(Diagnostic Reagent Grade) ASAHI KASEI ENZYMES T-243

CHOLESTEROL ESTERASE [CEBP Ⅱ]

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

Cholesterol Ester + H_2O ⟶ Cholesterol + Fatty Acid

★ Advantage
High liquid stability

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Preparation and Specification

Appearance : White to off white lyophilized powder
Specific activity : More than 10.0 U/mg solid

Properties

Substrate specificity	: See Table 1	
Molecular weight	: 63 kDa (SDS-PAGE)	
Isoelectric point	: 5.1 (estimated from amino acid sequence)	
Optimum pH	: 5.6	Figure 1
pH stability	: 4.0-8.0 (37℃, 60 min)	Figure 2
Optimum temperature	: 45℃	Figure 3
Thermal stability	: Stable at 45℃ and below (pH6.0, 30 min)	Figure 4
Liquid stability	: See Figure 5	
Adsorption on glass	: See Figure 6	
Effect of coexisting ions	: See Table 2	
Effect of detergents	: See Table 3	
Light stability	: See Table 4	

Applications for Diagnostic Test

This enzyme is useful for enzymatic determination of **total cholesterol**, **HDL-C**, and **LDL-C** coupled with cholestreol oxidase (T-84 and T-101).
This enzyme is suitable for assembling in liquid reagens.

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{CEBP Ⅱ}} \text{Cholesterol} + \text{FFA}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{CON Ⅱ}} \Delta^4\text{-Cholesten-3-one} + H_2O_2$$

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

FFA: Free fatty acid

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Table 1 Substrate specificity

Substrate (0.95mM)			Relative activity (%)
Cholesterol	Acetate	C 2:0	0.4
Cholesterol	Propionate	C 3:0	9.3
Cholesterol	Butyrate	C 4:0	27.8
Cholesterol	Palmitate	C16:0	19.8
Cholesterol	Stearate	C18:0	2.0
Cholesterol	Oleate	C18:1	100.0
Cholesterol	Linoleate	C18:2	80.6

Table 2 Effect of coexisting ions on CEBP Ⅱ activity

Coexisting ion (20 mM)	Relative activity (%)
None	100
NaCl	101
KCl	100
NH_4Cl	100
$CaCl_2$	101
$MgCl_2$	101
$MnCl_2$	103
$ZnCl_2$	105
EDTA	102
NaN_3	100
NaF	98

All samples contain 0.1% BSA

Table 3 Effect of detergents on CEBP II activity

Detergent (0.1%)	Relative activity (%)
None	100
Triton X-100	115
Adekatol TN-100	117
Adekatol SO-120	142
Newcol-707	115
Newcol-710	113
Emulgen 120	141
Emulgen 705	135
Deoxycholic acid	129
Tauroursodeoxycholic acid	134
Sodium dodecyl sulfate	127

Table 4 Light stability

Form of CEBP II	Light source	Residual activity (%)
Powder	LED	96.5
	Fluorescent	98.7
Solution	LED	94.1
(1mg/ml, water)	Fluorescent	98.4

After 24 hours of incubation

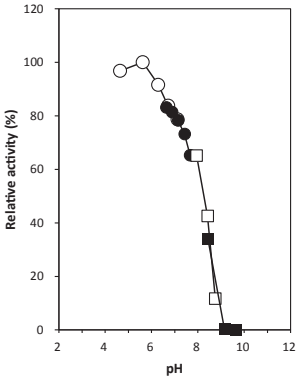

Fig. 1 Optimum pH

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

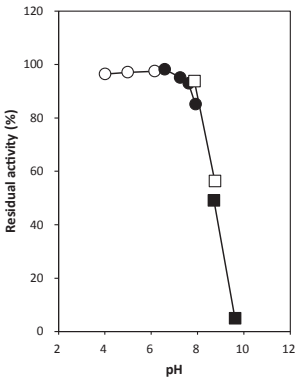

Fig. 2 pH stability

37℃, 60 min
○ : 3.3-Dimethylglutarate-NaOH buffer
● : Phosphate buffer
□ : Tris-HCl buffer
■ : Glycine-NaOH buffer

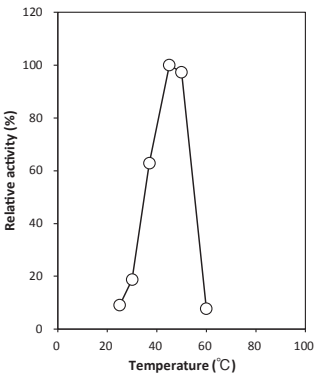

Fig. 3 Optimum temperature

pH 7.5
40 mM Phosphate buffer

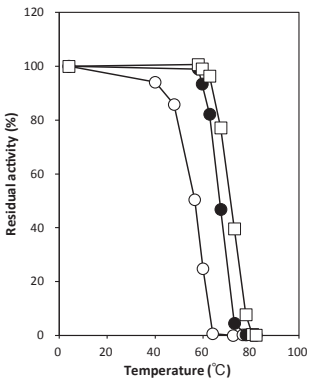

Fig. 4 Temperature stability

pH 6.0, 30 min
○ : Phosphate buffer
● : Phosphate buffer + 0.1% Emulgen 705
□ : Phosphate buffer
+ 0.1% Emulgen 705 & 10 mM NH_4Cl

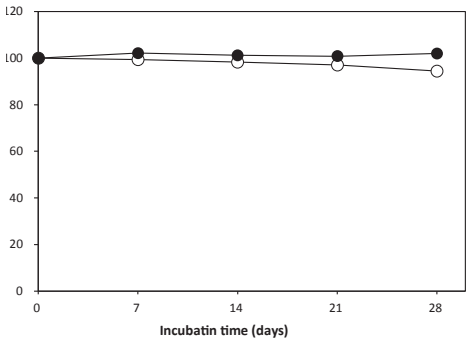

Fig. 5 Stability of CEBPⅡ in a liquid reagent

● : 4℃
○ : 37℃

Reagent composition: 0.5 U/mL CEBP II
50 mM BES buffer pH 6.5
0.4% Triton X-100
0.05% NaN_3
0.6 mM ADOS
7.5 U/mL POD

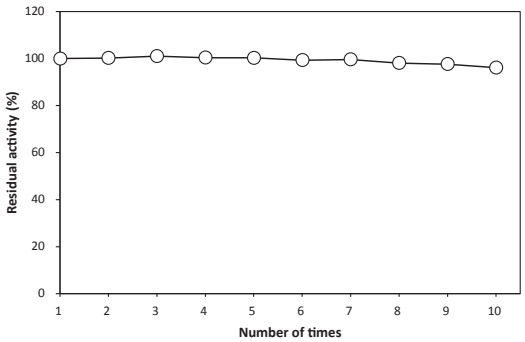

Fig. 6 Adsorption on glass

pH 7.0
10 mM Phosphate buffer containing 0.1% BSA
Solution of CEBPⅡ was repeatedly transferred into another test tube, and then the residual activity in the solution of each tube was measured.

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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:

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

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

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

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% 4-AA solution	0.30 ml
0.2% (W/V) Phenol solution	0.30 ml
100U/ml POD solution [1)]	0.30 ml
3% (W/V) Triton X-100 solution	0.30 ml
0.2U/ml CONⅡ solution [2)]	0.60 ml
Substrate solution [3)]	0.30 ml
Distilled water	0.30 ml

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

2): 0.2U/ml CONⅡ solution
Dissolve 2 U of CONⅡ with CONⅡ dilution buffer※)
※) : CONⅡ dilution buffer
0.1M 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
10mM KH_2PO_4-NaOH buffer pH 7.5 containing 0.1% (W/V) 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 pH5.2 #81-053
4-AA: NACALAI TESQUE, INC. Special grade #01907-52
POD: Sigma Chemical Co. Type Ⅱ #P-8250

■ 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 0.35 U/ml.

■ 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

$\triangle A/\text{min} = (As/\text{min} - Ab/\text{min}) \leqq 0.040$ Abs/min

■ Calculation

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

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.

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) Jap. J. Clin. Path., **24**, 650.

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CEBPⅡ 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% 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
POD1,000 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 社製
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
POD：シグマ製 Type Ⅱ #P–8250
4–AA：ナカライテスク製 特級 #01907–52

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約 0.35U/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。
Δ A/min =（As/min − Ab/min）≦ 0.040Abs/min

Ⅳ．計算

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

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/μ 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）