温する。

1.0mℓ	COD-POD溶液	(A)
1.0mℓ	DMA-MBTH溶液	(B)
0.1mℓ	酵素溶液	

②基質溶液(C)1.0mℓを加え反応を開始する。

③正確に37℃で10分間反応させた後, 1.0N HCl (D) 1.0mℓを加えて反応を停止させる。この液につき590nmにおける吸光度を測定する(OD test)。

④盲検は,反応混液①(但し酵素溶液の代わりに1.0mℓの基質溶液(C)を加えて調整)を37℃で10分間放置後, 1.0N HCl (D) 1.0mℓを加え, 次いで酵素溶液0.1mℓを加えて調整し,同様に吸光度を測定する(OD blank)。

5.計算式

$$U/mℓ = \frac{\Delta OD/min\ (OD\ test - OD\ blank) \times 4.1(mℓ) \times 希釈倍率}{39.0 \times 10(分) \times 0.1(mℓ)}$$

$$= \Delta OD \times 0.105 \times 希釈倍率$$

U/mg ＝U/mℓ×1/C

39.0 ：Indamine色素の上記測定条件下でのミリモル分子吸光係数 (㎠/micromole)

C ：溶解時の酵素濃度(c mg/mℓ)