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CHOLESTEROL ESTERASE [CEBP-M]

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

Cholesterol Ester + H_2O ⟶ Cholesterol + Fatty Acid

★ Advantage
High liquid stability

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	: 87 kDa (gel filtration) 62 kDa (SDS-PAGE)	
Isoelectric point	: pH 5.0 ± 0.2	
Optimum pH	: 7.0	Figure 1
pH stability	: 5.0-8.0 (37℃, 60 min)	Figure 2
Optimum temperature	: 45℃ (Phosphate buffer)	Figure 3
Thermal stability	: Stable at 55℃ and below (pH7.5, 10 min)	Figure 4
Liquid stability	: See Figure 5	
Effect of metal ions	: See Table 2	
Effect of detergents	: See Table 3	

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.

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

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

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

FFA: Free fatty acid

Table 1. Substrate specificity

Substrate (0.95mM)	Relative activity (%)
Cholesterol acetate	1.20
Cholesterol propionate	8.90
Cholesterol butyrate	17.7
Cholesterol palmitate	27.0
Cholesterol stearate	8.10
Cholesterol oleate	100
Cholesterol linolate	187

Table 2. Effect of metal ions on CEBP-M activity

Metal ion	Relative activity (%)
None	100
NaCl (100mM)	108
KCl (100mM)	106
NH_4Cl (100mM)	100
LiCl (100mM)	96.9
$MgCl_2$ (1mM)	99.5
$MnCl_2$ (1mM)	125
$CoCl_2$ (1mM)	96.4
$ZnCl_2$ (1mM)	105

Table 3. Effect of detergents on CEBP-M activity

Detergent	Relative activity (%)
None	100
Triton X-100 (0.1%)	106
Deoxycholic acid (0.05%)	116
SDS (0.05%)	134

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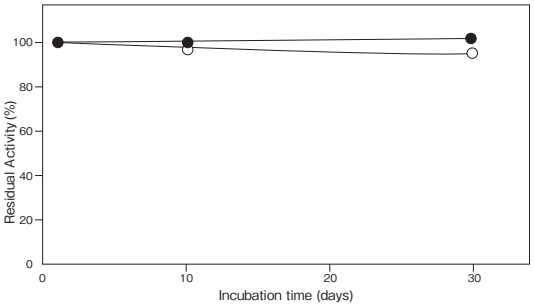
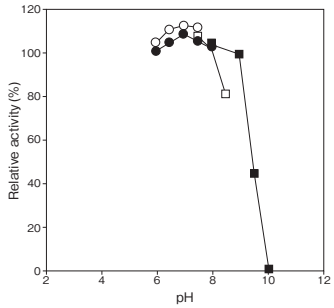
Fig.5 Stability of reconstituted CEBP-M



●: at 5℃
○: at 37℃

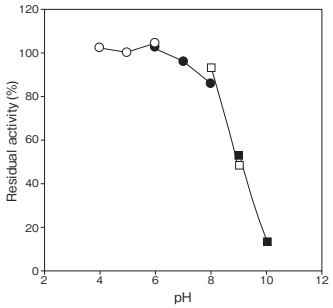
Storage conditions : 0.5U/ml CEBP-M
50mM BES buffer pH 6.5
0.4% Triton X-100
0.05% NaN_3
0.6mM ADOS
7.5U/ml POD

Fig.1 pH Optimum



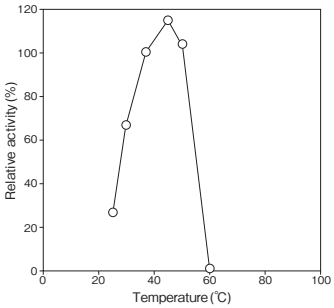
○: 3,3-Dimethylglutarate-NaOH buffer
●: Phosphate buffer
□: Tris-HCl buffer
■: Glycine-NaOH buffer

Fig.2 pH Stability



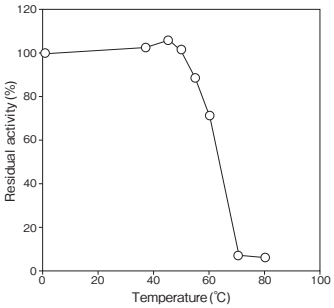
37℃, 60min
○: 3,3-Dimethylglutarate-NaOH buffer
●: Phosphate buffer
□: Tris-HCl buffer
■: Glycine-NaOH buffer

Fig.3 Optimum Temperature



pH 6.8
40 mM Phosphate buffer

Fig.4 Thermal Stability



pH 7.5, 10min.
40 mM Phosphate buffer

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:

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

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

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

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

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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
△A/min = (As/min − Ab/min) ≦ 0.040 Abs/min

■ **Calculation**

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

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.

CEBP–M 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 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)