(Diagnostic Reagent Grade) ASAHI KASEI ENZYMES T-250

CHOLESTEROL ESTERASE [CEN Ⅱ]

from Microorganism
(Steryl-ester acylhydrolase, EC 3.1.1.13)
(Sterol esterase)

Cholesterol ester + H_2O ⟶ Cholesterol + Fatty acid

Preparation and Specification

Appearance : White to pale brownish amorphous powder, lyophilized
Specific activity : More than 100 U/mg solid

Properties

Substrate specificity	: See Table 1	
Molecular weight	: 30kDa (SDS-PAGE)	
Isoelectric point	: pH 5.28 (estimated from amino acid sequence)	
Optimum pH	: 6.5	Figure 1
pH stability	: 6.0–11.0 (37℃, 60 min, in 0.1% BSA)	Figure 2
Optimum temperature	: 35-40℃ (Phosphate Buffer)	Figure 3
Thermal stability	: Stable at 45℃ and below (pH 8.0, 30min)	Figure 4
Storage stability	: At least one year at –20℃	Figure 6
Effect of coexisting ions	: See Table 2	
Effect of detergents	: See Table 3	
Activator	: Adekatol TN-100, Adekatol PC-8, Adelatol SO-120, Adekatol SO-135, Triton X-100	

Applications for Diagnostic Test

This enzyme is useful for enzymatic determination of **total cholesterol**, **HDL-C**, and **LDL-C** coupled with cholesterol oxidase (T-101).

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{CENⅡ}} \text{Cholesterol} + \text{FFA}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{CONⅡ-FD}} \text{Cholestenone} + H_2O_2$$

$$2\,H_2O_2 + \text{4-AA} + \text{Phenol} \xrightarrow{\text{POD}} \text{Quinoneimine dye} + 4\,H_2O$$

FFA: Free fatty acid

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Table 1. Substrate specificity

Substrate			Relative activity (%)	
			CEN Ⅱ	CEN(T-18)
Cholesterol	Acetate	C 2:0	22.6	21.1
Cholesterol	Propionate	C 3:0	27.7	28.1
Cholesterol	Butyrate	C 4:0	78.3	78.9
Cholesterol	Palmitate	C 16:0	37.9	42.2
Cholesterol	Stearate	C 18:0	13.2	13.0
Cholesterol	Oleate	C 18:1	100.0	100.0
Cholesterol	Linolate	C 18:2	80.9	78.1

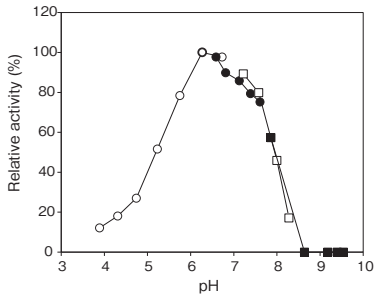
Table 2. Effect of various chemicals on CEN Ⅱ activity

Additives	Concentration	Relative activity (%)
None	-	100
NiCl	1mM	67
$MnCl_2$	1mM	101
$(NH_4)_2SO_4$	1mM	107
$MgCl_2$	1mM	106
ZnCl	1mM	87
$ZnSO_4$	1mM	81
$Ba(CH_3COO)_2$	1mM	106
$CaCl_2$	1mM	114
$MoSO_4$	1mM	69
$CuSO_4$	0.5mM	3
$CuCl_2$	0.5mM	0
$FeCl_3$	1mM	102
$CoCl_2$	1mM	86
Li_2CO_3	1mM	100
CH_3COOT	1mM	104
EDTA	1mM	101
KCl	0.1M	97
NaCl	0.1M	98
NaN_3	0.05%	102
NaF	20mM	98

Table 3. Effect of detergents on CEN Ⅱ activity

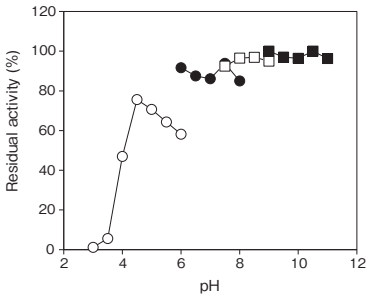
Detergent (0.3%)	Relative activity (%)
TritonX-100	100
Adekatol SO-120	61
Adekatol SO-135	69
Adekatol TN-100	82
Adekatol PC-8	81
Tween 20	1
Tween 40	1
Tween 80	1
Sodium Cholate	0
Brij 35	2
CHAPSO	0
Benzyltriethylammonium Chloride	1
SDS	0
Emulgen LS-110	30
Newcol 710	13
None	0

Fig.1 pH Optimum pH



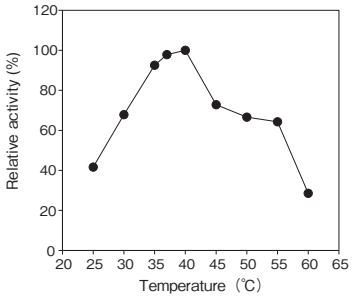
○ : Citrate buffer
● : Phosphate buffer
□ : Tris-HCl buffer
■ : Glycine-NaOH buffer

Fig.2 pH Stability



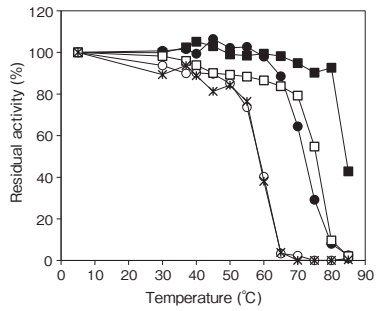
37℃, 60min. in 0.1% BSA
○ : Citrate buffer
● : Phosphate buffer
□ : Tris-HCl buffer
■ : Glycine-NaOH buffer

Fig.3 Optimum temperature



pH6.8
40mM Phosphate buffer

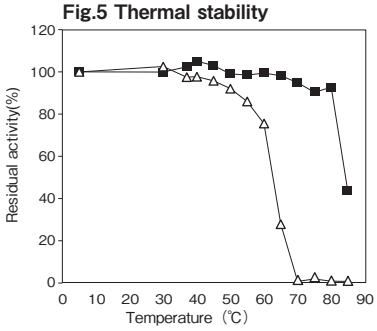
Fig.4 Thermal stability



Tris-HCl buffer pH8.0 30min
CEN II
○ : None
● : + 0.1% Newcol 710
□ : + 1mM $CaCl_2$
■ : + 0.1% Newcol 710 + 1mM $CaCl_2$

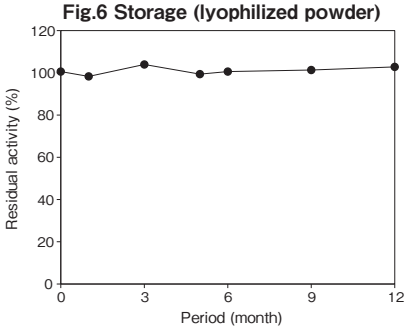
CEN(T-18)
＊ : None

Fig.5 Thermal stability



Tris-HCl buffer pH8.0
+0.1% Newcol 710
+1mM $CaCl_2$ 30min
■ : CEN II
△ : CE from Pseudomonas sp.

Fig.6 Storage (lyophilized powder)



−20℃

Assay

■ Principle

The assay is based on the increase in absorbance at 493 nm as the formation of quinoneimine dye proceeds in the following reactions:

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{CEN Ⅱ}} \text{Cholesterol} + \text{Fatty acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{CO}} \triangle^4\text{-Cholesten-3-one} + H_2O_2$$

$$2\,H_2O_2 + 4\text{-AA} + \text{Phenol} \xrightarrow{\text{POD}} \text{Quinoneimine dye} + 4\,H_2O$$

CO: Cholesterol oxidase

■ Unit definition

One unit is defined as the amount of enzyme which liberates 1 μmole of cholesterol per minute at 37℃ under the conditions specified in the assay procedure.

■ Reagents

1. Reaction mixture

0.2M KH_2PO_4–NaOH buffer pH 6.8	0.60 ml
0.35% (W/V) 4-AA solution	0.30 ml
0.2% (W/V) Phenol solution	0.30 ml
100 U/ml POD solution [1)]	0.30 ml
3% (W/V) Triton X-100 solution	0.30 ml
0.2 U/ml CONⅡ solution [2)]	0.60 ml
Substrate solution [3)]	0.30 ml
Distilled water	0.30 ml

1) : 100 U/ml POD solution
Dissolve 1000 U (PPU) of POD with 10 ml of distilled water.

2) : 0.2 U/ml CONⅡ solution
Dissolve 2 U of CONⅡ with CONⅡ dilution buffer [※)]

※) : CONⅡ dilution buffer
0.1 M KH_2PO_4–Na_2HPO_4 buffer pH 7.0
containing 0.05% (W/V) Triton X-100.

3) : Substrate solution
Calf serum

2. Enzyme dilution buffer
10 mM KH_2PO_4–NaOH buffer pH 7.5 containing 0.1% (W/V) bovine serum albumin (BSA).

3. Reagents
Triton X-100: The Dow Chemical Company
CONⅡ : Asahi Kasei Pharma Corporation #T-84
Calf serum: GIBCO Co. (USA)
BSA: Millipore Fraction V pH 5.2 #81-053
4-AA: NACALAI TESQUE, INC. Special grade #01907-52
POD: Sigma Chemical Co. Type Ⅱ #P-8250

■ Procedure

1. Pipette accurately 3.0 ml of reaction mixture into a small test tube and preincubate it at 37℃.
2. After 10 min, add 50 μl of enzyme solution and mix to start the reaction at 37℃.
   ※ In the case of a test blank, add 50 μl of enzyme dilution buffer in place of enzyme solution.
3. After starting the reaction, measure the rate of increase per minute in absorbance at 493 nm. The rate must be measured within the linear portion of the absorbance curve.

Absorbance sample : As/min
blank : Ab/min

$$\triangle A/\text{min} = (As/\text{min} - Ab/\text{min}) \leqq 0.050\ \text{Abs/min}$$

■ Calculation

$$\text{Activity (U/mg of powder)} = \frac{\triangle A/\text{min}}{12.0 \times 1/2} \times \frac{3.05}{0.05} \times \frac{1}{X}$$

■ Enzyme solution

Accurately weigh about 20 mg of the sample and add enzyme dilution buffer to make a total of 20 ml. Dilute it with enzyme dilution buffer to adjust the concentration to within 0.3–0.5 U/ml.

12.0 : millimolar extinction coefficient of quinoneimine dye at 493 nm (cm^2/μmole)
1/2 : a multiplier derived from the fact that 2 mole of H_2O_2 produce 1 mole of quinoneimine dye
3.05 : final volume (ml)
0.05 : volume of enzyme solution (ml)
X : concentration of the sample in enzyme solution (mg/ml)

Storage

Storage at −20℃ in the presence of a desiccant is recommended. Enzyme activity will be retained for at least one year under this condition (Figure 5).

References

1. Bradford, M. B., (1976) Anal. Biochem., **72**, 248–254.
2. Allain, C. C., Poon, L. S., Chan, C. S. G., Richmond, W. and Fu, P.C. (1974) Clin. Chem., **20**, 470–475.
3. Kameno, Y., Nakano, N. and Baba, S. (1976) Japanese Journal of Clinical Pathology, **24**, 650.

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CENⅡ 活性測定法（Japanese）

Ⅰ．試薬液

1. 反応試薬混合液

0.2M KH_2PO_4–NaOH 緩衝液　pH6.8	0.60 ml
0.35%（W/V）4–AA 溶液	0.30 ml
0.2%（W/V）フェノール溶液	0.30 ml
100U/ml POD 溶液 [1)]	0.30 ml
3%（W/V）トリトン X–100 溶液	0.30 ml
0.2U/ml CONⅡ溶液 [2)]	0.60 ml
基質溶液 [3)]	0.30 ml
精製水	0.30 ml

1）: 100U/ml POD 溶液
　POD 1,000 単位（PPU）を精製水 10ml で溶解する。
2）: 0.2U/ml CONⅡ溶液
　CONⅡ 2 単位（U）を CONⅡ溶解用液※) 10ml で溶解する。
　※）: CONⅡ溶解用液
　　0.05%（W/V）トリトン X–100 を含む 0.1M KH_2PO_4–Na_2HPO_4 緩衝液 pH7.0
3）: 基質溶液
　仔牛血清液

2. 酵素溶解希釈用液
　0.1%（W/V）BSA を含む 10mM KH_2PO_4–NaOH 緩衝液 pH7.5

3. 試薬
　トリトン X–100：Dow Chemical 製
　CONⅡ（コレステロール酸化酵素）：
　　旭化成ファーマ製 #T–84
　仔牛血清液（Calf serum）：GIBCO（USA）製
　BSA: Millipore 製　Fraction V pH5.2 #81–053
　4–AA：ナカライテスク製　特級　#01907-52
　POD：シグマ製　Type Ⅱ　#P–8250

Ⅱ．酵素試料液

検品約 20mg を精密に量り、酵素溶解希釈用液に溶解して全容 20ml とする。
その液を酵素溶解希釈用液で 0.3～0.5U/ml 濃度となるように適宜希釈する。

Ⅲ．測定操作法

1. 小試験管に反応試薬混合液を 3.0ml 正確に分注して 37℃で予備加温する。
2. 10 分経過後、酵素試料液 50 μl を正確に加えて混和し、37℃で反応を開始する。
　※盲検は酵素試料液の代わりに酵素溶解希釈用液 50 μl を加える。
3. 反応開始後、493nm における吸光度を測定して直線的に反応している 1 分間当たりの吸光度変化を求める。
　求められた吸光度変化を試料液は As/min、盲検液は Ab/min とする。
　$\Delta A/min = (As/min - Ab/min) \leqq 0.050$ Abs/min

Ⅳ．計算

$$活性\ (U/mg) = \frac{\Delta A/min}{12.0 \times 1/2} \times \frac{3.05}{0.05} \times \frac{1}{X}$$

12.0：キノンイミン色素の 493nm におけるミリモル分子吸光係数（$cm^2/\ \mu mole$）
1/2：H_2O_2 2 モルからキノンイミン色素 1 モルが生成することによる係数
3.05：反応総液量（ml）
0.05：反応に供した酵素試料液量（ml）
X　：酵素試料液中の検品濃度（mg/ml）