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Cholesterol oxidase with high catalytic activity from *Pseudomonas aeruginosa*: Screening, molecular genetic analysis, expression and characterization

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An extracellular cholesterol oxidase producer, *Pseudomonas aeruginosa* strain PA157, was isolated by a screening method to detect 6β-hydroperoxycholest-4-en-3-one-forming cholesterol oxidase. On the basis of a putative cholesterol oxidase gene sequence in the genome sequence data of *P. aeruginosa* strain PAO1, the cholesterol oxidase gene from strain PA157 was cloned. The mature form of the enzyme was overexpressed in *Escherichia coli* cells. The overexpressed enzyme formed inclusion bodies in recombinant *E. coli* cells grown at 20°C and 30°C. A soluble and active PA157 enzyme was obtained when the recombinant cells were grown at 10°C. The purified enzyme was stable at pH 5.5 to 10 and was most active at pH 7.5–8.0, showing optimal activity at pH 7.0 and 70°C. The enzyme retained about 90% of its activity after incubation for 30 min at 70°C. The enzyme oxidized 3β-hydroxysteroids such as cholesterol, β-cholestanol, and β-sitosterol at high rates. The K_m value and V_{max} value for the cholesterol were 92.6 μM and 15.9 μmol/min/mg of protein, respectively. The V_{max} value of the enzyme was higher than those of commercially available cholesterol oxidases. This is the first report to characterize a cholesterol oxidase from *P. aeruginosa*.

[**Key words:** Cholesterol; Cholesterol oxidase; *Pseudomonas aeruginosa*; Cholest-4-en-3-one; 6β-Hydroperoxycholest-4-en-3-one; Vanillyl-alcohol oxidase family]

Cholesterol oxidases (EC 1.1.3.6) are flavoenzymes that generally catalyze the oxidation of cholesterol (cholest-5-en-3β-ol) to cholest-4-en-3-one with the reduction of oxygen to hydrogen peroxide (1,2). They contain a single molecule of flavin adenine dinucleotide (FAD) as the redox cofactor. Cholesterol oxidase plays important roles in several biological processes (3,4). A wide variety of microorganisms can metabolize cholesterol and produce a cholesterol oxidase that is involved in the first step of cholesterol metabolism (5). Some pathogenic bacteria possess cholesterol oxidases that are major membrane-damaging factors and are therefore implicated in the pathogenicity of these bacteria (6,7). In addition, *Streptomyces natalensis* produces a cholesterol oxidase (PimE) that acts as a signaling protein for the biosynthesis of an antifungal antibiotic, polyene macrolide pimaricin (8,9).

Microbial cholesterol oxidase is employed in a variety of applications. Cholesterol oxidase is widely used for the clinical determination of cholesterol levels in serum (10,11). In addition, cholesterol oxidase possesses larvicidal and insecticidal activity that is a vital part of pest control strategies involving transgenic crops (12,13). Moreover, cholesterol oxidase has been used as a biocatalyst that provides valuable steroidal compounds (14–16) and non-steroidal chiral compounds (17,18). Cholesterol oxidase from *Chromobacterium* sp. DS-1 can be used to reduce oxysterol cytotoxicity in human fibroblasts (19).

The crystal structures of cholesterol oxidases have revealed that they belong to two distinct structural classes (class I and class II) belonging to different protein families. The class I enzyme contains the FAD cofactor non-covalently bound to the enzyme and belongs to the glucose/methanol/choline (GMC) oxidoreductase family. The class II enzyme contains the cofactor covalently linked to the enzyme and belongs to the vanillyl-alcohol oxidase (VAO) family (20,21). These two forms of enzymes have no significant sequence homology. The class I enzymes have been identified mostly in actinomycetes such as *Streptomyces* spp., *Rhodococcus* spp. and *Brevibacterium sterolicum*. On the other hand, class II enzymes have been found not only in several actinomycetes but also in beta- and gamma-proteobacteria such as *Burkholderia cepacia* ST-200 (22,23) and *Chromobacterium* sp. DS-1 (24,25).

Among class II enzymes, cholesterol oxidases from *B. cepacia* ST-200 and *Chromobacterium* sp. DS-1 oxidize cholesterol to 6β-hydroperoxycholest-4-en-3-one (HCEO), but not to the cholest-4-en-3-one (CEO) produced by most cholesterol oxidases (24,26). These enzymes exhibit relatively higher stability against high temperatures, organic solvents, and detergents than commercially available cholesterol oxidases. The characterization of HCEO-forming cholesterol oxidases has been reported based only on these two enzymes.

In this paper, we report the screening of a cholesterol oxidase from *Pseudomonas aeruginosa* strain PA157, the cloning of the cholesterol oxidase gene, the expression of the gene in *Escherichia coli*, and the purification and partial characterization of the enzyme produced by the recombinant *E. coli*.

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MATERIALS AND METHODS

Strains, media and plasmids *E. coli* DH5α and *E. coli* Rosetta (DE3) pLysS (Novagen, Madison, WI, USA) were used as hosts for the recombinant plasmids. pBlueScript II SK+ (pBSII) and pET-21a(+) were obtained from Toyobo Biochemical (Osaka, Japan) and Novagen, respectively. A screening medium consisting of 1% Bacto Tryptone (Difco Laboratories, Detroit, MI, USA), 0.5% Bacto yeast extract (Difco), 1% NaCl, 64 mM sodium cholate, 0.3% Triton X-100, and 0.9 mM cholesterol was used for the screening of microorganisms that produce HCEO-forming cholesterol oxidase (24). *E. coli* strains were grown in Luria–Bertani broth (LB medium). When necessary, the medium was solidified with 1.5% (w/v) agar and supplemented with appropriate antibiotics. The recombinant *E. coli* Rosetta strain was grown in LB+AC medium, which has the same composition as LB medium except that the medium was supplemented with 50 μg/ml ampicillin and 25 μg/ml chloramphenicol.

Isolation of cholesterol-oxidase-producing bacteria Bacteria producing HCEO-forming cholesterol oxidases were screened by the detection of turbid halos around bacterial colonies as described previously (24). These halos were formed due to the low solubility of HCEO. About 500 soil samples were collected from the northern Kanto region in Japan. A small amount of each soil sample was spread on a screening medium agar plate. The plates were incubated at 30°C for few days. Microorganisms in colonies around which turbid halos formed were isolated by repeated single-colony isolation on the screening medium agar.

Taxonomic studies The isolated strain was identified according to Bergey's manual of Systematic Bacteriology (27). Physiological and biochemical characteristics were determined using the API 20NE tests (bioMérieux, Lyon, France). Catalase and oxidase tests were performed as described by Barrow and Feltham (28). The 16S ribosomal DNA (rDNA) sequencing analysis was performed as described previously (24).

Cloning and sequence analysis of the cholesterol oxidase gene A putative cholesterol oxidase gene sequence of *P. aeruginosa* PAO1 determined via an analysis of its genome sequence was deposited in the National Center for Biotechnology Information (NCBI) database under accession number NC_002516.2. PCR primers were designed based on the sequence of *P. aeruginosa* PAO1. PCR was performed to amplify the fragment, including the entire cholesterol oxidase gene, using PrimeSTAR HS DNA polymerase (Takara Bio), genomic DNA of strain PA157 as the template, and primers PAW-S, 5′-CCAGAATTCTCGATTACCACTACGACACT-3′ (*Eco*RI site underlined) and PAW-AS, 5′-TAACTCGAGTTCGTGCGACACCTCGATAA-3′ (*Xho*I site underlined). The thermal cycling parameters were 94°C for 1 min, followed by 30 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min. The obtained PCR fragment was cloned into the same sites in pBSII SK+ and the resulting recombinant plasmid was designated pBSpacox. The DNA and the amino acid sequences were analyzed using the BLAST network service on the NCBI website (29). The phylogenetic tree based on the amino acid sequences of cholesterol oxidases was constructed by the neighbor-joining method using GENETYX software (GENETYX, Tokyo, Japan).

Construction of pETpacox A sense primer was designed to incorporate the restriction enzyme site *Nde*I and remove the signal peptide. The sense primer PAdelS-S: 5′- GCCCATATGAGCAGTTGTCCGGCGCCG -3′ (*Nde*I site underlined) and the antisense primer PAW-AS were used to amplify the cholesterol oxidase gene from pBSpacox as the template. The recombinant enzyme contained an additional Met at the N-terminal sequence of the putative mature enzyme in order to create the *Nde*I site. The obtained PCR fragment digested with the restriction enzymes (*Nde*I and *Xho*I) was cloned into the same sites in pET-21a(+). The resulting plasmid was called pETpacox. It was confirmed by resequencing that there was no mutation in the cholesterol oxidase gene on the pETpacox.

Purification of the recombinant cholesterol oxidase The *E. coli* Rosetta strain harboring pETpacox was grown in 200 ml of LB+AC medium at 30°C. When the culture reached an OD_{660} of approximately 0.5, protein expression was induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After incubation for another 48 h at 10°C, the cells were harvested by centrifugation (6000 ×*g*, 15 min, 4°C). The cell pellet was resuspended with 10 ml of 10 mM Tris–HCl (pH 8.0) and disrupted by using the ultrasonic disruptor UD-200 (Tomy Seiko, Tokyo, Japan). Cell debris was removed by centrifugation at 10,000 ×*g*, 10 min, 4°C. The supernatant was loaded on a column (2.5 by 8 cm) of DEAE-cellulose DE52 (Whatman, Maidstone, UK) equilibrated with the Tris–HCl buffer. The recombinant enzyme was eluted with a linear gradient of NaCl concentrations of 0–400 mM in 400 ml of the Tris buffer. The fractions with the enzyme activity were pooled. This solution was dialyzed against 10 mM sodium acetate buffer (pH 5.5) at 4°C and put on a column (2.5 by 6 cm) of CM-cellulose CM52 (Whatman) equilibrated with the sodium acetate buffer. The enzyme was eluted with a linear gradient of NaCl concentrations of 0–400 mM in 400 ml of the sodium acetate buffer. The enzyme solution was dialyzed against 10 mM Tris–HCl (pH 8.0) at 4°C and kept at 4°C until it was used.

Assay of the cholesterol oxidase activity Cholesterol oxidase activity was determined by monitoring H_2O_2 production using the horseradish peroxidase assay as described previously (22). Assay mixture (total 1 ml) contained 50 mM sodium phosphate buffer (pH 7.0), 64 mM sodium cholate, 0.34% Triton X-100, 1.4 mM 4-aminoantipyrine, 21 mM phenol, 5 units of horseradish peroxidase (Toyobo Co., Ltd., Tsuruga, Japan), and 0.89 mM of cholesterol. The reaction was started by adding an appropriate amount of enzyme to the assay mixture. The development of a red color was monitored at 500 nm. One unit of enzymatic activity corresponds to the amount of enzyme that oxidizes one μmol of cholesterol per minute, at 30°C. Unless otherwise stated, the enzyme activity was determined by this method. To estimate the optimum pH for the cholesterol oxidase activity, the enzyme activity was determined by monitoring the consumption of oxygen. Assay mixture (total 1 ml) contained 64 mM sodium cholate, 0.34% Triton X-100 and 0.89 mM of cholesterol and 100 mM of each buffer solution. Initial velocity of oxygen consumption was measured at 30°C as described previously (24). To investigate the optimum temperature for the activity, the enzyme activity was examined by measuring the formation of HCEO. Assay mixture (total 1 ml) contained 50 mM Tris–HCl buffer (pH 8.0), 64 mM sodium cholate, 5% isopropanol and 0.25 mM of cholesterol. The reaction was initiated by adding an appropriate amount of enzyme to the assay mixture. After 3–5 min of incubation at 30°C, 2 ml of ethanol was added to the mixture and the formation of HCEO was determined by measuring the A_{249} of λ_{max} value of HCEO, as described previously (24).

Amino acid sequencing The N-terminal amino acid sequence of the enzyme was determined using an automated protein sequencer (model G1005A; Hewlett–Packard, Palo Alto, CA, USA).

Determination of protein concentration Protein concentrations were determined using the method of Bradford (30) and bovine serum albumin as the standard.

Steady-state kinetics Cholesterol oxidation activity was assayed by measuring H_2O_2 generation with the assay solution containing 0–1 mM cholesterol. The K_m and V_{max} values were determined by extrapolation using the Lineweaver–Burk plot. The mean values and standard deviations of the K_m and V_{max} values were obtained from three independent experiments.

Isolation of the cholesterol oxidation product The cholesterol oxidation product of PA157 cholesterol oxidase was isolated in a manner similar to that described previously (24). The purified cholesterol oxidase from strain PA157 (1 unit) was incubated in 100 ml of the reaction mixture consisting of 50 mM phosphate buffer, pH 7.0, containing 64 mM sodium cholate, 0.34% (vol/vol) Triton X-100 and 0.9 mM cholesterol, at 30°C for 24 h. The reaction mixture was extracted twice with 1 vol. of chloroform. Cholesterol oxidation product in the concentrated chloroform extract was purified by preparative thin-layer chromatography (TLC) using a 2-mm silica gel 60F254 preparative TLC plate (E. Merck AG, Darmstadt, Germany) as described previously (24).

Molecular mass of the cholesterol oxidation product Molecular mass of the cholesterol oxidation product was determined by Fast atom bombardment (FAB) mass spectrometry using an MS-700 mass spectrometer/data system (JEOL Ltd., Tokyo, Japan) as described previously (24).

Nucleotide sequence accession number The nucleotide sequences of the 16S ribosomal ribonucleic acid (rRNA) gene and the cholesterol oxidase gene of strain PA157 have been deposited in the GenBank/EMBL/DDBJ database under accession numbers AB920753 and AB920752, respectively.

Materials Triton X-100 and Triton-405 were purchased from Sigma Chemicals. Tween 20, sodium cholate, sodium dodecyl sarcosinate (sarcosyl), sodium dodecyl sarcosinate (SDS) and laurylbenzenesulfonate (LBS) were from Wako Pure Chemical Industries, Osaka, Japan, and sodium polyoxyethylene alkyl (C12-13) ether sulfate (Emal 20CM) was from Kao Corp., Tokyo, Japan. The organic solvents used were of the highest grade.

RESULTS

Isolation and identification of a cholesterol-oxidase-producing bacterium We screened for microorganisms that produce HCEO-forming cholesterol oxidases on the screening agar medium as described previously (24). As a result, strain PA157 formed a halo around its colonies on the agar medium. We analyzed the 16S rDNA sequence of strain PA157 as described by Lane (31). A sequence similarity search of the 16S rDNA sequence was performed using the BLAST program. The 1433 bp sequence of strain PA157 showed a high sequence similarity of 99.3% with that of *P. aeruginosa* PAO1 (NCBI accession no. NC_002516.2). In addition, the sequence of strain PA157 exhibited slightly less similarity to the sequences of *Pseudomonas stutzeri* (97.2%, NC_009434.1), *Pseudomonas mendocina* (96.4%, NC_009439.1), and *Pseudomonas monteilii* (96.2%, NC_023076.1).

The cells were straight rods measuring 0.6 to 0.8 by 1.3–1.8 μm and were gram-negative, motile, and non-spore-forming. The colonies were circular and pale yellow on the LB agar. Strain PA157

reduced nitrate and degraded gelatin. Catalase, oxidase, arginine dihydrolase, and urease tests were positive. However, tests for indole production and hydrolysis of *O*-nitrophenyl-β-D-galactopyranoside and esculin were negative. Strain PA157 assimilated glucose, mannitol, *N*-acetylglucosamine, gluconate, *N*-capric acid, malate, and citrate, but not arabinose, mannose, maltose, adipiate, and phenyl acetate. These results were identical to those for *P. aeruginosa*.

Cloning and nucleotide sequence of the cholesterol oxidase gene When strain PA157 was grown in the screening medium at 30°C for 24 h, the enzyme activity in the supernatant was less than 0.01 U/ml. This activity was much lower than those of other cholesterol-oxidase-producing bacteria such as *B. cepacia* ST-200 (22) and *Chromobacterium* sp. DS-1 (24). Thus, we tried to clone and express a cholesterol oxidase gene from PA157 in *E. coli*. On the basis of a putative cholesterol oxidase gene sequence of *P. aeruginosa* PAO1, we cloned the cholesterol oxidase gene from strain PA157. *E. coli* DH5α was transformed with plasmids containing the entire cholesterol oxidase gene, pBSpacox. Sequence analysis of the cloned gene identified an open reading frame of 1791 bp that encoded a protein of 597 amino acids with an estimated molecular mass of 65,344 Da and an isoelectric point of 5.96.

Several amino acid sequences were found to exhibit significant similarities to PA157 cholesterol oxidase via a BLAST search. A phylogenetic tree was constructed by using amino acid sequences of PA157 cholesterol oxidase and other bacterial cholesterol oxidases (Fig. 1). PA157 cholesterol oxidase showed high similarity to the cholesterol oxidases (98–99%) from *P. aeruginosa* strains. Lower similarities were found in cholesterol oxidases from *Chromobacterium* sp. DS-1 (58%), *Burkholderia* spp. (57–58%), *Rhodococcus erythropolis* (45%) and *B. sterolicum* (42%).

The N-terminal amino acid sequence of the cholesterol oxidase contained the distinctive twin-arginine motif-like sequence SRRSFLG between Ser-23 and Gly-29 (Fig. 2). This signal sequence consensus motif is essential for the secretion of a twin arginine translocase (Tat) system which secretes fully folded proteins across the cytoplasmic membrane in bacteria (32,33). It was speculated that the signal peptidase recognition sequence (Ala-X-Ala) was AAA between Ala-54 and Ala-56. Thus, the signal peptide seemed to be proteolytically processed between Ala-56 and Ser-57 upon excretion and secretion in strain PA157. The predicted mature polypeptide consisted of 541 amino acid residues, with a molecular mass of 59,626 Da.

Expression and purification of the recombinant cholesterol oxidase When *E. coli* Rosetta harboring pETpacox grew on LB+AC medium supplemented with 0.5 mM IPTG at 30°C, the enzyme activity in sonicated lysate of this strain was extremely low. Thus, we examined the formation of inclusion bodies by the expressed cholesterol oxidase at various temperatures. The recombinant clones were cultivated in LB+AC medium at 30°C until the optical density at 660 nm of the culture reached about 0.5. After IPTG was added to reach a concentration of 0.5 mM, the cultivation was continued for another 48 h at 10°C, 20°C or 30°C. As a result, the enzyme activities in sonicated lysate of the recombinant clones grown at 10°C, 20°C and 30°C were 1.1, 0.18 and 0.015 U/mg, respectively. The cells were harvested by centrifugation and disrupted by sonication. The supernatant and precipitation after centrifugation of the disrupted cells were separately collected as the soluble and insoluble fractions, respectively. The proteins contained in the soluble and insoluble fractions were analyzed by SDS-PAGE as shown in Fig. 3. When the recombinant clones were grown at 20°C and 30°C, a large amount of the expressed enzyme was found in the insoluble fraction but not in the soluble fraction. Thus, the expressed protein seemed to form insoluble inclusion bodies. By contrast, the expressed protein from the recombinant clones grown at 10°C was found in the soluble fraction. Therefore, the recombinant clones were cultivated at 10°C to purify the functional cholesterol oxidase. Table S1 summarizes the purification steps used. The DE52 column was effective for purifying the enzyme. The overall purification of the enzyme from 200 ml of the culture was 11-fold, with an activity yield of 26%. The total amount of the purified enzyme with a specific activity of 11.6 U/mg-protein was 2.34 mg of protein. SDS-PAGE was performed, and the purified preparation gave a single band (Fig. S1). The molecular mass was estimated to be

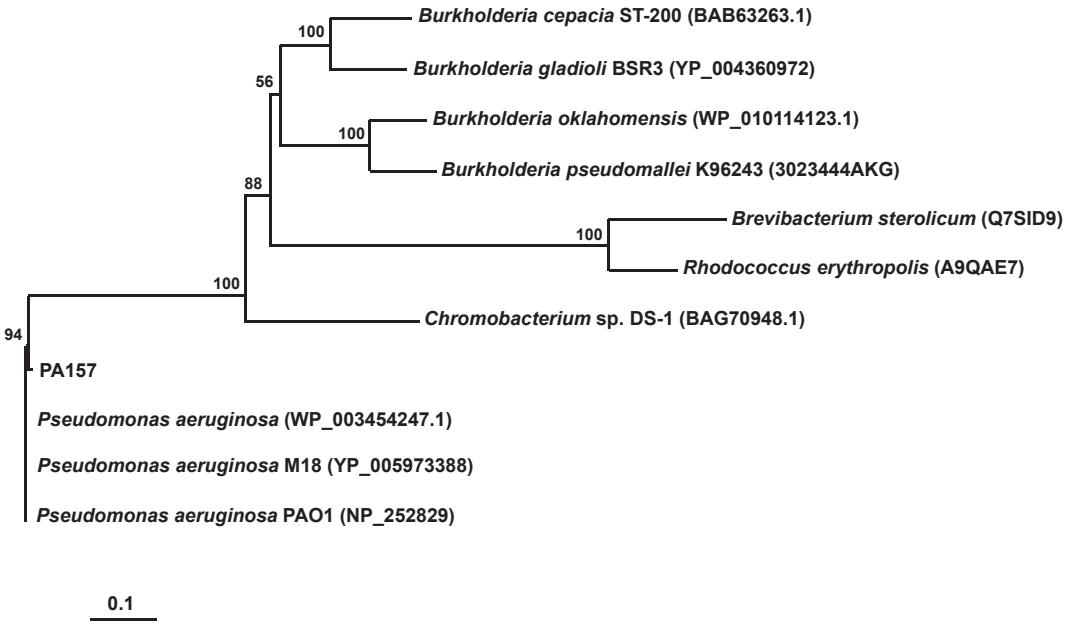

FIG. 1. Phylogenetic tree of the cholesterol oxidase from strain PA157. The deduced amino acid sequence of PA157 cholesterol oxidase was compared with other homologous sequences. A phylogenetic tree was constructed using the neighbor-joining method. The scale bar represents 0.1 substitutions per amino acid position. Bootstrap values greater than 50% are shown as percentages on branches. The GenBank/EMBL/DDBJ accession numbers are shown in parentheses.

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PA157                          11 FVGDPDQESGGLSRRSFLGKSATLGAVGLVAGWTPAFVIQPAEAAASSCPAPAGFPA 67
Burkholderia cepacia ST-200     1    MSQDFRDEPASRRAFLADMAKLAAAGVVTGWTPLYQIAANARTADAPPPGFPADI 55
Chromobacterium sp. DS-1        1 MSSNDAEKPLAASRRDFLTGSLKLASAGALAGWLPAERIMAGRLTCSQPNNFPAEIP 57
Brevibacterium sterolicum       1    MTLNDEQLRLSRRGFLTAGAAGAGVLAAGALGGWTPAFAVPAGSAGSLGSLGSTG 60
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FIG. 2. N-terminal amino acid sequences of class II cholesterol oxidases. These sequences include enzymes from PA157 (accession no. AB920752), *Burkholderia cepacia* ST-200 (no. BAB63263.1), *Chromobacterium* sp. DS-1 (no. BAG70948.1), and *Brevibacterium sterolicum* (no. Q7SID9). Putative twin-arginine motifs are underlined. The open arrowhead indicates the predicted cleavage site of the PA157 enzyme. The filled arrowheads indicate the reported cleavage sites of the enzymes.

59 kDa. The N-terminal amino acid sequence of the expressed protein was SSCPA, which was identical to the N-terminal sequence of the predicted mature enzyme. The N-terminal methionine residue of the recombinant enzyme seemed to be removed by methionyl aminopeptidase of *E. coli*.

Effect of pH, temperature and chemicals on cholesterol oxidase activity The recombinant enzyme displayed >95% of its maximal activity in the pH range of 7.0–8.0, with the optimal pH at pH 7.5 (Fig. 4A). The enzyme was stable from pH 5.5 to 11 in the tested conditions (Fig. 4A). The enzyme had an optimal temperature of 70°C (Fig. 4B). The enzyme was stable at temperatures from 4°C to 70°C after incubation for 30 min (Fig. 4B). The enzyme lost almost all activity after 30 min at 75°C.

The effects of metal ions on the cholesterol oxidase activity were determined by measuring enzyme activity at 30°C for 1 h in the presence of various metal ions. At concentrations of 1 mM, Ca^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+} Fe^{2+} and Zn^{2+} had no obvious effect on the enzyme activity. The addition of Ag^{+} and Cu^{2+} at 1 mM reduced the activity to 76% and 78% of that without a metal ion, respectively. A chelating agent, EDTA, had no influence on the enzyme activity.

The enzyme was stable and partially activated in the presence of 0.5% (wt/vol) detergents including Tween 20, Triton X-100, and sarcosyl (Table 1). The enzyme was partially inactivated by the addition of Emal 20CM and completely inactivated by the addition of SDS and LBS. The enzyme was also stable in the presence of 50% (vol/vol) organic solvents, including chloroform, toluene, *p*-xylene, and cyclooctane. The addition of methanol partially inhibited the enzyme. The addition of acetone and isopropanol markedly inactivated the enzyme.

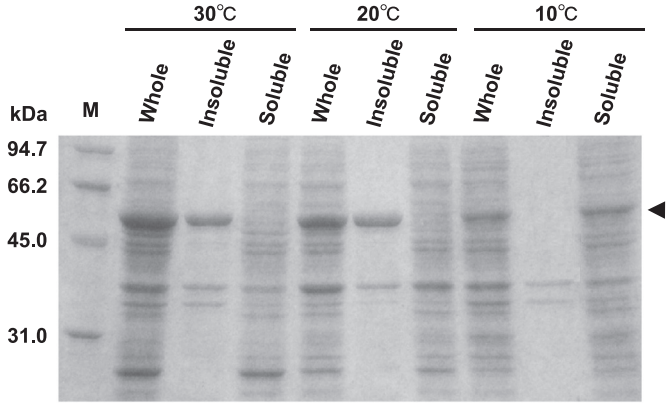

FIG. 3. SDS-PAGE analysis of soluble and insoluble fractions of the *E. coli* Rosetta harboring pETpacox grown at 10°C, 20°C or 30°C. The recombinant *E. coli* Rosetta strain was cultivated for about 3 h at 30°C. After the addition of IPTG, 48 h of additional incubation was performed at 10°C, 20°C or 30°C. The harvested cells were disrupted by sonication. The supernatant and precipitate of disrupted cells were collected by centrifugation as soluble and insoluble fractions, respectively. Samples containing about 0.02 mg proteins were applied on SDS-12.5% (wt/vol) polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue R-250 (CBB). Lane M, molecular size markers (kilodaltons); whole, whole disrupted cells without separation of the supernatant and precipitation by centrifugation; soluble, soluble fraction; insoluble, insoluble fraction. The arrowhead indicates the size of the recombinant cholesterol oxidase.

Enzyme kinetics and substrate specificity The enzyme exhibited oxidation activities with most 3β-hydroxysteroids (Table 2). The highest oxidation activity was obtained with cholesterol. The K_m and V_{max} values for cholesterol estimated from the Lineweaver–Burk plots were 92.6 ± 3.0 μM and 15.9 ± 1.0 μmol/min/mg of protein, respectively. β-Cholestanol and β-sitosterol were oxidized rapidly, as was cholesterol. Only minimal activity was observed with β-stigmasterol and pregnenolone as substrates. No activity was observed for epiandrosterone and dehydroepiandrosterone.

Analysis of the cholesterol oxidation product The R_f values were analyzed as described previously (26). The R_f values of the product with *n*-hexane:diethyl ether systems in the ratio of 3:1, 1:1, and 1:3 (vol/vol) were 0.56, 0.30, and 0.10, respectively. These R_f values of the product were the same as those of standard HCEO. The molecular mass of the product analyzed by FAB mass spectrometry was 417.3 (M + H), and the λ_{max} value was 249 nm. These results were also identical to those previously reported for HCEO (26).

DISCUSSION

Strain PA157, which produced a cholesterol oxidase, was isolated from humus soil by a method designed specifically to detect HCEO-forming cholesterol oxidase producers (24). Based on its morphological and physiological characteristics as well as its 16S rDNA sequence, strain PA157 was classified as *P. aeruginosa*. The characterization of cholesterol oxidase from *P. aeruginosa* has not been previously reported. This is the first report on the cloning, expression, and characterization of cholesterol oxidase from *P. aeruginosa*.

The amino acid sequence of PA157 cholesterol oxidase showed significant similarity to those of class II cholesterol oxidases not only from *P. aeruginosa* strains but also from other bacteria including *Chromobacterium* sp. DS-1, *Burkholderia* spp., *R. erythropolis* and *B. sterolicum*. Among class II enzymes, cholesterol oxidases from *Chromobacterium* sp. DS-1, *B. cepacia* ST-200 and *B. sterolicum* have been characterized (2,22,24,34). Among these enzymes, cholesterol oxidase from strain DS-1 was highly stable at high temperatures and in the presence of detergents and organic solvents relative to the other cholesterol oxidases (24).

The structures of the cholesterol oxidases from *B. sterolicum* and *Chromobacterium* sp. DS-1 have been determined by X-ray crystallography (35–37). These structures indicated that the FAD was covalently attached to the imidazole ND1 atom of a histidine residue corresponding to His121 of *B. sterolicum* oxidase and His107 of DS-1 oxidase. In the case of *B. sterolicum* oxidase, Glu475 and Arg477 were found to adopt two distinct conformations and play an important role in catalysis. This Glu–Arg pair was assumed to function as a gate for oxygen access (36). In addition, Glu475 was suggested to be the base for proton abstraction of the steroid C3-OH. Comparative amino acid sequence analysis showed that the

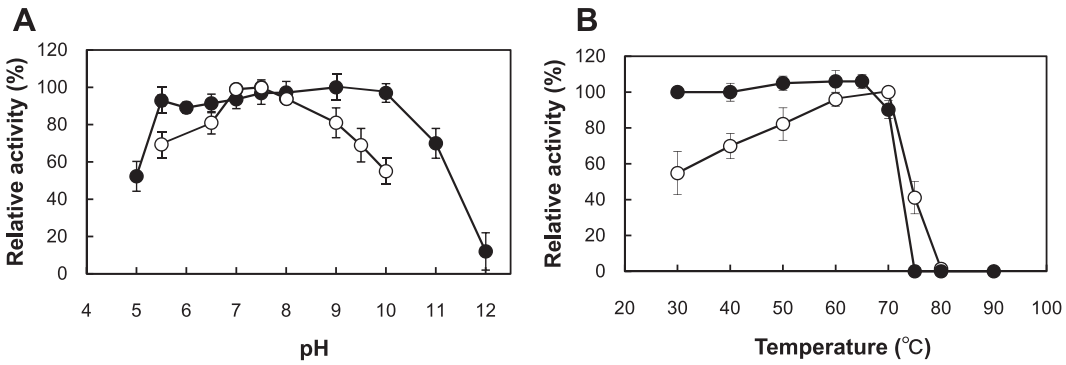

FIG. 4. Effects of pH and temperature on activity and stability of the recombinant cholesterol oxidase. (A) Effect of pH: Enzyme activity (open circles) was determined by monitoring the consumption of oxygen at 30°C. The pH stability (closed circles) was determined after incubation at 30°C for 1 h under the pH conditions indicated. The residual activity was examined by measuring H_2O_2 generation at 30°C. The buffer systems (100 mM) used were CH_3COOH–CH_3COONa (pH 5.0–5.5), 2-morpholinoethanesulfonic acid-NaOH (pH 5.5–7.5), Tris–HCl (pH 7.5–9.0), Na_2CO_3–$NaHCO_3$ (pH 9.0–11.0), and NaCl–NaOH (pH 11.0–12.0). (B) Effect of temperature: Enzyme activity (open circles) was determined by monitoring the HCEO formation at pH 7.0. Thermal stability (closed circles) was assayed after incubation of the enzyme (0.2 U/ml) dissolved in 100 mM phosphate buffer (pH 7.0) for 30 min at the temperatures indicated, and the relative activity was examined by measuring H_2O_2 generation at 30°C. Data are the means of experiments performed in triplicate. Error bars represent the standard deviation.

amino acid residues His-121, Glu-475 and Arg-477 of the *B. sterolicum* enzyme were conserved in the sequence of the PA157 enzyme as the corresponding amino acid residues His-120, Glu-458 and Arg-460, respectively.

In bacteria, there are two major export pathways for the transport of proteins across the cytoplasmic membrane (32). The general secretion (Sec) system transports proteins in a more or less unfolded state using energy provided by adenosine triphosphate (ATP) hydrolysis and the transmembrane proton electrochemical gradient. By contrast, the Tat system uses the transmembrane proton electrochemical gradient to achieve the export of folded proteins across the cytoplasmic membrane. The consensus motif (S/TRRxFLK) within the Tat signal sequence is essential for the secretion of proteins via this system (32,33). In this consensus motif, the consecutive arginine residues are almost invariant, the other amino acids are found with a frequency exceeding 50%, and x represents any polar amino acid (32). The Tat signal sequence consensus motif-like sequence SRRSFLA was found in the N-terminal sequence of the PA157 cholesterol oxidase, although the amino acid residue Lys in the ideal consensus motif was replaced by Gly in the sequence of PA157 cholesterol oxidase (Fig. 2). These consensus motif-like sequences were also found in the signal sequences of cholesterol oxidases from *B. cepacia* ST-200 (SRRAFLA between Ser-11 and Ala-17), *Chromobacterium* sp. DS-1 (SRRDFLT between Ser-13 and Thr-19) and *B. sterolicum* (SRRGFLT between Ser-11 and Thr-17).

Soluble and active recombinant class II cholesterol oxidases from *Chromobacterium* sp. DS-1, *B. cepacia* ST-200 and *B. sterolicum* were obtained from the recombinant *E. coli* grown at 25–30°C (23,25). In this study, a soluble and active enzyme could be obtained by cultivation of the recombinant clones not at 20°C or 30°C but at 10°C. The cultivation of a recombinant *E. coli* at reduced temperatures is a widespread technique for avoiding the *in vivo* aggregation of recombinant proteins and obtaining soluble and functional proteins (38). This approach was found to be effective in increasing the solubility of the PA157 cholesterol oxidase.

PA157 oxidase showed thermal stability up to 70°C after incubation for 30 min. The enzyme retained about 90% of its activity at 70°C after 30 min. The thermal stability of the PA157 enzyme was higher than those of commercially available cholesterol oxidases because most commercial cholesterol oxidases lost most of their original activity at 60–70°C after 30 min (4,25). We previously reported on *Chromobacterium* sp. DS-1 cholesterol oxidase, which

TABLE 1. Effects of detergents and organic solvents on the stability of the recombinant cholesterol oxidase.

Reagent group	Reagent[a]	Relative activity[b]
Detergent	None	100
	Tween 20	171 ± 26
	Triton X-100	177 ± 23
	Triton X-405	155 ± 17
	Sodium cholate	114 ± 14
	Sarcosyl	154 ± 16
	Emal 20CM	68 ± 5
	SDS	ND[c]
	LBS	ND
Organic solvent	None	100
	Methanol	65 ± 6
	Ethanol	31 ± 8
	Acetone	2 ± 0.4
	Isopropanol	6 ± 0.7
	Chloroform	89 ± 3
	Toluene	112 ± 1
	p-Xylene	102 ± 4
	Cyclooctane	108 ± 1

[a] Sarcosyl, sodium dodecyl sarcosinate; Emal 20CM, sodium polyoxyethylene alkyl ether sulfate; SDS, sodium dodecyl sulfate; LBS, sodium laurylbenzenesulfonate.

[b] To investigate enzymatic stability in the presence of detergents, the enzyme (0.2 U/ml) was incubated with 50 mM sodium phosphate (pH 7.0) containing each 0.5% detergent at 30°C for 1 h. The residual activity of the enzyme was measured by adding 25 µl of enzyme solution to 1 ml of the assay solution. To investigate enzymatic stability in the presence of organic solvents, each organic solvent (1 ml) was added to 2 ml of the enzyme solution containing 0.2 U/ml of each enzyme and 50 mM sodium phosphate (pH 7.0). This enzyme solution containing an organic solvent was incubated at 37°C for 24 h. In both cases, the residual activity of the enzyme was determined by adding 25 µl of enzyme solution to 1 ml of the assay solution for the measurement of H_2O_2 generation. Activity without heating in each detergent or organic solvent was defined as 100%. Results are mean values and standard deviations from three independent experiments.

[c] ND, not detected.

TABLE 2. Substrate specificity of cholesterol oxidase.

Substrate	Systematic name	Relative activity (%)[a]
Cholesterol	Cholest-5-en-3β-ol	100
β-Cholestanol	5α-Cholestan-5-en-3β-ol	96 ± 3
β-Sitosterol	Sitost-5-en-3β-ol	81 ± 5
β-Stigmasterol	Stigmast-5-en-3β-ol	42 ± 5
Pregnenolone	3β-Hydroxypregn-5-en-20-one	47 ± 4
Epiandrosterone	5α-Androstan-3β-ol-17-one	ND[b]
Dehydroepiandrosterone	3β-Hydroxyandrost-5-en-17-one	ND

[a] Enzyme activity was determined by measuring H_2O_2 generation. Activity obtained with cholesterol was defined as 100%. Results are mean values and standard deviations from three independent experiments.

[b] ND, not detected.

exhibits the highest thermal stability among all cholesterol oxidases reported so far (25). DS-1 cholesterol oxidase retained about 80% of its activity after incubation for 30 min at 85°C. The thermal stability of PA157 oxidase is second only to that of the DS-1 oxidase. Since cholesterol is an insoluble compound, detergents or organic solvents are often added to the reaction solution to act as a solubilizer. However, detergents or organic solvents often inactivate cholesterol oxidases as well as most enzymes. Most cholesterol oxidases were unstable in the presence of hydrophilic solvents (25). Commercially available cholesterol oxidases from *Nocardia erythropolis*, *Nocardia* sp., *Cellulomonas* sp. and *Streptomyces* sp. lost most of their activity with the addition of hydrophilic solvents such as methanol and ethanol. The stability of the PA157 oxidase against these solvents was higher than those of the commercial enzymes. In terms of its stability in detergents, PA157 oxidase was significantly activated by the addition of Tween 20, Triton X-100, sodium cholate, and sarcosyl. A slight activation of cholesterol oxidase due to the addition of detergents was found in other cholesterol oxidases (25). However, the extent of the activation of PA157 oxidase was greater than those of the other cholesterol oxidases. In terms of substrate specificity, PA157 oxidase did not oxidize epiandrosterone and dehydroepiandrosterone. By contrast, the cholesterol oxidases of *Chromobacterium* sp. DS-1 and *B. cepacia* ST-200 oxidized these two sterols (22,24). The PA157 oxidase slowly oxidized pregnenolone, although the DS-1 enzyme showed a significant preference for pregnenolone. The V_{max} value for cholesterol by PA157 cholesterol oxidase was 15.9 μmol/min/mg of protein. The V_{max} values of other cholesterol oxidases determined by a method similar to that used in this study and their sources were as follows: 10.4 μmol/min/mg, *Chromobacterium* sp. DS-1; 7.0 μmol/min/mg, *B. cepacia* ST-200; 5.2 μmol/min/mg, *Nocardia* sp.; 3.9 μmol/min/mg, *Streptomyces* sp. SA-COO; and 3.0 μmol/min/mg, *N. erythropolis* (25). Thus, PA157 cholesterol oxidase showed a higher V_{max} value than these enzymes.

There are two types of cholesterol oxidase that differ in terms of what they produce from cholesterol (CEO or HCEO) (26). Among HCEO-forming enzymes, the cloning of a cholesterol oxidase gene was reported only for *B. cepacia* strain ST-200 and *Chromobacterium* sp. DS-1. In addition to these two enzymes, it was suggested that PA157 cholesterol oxidase also produces HCEO from cholesterol in the present study.

The PA157 cholesterol oxidase is relatively stable at high temperatures and in the presence of various detergents or organic solvents. In addition, the enzyme exhibited a relatively high V_{max} value. Thus, the PA157 enzyme possessed features that are useful for clinical applications.

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