(Diagnostic Reagent Grade)

T-101

CHOLESTEROL OXIDASE [CON Ⅱ-FD]

Lyophilized type

from *Rhodococcus* sp.
(Cholesterol: oxygen oxidoreductase, EC 1.1.3.6)

Cholesterol + O_2 ⟶ $\triangle^4$-Cholesten-3-one + H_2O_2

C

Preparation and Specification

Appearance : Yellowish amorphous powder, lyophilized
Specific activity : More than 15 U/mg solid

Properties

Substrate specificity	: See Table 1	
Molecular weight	: 61.8 KDa (SDS-PAGE)	
Isoelectric point	: pH 4.5	
Michaelis constant	: Cholesterol 6.0 × 10^{-5}M	
Optimum pH	: 7.0–7.5	Figure 1
pH stability	: 5.7–7.8 (65℃, 10 min)	Figure 2
Optimum temperature	: 50℃ (Tris-HCl buffer)	Figure 3
Thermal stability	: Stable at 65℃ and below (pH 7.0, 10 min)	Figure 4 and Figure 5
Effects of detergents	: See Table 2	

Applications for Diagnostic Test

This enzyme is useful for enzymatic determination of **total cholesterol**, **HDL-C**, and **LDL-C** when coupled with cholesterol esterase (T-18 and T-98).

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

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

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

FFA: Free fatty acid

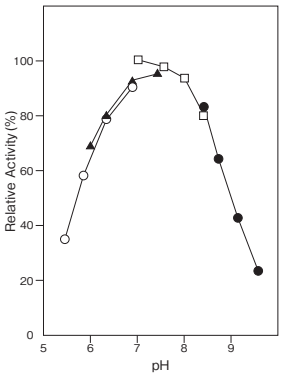
Table 1. Substrate specificity

Substrate (1mM)	Relative activity (%)
Cholesterol	100
β-Cholesterol	93
Pregnenolone	98
Dehydro-iso-androsterone	10
β-Sitosterol	94
Stigmasterol	66
Androsterol	2
Teststerone	1
Cholic acid	3

Table 2. Effect of detergents on CON Ⅱ-FD activity

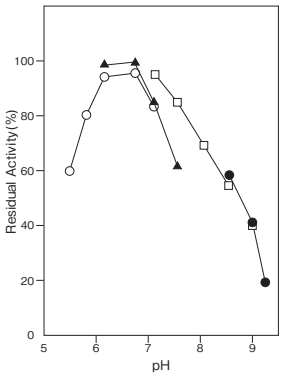
Detergents (0.1%)	Relative activity (%)
Triton X-100	100
Emulgen 810	101
Emulgen 911	113
Emulgen 709	107
Emulgen 109P	118
Adekatol SO-120	100
RHEODOL 460	63
SM 1080	122

Fig.1 pH Optimum



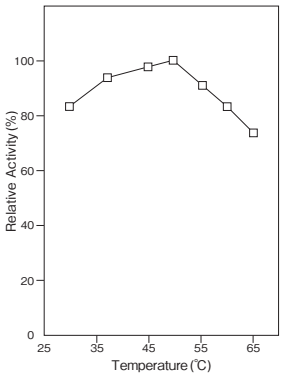
○ : MES buffer
▲ : PIPES buffer
□ : Tris-HCl buffer
● : Glycine-NaOH buffer

Fig.2 pH Stability



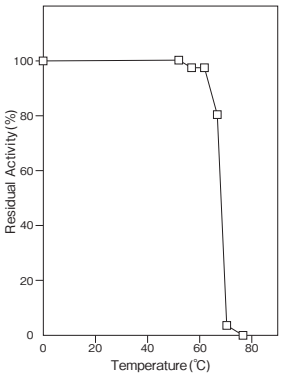
65℃, 10 min.
○ : MES buffer
▲ : PIPES buffer
□ : Tris-HCl buffer
● : Glycine-NaOH buffer

Fig.3 Optimum Temperature



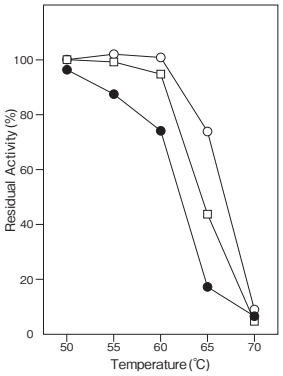
pH 7.0
100 mM Tris-HCl buffer

Fig.4 Thermal Stability



pH 7.0, 10 min.
100 mM Tris-HCl buffer

Fig.5 Thermal Stability



100mM, 10min.
○ : MES buffer(pH5.95)
□ : Tris-HCl buffer(pH7.63)
● : Glycine-NaOH buffer(pH8.95)

Assay

■ Principle

The assay is based on the increase in absorbance at 240 nm as $\triangle^4$-cholesten-3-one is produced in the following reaction:

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

■ Unit definition

One unit is defined as the amount of enzyme which liberates 1 μmole of $\triangle^4$-cholesten-3-one per minute at 37℃ under the conditions specified in the assay procedure.

■ Reagents

1. Substrate solution (6 mM cholesterol solution)
   Dissolve 232 mg of cholesterol with isopropanol to make a total of 100 ml.

2. Enzyme dilution buffer
0.1 M KH_2PO_4–Na_2HPO_4 buffer pH 7.0 containing 0.05% (W/V) Triton X-100
※ Prepare the enzyme dilution buffer two days before use and keep it in the refrigerator until use.
3. Reagents
Cholesterol : NACALAI TESQUE, INC. Special grade #08721
Triton X-100 : The Dow Chemical Company

■ **Enzyme solution**

Accurately weigh about 20 mg of the sample and add enzyme dilution buffer to make a total of 20 ml. After 1–1.5 hour incubation at room temperature, dilute it with enzyme dilution buffer to adjust the concentration to within 0.1–0.2 U/ml.

■ **Procedure**

1. Pipette accurately 3.0ml of enzyme dilution buffer and 50 μl of enzyme solution and preincubate at 37℃.
※ In the case of a test blank, add 50 μl of enzyme dilution buffer in place of enzyme sdution.
2. After 5 min, add 50 μl of substrate solution and mix to start the reaction at 37℃.
3. After starting the reaction, measure the rate of increase per minute in absorbance at 240nm. The rate must be measured within the linear portion of the absorbance curve.
Absorbance sample : As/min
blank : Ab/min
0.010 Abs/min ≦ △ A/min = (As/min-Ab/min) ≦ 0.060 Abs/min

■ **Calculation**

$$\text{Activity (U/mg)} = \frac{\triangle A/\text{min}}{12.2} \times \frac{3.10}{0.05} \times \frac{1}{X}$$

12.2 : millimolar extinction coefficient of $\triangle^4$-Cholesten-3-one at 240 nm (cm^2 / μmole)
3.10 : 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.

References

1. Richmond, W. (1973) Clin. Chem., **19**, 1350.
2. Flegg, H. M. (1973) Ann. Clin. Biochem., **10**, 79.
3. Alain, C. C. et. al. (1973) Clin. Chem., **20**, 470.
4. Tarbutton, P. N. and Gunter, C. R. (1974) Clin. Chem., **20**, 724.
5. Nomoto, S. (1976) Rinsho Kensa, **20**, 688.
6. Kameno, K., Nakano, N. and Baba, S. (1976) Jap. J. Clin. Path., **24**, 650.

CON Ⅱ-FD 活性測定法 (Japanese)

Ⅰ. 試薬液

1. 基質溶液 (6mM コレステロール溶液)
コレステロール 232mg をイソプロパノールに溶解して全容 100ml とする。
2. 酵素溶解希釈用液
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
※酵素溶解希釈用液は使用する 2 日前に調製し、使用まで冷蔵保存する。
3. 試薬
コレステロール：ナカライテスク製　特級 #08721
トリトン X-100：Dow Chemical 製

Ⅱ. 酵素試料液

検品約 20mg を精密に量り、酵素溶解希釈用液で溶解して全容 20ml とする。室温にて 1〜1.5 時間放置し、その液を酵素溶解希釈用液で約 0.1–0.2U/ml 濃度となるように適宜希釈する。

Ⅲ. 測定操作法

1. 小試験管に酵素溶解希釈用液 3.0ml と酵素試料液 50 μl を正確に加え 37℃で予備加温する。
※盲検は酵素試料液の代りに酵素溶解希釈用液 50 μl を加える。
2. 5 分経過後、基質溶液 50 μl を正確に加えて混和し、37℃で反応を開始する。
3. 反応開始後、240nm 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。
0.010 Abs/min ≦ △ A/min = (As/min-Ab/min) ≦ 0.060 Abs/min

Ⅳ. 計算

$$\text{活性 (U/mg)} = \frac{\Delta A/\text{min}}{12.2} \times \frac{3.10}{0.05} \times \frac{1}{X}$$

12.2: Δ^4– コレステン –3– オンの 240nm におけるミリモル分子吸光係数 (cm^2 / μmole)
3.10: 反応総液量 (ml)
0.05: 反応に供した酵素試料液量 (ml)
X : 酵素試料液の検品濃度 (mg/ml)