ORIGINAL

Properties of Cholesterol Oxidase Produced by *Rhodococcus equi* MIL 1037[†]

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Cholesterol oxidase produced by *Rhodococcus equi* MIL 1037 was purified from the supernatant solution of the culture broth by ammonium sulfate fractionation, DEAE- and CM-cellulose column chromatography, hydroxyapatite column chromatography and Sephadex G-75 gel filtration. The resulting enzyme was shown homogeneous by disc gel electrophoresis. The molecular weight of the enzyme was estimated as 48,000 by SDS-gel electrophoresis. The enzyme contained 453 amino acid residues including 40 moles of alanine and 16 moles of tryptophan. The enzyme was specific to 3 β -hydroxysteroids and its relative activity (RA) was 37.5 for β -sitosterol, 25 for 5 α -cholestan-3 β -ol, 25 for pregnenolone and 0.1 for stigmasterol on the basis of 100 for cholesterol. The *K_m* value for the oxidation of cholesterol was 0.33 mM. The enzyme was inhibited by CuSO₄, AgNO₃ and HgCl₂, and partially inhibited by *p*-chloromercuribenzoic acid, but unaffected by metal chelating reagents.

1 Introduction

Cholesterol oxidases [EC 1.1.3.6] from microorganisms are useful clinically for the determination of cholesterol in serum. Their properties from *Nocardia*^(1,2), *Brevibacterium*⁽³⁾, *Streptomyces*^(4),5), *Schizophyllum*⁽⁶⁾, *Corynebacterium*⁽⁷⁾, *Streptoverticillium*⁽⁸⁾ and *Arthrobacter*⁽⁹⁾ have been studied by many investigators. On the other hand, these enzymes oxidize cholesterol at the first step in the microbial decomposition of it⁽¹⁰⁾. In the previous papers^(11,12), we have isolated *Rhodococcus equi* MIL 1037 capable of degrading cholesterol and β -sitosterol (campesterol) selectively to 20-carboxy-pregnane derivatives in the presence of α , α' -dipyridyl. These acids can be very useful intermediates for the production of corticosteroid hormones. Moreover, k-3, the mutant of MIL 1037, accumulated these acids without the chelating reagent. Therefore, it is significative to investigate the enzymes participating transformation of steroids in these strains.

In this paper, the purification and properties of cholesterol oxidase produced by *R. equi* MIL

1037 are described as a part of studies on the microbial transformation of steroids.

2 Experimental

2.1 Microorganism

R. equi MIL 1037 which was able to use kerosene or cholesterol as a sole source of carbon was isolated from soil. This strain was found to be capable of cleaving the side-chain of β -sitosterol⁽¹¹⁾ and to produce abundantly cholesterol oxidase in the culture broth.

2.2 Cultivation

The cultivation of MIL 1037 for cholesterol oxidase production was carried out in a 500mL Erlenmeyer flask containing 100mL of modified nutrient medium (0.5% glucose, 0.5% beef extract, 1% corn steep liquor and 0.2% K₂HPO₄, pH 7.2) at 27°C, 220 rpm.

2.3 Assay of the enzyme activity

Assay method 1 : The oxidizing activity of cholesterol oxidase was assayed by estimating the initial rate of oxygen consumption at 30°C with a Clark oxygen electrode (Oxygraph 8, Central Kagaku Co. Ltd.). After the solution (total 1.0mL) containing 10 mM of phosphate buffer (pH 7.0) and the enzyme was saturated with air, the reaction was initiated by the addi-

[†] Studies on Microbial Transformations (XXV)

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tion of cholesterol (1 μ mole in 0.02mL of ethanol). One unit of cholesterol oxidase was defined as the amount of the enzyme oxidizing 1 μ mole of cholesterol (corresponding the consumption of 1 μ mole of oxygen) per min.

Assay method 2 : Cholest-4-en-3-one producing activity of the enzyme was assayed by the method of Stadtman¹³⁾ with modifications. After 0.9mL of 10mM phosphate buffer (pH 7.0) was mixed with 0.1mL of enzyme solution, 0.02mL of cholesterol solution (50 mM in ethanol) was added and incubated at 30°C for 15 min. At the end of the incubation, 4.0mL of ethanol was added to the reaction mixture and the amount of cholest-4-en-3-one was determined by measuring absorbance at 240 nm.

2.4 Determination of protein concentration

Concentrations of protein were measured by the method of Lowry *et al.*¹⁴⁾ using bovine serum albumin as a standard, and by the measurement of absorbance at 280 nm.

2.5 Electrophoresis

Polyacrylamide gel disc electrophoresis was performed by the method of Davis¹⁵⁾. The molecular weight of the purified enzyme was estimated by SDS-polyacrylamide gel electrophoresis according to the method of Weber and Osborn¹⁶⁾.

2.6 Product analysis

The reaction mixture was extracted with ethyl acetate and analyzed by GLC (Hitachi 663, 1.5% OV-17, 285°C). Further purification was performed by column chromatography (Wakogel C-200) with *n*-hexane/ethyl acetate (25 : 1, vol/vol). IR and Ms spectra of the purified product were also measured in the same way as in the previous paper¹⁷⁾.

2.7 Amino acid analysis

Amino acid analysis was performed with a Hitachi amino acid analyzer, Model 835. The enzyme was hydrolyzed in 6N HCl in a sealed evacuated tube at 110°C for 24, 48 and 72 h¹⁸⁾. Methionine and cysteine were determined according to the method of Moore¹⁹⁾. Tryptophan was measured after hydrolysis in 4N methanesulfonic acid²⁰⁾.

2.8 Materials

Cholesterol and cholest-4-en-3-one were purchased from Wako Pure Chemical Industries Ltd. and Sigma Co., respectively. Other ster-

oids used were obtained from Tokyo Chemical Industries Ltd. and Sigma Chemical Co. Other chemicals were of the highest purity commercially available.

2.9 Purification of the enzyme

All operations were carried out at 0~4°C, unless otherwise specified. The enzyme activity was measured by "Assay method 1".

Step 1 : Ammonium sulfate fractionation : From the culture filtrate (10L), the precipitate with ammonium sulfate (40 ~ 60% saturation) was collected by centrifugation and dissolved in 400mL of 10 mM Tris-HCl (pH 8.0) and dialyzed against the same buffer for 48 h (5L $\times$ 5 times).

Step 2 : DEAE-cellulose column chromatography : The dialyzed solution (550mL) was applied to a DEAE-cellulose column (2.6 $\times$ 60 cm) previously equilibrated with 10 mM Tris-HCl buffer, pH 8.0. The column was eluted with the same buffer at a flow rate of 60mL/h. The active fractions were concentrated and dialyzed against 10 mM sodium phosphate buffer (pH 7.0) for 48h.

Step 3 : CM-cellulose column chromatography : The dialyzed solution (150mL) obtained from Step 2 was applied to a CM-cellulose column (2.6 $\times$ 60 cm) previously equilibrated with 10 mM sodium phosphate buffer, pH 7.0. The charged column was washed with the same buffer and eluted with 25 mM of the same buffer. The active fractions were concentrated and dialyzed for 48 h against 10 mM of the same buffer.

Step 4 : Hydroxyapatite column chromatography : The dialyzed solution was applied to a hydroxyapatite column (5.0 $\times$ 15 cm) pre-equilibrated with 10 mM sodium phosphate buffer, pH 7.0. The charged column was washed with 50 mM of the same buffer and eluted with 100 mM of the same buffer. The active fractions were concentrated and dialyzed for 48 h against 10 mM of the same buffer.

Step 5 : Sephadex G-75 gel filtration : The dialyzed solution (5mL) from Step 4 was applied to a Sephadex G-75 column (2.6 $\times$ 60cm) previously equilibrated with 10 mM sodium phosphate buffer (pH 7.0). The column was eluted with the same buffer and then the active fractions were concentrated and dialyzed for 48h

Table-1 Summary of purification of cholesterol oxidase from *R. equi* MIL 1037.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Culture supernatant	27,000	3,750	0.14	100
(NH ₄) ₂ SO ₄ ppt	5,600	2,250	0.40	60.0
DEAE-cellulose	800	1,750	2.19	46.7
CM-cellulose	320	1,350	4.22	36.0
Hydroxyapatite	75	850	11.3	22.6
Sephadex G-75	22	470	21.4	12.5

Fig.-1 Disc electrophoresis of purified cholesterol oxidase.

against the same buffer. After this process, the preparation of cholesterol oxidase gave a single band in disc electrophoresis on a polyacrylamide gel column (Fig.-1).

A summary of the purification procedures for the cholesterol oxidase is shown in Table-1. From the culture filtrate, this enzyme was purified 153-fold and recovery of the activity was 12.5%.

3 Results and Discussion

3.1 Molecular weight

The molecular weight of the enzyme was esti-

Relative mobility expresses the distance of migration relative to that of bromophenol blue. s, Cholesterol oxidase; a, cytochrome c monomer (M.W., 12,300); b, dimer (M.W., 24,600); c, trimer (M.W., 36,900); d, tetramer (M.W., 49,200); e, hexamer (M.W., 73,800).

Fig.-2 Estimation of molecular weight of cholesterol oxidase by SDS-polyacrylamide gel electrophoresis.

mated from the R_f value on SDS-polyacrylamide gel electrophoresis. As shown in Fig.-2, the molecular weight of this enzyme was found to be approximately 48,000. This value is similar to that of *Schizophyllum* (53,000)⁶⁾ but comparatively small than those of cholesterol oxidases from other microbes (56,000–61,000)^{2), 5), 7)–9)} mentioned above excepting *Brevibacterium* (32,500)³⁾.

3.2 Optimum pH and pH stability

As shown in Fig.-3, the enzyme had a very common optimum pH (7.5 for cholesterol) observed in other microbial cholesterol oxidases (pH 7.0–7.5) excepting for *Schizophyllum* (pH 4–5)⁶⁾. The enzyme was stable over the pH range of 7.0–9.0 for 24 h at 4°C, and was comparatively unstable in acidic pH than others^{1), 3), 7)–9)}.

Citrate buffer, acetate buffer, phosphate buffer, borate buffer and carbonate buffer were used. Optimum pH : The enzyme activity was measured by "Assay method 2" with 0.21 unit of the enzyme except for using each of above buffers (0.1 mM) instead of 10 mM phosphate buffer. The reaction was carried out at indicated pH for 5 min at 30°C. pH Stability : To the enzyme solution (0.1 mL, 1.1 unit), 0.4 mL of 50 mM of each buffer was added and incubated at indicated pH for 24 h at 4°C. After the incubation, 4.5 mL of 0.1 M phosphate buffer (pH 7.0) was added and the activity was measured by "Assay method 1". Symbols : ●, optimum pH ; ○, pH stability.

Fig. -3 Effect of pH on the enzyme activity of cholesterol oxidase.

3.3 Optimum temperature and thermostability

The enzyme reaction was carried out at various temperatures at pH 7.0 for 5 min. As shown in Fig.-4, optimum temperature of the purified cholesterol oxidase was 45°C. It was lower than the enzymes from *Streptomyces*⁴⁾ and *Arthrobacter*⁶⁾ (50°C), but higher than *Corynebacterium*'s (40~42°C)⁷⁾. The purified enzyme was stable at temperature below 35°C for 1 h at pH 7.0 but, while about 90% of the activity remained at 40°C, 30% at 50°C, and the enzyme completely lost the activity at 60°C. This enzyme was more labile against heat than the enzymes from *Brevibacterium*³⁾, *Nocardia*¹⁾ and *Corynebacterium*⁷⁾ which maintain the activities more than 98% after the treatment at 50°C.

3.4 Effect of salts and other chemicals

Effect of various salts and other chemical reagents on the enzyme activity is summarized in

Optimum temperature : The enzyme activity was measured by "Assay method 2" except for the reaction temperature. The reaction was carried out at indicated temperature for 5 min. Thermostability : The enzyme in 10 mM phosphate buffer (0.21 unit/mL, pH 7.0) was kept at the indicated temperature for 1 h. Residual activity was measured by "Assay method 1". Symbols : ●, optimum temperature ; ○, thermostability.

Fig. -4 Effect of temperature on the enzyme activity of cholesterol oxidase.

Table-2 and 3. This enzyme was considerably inhibited by CuSO_4 , AgNO_3 and HgCl_2 at higher concentration than 1 mM. The enzyme activity reduced by the latter two salts was almost completely recovered by adding either glutathione or dithiothreitol. SH-group-inhibitor of *p*-chloromercuribenzoic acid (*p*-CMB) partially inhibited the activity. This is in accord with the results in *Brevibacterium*³⁾, *Corynebacterium*⁷⁾ and *Arthrobacter*⁶⁾ enzymes, but is different from the results in *Streptomyces*⁴⁾ and *Streptovercillium*⁸⁾ enzymes which are not inhibited by *p*-CMB. *p*-Chloromercuribenzenesulfonic acid and monoiodoacetic acid, however, failed to inhibit the enzyme activity. No metal chelating reagents such as EDTA, *o*-phenanthroline, α , α' -dipyridyl, 8-hydroxyquinoline and KCN significantly inhibited the activity of the enzyme. Therefore, it is likely that no metal ions are associated with the action of the enzyme.

3.5 Identification of the product

The retention time (9.5 min) of the product coincided with that of cholest-4-ene-3-one in

Table-2 Effect of salts on cholesterol oxidase.

Salts	Relative activity (%) Concentration		
	10 mM	1 mM	0.1 mM
CaCl ₂	61	98	108
MgCl ₂	98	100	112
FeCl ₃	22	95	100
FeSO ₄	15	71	97
CuSO ₄	6	14	84
Na ₂ MoO ₄	17	97	100
CoCl ₂	38	82	101
NiCl ₂	79	93	104
MnCl ₂	22	84	84
AgNO ₃	0	5	52
+ 10 mM glutathione	100	—	—
+ 10 mM DTT*	94	—	—
HgCl ₂	0	10	56
+ 10 mM glutathione	98	—	—
+ 10 mM DTT*	96	—	—

The mixture (1.0 mL, 10 mM phosphate buffer, pH 7.0) of salt and enzyme (5 μ g) was incubated at 30°C for 15 min, and then the residual activity was measured by "Assay method 2". The absorbance of control at 240 nm (0.294) was basis (100%).

* Dithiothreitol.

Table-3 Effect of chemical reagents on cholesterol oxidase.

Chemicals	Relative activity (%) Concentration	
	10 mM	1 mM
<i>p</i> -CMB*	—	72
<i>p</i> -CMS**	—	103
Monoiodoacetic acid	—	108
EDTA	100	—
<i>o</i> -Phenanthroline	100	—
α, α' -Dipyridyl	100	—
8-Quinolol	100	—
KCN	100	—

The mixture (1.0 mL, 10 mM phosphate buffer, pH 7.0) of chemical reagent and enzyme (5 μ g) was incubated at 30°C for 15 min, and then the residual activity was measured by "Assay method 2". The absorbance of control at 240 nm (0.294) was basis (100%).

* *p*-Chloromercuribenzoic acid.

** *p*-Chloromercuribenzenesulfonic acid.

GLC analysis. Ms of the purified product revealed an ion peak of m/z 384 in agreement with the formula C₂₇H₄₄O. IR spectrum of it completely agreed with that of cholest-4-ene-3-one. From these results, the product of the reaction was confirmed as cholest-4-ene-3-one.

3.6 *K_m* value

The reaction was found to follow the normal Michaelis-Menten kinetics, and the Lineweaver-Burk plot indicated the *K_m* value of the enzyme toward cholesterol to be 3.3×10^{-4} M (Fig.-5). Dissolved oxygen was not rate limiting factor in this reaction, because the plot was linear in the region of 0.1 ~ 0.4 mM of cholesterol. This *K_m* value was identical with that of *Schizophyllum*'s enzyme⁶⁾ and lower than that of either *Streptomyces*'s or *Streptoverticillium*'s enzyme [(4.0-6.7) $\times 10^{-4}$ M]^{4), 8)}, but the enzymes from *Nocardia*^{1), 2)}, *Corynebacterium*⁷⁾ and *Arthrobacter*⁹⁾ was reported to have remarkably lower *K_m* values (1.97-30.8 μ M) than *Rhodococcus*'s enzyme purified in this study.

3.7 Amino acids analysis

The amino acid composition of the purified enzyme, expressed as residues per 48,000 molecular weight, is shown in Table-4. The number of residues per molecule of protein was 453 including 40 moles of alanine and 16 moles of tryptophan. Although this enzyme has higher content of threonine than the enzymes from other microbes^{3), 6), 7)}, it has a common amino acids composition, while that from *Brevibac-*

To 1.0 mL of the enzyme solution (0.11 unit, 10 mM phosphate buffer, pH 7.0), 0.02 mL of cholesterol/ethanol solution was added to give the final concentration as indicated. The enzyme activity was measured by "Assay method 1".

Fig.-5 Lineweaver-Burk plot for cholesterol oxidase.

Table-4 Amino acid composition of cholesterol oxidase.

Amino acid	mol%	Integral number of residues
Aspartic acid	10.33	47
Threonine ^{a)}	9.34	42
Serine ^{a)}	7.67	35
Glutamic acid	6.77	31
Glycine	12.87	58
Alanine	8.89	40
Valine ^{b)}	7.84	35
Isoleucine ^{b)}	4.63	21
Leucine ^{b)}	5.85	26
Tyrosine	2.46	11
Phenylalanine	3.47	16
Lysine	4.99	23
Histidine	0.94	4
Arginine	3.25	15
Proline	4.65	21
Tryptophan ^{c)}	3.48	16
Half-cystine ^{d)}	1.27	6
Methionine ^{e)}	1.33	6
Total	100.03	453

The number of residues per molecule of protein was calculated on the basis of the molecular weight of 48,000.

^{a)} Extrapolated to zero time of hydrolysis.

^{b)} Adopted from the value of 72 h hydrolysis.

^{c)} Hydrolysis in 4 N methanesulfonic acid ⁽²⁰⁾.

^{d)} Determined as cysteic acid ⁽¹⁹⁾.

^{e)} Determined as methionine sulfone ⁽¹⁹⁾.

*terium*³⁾ is unusual in that it contains neither alanine nor tryptophan residue and has a high content of proline.

3.8 Substrate specificity

The relative oxidation rate of the enzyme for various 3-hydroxysteroids were measured. As shown in **Table-5**, the enzyme was specific for 3 β -OH steroids and did not attack 3 α -OH ones. Of the steroids tested, cholesterol was oxidized most efficiently. The enzyme was more active for steroids having double bond at the 5 position [cholesterol and dehydroepiandrosteron (DHEA)] than 5 α -steroids (5 α -cholestan-3 β -ol and epiandrosterone), and inactive for 5 β -steroids (A/B *cis* : 5 β -cholestan-3 β -ol and 5 β -androstan-3 β -ol-17-one) in either C₂₇ or C₁₉ steroids. In

Table-5 Relative rates of oxidation of various hydroxy steroids by cholesterol oxidase.

Substrate	Relative rate (%)
3 β -OH type	
Cholesterol	100
β -Sitosterol	37.5
5 α -Cholestan-3 β -ol	25.0
Pregnenolone	25.0
Dehydroepiandrosterone	18.8
Epiandrosterone	6.3
Stigmasterol	0.1
Diosgenin	0.0
Ergosterol	0.0
5 β -Cholestan-3 β -ol	0.0
5 β -Androstan-3 β -ol-17-one	0.0
3 α -OH type	
5 β -Cholestan-3 α -ol	0.0
5 α -Cholestane-3 α -ol	0.0
5 β -Androstan-3 α -ol-17-one	0.0
Lithocholic acid	0.0
Cholic acid	0.0

The enzyme activity was measured by "Assay method 1" except for using the indicated steroid instead of cholesterol.

Δ^8 steroids, the enzyme activity for steroids having branch (β -sitosterol) and double bond (stigmasterol) in the side chain at the 17 position and for steroids having comparatively short or no side chain (pregnenolone and DHEA) drastically reduced. Moreover, the activity for ergosterol and diosgenin could not be recognized. From these results, the substrate specificity of the enzyme is similar to that of *Brevibacterium*³⁾ but remarkably different from those of *Streptomyces*⁵⁾ and *Streptoverticillium*⁸⁾ whose enzymes can oxidize 5 α -cholestan-3 β -ol well [relative activity (RA) : 101% and 96%, respectively]. The enzymes from *Corynebacterium*⁷⁾ and *Arthrobacter*⁹⁾ can oxidize ergosterol and the latter enzyme has activity even for diosgenin. The enzyme of *Nocardia erythropolis*²⁾ has high RA (82%) for pregnenolone. On the other hand, a double bond in the 5 or 4 position of the steroid is essential for the enzyme from *Nocardia* sp.¹⁾, which cannot oxidize 5 α -cholestan-3 β -ol. Cholesterol oxidase of *Rhodococcus*, therefore, is found to have a comparatively high specificity for cholesterol than the

enzymes from other microbes except that the enzyme of *Nocardia* sp¹⁾ is inactive for the 5 α -steroids (the enzyme of *Rhodococcus* purified in this study showed RA of 25% even for 5 α -cholestan-3 β -ol as shown in Table-5).

Cholesterol oxidase is thought to react with the substrate at the first step in the microbial decomposition of steroids¹⁰⁾, and we reported that β -sitosterol was better substrate than cholesterol to produce 20-carboxy-pregna-1,4-dien-3-one (BNC) by *Rhodococcus equi* MIL 1037^{11),21)}. Namely, maximal yields (%) of BNC from β -sitosterol, cholesterol and 5 α -cholestan-3 β -ol were 56, 48 and 27, respectively, while cholesterol oxidase of this strain was not so active for β -sitosterol (RA : 37.5%). Consequently, the oxidation of β -sitosterol by cholesterol oxidase of this strain did not directly participate in the productivity of BNC.

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Rhodococcus equi MIL 1037 の生産する コレステロールオキシダーゼの性質

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Rhodococcus equi MIL 1037 の生産するコレステロールオキシダーゼをその培養上清から硫酸分画, DEAE-セルロース, CM-セルロース, ヒドロキシアパタイト, さらに, セファデックス G-75 カラムによって, ディスク電気泳動で単一バンドを示すまでに分離精製した。本酵素は SDS-ゲル電気泳動で分子量 48,000 と推定され, Ala 40 残基, Trp 16 残基を含む 453 個のアミノ酸残基よりなっていた。また, 本酵素は 3 β -ヒドロキシステロイドに特異的で, コレステロールを 100 とした時の比活性は, β -シトステロール 37.5, 5 α -コレスタン-3 β -オール 25, ステイグマステロール 0.1 であり, コレステロールに対する K_m 値は 0.33 mM であった。さらに, 本酵素は CuSO₄, AgNO₃, HgCl₂ によって完全に阻害され, p-クロロ安息香酸水銀 (II) では部分的な阻害を受けたが, 種々のキレート剤では阻害されなかった。