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Purification and characterization of cholesterol oxidase from *Pseudomonas* sp. and taxonomic study of the strain

Sang-young Lee[1], Hae-ik Rhee[2], Weon-chan Tae[1], Jeong-chul Shin[1], and Boo-kil Park[1]

[1] Department of Food Science and Technology, Kangweon National University, Chuncheon 200-701, Republic of Korea
[2] Department of Applied Biotechnology, Kangweon National University, Chuncheon 200-701, Republic of Korea

Summary. A cholesterol-oxidase-producing microorganism, strain COX629, isolated from soil was identified as *Pseudomonas* sp. The cholesterol oxidase produced by *Pseudomonas* sp. strain COX629 was purified 2400-fold to homogeneity in an overall yield of 60% from culture broth. The enzyme was a monomer with a molecular weight of 56000, as estimated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Sephadex G-150 gel column chromatography. The enzyme showed optimum activity at pH 7.0 and was stable over a rather wide pH range of 4.0 to 11.0. The enzyme showed a high substrate specificity for 3β-hydroxysteroids and the K_m value for the oxidation of cholesterol by this enzyme was about 0.2 m*M*. A characteristic of the enzyme is marked stability at high temperature.

Introduction

Cholesterol oxidase (EC 1.1.3.6) catalyses the oxidation of cholesterol (5-cholesten-3β-ol) to 4-cholesten-3-one with the reduction of oxygen to hydrogen peroxide. Since the first report on cholesterol oxidation in microorganism (Turfitt 1944), the same reaction has been reported in a number of microorganisms such as *Mycobacterium* sp. (Stadtman et al. 1954), *Nocardia restricus* (Richmond 1973; Sih and Wang 1965), *Brevibacterium sterolicum* (Uwajima et al. 1973) and *Streptomyces violascens* (Kamei et al. 1978). Moreover, this enzyme has been purified and partially characterized from several microorganisms (Uwajima et al. 1973; Tomioka et al. 1976; Kamei et al. 1978; Fukuyama and Miyake 1979), and mainly used for determination of cholesterol by coupling with peroxidase in clinical specimens (Allain et al. 1974). To select a potent cholesterol oxidase producer we initiated the screening for cholesterol-oxidase-producing microorganisms from soil. Here we present the taxonomic characteristics of a strain isolated from soil that exhibited markedly high activity of cholesterol oxidase in culture broth and the purification and characterization of extracellular cholesterol oxidase from the organism.

Offprint requests to: S. Lee

Materials and methods

Chemicals. Sephadex G-150 and DEAE-cellulose were purchased from Pharmacia, Uppsala, Sweden. Cholesterol, horseradish peroxidase and Triton X-100 were obtained from Sigma Chemical Co., St. Louis, Mo, USA. Molecular weight standard proteins were from Bio-Rad Laboratories, Richmond, Calif, USA. All other chemicals were from commercial sources and were of analytical grade.

Microorganism and culture conditions. Cholesterol-oxidase-producing microorganisms were isolated by the following procedure. Soil suspension was spread onto agar plates containing isolation medium, which was composed of 1.0% glycerol, 0.5% corn steep liquor, 0.1% KH_2PO_4, 0.1% $NaNO_3$ and 0.05% $MgSO_4$ (pH 7.3) and was solidified with 1.5% agar. The plates were incubated at 30° C for about 24 h and bacterial colonies that appeared were replica-plated by toothpick onto the isolation medium plates. The replica plates were incubated at 30° C for 24 h. To select the cholesterol-oxidase-producing strains, we used a colony staining method on agar plates. Filter papers dipped into 0.5% cholesterol, 1.76% 4-aminoantipyrine, 6% phenol and 3000 units/l horseradish peroxidase in 100 m*M* potassium phosphate buffer, pH 7.0 (KPB), were placed on colonies grown on agar medium and incubated at 30° C. The cholesterol oxidase activity of the test colonies was indicated by a red colour, due to the formation of quinoneimine dye. Strains able to produce the red colour were selected and cultivated at 30° C in 5 ml liquid isolation medium with constant shaking. The cells were collected by centrifugation, and the culture broth was assayed for extracellular activity of cholesterol oxidase. A bacterial strain, named strain COX629, was se-

lected. The medium used for the purification of the enzyme was a nutrient medium (1.0% peptone, 0.4% yeast extract, 0.1% glucose, 0.5% NaCl, pH 7.2) and the cells were grown at 30° C for 24 h with reciprocal shaking in 3-l erlenmeyer flasks containing 1000 ml medium.

Characterization and identification of strain COX629. Taxonomic characteristics of strain COX629 were investigated by the procedures described by Cowan (1974), Starr et al. (1981), and Palleroni (1984).

Determination of cholesterol oxidase activity. Cholesterol oxidase activity was assayed by the method of Allain et al. (1974). One unit of cholesterol oxidase activity was defined as the amount of enzyme which catalysed the formation of 1 μmol hydrogen peroxide/min at 37° C.

Protein determination. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Determination of molecular weight. Molecular weight was estimated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) and by gel filtration on a calibrated column of Sephadex G-150

Results

Characterization of the cholesterol-oxidase-producing bacterium strain COX629

Strain COX629 was isolated from the soil as described above. This strain was a Gram-negative, rod-shaped (1.2–1.5 μm × 2.1–2.5 μm), obligately aerobic, motile bacterium with peritrichous flagella. This strain reacted positively to the following tests: nitrate reduction, oxidase, catalase, oxidation-fermentation and hydrolysis of gelatine and Tween 80. It reacted negatively to the following tests: indole production, urease, phenylalanine deaminase, starch hydrolysis and pyoverdin production. This strain was able to grow in medium containing 4% NaCl and utilized citrate or acetate as the sole carbon source. The temperature range for growth was 4°–41° C, and the pH range was 4–10. The above characteristics of the strain seem to fit the genus *Pseudomonas* according to Palleroni (1984). A scanning electron micrograph of this strain is shown in Fig. 1.

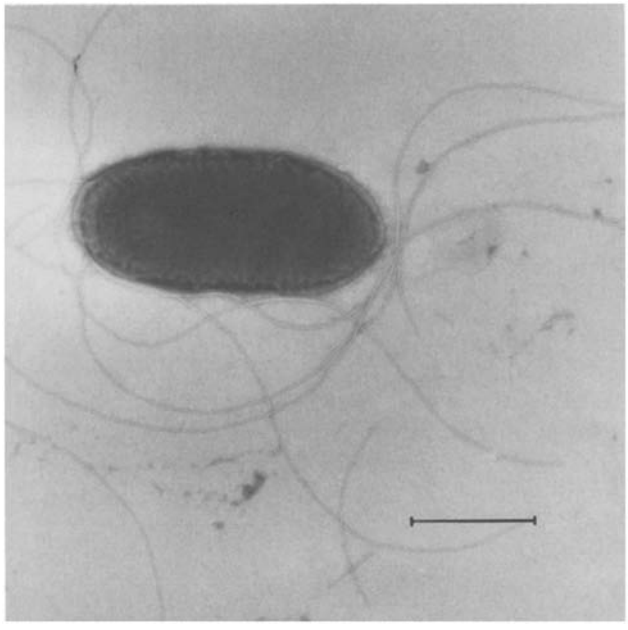

Fig. 1. Scanning electron micrograph of *Pseudomonas* sp. strain COX629. *Bar*, 1.0 μm

Purification of cholesterol oxidase

All purification steps were conducted at 0°–5° C, unless otherwise specified.

Step 1. Preparation of crude enzyme solution. The culture broth (10 l) was centrifuged at 10000 rpm for 10 min and the clear supernatants obtained were used for purification of the enzyme.

Step 2. DEAE-Cellulose column chromatography. The culture broth (10200 mg protein, 10 l) was directly applied to a DEAE-cellulose column (4 × 40 cm) equilibrated with 10 m*M* KPB, and the column was washed extensively with 500 ml of the same buffer. The cholesterol oxidase was not absorbed on the column under the conditions employed. The active fractions were pooled, and then concentrated by ultrafiltration on a Pellicon Membrane PTGC OLC M2 (Millipore, Bedford, Mass, USA).

Step 3. Cholesterol affinity column chromatography. Commercial cholesterol was recrystallized in 50% ethanol, and the recrystallized cholesterol was used as the adsorbent of the enzyme. The concentrate (508 mg protein, 100 ml) was loaded onto a cholesterol affinity column (1.2 × 30 cm) equilibrated with 10 m*M* KPB. After washing the column with 100 ml of 10 m*M* KPB, cholesterol oxidase was eluted with 0.1% Triton X-100 in 10 m*M* KPB. To remove cholesterol and Triton X-100 in the enzyme solution, eluates were passed through a Sephadex G-150 column (1.6 × 90 cm) equilibrated with 10 m*M* KBP. The active fractions were combined, concentrated as above and used for the characterization of cholesterol oxidase.

Table 1. Summary of the purification of the cholesterol oxidase from *Pseudomonas* sp. strain COX629

Step	Protein (mg)	Specific activity (μmol/min/mg protein)	Total activity (μmol/min)	Yield (%)	Fold
Culture broth	10200	0.012	115.3	100	1
DEAE-cellulose	508	0.17	87.9	76	15
Cholesterol	2.6	27.9	74.1	64	2471

The procedures for purification of the enzyme are summarized in Table 1 and Fig. 2. The cholesterol oxidase was purified approximately 2400-fold from culture broth with an activity yield of 60% (Table 1). The purified enzyme is apparently homogeneous as judged by SDS-PAGE (Fig. 2, lane C).

Some properties of the cholesterol oxidase

Molecular mass. The molecular weight of the enzyme was estimated to be 56000 by gel filtration on a Sephadex G-150 column (1.6 × 90 cm). On SDS-PAGE, the enzyme migrated as a single protein band with a molecular weight of 56000 (Fig. 2, lane C), indicating that the cholesterol oxidase of *Pseudomonas* sp. consists of a single polypeptide chain with a molecular weight of about 56000.

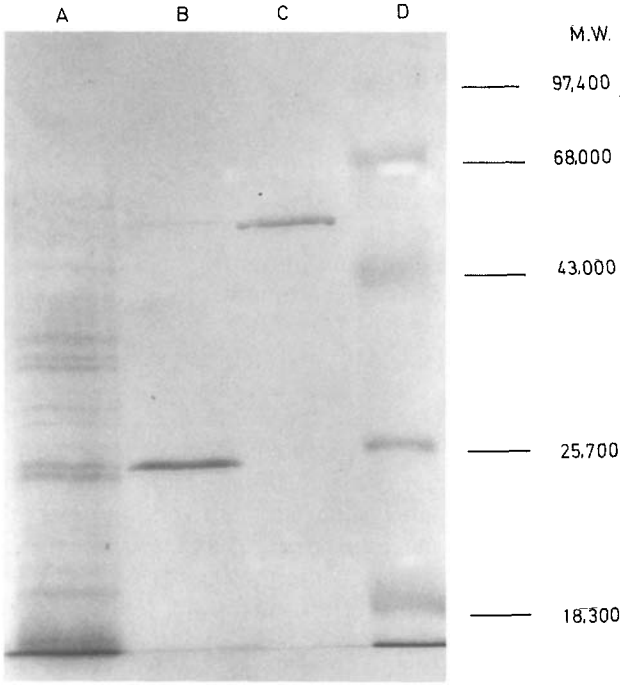

Fig. 2. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of cholesterol oxidase at the various purification steps. *Lane A,* culture broth; *lane B,* after DEAE-cellulose; *lane C,* after cholesterol and *lane D,* standard proteins. The standard proteins and their molecular weights (M.W.) were as follows: phosphorylase b (97400); bovine serum albumin (68000); ovalbumin (43000) and α-chymotrypsinogen (25700). The gel was stained for protein with Coomassie Brilliant Blue R-250

Effects of pH on activity and stability. The purified enzyme was most active at pH 7.0, when activity was assayed in 100 m*M* potassium phosphate buffer at 37° C. On either side of this pH, the activity was rapidly lost and the activities at pH 5.0 and pH 9.0 were one-third of that at pH 7.0 (data not shown). To determine the pH stability of the enzyme, 2.5 μg enzyme in 0.5 ml of 10 m*M* Tris-malate buffer was incubated at various pH values for 1 min at 60° C and the remaining activity was assayed under the standard conditions. No appreciable loss in activity was found between pH 5.0 and pH 8.0. Twenty-five percent activity loss was observed at pH 10 (data not shown).

Substrate specificity and reaction kinectis. The relative oxidation rates of various steroids by the enzyme were measured by the formation of hydrogen peroxide. The purified enzyme specifically oxidized 3β-hydroxy groups in steroids (cholesterol, dihydrocholesterol, ergosterol, stigmasterol, stigmastanol, 7-dehydrocholesterol) (Table 2). Cholesterol and dihydrocholesterol (5-cholestan-3β-ol) were the most suitable substrates among the steroids tested. Neither steroids lacking the 3β-hydroxy group (testosterone, 5-cholestene) nor esterified sterol (cholesteryl linoleate) was oxidized by the enzyme. The Michaelis constant (K_m) of the enzyme for cholesterol was determined at pH 7.0 in 100 m*M* KPB. The substrate saturation curves followed the usual Michaelis-Menten kinetics. The K_m value for cholesterol was calculated to be 0.2 m*M* from Lineweaver-Burk plots.

Effect of metal ions. The effects of metal ions on the enzyme activity were tested. Among the metal

Table 2. Substrate specificity of the cholesterol oxidase from *Pseudomonas* sp. strain COX629

Substrate (0.5 m*M*)	Relative activity (%)
Cholesterol	100
Dihydrocholesterol	78
Ergosterol	51
Stigmasterol	40
Stigmastanol	32
7-Dehydrocholesterol	24
Testosterone	0
5-Cholestene	0
Dehydroisoandrosterone	0
Cholesteryl linoleate	0

The activity was determined as described under Materials and methods. The activity for cholesterol was taken as 100%

ions tested, only Mn^{++} activated the enzyme, the activation being 20% at 0.5 m*M* and 50% at 2.0 m*M*. On the other hand, the enzymatic activity was markedly inhibited by various metal ions such as Fe^{++}, Zn^{++} and Hg^{++} (data not shown).

Heat stability. Ten micrograms of enzyme was incubated in 2.0 ml of 100 m*M* KPB for several minutes at various temperatures. After treatment, the enzyme solutions were immediately chilled to 20° C and the remaining activities were assayed. The enzyme retained its full activity on heating at 70° C for 5 min, and about 85% of the original activity after heating at 70° C for 30 min, although considerable activity was lost by heating at 80° C for 5 min (Fig. 3).

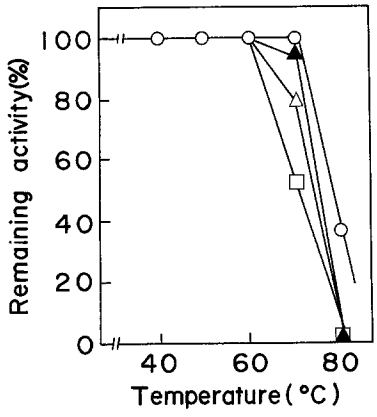

Fig. 3. Effect of temperature on the stability of cholesterol oxidase activity. The enzyme (10 μg) was incubated in 2.0 ml of 100 m*M* potassium phosphate buffer (pH 7.0) at various temperatures for 5 (○), 10 (▲), 30 (△) and 60 (□) min, and the remaining activity was measured. The activity at 40° C for 5 min was taken as 100%

Discussion

The cholesterol-oxidase-producing microorganism, *Pseudomonas* sp. strain COX629, was isolated from soil. The properties of the enzyme purified from strain COX629 were largely different from those reported for several other microorganisms. The cholesterol oxidase from strain COX629 has a molecular weight of 56 000 and is a monomer. On the other hand, the molecular weight of the enzyme from *S. violascens* (Kamei et al. 1978), *B. sterolicum* (Uwajima et al. 1973) and *Schizophyllum commune* (Fukuyama and Miyake 1979) are reported to be 61 000, 31 000 and 53 000 respectively. All of these enzymes consist of a single polypeptide chain, whilst the molecular weights are apparently distinct from the origin of the enzymes. In substrate specificity, the 3β-hydroxy configuration of the steroid was essential for the substrates of these enzymes. However, the enzymes showed some differences in their substrate specificities as follows. The 3β-hydroxysteroid oxidase from *B. sterolicum* (Uwajima et al. 1973) cannot oxidize dihydrocholesterol, which is the preferred substrate of the enzymes from *Streptomyces violascens* (Tomioka et al. 1976), *Streptoverticillium cholesterolicum* (Inouye et al. 1982) and strain COX629. The length of the C17 side chain on the steroid is important for efficient oxidation by the cholesterol oxidases. In contrast to the enzymes from *Streptomyces violascens* and *Streptoverticillium cholesterolicum,* cholesterol oxidases from strain COX629 and *Corynebacterium cholesterolicum* (Shirokane et al. 1977) cannot oxidize dehydroisoandrosterone (3β-hydroxy-5-androsten-17-one) effectively. Therefore, the 3β-hydroxy configuration and the presence of a side-chain containing more than two carbon atoms at the C17 position of the steroids is essential for substrates of strain COX629 enzyme.

A characteristic of the cholesterol oxidase of strain COX629 is marked stability under extreme conditions such as high temperatures and rather acidic and basic pHs. The heat stability of this enzyme gives it a great advantage for clinical uses. Detailed investigations on the heat stability will be described in a subsequent report.

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