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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Cloning, sequence analysis, and expression of a gene encoding *Chromobacterium* sp. DS-1 cholesterol oxidase

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Abstract *Chromobacterium* sp. strain DS-1 produces an extracellular cholesterol oxidase that is very stable at high temperatures and in the presence of organic solvents and detergents. In this study, we cloned and sequenced the structural gene encoding the cholesterol oxidase. The primary translation product was predicted to be 584 amino acid residues. The mature product is composed of 540 amino acid residues. The amino acid sequence of the product showed significant similarity (53–62%) to the cholesterol oxidases from *Burkholderia* spp. and *Pseudomonas aeruginosa.* The DNA fragment corresponding to the mature enzyme was subcloned in the pET-21d(+) expression vector and expressed as an active product in *Escherichia coli*. The cholesterol oxidase produced from the recombinant *E. coli* was purified to homogeneity. The physicochemical properties were similar to those of native enzyme purified from strain DS-1. K_m and V_{max} values of the cholesterol oxidase were estimated from Lineweaver–Burk plots. The V_{max}/K_m ratio of the enzyme was higher than those of commercially available cholesterol oxidases. The circular dichroism spectral analysis of the recombinant DS-1 enzyme and *Burkholderia cepacia* ST-200 cholesterol oxidase showed that the conformational stability of the DS-1 enzyme was higher than that of *B. cepacia* ST-200 enzyme at higher temperatures.

Keywords Cholesterol oxidase · Cloning · Thermal stability · Organic solvent · Detergent · *Chromobacterium*

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Introduction

Cholesterol oxidase (EC 1.1.3.6) is a flavin adenin dinucleotide (FAD)-dependent enzyme that in most cases catalyzes the oxidation of cholesterol (cholest-5-en-3β-ol) using oxygen as an electron acceptor to form cholest-4-en-3-one (CEO) and hydrogen peroxide (Smith and Brooks 1974; Uwajima et al. 1974). On the other hand, some cholesterol oxidases from *Burkholderia cepacia* strain ST-200, *Pseudomonas* spp., and *Chromobacterium* sp. strain DS-1 oxidize cholesterol to 6β-hydroperoxycholest-4-en-3-one (HCEO) but not the CEO produced by most cholesterol oxidases (Doukyu and Aono 1999; Doukyu et al. 2008). Cholesterol oxidases have been isolated from various microorganisms such as *Arthrobacter* (Liu et al. 1988), *Brevibacterium* (Uwajima et al. 1974),

Burkholderia (Doukyu and Aono 1998), *Chromobacterium* (Doukyu et al. 2008), *Cellulomonas* (Srisawasdi et al. 2008), *Corynebacterium* (Shirokane et al. 1977), *Mycobacterium* (Smith et al. 1993), *Nocardia* (Richmond 1973), *Pseudomonas* (Lee et al. 1989), *Rhodococcus* (Johnson and Somkuti 1991), *Schizophyllum* (Fukuyama and Miyake 1979), *Streptomyces* (Tomioka et al. 1976), and *Streptoverticillium* (Inouye et al. 1982).

With only the exception of glucose oxidase, cholesterol oxidase is the most widely used enzyme in clinical laboratories. It is used for the determination of cholesterol concentrations in serum and other clinical samples and as a probe to study membrane structure (MacLachlan et al. 2000). In addition, cholesterol oxidase shows insecticidal activity that is a vital part of pest control strategies employing transgenic crops (Cho et al. 1995; Purcell et al. 1993). Moreover, cholesterol oxidase has been used for the optical resolution of non-steroidal compounds and allylic alcohols (Biellmann 2001; Dieth et al. 1995) and for the bioconversion of a number of 3β-hydroxysteroids in the presence of organic solvents (Aono and Doukyu 1996; Aono et al. 1994; Kazandjian et al. 1986). Since cholesterol is an insoluble compound, detergents or organic solvents are often added to the reaction solution to act as a solubilizer, and because detergents or organic solvents often inactivate cholesterol oxidases as well as most enzymes (Doukyu and Aono 2001; Isobe et al. 2003; Pollegioni et al. 1999), a detergent- and organic solvent-tolerant cholesterol oxidase would be useful for the applications described above. A detergent- and organic solvent-tolerant cholesterol oxidase has been reported from *B. cepacia* strain ST-200 (Doukyu and Aono 1998; Doukyu and Aono 2001), and a detergent-tolerant cholesterol oxidase has been reported from γ-Proteobacterium Y-134 (Isobe et al. 2003).

We previously reported the screening and purification of an extracellular cholesterol oxidase from *Chromobacterium* sp. strain DS-1 and listed some of its properties (Doukyu et al. 2008). This was the first report of cholesterol oxidase from the genus *Chromobacterium*. Most cholesterol oxidases from various bacterial sources such as *Brevibacterium* sp., *Streptomyces* sp., *Cellulomonas* sp., *Nocardia* sp., *Nocardia erythropolis*, *Pseudomonas fluorescens*, and *B. cepacia* ST-200 lost most of their activity after incubation for 30 min at 60–80°C (Doukyu and Aono 2001; Doukyu et al. 2008). By contrast, the enzyme from strain DS-1 retained 80% of its original activity even at 85°C after 30 min. As far as we can tell, cholesterol oxidase from strain DS-1 exhibits the highest thermal stability among all cholesterol oxidases reported so far. Furthermore, DS-1 cholesterol oxidase was more stable in the presence of various organic solvents and detergents than commercially available cholesterol oxidases, including a detergent- and organic solvent-tolerant cholesterol oxidase from *B. cepacia* ST-200. Cholesterol oxidase from strain DS-1 oxidized cholesterol to HCEO. A gene sequence of an HCEO-forming cholesterol oxidase has been reported only from *B. cepacia* strain ST-200 (Doukyu and Aono 2001); no other gene sequence of HCEO-forming cholesterol oxidase has been reported so far.

In this paper, we report the cloning of a cholesterol oxidase gene from *Chromobacterium* sp. strain DS-1, expression of the gene in *Escherichia coli*, and the purification and partial characterization of the enzyme produced by the recombinant *E. coli*. This is the first report concerning cloning of a gene encoding cholesterol oxidase from the genus *Chromobacterium*.

Materials and methods

Chemicals and enzymes

ExTaq polymerase and PrimeSTAR HS DNA polymerase were purchased from Takara Bio (Kyoto, Japan). Commercially available cholesterol oxidases were obtained as follows: *N. erythropolis* (Fluka, Steinheim, Germany), *Nocardia* sp. (Calbiochem, San Diego, CA, USA), *P. fluorescens* (Sigma Chemicals, St Louis, MO, USA), *Streptomyces* sp. SA-COO (Toyobo, Tsuruga, Japan). Cholesterol oxidase from *B. cepacia* strain ST-200 was purified as described previously (Doukyu and Aono 2001) and used in this study. Triton X-100 was purchased from Sigma Chemicals; Tween 20, sodium dodecyl sarcosinate (sarcosyl), and sodium dodecyl sarcosinate (SDS) were purchased from Wako Pure Chemical Industries, Osaka, Japan; and sodium polyoxyethylene alkyl (C12–13) ether sulfate (Emal 20CM) was obtained from Kao Corp., Tokyo, Japan. Organic solvents used were of the highest quality commercially available.

Strains and media

Chromobacterium sp. strain DS-1 (Doukyu et al. 2008) was used as the source of DNA encoding the cholesterol oxidase. The vector plasmid, pHSG398, was purchased from Takara Bio (Kyoto, Japan). pBluescript II SK+ (pBSII) was purchased from Toyobo Biochemical (Osaka, Japan). pET-21d(+) was produced by Novagen (Madison, WI, USA). *E. coli* DH5α (*supE44*, Δ*lac*U169 (φ80*lacZ*Δ*M15*), *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*) was used for cloning and expression of the gene. *E. coli* Rosetta (DE3) pLysS [F^-, *omp*T, *hsd*S_B(R_B^- m_B^-), *gal*, *dcm*, *lacYI* (DE3), pLysSRARE (Cam^R)] (Novagen) was used for expression of the gene. *E. coli* strains were grown in modified Luria broth (LB medium) consisting of 1% Bacto tryptone (Difco Laboratories, Detroit, MI, USA),

0.5% Bacto yeast extract (Difco), and l% NaCl. When necessary, the medium was solidified with 1.5% (wt vol^{-1}) agar and supplemented with appropriate antibiotics. LB agar medium supplemented with 64 mM sodium cholate, 0.3% Triton X-100, and 0.9 mM cholesterol (LBC agar medium) was also used for the isolation of a transformant containing the cholesterol oxidase gene.

Amino acid sequence

Extracellular cholesterol oxidase from strain DS-1 was purified from the culture supernatant (Doukyu et al. 2008).The amino acid sequence was determined using an automated protein sequencer (model G1005A; Hewlett-Packard, Palo Alto, CA, USA). For determination of the amino acid sequence of internal regions of the cholesterol oxidase, the purified enzyme was digested with lysyl endopeptidase from *Achromobacter lyticus* M497-1 (Wako Pure Chemical Industries). The digestion mixture was analyzed by SDS-PAGE. Peptides were electroblotted from the gel onto a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA, USA). The N-terminal amino acid sequences of selected peptides were determined as described above.

Genetic analysis

DNA manipulations, including preparation of plasmids, restriction enzyme digestion and ligation, and transformation of *E. coli*, were carried out by standard methods (Sambrook et al. 1989). Southern hybridization was performed by means of a DNA labeling and detection kit (Roche, Basel, Switzerland). Nucleotide sequences of the cloned DNA fragments were determined with a DNA sequencing system (Prism 377; Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The dideoxy chain termination method was used to sequence the gene. The DNA and the predicted protein sequences were analyzed using the BLAST (Altschul et al. 1990) network service from the National Center for Biotechnology Information. The phylogenetic tree based on the comparison of deduced amino acid sequences was constructed using the neighbor-joining method with GENETYX software (GENETYX Co., Tokyo, Japan).

PCR conditions for cloning of the cholesterol oxidase gene

PCR primers, primer 1, 5′-CCIAA(C/T)AA(C/T)TT(C/T)CCIGCIGA(A/G)AT(A/C /T)CC-3′ and primer 2, 5′-GTIACIC(T/G)IA(A/G)IGTIGTIGG(C/T)TTIAC(A/G)TA-3′, were designed based on N-terminal and internal amino acid sequences of the cholesterol oxidase, respectively. A DNA fragment (1 kb) was amplified from the chromosomal DNA of strain DS-1 by PCR using ExTaq polymerase and the primers. Amplification (30 cycles of 1 min at 96°C for denaturation, 1 min at 45°C for annealing, 1 min at 72°C for extension) was carried out using the GeneAmp PCR system 2400 (Perkin-Elmer Applied Biosystems). The amplified fragment was blunted and ligated into the *Sma*I site of pHSG398.

For identification of the entire nucleotide sequence of the cholesterol oxidase gene, the chromosomal DNA was digested with *Sma*I and ligated with T4 DNA ligase to form circularized DNA. Then, inverse PCR was performed to amplify a fragment using ExTaq polymerase, the *Sma*I-self-circularized DNA molecule as a template, and appropriate primers P-S, 5′-GACAACCTGCCCGACGAAGT-3′ and P-AS, 5′-CGCACCTTATAACCGTTGTC-3′. These primers were designed based on the nucleotide sequence of the DNA insert ligated into the *Sma*I site of pHSG398. The PCR conditions were as follows: 1 min at 94°C for denaturation, 1 min at 50°C for annealing, 2 min at 72°C for extension, for 30 cycles.

For isolation of the entire cholesterol oxidase gene, primers were designed according to the determined sequence. PCR was performed to amplify the fragment, including the entire cholesterol oxidase gene using PrimeSTAR HS DNA polymerase with high fidelity, genomic DNA as the template, and primers CO-S, 5′-GGTCTAGATAGCTAAATCAGCGATCCGC-3′ (*Xba*I site underlined) and CO-AS, 5′-GGGGTACCCGGCGAATACCGCTTCGAAA-3′ (*Kpn*I site underlined). The PCR conditions were as follows: 1 min at 94°C for denaturation, 1 min at 52°C for annealing, 2 min at 72°C for extension, for 30 cycles. The amplified fragment was inserted into the same sites in pBSII SK+.

Construction of pETcox

The cholesterol oxidase gene was amplified from pBScox by designing a set of primers with incorporated restriction enzyme sites *Nco*I/*Bam*HI (Fig. 1). The primers COR-S: 5′-TACCATGGCTACTTGCAGCCAACCCAATAA -3′ (*Nco*I site underlined) and COR-AS: 5′-AAGGATCCTTAGCTCGCGAGATCGTAAA -3′ (*Bam*HI site underlined) were used to clone a DNA fragment encoding the complete cholesterol oxidase gene except the signal peptide. As a consequence of the introduction of *Nco*I site, the recombinant enzyme contained an extra Met-Ala at the N-terminal end. The amplified gene and plasmid pET-21d (+) were digested with the same restriction enzymes, *Nco*I and *Bam*HI. The ligated plasmid (pETcox) was used to transform the *E. coli* Rosetta strain.

Purification of the recombinant cholesterol oxidase

The *E. coli* Rosetta strain harboring pETcox was grown in 100 ml of LB medium supplemented with 100 μg ml^{-1} ampicillin and 25 μg ml^{-1} chloramphenicol on a rotary

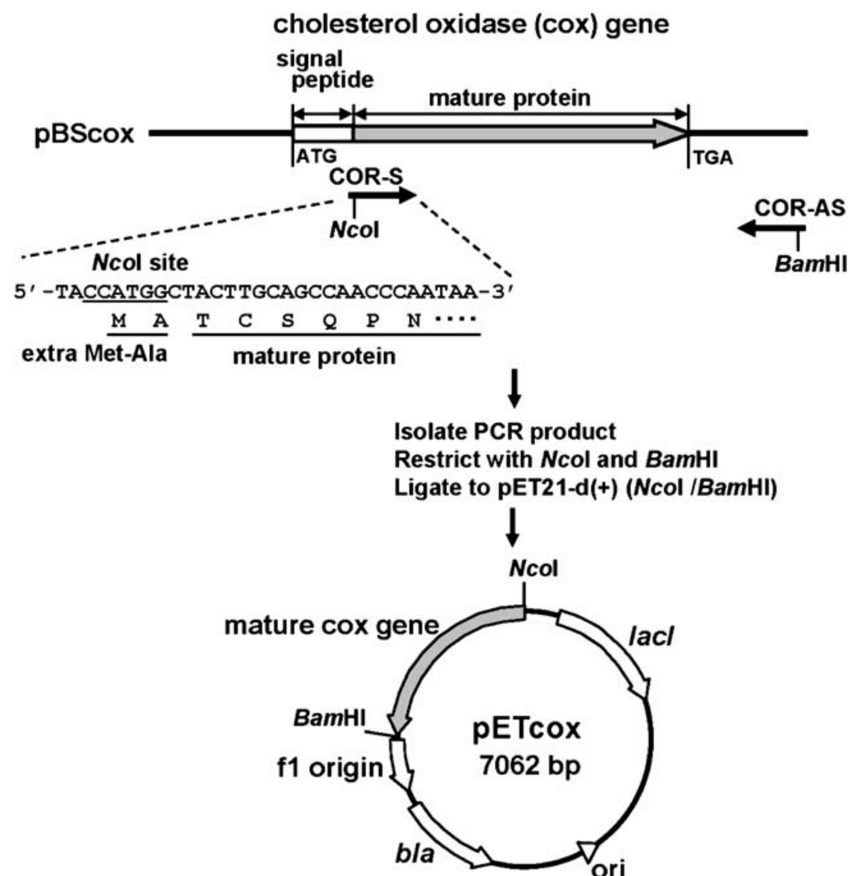

Fig. 1 Scheme of the construction of pETcox derived from the expression vector pET21-d(+)

shaker (120 rpm) at 30°C. The expression of the cholesterol oxidase gene was induced by adding 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) when the cell density reached an A_{600} of ca. 0.5. After incubation during 12 h at 30°C, the cells were harvested by centrifugation (6,000×*g*, 15 min, 4°C) and resuspended with 10 ml of 10 mM Tris–HCl (pH 8.0). The resuspended cells were lysed using an ultrasonic disruptor UD-200 (Tomy Seiko, Tokyo, Japan) at 60 W intermittently in an ice bath. The sonicated lysate was cleared by centrifugation (10,000×*g*, 10 min, 4°C), and then the cleared cell lysate was heated at 70°C for 30 min and centrifuged (8,000×*g*, 20 min, 4°C). The supernatant was loaded on a column (2.5 by 8 cm) of DEAE-cellulose DE52 (Whatman, Maidstone, England) equilibrated with the Tris–HCl buffer. The column was washed with 100 ml of the Tris buffer and then eluted with a linear gradient of NaCl concentrations of 0 to 250 mM in 400 ml of the Tris buffer. The fractions with the cholesterol oxidase activity were pooled.

Determination of protein concentration

The protein concentration was determined using the method of Bradford (1976) and bovine serum albumin as the standard.

SDS-polyacrylamide gel electrophoresis of proteins

Samples were dissolved in a solution containing 1% (wt vol^{-1}) sodium dodecyl sulfate (SDS), 2.5% (vol vol^{-1}) β-mercaptoethanol, 30% (vol vol^{-1}) glycerol, and 30 mM Tris–HCl (pH 6.8) and heated in a boiling water bath for 5 min. The samples were run on a SDS-polyacrylamide gel as described by Laemmli (1970).

Assay of the cholesterol oxidase activity

Cholesterol oxidation activity was assayed by measuring H_2O_2 generation accompanying the oxidation of cholesterol as described previously (Doukyu and Aono 1998). One unit of enzymatic activity was defined as the amount required to oxidize 1 μmol cholesterol min^{-1} at 30°C. Unless otherwise stated, this is the method we used for the determination of enzyme activity in this study. To investigate the optimum pH for the enzyme activity, we estimated the cholesterol oxidase activity by monitoring the consumption of oxygen, and to investigate the optimum temperature for the enzyme activity, we estimated the cholesterol oxidase activity by measuring the A_{249} of λ_{max} value of HCEO, as described previously (Doukyu et al. 2008).

Determination of K_m and V_{max} values of the cholesterol oxidase

Cholesterol oxidation activity was assayed by measuring H_2O_2 generation. The K_m and V_{max} values were estimated from Lineweaver–Burk plots of data obtained with the assay solution containing 0 to 1 mM cholesterol.

CD spectral analysis of the cholesterol oxidase

The circular dichroism (CD) spectra analysis of the cholesterol oxidase was conducted using a J-820 spectropolarimeter (JASCO, Tokyo, Japan). The measurement of CD spectra from 195 to 250 nm was performed at each temperature (60–95°C at 5°C intervals) using a 0.1-cm path-length cell. The concentration was set at 0.2 mg ml^{-1}. The top of the cell was completely closed using a cap to minimize evaporation. Data pitch, bandwidth, response, scanning speed, and accumulation were set at 0.1 nm, 1 nm, 8 s, 50 nm per min, and four times, respectively. The secondary structure was analyzed using three component model (α-helix, turn, and random coil) reference spectra (Yang et al. 1986).

Nucleotide sequence accession number

The nucleotide sequence of the cholesterol oxidase gene of strain DS-1 has been deposited in the GenBank/EMBL/DDBJ database under the accession number AB456533.

Results

Cloning of the cholesterol oxidase gene

The N-terminal amino acid sequence of the cholesterol oxidase purified from culture supernatant was TCSQPNNFPAEI PLYKQSFKN as described previously (Doukyu et al. 2008). The enzyme was cleaved with lysyl endopeptidase, and the resulting peptides were fractionated by SDS-PAGE. Among several peptides separated on the gel, the N-terminal amino acid sequence of 22 kDa was determined. The sequence obtained was NLLLYVKPTTLRVTANGYAV. The sense primer (primer 1) and the antisense primer (primer 2) were designed based on the determined N-terminal amino acid sequences of the mature enzyme and the 22-kDa peptide, respectively. Inosine was incorporated into the primers at positions of four-base redundancy to reduce the complexity of the primer mixtures. A DNA fragment of about 1 kb was amplified from the chromosomal DNA of strain DS-1 by PCR with a combination of primer 1 and primer 2. The amplified fragment was ligated into the *Sma*I site of pHSG398. This plasmid was designated pHSGcox. The nucleotide sequence of the insert was analyzed to confirm that the amplified DNA corresponded to the determined amino acid sequences.

The DNA inserted in pHSGcox was digested with *Bam*HI and *Sac*I at both of the sites derived from the original plasmid pHSG398. The excised fragment (1 kb) was recovered and labeled with digoxigenin. Chromosomal DNA of DS-1 was digested with various restriction enzymes and analyzed by Southern hybridization, using the digoxigenin-labeled DNA as a probe, to assist in the choice of restriction enzymes for the inverse PCR. The results allowed us to create a physical map of the vicinity of the cholesterol oxidase gene (data not shown).

Chromosomal DNA of strain DS-1 digested with *Sma*I showed a single hybridization band of 3 kb. DNA fragments of 2–4-kb long were recovered from the *Sma*I digest of the chromosomal DNA and were ligated to form a self-circularized DNA molecule. A DNA fragment of 2.1 kb was amplified from the self-circulated molecules as the template and primers P-S and P-AS by inverse PCR. The sequence of the 2.1-kb DNA fragment included the sequence of 5′ and 3′ flanking regions of the cholesterol oxidase gene. The fragment (2.0 kb) containing the entire cholesterol oxidase gene was amplified using chromosomal DNA as the template and the primers CO-S and CO-AS and inserted into the *Xba*I/*Kpn*I sites of pBSII in the same direction as *Plac* of the vector. *E. coli* DH5α was transformed with the resulting plasmids, pBScox. A clone, grown on LBC agar containing ampicillin, formed a turbid halo around its colony (Fig. 2) and contained a recombinant plasmid with a 2.0-kb *Xba*I/*Kpn*I insert. The halo was formed because of the low solubility of HCEO generated by the cholesterol oxidase from cholesterol, as described previously (Doukyu and Aono 2001; Doukyu et al. 2008).

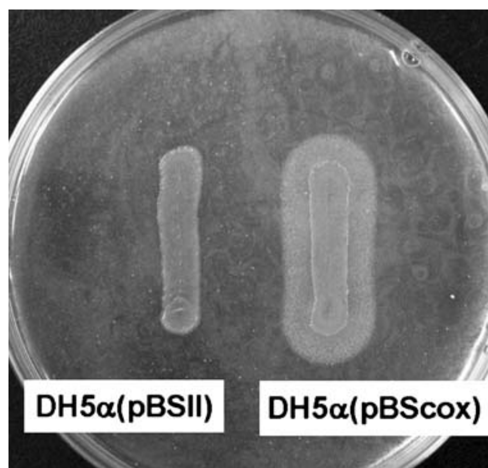

Fig. 2 Turbid halo around colonies of *E. coli* DH5α carrying pBScox. *E. coli* DH5α cells carrying pBluescript II SK+ (*left side*) or pBScox (*right side*) were grown on LBC agar containing 50 μg ml^{-1} ampicillin at 30°C overnight

Nucleotide sequence of the cholesterol oxidase gene

The nucleotide sequence of the cholesterol oxidase gene contains an open reading frame of 2,004 bp within a TAG at nucleotide position 295 and a TAG codon at nucleotide position 2,164 (Fig. 3). We found a structural gene of 1,755 bp, coding for a polypeptide consisting of 584 amino acid residues, with the ATG initiation codon at nucleotide position 412 and the TGA termination codon at position 2,164. A potential ribosome-binding site (GGAG) was observed seven bases upstream of this ATG. The nucleotide sequence of the structural gene coded for the N-terminal and the internal amino acid sequences that were determined for the cholesterol oxidase purified from DS-1. The N-terminal amino acid sequence showed that the signal peptide was proteolytically processed between Leu-44 and Thr-45 upon excretion and secretion in strain DS-1. The mature polypeptide consisted of 540 amino acid residues, with a molecular mass of 58,424 Da. This is nearly 58 kDa, the mass of the native enzyme as previously estimated by SDS-PAGE (Doukyu et al. 2008).

Sequence comparison of cholesterol oxidases

We found several amino acid sequences that showed significant similarities to DS-1 cholesterol oxidase via a BLAST search. An alignment of the amino acid sequence of DS-1 cholesterol oxidase with corresponding sequences from other bacterial cholesterol oxidases was used to construct the phylogenetic tree (Fig. 4). DS-1 cholesterol oxidase displayed relatively high similarity to the cholesterol oxidases (53–62%) from *Burkholderia* spp. and *Pseudomonas aeruginosa* strains (Table 1). A lower similarity was found in cholesterol oxidases from *Streptomyces ambofaciens* (47%), *Rhodococcus erythropolis* (44%), and *Brevibacterium sterolicum* (42%).

Expression and purification of the recombinant cholesterol oxidase

Overexpression of the recombinant cholesterol oxidase was attempted by using the pET system. The nucleotide sequence of DS-1 cholesterol oxidase contained codons

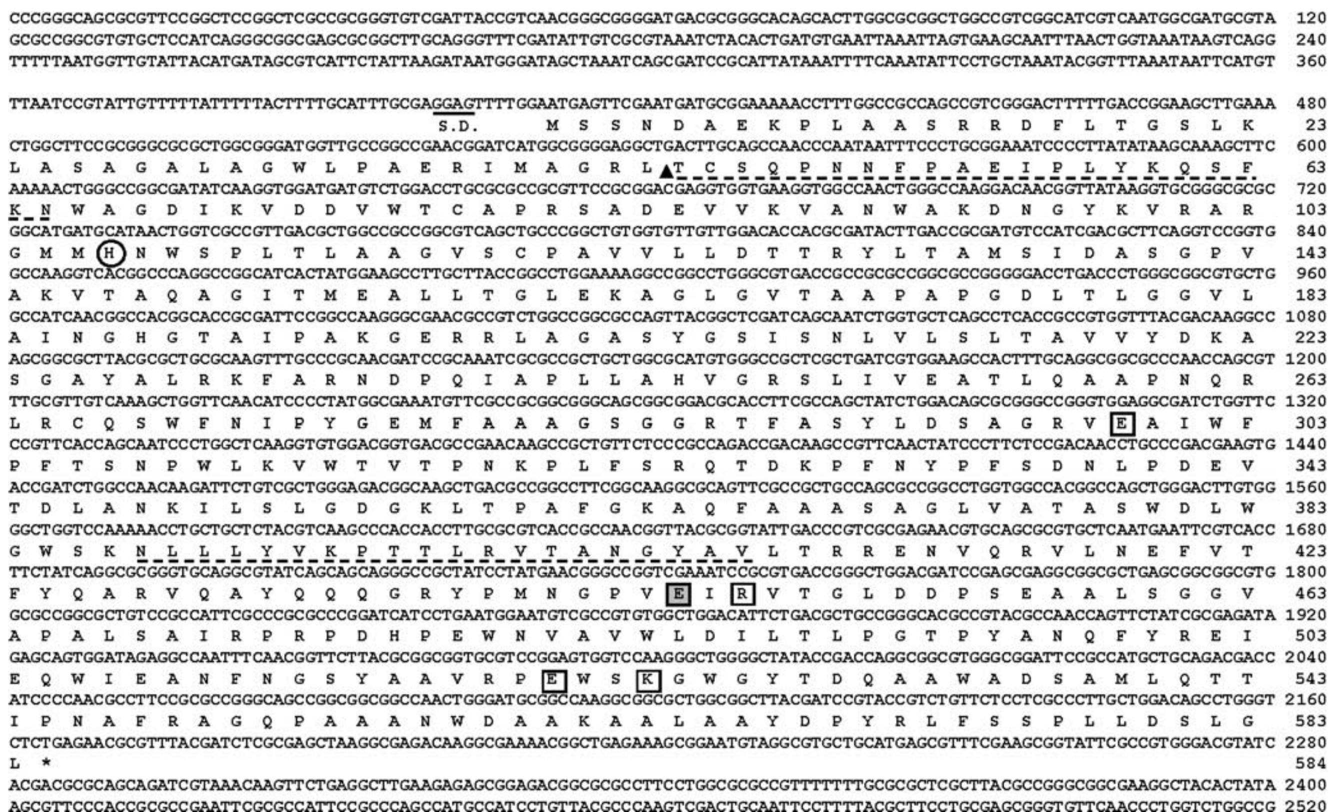

Fig. 3 Nucleotide sequence of the cholesterol oxidase gene from strain DS-1 and the deduced amino acid sequence. *Numbers on the right* denote nucleotide positions and amino acid positions. The N-terminal and the internal amino acid sequences determined through analysis of the mature cholesterol oxidase from strain DS-1 are *underlined with dotted lines*. The *vertical arrowheads* indicate the cleavage sites of the enzyme in strain DS-1. The Shine–Dalgarno sequence (SD) is *underlined*. A putative histidine residue (His107) that was covalently bound to a FAD cofactor is *enclosed in a circle*. A putative glutamic acid residue, Glu445, that may act as the base for both the oxidation and the isomerization steps of the catalytic reaction is *enclosed in a shadowed box*. Putative hydrophilic amino acid residues lining the cavity near the FAD cofactor (Glu299, Arg447, Glu518, and Lys524) are *enclosed in a box*, respectively

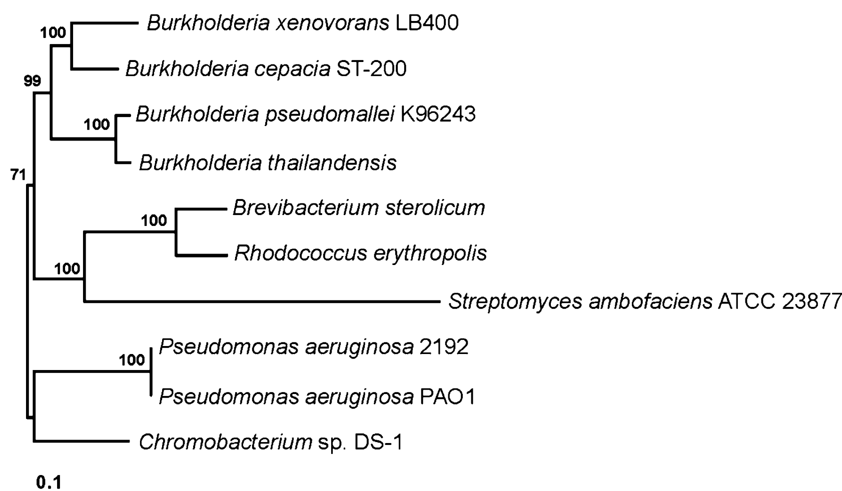

Fig. 4 Phylogenetic tree of homologues of the cholesterol oxidase from strain DS-1. A phylogenetic tree based on the comparison of the deduced amino acid was constructed using the neighbor-joining method. The scale bar represents 0.1 substitutions per amino acid position. Bootstrap values greater than 40% are shown as percentages on branches

(ten of Pro codon ccc, four of Ile codon gga, one of Arg codon agg, and one of Arg codon cga) rarely used in *E. coli*. The translation of a gene might be limited by the codon usage. Therefore, we used an *E. coli* Rosetta host strain, BL21 derivative, that was able to supply tRNAs for these rare codons on a compatible plasmid. We confirmed that there was no mutation in the cholesterol oxidase gene on the pETcox by resequencing. The cholesterol oxidase activity of the sonicated lysate of *E. coli* Rosetta harboring pETcox grown on LB medium supplemented with antibiotics and IPTG was 2.28 U mg^{-1}. This activity was about fivefold higher than that (0.45 U mg^{-1}) of *E. coli* DH5α harboring pBScox grown under the same conditions. Table 2 summarizes the purification steps used. High thermal stability of DS-1 enzyme made it possible to easily remove most proteins from *E. coli* by the heat treatment at 70°C. The cholesterol oxidase activity passed through the DE52 column. This step was effective for purifying the enzyme. In the end, the enzyme was purified 7.3-fold from the cell lysate. The specific activity of the purified cholesterol oxidase was 16.7 U mg^{-1} of protein. This value was slightly higher than that observed for the purified enzyme of strain DS-1 (13.9 U mg^{-1}; Doukyu et al. 2008). The purified preparation gave a single band by SDS-PAGE (Fig. 5). The molecular mass was estimated to be 58 kDa. The N-terminal amino acid sequence of the expressed protein was ATCSQPNN. This result indicated that the methionine of the N-terminal of the recombinant enzyme seemed to be removed by methionyl aminopeptidase of *E. coli*.

Prosthetic groups

The recombinant enzyme solution was yellow, like a typical flavoprotein, exhibiting two absorption maxima, at 355 and 450 nm. Most cholesterol oxidases contain 1 mol of FAD per mole of protein as a prosthetic group (Coulombe et al. 2001; Doukyu and Aono 1998; Kamei et al. 1978; Uwajima et al. 1974). A solution of the recombinant enzyme (1.0 mg ml^{-1}) showed an absorbance of 0.198 at 450 nm. In this solution, the molarity of the protein was estimated to be 19.0 mM based on the molecular mass (58 kDa) determined by SDS-PAGE. The molar adsorption coefficient of FAD (ε=11.3×10^3 M^{-1} cm^{-1}) was employed to calculate the concentration of FAD, 17.4 mM. This result

Table 1 Comparison of the amino acid sequence of the DS-1 cholesterol oxidase with corresponding sequences from GenBank

Original organism	Protein	Identity (%)	Accession number
Burkholderia cepacia ST-200	Cholesterol oxidase	61.6	Q93RE1
Burkholderia pseudomallei K96243	Putative uncharacterized protein	58.5	3023444AKG
Burkholderia xenovorans LB400	Putative uncharacterized protein	57.8	Q13UN0
Pseudomonas aeruginosa 2192	Putative uncharacterized protein	56.2	A3LFN5
Pseudomonas aeruginosa PAO1	Putative uncharacterized protein	56.0	PA4140
Burkholderia thailandensis	Putative uncharacterized protein	53.1	Q2T0X3
Streptomyces ambofaciens ATCC 23877	Putative uncharacterized protein	46.9	A3KHX8
Rhodococcus erythropolis	Putative uncharacterized protein	43.6	A9QAE7
Brevibacterium sterolicum	Cholesterol oxidase (BCO2[a])	41.9	Q7SID9

[a] Two forms of cholesterol oxidase have been identified in *Brevibacterium sterolicum*, one containing the FAD cofactor non-covalently bound to the enzyme (BCO1) and the other containing the cofactor covalently linked to the enzyme (BCO2)

Table 2 Summary of the purification procedure for the recombinant cholesterol oxidase

Step	Total activity (U)	Total protein (mg)	Specific activity[a] (U mg^{-1})	Purification (fold)	Yield (%)
Cell lysate[b]	185	81.1	2.28	1	100
Heat treatment at 70°C	123	21.5	5.72	2.5	66
DEAE-cellulose DE52	74.0	4.43	16.7	7.3	40

[a] Cholesterol oxidation activity was assayed by measuring H_2O_2 generation
[b] Cell lysate was obtained from 100 ml of the culture of *E. coli* Rosetta harboring pETcox

indicating that the recombinant enzyme contained 1 mol of FAD per mole of protein was similar to that of the enzyme purified from strain DS-1 (Doukyu et al. 2008).

Physicochemical properties of the recombinant cholesterol oxidase

The enzyme was most active at pH 7.0 to 7.5 (Fig. 6a). The recombinant enzyme was stable from pH 3.0 to 11 after incubation for 1 h at 30°C (Fig. 6a). The optimum temperature at pH 7.0 was 65°C (Fig. 6b). Thermal stability was examined by incubating the enzyme in sodium phosphate (pH 7.0) buffer at various temperatures for 30 min (Fig. 6b). The enzyme was stable at temperatures from 4°C to 85°C. The enzyme retained about 79% and 27% of its activity after incubation for 30 min at 85°C and 90°C, respectively. However, the enzyme lost almost all activity after 30 min at 100°C.

We examined the effect of metal ions on the cholesterol oxidase activity by measuring enzyme activity at 30°C for 1 h in the presence of various metal ions. At a concentration of 1 mM, Ag^{+}, Ca^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, and Fe^{2+} scarcely influenced the enzyme activity. However, the addition of 1 mM Zn^{2+} reduced the activity to 68% of that without a metal ion. A chelating agent, EDTA, did not show a significant inhibitory effect on the enzyme activity.

The enzyme was stable in the presence of 0.5% (wt vol^{-1}) detergents, including Tween 20, Triton X-100, sodium cholate, sarcosyl, and Emal 20CM after 1 h at 45°C (Table 3). In the presence of SDS, the enzyme activity was only 12% of that without a detergent. The enzyme was stable also in the presence of 50% (vol vol^{-1}) organic solvents, including dimethylsulfoxide, methanol, ethanol, isopropanol, ethyl acetate, butanol, chloroform, toluene, *p*-xylene, and cyclooctane after 24 h at 37°C. Treatment with acetone markedly inactivated the enzyme.

The pH and temperature activity and stability profiles, the activity in metal ions, and the stability in the presence of detergents and organic solvents of the recombinant enzyme were similar to those of native enzyme purified from strain DS-1 (Doukyu et al. 2008).

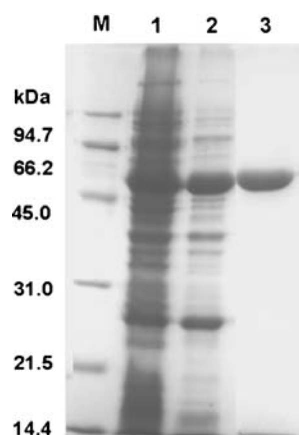

Fig. 5 SDS-PAGE of cholesterol oxidase during the purification procedure. Samples containing 0.04 U of cholesterol oxidase were applied on SDS-12.5% (wt vol^{-1}) polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue R-250 (CBB). *Lane M* molecular size markers (kilodaltons), *lane 1* cleared sonicated lysate, *lane 2* cleared lysate after the heat treatment at 70°C, *lane 3* fraction from the DEAE-cellulose DE52 column

K_m and V_{max} values of cholesterol oxidases

We estimated the K_m and V_{max} values of the recombinant DS-1 enzyme and cholesterol oxidases from other bacterial origins from Lineweaver–Burk plots (Table 4) and found that the values were similar to those of the native enzyme purified from strain DS-1 (Doukyu et al. 2008). The K_m value of the recombinant enzyme was lower than those of the enzymes from *B. cepacia* ST-200, *P. fluorescens*, and *Streptomyces* sp. SA-COO, but not those from *Nocardia* species. The V_{max} value and V_{max}/K_m value (which was tentatively regarded as an enzyme efficiency) of the recombinant enzyme were the highest among the enzymes tested.

CD spectral analysis

The amino acid sequence of DS-1 cholesterol oxidase showed significant similarity to that of cholesterol oxidase from *B. cepacia* ST-200 (Table 1). The enzyme from *B. cepacia* ST-200 was stable from 4°C to 70°C but lost almost all activity after 30 min at 80°C, as reported previously (Doukyu et al. 2008). Therefore, the thermal stability of DS-1 cholesterol oxidase was higher than that of the enzyme from *B. cepacia* ST-200. The conformational transitions of the recombinant DS-1 enzyme and *B. cepacia* ST-200 enzyme were monitored by measuring the CD spectra at various temperatures (Fig. 7), and small changes of the CD spectra of DS-1 cholesterol oxidase were encountered at temperatures between 85°C and 90°C.

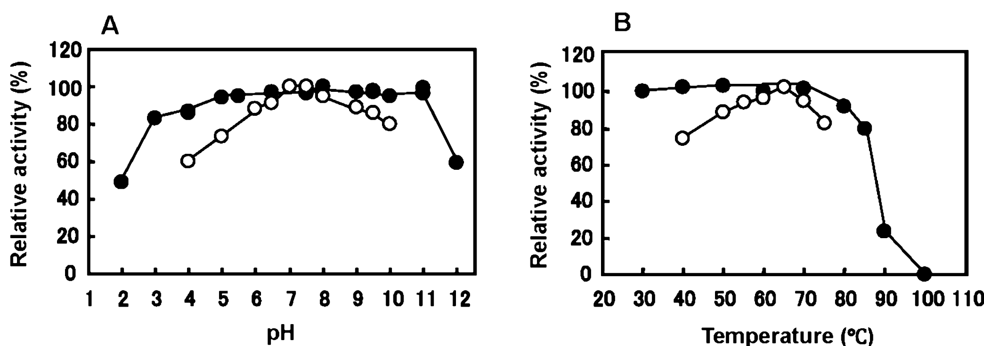

Fig. 6 Effects of pH and temperature on activity and stability of the recombinant cholesterol oxidase. **a** Effect of pH: enzyme activity (*open circles*) was assayed by following the consumption of oxygen at 30°C under the various pH conditions indicated in the figure. The pH stability (*closed circles*) was assayed after incubation at 30°C for 1 h under the various pH conditions indicated. The residual activity was examined by monitoring H_2O_2 generation at 30°C. The buffer systems (100 mM) used were glycine–HCl (pH 2.0–3.0), citrate–sodium citrate (pH 3.0–4.0), CH_3COOH–CH_3COONa (pH 5.0–5.5), NaH_2PO_4–Na_2HPO_4 (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 assayed by following the formation of HCEO at pH 7.0 at the temperatures indicated in the figure. Thermal stability (*closed circles*) was assayed after incubation of the enzyme (0.2 U ml^{-1}) dissolved in 100 mM phosphate buffer (pH 7.0) for 30 min at the temperatures indicated, and the relative activity was assayed by monitoring H_2O_2 generation at 30°C

Table 3 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	102±4
	Triton X-100	102±2
	Sodium cholate	100±1
	Sarcosyl	110±5
	Emal 20CM	107±10
	SDS	12±2
Organic solvent	None	100
	Dimethylsulfoxide	99±2
	Methanol	108±6
	Ethanol	111±8
	Acetone	15±10
	Isopropanol	116±5
	Ethyl acetate	98±3
	Butanol	99±5
	Chloroform	115±6
	Benzene	112±12
	Toluene	110±6
	p-Xylene	102±10
	Cyclooctane	102±6

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

[b] For the investigation of enzymatic stability in the presence of detergents, cholesterol oxidase solution (0.2 U ml^{-1} in 50 mM sodium phosphate [pH 7.0]) containing each 0.5% detergent was incubated at 45°C for 1 h. For the investigation of enzymatic stability in the presence of organic solvents, each organic solvent (1 ml) was added to 2 ml of the enzyme solution described above. The mixture was incubated at 37°C with shaking for 24 h. In both cases, the residual activity in the buffer was measured by following the formation of H_2O_2. The relative residual activity was calculated as a percentage of the enzyme activity without heating in each detergent or organic solvent. Mean values and standard deviations for three independent experiments are shown

Further increases in temperature had a significant impact on protein structure. The percentages of α-helix, β-sheet, turn, and random coil of DS-1 enzyme were as follows: at 60°C, 19.7%, 48.0%, 3.4%, and 28.9%; and at 95°C, 30.1%, 16.6%, 18.7%, and 34.6%, respectively. Thus, the increase of the α-helix, the turn, and the random coil and the decrease of the β-sheet were observed accompanying the conformational changes at high temperatures. Big changes of the CD spectra of *B. cepacia* ST-200 enzyme were observed at temperatures between 75°C and 80°C. The percentages of α-helix, β-sheet, turn, and random coil of *B. cepacia* ST-200 enzyme were as follows: at 60°C, 26.2%, 53.0%, 0%, and 20.8%; and at 80°C, 78.9%, 0% 0%, and 21.1%, respectively. In the case of *B. cepacia* ST-200 enzyme, the α-helix was significantly increased, and the β-sheet was completely lost at 80°C.

Table 4 Michaelis constant and maximum velocity of cholesterol oxidases

Source	K_m[a] (μM)	V_{max}[a] (μmol min^{-1} mg^{-1})	V_{max} K_m^{-1}
E. coli Rosetta(pBScox)	26.2±0.9	10.4±0.7	0.40
Nocardia sp.	18.4±1.7	5.2±0.3	0.28
Nocardia erythropolis	18.8±1.6	3.0±0.4	0.16
Burkholderia cepacia ST-200	76.8±1.6	7.0±0.8	0.09
Pseudomonas fluorescens	183±11	6.7±1.2	0.04
Streptomyces sp. SA-COO	315±17	3.9±0.9	0.01

[a] Cholesterol oxidation activity was assayed by measuring H_2O_2 generation. The K_m and V_{max} values were estimated from Lineweaver–Burk plots of data obtained with the assay solution containing 0 to 1 mM cholesterol

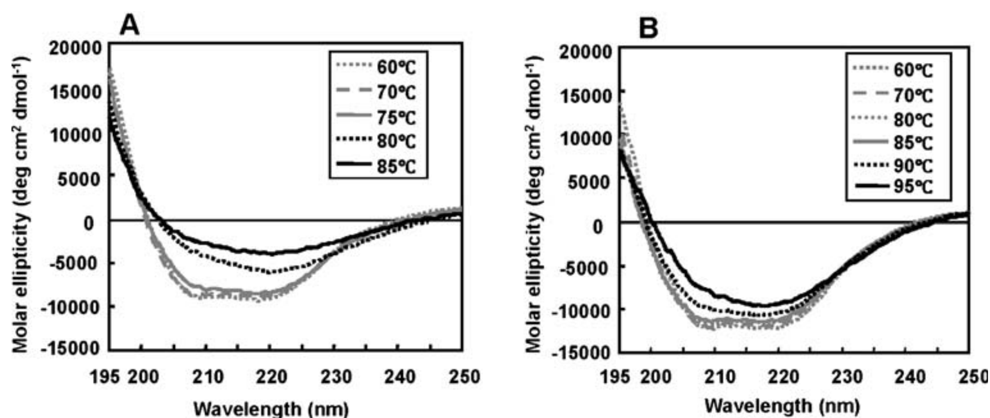

Fig. 7 CD spectra of the recombinant cholesterol oxidase and *B. cepacia* ST-200 cholesterol oxidase at various temperatures. The CD spectra of cholesterol oxidases were measured between wavelengths of 195 nm and 250 nm. **a** The CD spectra of *B. cepacia* ST-200 cholesterol oxidase were measured at temperatures between 60°C and 85°C. **b** The CD spectra of DS-1 cholesterol oxidase were measured at temperatures between 60°C and 95°C

Discussion

There are two types of cholesterol oxidase that are different in their products (CEO or HCEO) from cholesterol (Doukyu and Aono 1999). CEO-forming cholesterol oxidase oxidizes cholesterol to CEO, with the consumption of 1 mol of O_2 and the formation of 1 mol of H_2O_2 for every 1 mol of cholesterol oxidized. CEO-forming cholesterol oxidases have been reported from various microorganisms, including the genus *Brevibacterium*, *Nocardia*, and *Streptomyces* (Doukyu and Aono 1999; MacLachlan et al. 2000). On the other hand, HCEO-forming cholesterol oxidase oxidizes cholesterol to HCEO, with the consumption of 2 mol of O_2 and the formation of 1 mol of H_2O_2 for every 1 mol of cholesterol oxidized. In a previous study, we showed that DS-1 cholesterol oxidase was the latter type of enzyme (Doukyu et al. 2008). HCEO-forming cholesterol oxidases have been reported from *B. cepacia* strain ST-200, *Pseudomonas* spp., and *Chromobacterium* sp. strain DS-1 (Doukyu and Aono 1999). Among these HCEO-forming enzymes, the cloning of a cholesterol oxidase gene was reported only from *B. cepacia* strain ST-200.

The sequence of a cholesterol oxidase gene from *Chromobacterium* has not been reported previously. Therefore, this is the first report of a gene encoding cholesterol oxidase from the genus *Chromobacterium*. Two forms of cholesterol oxidase have been identified in *B. sterolicum*, one containing the FAD cofactor non-covalently bound to the enzyme (BCO1) and another containing the cofactor covalently linked to the enzyme (BCO2; Croteau and Vrielink 1996). These two enzymes catalyze the same chemical reaction, although they have no significant sequence homology. BCO1 shows remarkable similarities (52% to 90%) to cholesterol oxidases or hypothetical proteins from actinomycetes, such as *Rhodococcus* and *Streptomyces*. These sequences contain a consensus sequence for FAD-binding, Gly-X-Gly-X-X-Gly, in the N-terminal region of the mature cholesterol oxidases (Croteau and Vrielink 1996; Ohta et al. 1991). The amino acid sequence of cholesterol oxidase from *Chromobacterium* sp. strain DS-1 did not show similarity to BCO1 or other proteins that have significant homology with BCO1, and it did not contain the consensus sequence for FAD-binding described above. By contrast, the sequence of DS-1 enzyme showed significant similarity (42% to 62%) to BCO2 and other cholesterol oxidases from *R. erythropolis*, *S. ambofaciens*, *Burkholderia* spp., and *P. aeruginosa*. The structure of BCO2 has been determined by X-ray crystallography and refined to high resolution (Coulombe et al. 2001). The structure suggested that the FAD cofactor was covalently bound to an active-site histidine (His121) via the C8α group of the flavin isoalloxazine ring. In addition, Glu475, located at the active-site cavity, was predicted to act as the base for both the oxidation and the isomerization steps of the catalytic reaction. Moreover, the structure indicated that the highly hydrophilic residues (Glu311, Glu432, Arg477, Glu551, Lys554, and Asn516) lining the cavity near the FAD cofactor probably play an important role in the reactivity of the cofactor. By a comparative amino acid sequence analysis, the amino acid residues His121, Glu475, Glu311, Arg477, Glu551, and Lys554 of BCO2 were conserved in the sequence of DS-1 enzyme as corresponding amino acid residues His107, Glu445, Glu299, Arg447, Glu518, and Lys524, respectively (Fig. 3). However, the amino acid residues Glu432 and Asn516 of BCO2 were replaced by corresponding amino acid residues of Ala402 and Asp486 in the sequence of DS-1 enzyme, respectively.

The amino acid sequence of cholesterol oxidase from strain DS-1 showed significant similarity to that of cholesterol oxidase from *B. cepacia* ST-200. The thermal stability of DS-1 cholesterol oxidase was higher than that of the enzyme from *B. cepacia* ST-200 (Doukyu et al. 2008). CD spectral analysis of the DS-1 enzyme and *B. cepacia* ST-200 enzyme at various temperatures showed that the conformational stabilities of these two enzymes were closely correlated with their respective thermal stabilities. As expected, the conformational stability of the DS-1 enzyme was higher than that of *B. cepacia* ST-200 enzyme

at higher temperatures. In both enzymes, the transitions of the β-sheet to other secondary structures were observed at higher temperatures. Especially, the loss of the β-sheet of *B. cepacia* ST-200 enzyme was more distinctive than that of the DS-1 enzyme. The thermal stability of these enzymes might depend on the structural stability of the β-sheet at high temperatures.

Native cholesterol oxidase purified from strain DS-1 was more stable at high temperatures and in the presence of various organic solvents and detergents than were commercially available cholesterol oxidases (Doukyu et al. 2008). The recombinant enzyme of strain DS-1 also possessed these useful features for clinical applications and other reactions containing various organic solvents and detergents. In this study, we found that the catalytic efficiency of the recombinant enzyme from strain DS-1 was higher than those of the commercial enzymes. Moreover, we improved the production of the enzyme by recombinant *E. coli*, as compared with that by strain DS-1. The enzyme yield from the same culture volume was about 148-fold higher than that reported for strain DS-1 (Doukyu et al. 2008). The overproduction of the protein could allow its production on an industrial scale and shows its potential as a commercial enzyme.

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