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Purification, characterization, and molecular cloning of organic-solvent-tolerant cholesterol esterase from cyclohexane-tolerant *Burkholderia cepacia* strain ST-200

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Abstract Extracellular cholesterol esterase of *Burkholderia cepacia* strain ST-200 was purified from the culture supernatant. Its molecular mass was 37 kDa. The enzyme was stable at pH 5.5–12 and active at pH 5.5–6, showing optimal activity at pH 7.0 at 45°C. Relative to the commercially available cholesterol esterases, the purified enzyme was highly stable in the presence of various water-miscible organic solvents. The enzyme preferentially hydrolyzed long-chain fatty acid esters of cholesterol, except for that of cholesteryl palmitate. The enzyme exhibited lipolytic activity toward various *p*-nitrophenyl esters. The hydrolysis rate of *p*-nitrophenyl caprylate was enhanced 3.5- to 7.2-fold in the presence of 5–20% (vol/vol) water-miscible organic solvents relative to that in the absence of organic solvents. The structural gene encoding the cholesterol esterase was cloned and sequenced. The primary translation product was predicted to be 365 amino acid residues. The mature product is composed of 325 amino acid residues. The amino acid sequence of the product showed the highest similarity to the lipase LipA (87%) from *B. cepacia* DSM3959.

Keywords Cholesterol esterase · Organic-solvent tolerance · *Burkholderia cepacia* · Purification · Cloning

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Introduction

Cholesterol esterase (EC3.1.1.13) catalyzes the de-esterification of cholesterol esters. Cholesterol esterase is a valuable enzyme used for the measurement of total serum cholesterol in combination with cholesterol oxidase (EC1.1.3.6). It is known that several microorganisms produce cholesterol esterases. Cholesterol esterases have been isolated from several sources, such as *Pseudomonas fluorescence* (Uwajima and Terada 1976), *Staphylococcus aureus* (Harvie 1977), *P. aeruginosa* (Sugihara et al. 2002), *Fusarium oxysporum* (Okawa and Yamaguchi 1977), *Saccharomyces cerevisiae* (Taketani et al. 1981), and *Streptomyces lavendulae* (Kamei et al. 1977). Lipases from *P. pseudoalcaligenes* and *P. cepacia* DSM3401 were found to possess cholesterol esterase activity (Svendsen et al. 1995). In addition to microbial cholesterol esterase, cholesterol esterase has been examined in a variety of mammalian tissues (Anderson and Sando 1991; Kissel et al. 1989; Kyger et al. 1989). The genes encoding cholesterol esterase from human lysosome (Anderson and Sando 1991), bovine pancreas (Kissel et al. 1989), and rat pancreas (Kyger et al. 1989) have been cloned and sequenced. However, the gene of microbial cholesterol esterase has been cloned and sequenced only from *Streptomyces lavendulae* H646-SY2 (Nishimura and Sugiyama 1994).

The bioconversion of water-insoluble substrates such as steroids has been hindered because of their low solubility in aqueous media. Organic solvents are usually employed to the solution to solubilize water-immiscible hydrophobic compounds. However, many enzymes can easily be inactive in organic solvents (Antonini et al. 1981; Carrea 1984). Organic-solvent-tolerant microorganisms are useful in screening for extracellular enzymes in the presence of organic solvents. Organic-solvent-tolerant protease, lipase, and amylase have been found in organic-solvent-tolerant microorganisms (Doukyu et al. 2003; Ogino et al. 1994, 1995). We previously isolated a cyclohexane-tolerant and cholesterol-oxidizing

Burkholderia cepacia strain ST-200 (Doukyu and Aono 1998). Strain ST-200 produces an interesting extracellular cholesterol oxidase. This enzyme is highly stable in the presence of organic solvents or detergents, and is also thermo-stable (Doukyu and Aono 2001). In the course of the present study, it was found that the strain ST-200 showed cholesterol esterase activity in the culture supernatant and seemed to have the conversion system of cholesterol ester.

In this paper, we report the purification of the cholesterol esterase from strain ST-200, the partial characterization of the enzyme, and the cloning of the cholesterol esterase.

Materials and methods

Strains and media

Burkholderia cepacia strain ST-200 (Doukyu and Aono 1998) was used as the source of DNA encoding the cholesterol esterase. The vector plasmid, pBluescript II (pBSII) KS+, was purchased from Toyobo Biochemicals (Osaka, Japan). pGEM-T Easy vector was purchased from Promega (Madison, WI, USA). *E. coli* XL1-Blue {*hsd*R17, *sup*E44, *rec*A1, *end*A1, *gyr*A46, *thi*, *rel*A1, *lac*/F' [*pro*AB$^+$, *lac*I^q, *lac*ZΔM15::Tn10 (*tet*^{r})]} was used for the cloning and expression of the gene.

Escherichia coli strains were grown at 37°C in Luria broth (LB medium) consisting of 1% Bacto tryptone (Difco Laboratories, Detroit, MI, USA), 0.5% Bacto yeast extract (Difco), and 1% NaCl. When necessary, the medium was solidified with 1.5% (w/v) agar and supplemented with ampicillin (50 μg/ml).

Purification of cholesterol esterase from strain ST-200

Strain ST-200 was cultivated in a jar fermenter at 30°C for 24 h in LB medium with 0.05% (wt/vol) soybean oil, and then the culture was centrifuged (25 min, 8,000*g*, 4°C). Proteins in the supernatant (6 l) were extracted with $(NH_4)_2SO_4$ (80% saturation) at 4°C overnight. The precipitate was recovered by centrifugation (12,000*g*, 25 min, 4°C) and dissolved in 10 mM Tris–HCl (pH 9.0). This solution was dialyzed against the same buffer at 4°C, and a fourfold volume of ice-cold acetone was added to the solution. The precipitate was recovered by centrifugation (12,000*g*, 25 min, 4°C) and dissolved in 10 mM Tris–HCl (pH 9.0) and then loaded onto a column (2.5 cm ϕ×8 cm) of DEAE-cellulose DE52 (Whatman, Maidstone, England) that had been equilibrated with 10 mM Tris–HCl (pH 9.0). Proteins were eluted with a linear gradient of 0–200 mM NaCl in 10 mM Tris–HCl (pH 9.0) at a flow rate of 60 ml/h. The enzyme solution was treated with 2% (wt/vol) sodium cholate for 12 h at 4°C and then purified by gel filtration on a Sephadex G-100 column (2 cm ϕ×100 cm) that had been equilibrated with 10 mM Tris–HCl (pH 9.0) and 2% (wt/vol) sodium cholate. Active fractions were dialysed against 10 mM Tris–HCl (pH 9.0) and concentrated by use of an ultrafiltration membrane with a molecular cutoff of 3 kDa.

Measurement of protein concentration

Protein concentration was measured by the method of Bradford (1976) with bovine serum albumin as the standard.

SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done on a 12.5% (wt/vol) polyacrylamide gel by the method of Laemmli (1970).

Amino acid sequence

Extracellular cholesterol esterase from ST-200 was purified from the culture supernatant as described above. The N-terminal amino acid sequence of the purified cholesterol esterase was determined using an automated protein sequencer (model G1005A; Hewlett-Packard, Palo Alto, CA, USA). To determine the amino acid sequence of internal regions of the cholesterol esterase, the purified enzyme was cleaved with CNBr. 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. PCR primers were designed based on the determined amino acid sequences to amplify part of the cholesterol esterase gene as described below.

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 and colony hybridization were 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 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.

Cloning of the cholesterol esterase gene

N-terminal and the internal amino acid sequences of the cholesterol esterase were determined as described above. PCR primers were designed based on the amino acid sequences. A DNA fragment was amplified from the chromosomal DNA of ST-200 by PCR with 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, Foster City, CA, USA). The amplified fragment was ligated into the pGEM-T easy vector. The insert was restricted by *Eco*RI and ligated into the *Eco*RI site of pBSII KS+. The nucleotide sequence of the insert was analyzed to confirm that the amplified DNA corresponded to the determined amino acid sequences. The DNA insert containing part of the cholesterol esterase gene was recovered from the plasmid and labeled with digoxigenin. Chromosomal DNA of strain ST-200 was digested with various restriction enzymes and analyzed by Southern hybridization, using digoxigenin-labeled DNA as a probe. A physical map of the vicinity of the cholesterol esterase gene was established by Southern hybridization analysis. The chromosomal DNA was digested with *Bam*HI and *Sac*I, and the fragments were inserted into the same sites in pBSII KS+. *E. coli* XL1-Blue was transformed with the resulting plasmids and grown on LB agar containing ampicillin. A transformant containing the cholesterol esterase gene was identified by the colony hybridization with the probe.

Assay of cholesterol esterase activity

Cholesterol esterase activity was assayed by Allain's method (Allain et al. 1974). The reaction mixture was composed of 0.11 M potassium phosphate buffer (pH 7.0), 20 mM cholesteryl linoleate, 1.5 mM 4-aminoantipyrine, 22 mM phenol, 0.33% (wt/vol) Triton X-100, 5 U/ml peroxidase, and 0.6 U/ml cholesterol oxidase. The reaction was carried out at 37°C for 2–10 min. The produced color was measured at 500 nm. One unit of enzymatic activity was defined as the amount required to hydrolyze 1 μmol of cholesterol ester min^{-1} at 37°C.

In the investigation of the optimal pH, the reaction solution containing 20 mM cholesteryl linoleate and various buffer systems described in Fig. 2 was incubated at 37°C for 2–10 min. The reaction was then stopped by boiling the reaction solution for 5 min. The amount of produced free cholesterol was measured by Allain's method (Allain et al. 1974). In case of the optimal temperature, the reaction solution containing 20 mM cholesteryl linoleate and 100 mM potassium phosphate buffer (pH 7.0) was incubated at various temperatures for 30 min. The free cholesterol amount was measured in the same way as the optimal pH.

Commercial cholesterol esterase preparations

The following commercial cholesterol esterase preparations were used in this study: esterase from *Schizophyllum commune* (Toyobo, Tsuruga, Japan), *Pseudomonas* sp. (Toyobo), porcine pancreas (Sigma Chemical, St. Louis, MO, USA), and bovine pancreas (Wako Chemical, Osaka, Japan). These commercial esterases are abbreviated in this report as Sch, Pse, Por, and Bov, respectively.

Nucleotide sequence accession number

The nucleotide sequences of cholesterol esterase (*che*S) and its putative chaperon (*lim*S) of strain ST-200 have been deposited in the DDBJ/EMBL/GenBank databases under accession number AB175022.

Results

Purification of ST-200 cholesterol esterase

In the absence of sodium cholate, cholesterol esterase from strain ST-200 eluted at the void volume of Sephadex G-100 gel filtration, and seemed to exist as aggregates. Therefore, the fractions eluted from DEAE chromatography with enzyme activity were combined and then precipitated with 80% acetone. The precipitates were then dissolved with a buffer containing 2% (wt/vol) sodium cholate.

Table 1 summarizes the purification of the cholesterol esterase. The enzyme was purified 6.1-fold on the basis of the initial activity and was mostly homogeneous as judged by SDS-PAGE (Fig. 1). SDS-PAGE showed a single band at 37 kDa.

Properties of ST-200 cholesterol esterase

The enzyme showed its optimum pH from pH 5.0 to 7.5, and was stable between pH 5.5 and 12 for 24 h at 37°C (Fig. 2). The activity was highest at 45°C at pH 7.0 (Fig. 3). The enzyme was stable at temperatures from 4 to 65°C, and lost almost all activity at 70°C. Table 2 lists the effects of some typical metal ions. Cholesterol esterase activity was inhibited by heavy metal ions such as Cu^{2+}, Ag^{+}, and Hg^{2+}. No inhibition was observed with other cations.

Substrate specificity

Table 3 shows the relative rates of hydrolysis of different cholesteryl esters by ST-200 cholesterol esterase. The rates of hydrolysis were in the order: linoleate > oleate > decylate > capronate > butyrate > palmitate >

Table 1 Purification of cholesterol esterase from *Burkholderia cepacia* strain ST-200

Steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
$(NH_4)_2SO_4$ precipitation	488	592	0.82	1.0	100
80% acetone precipitation	292	181	1.6	2.0	60
DEAE-cellulose	35	15	2.3	2.8	7.1
Sephadex G-100	8.7	1.7	5.1	6.2	1.8

$(NH_4)_2SO_4$ precipitation was obtained from 24 l of the culture supernatant

acetate. The enzyme preferentially hydrolyzed long-chain fatty acid esters of cholesterol, except that of cholesteryl palmitate. Table 3 also shows the relative rates of hydrolysis of *p*-nitrophenyl esters by the enzyme. The enzyme exhibited lipolytic activity toward various *p*-nitrophenyl esters, showing the highest activity for myristate ester.

Effects of organic solvents on the stability of cholesterol esterase

Table 4 shows the stability of the purified enzyme of strain ST-200 and commercially available cholesterol esterases in the presence of various aqueous organic solvents. Highly polar solvents with log P_{ow} values (less than 2) inactivate most enzymes through structural denaturation (Laane et al. 1987). The enzyme of ST-200 was stable in the presence of dimethylsulfoxide (DMSO), dimethylformamide (DMF), ethanol, and 2-propanol. The enzyme of ST-200 was partially inactivated by the addition of methanol and acetone. All of the commercial cholesterol esterases were markedly inactivated by the addition of solvents except DMSO. DMSO partially inactivated the enzymes of Sch and Pse. The enzyme of ST-200 was highly stable in water-miscible organic solvents relative to the commercial enzyme.

Effects of organic solvents on the lipolytic activity of cholesterol esterase

Table 5 shows the lipolytic activity toward *p*-nitrophenyl caprylate by ST-200 cholesterol esterase in the presence of various water-miscible organic solvents. The activity in the presence of 5% (vol/vol) to 20% (vol/vol) was 3.5- to 7.2-fold higher than that found in the absence of organic solvent. The enzyme showed high activity even in the presence of 50% (vol/vol) DMSO, DMF, or methanol. The activities were remarkably lowered by the addition of 50% (vol/vol) ethanol, 2-propanol, or acetone.

Cloning of ST-200 cholesterol esterase gene

The N-terminal amino acid sequence of the cholesterol esterase was found to be APADNYAATR. The enzyme was cleaved with CNBr, and the resulting peptides were fractionated by SDS-PAGE. Among several peptides separated on the gel, the N-terminal amino acid sequence of 6 kDa was determined. The sequence obtained was PANALDPSTLAL.

PCR primers were designed based on the determined amino acid sequences. Inosine was incorporated into the primers at positions of three- and four-base redundancy to reduce the complexity of the primer mixtures from several hundred thousand to fewer than four different sequences. The sense primer sequence (primer 1) (5′-GCICCIGCIGA(C/T)AA(C/T)TAIGCIGC-3′) corresponded to the N-terminal amino acid sequence of the mature enzyme. Antisense primer (primer 2) (5′-TAI(C/G)(A/T)IGGITCIA(A/G)IGCITTIGCIGG-3′) was designed based on the determined N-terminal amino acid sequence of the 6 kDa peptide.

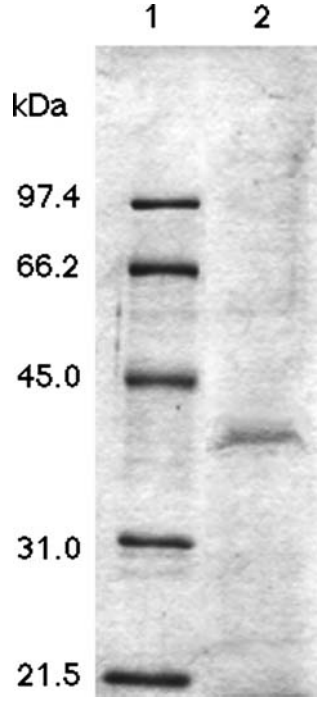

Fig. 1 SDS-polyacrylamide gel electrophoresis of cholesterol esterase after final preparation obtained following Sephadex G-100 gel chromatography. Samples containing 15 mU of cholesterol esterase were electrophoresed on a 12.5% SDS-polyacrylamide gel. The gel was stained with Coomasie Brilliant Blue R-250. *Lane 1* molecular size markers; *lane 2* final preparation obtained following Sephadex G-100 gel chromatography

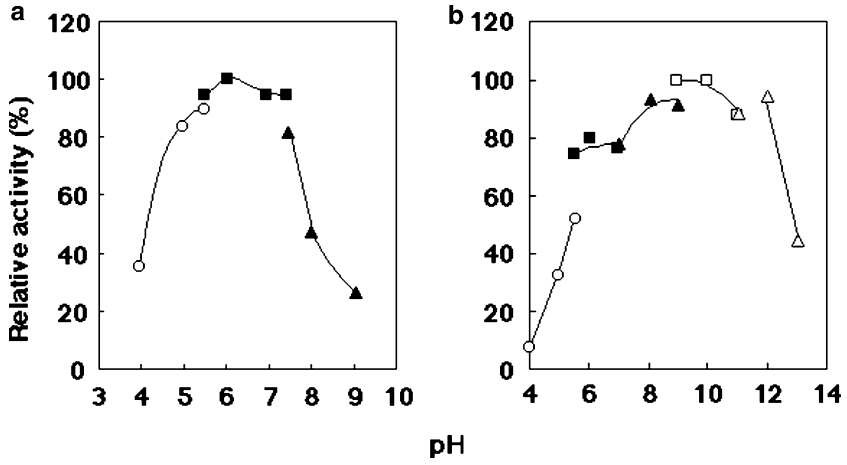

Fig. 2 Effect of pH on cholesterol esterase activity and stability. **a** Enzyme activity was assayed by measuring the free cholesterol at 37°C under various pH conditions. **b** Enzyme preparation (0.2 U/ml) was incubated at 37°C for 12 h under various pH conditions. Residual activity was estimated by Allain's method. The buffer systems (0.2 M) used were CH_3COOH–CH_3COONa (pH 4–5.5) (*open circle*), KH_2PO_4–K_2HPO_4 (pH 5.5–7.5) (*filled square*), Tris–HCl (pH 7.5–9) (*filled triangle*), Na_2CO_3–$NaHCO_3$ (pH 9–11) (*open square*), NaOH–NaCl (pH 11–13) (*open triangle*)

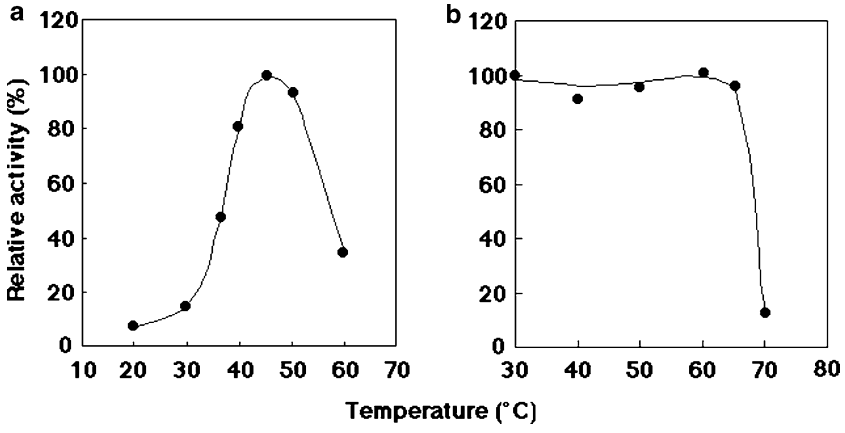

Fig. 3 Effect of temperature on cholesterol esterase activity and stability. **a** Enzyme activity was assayed by measuring the free cholesterol at pH 7.5 at the temperature indicated. **b** Enzyme dissolved in 100 mM potassium phosphate buffer (pH 7.0) was incubated for 30 min at the temperatures indicated, and the residual activity was estimated by Allain's method at pH 7.5 at 37°C

A DNA fragment of about 750 bp was amplified from the chromosomal DNA of ST-200 by PCR with a combination of primer 1 and primer 2. The amplified fragment was ligated into the TA cloning site of pGEM-T Easy. This plasmid was designated pGEMcep.

The DNA inserted in pGEMcep was digested with *Eco*RI at both of the sites derived from the original plasmid pGEM-T Easy. The excised fragment (750 bp) was recovered and labeled with digoxigenin. Chromosomal DNA of ST-200 was digested with various restriction enzymes and analyzed by Southern hybridization, using the digoxigenin-labeled DNA as a probe. The results allowed us to create a physical map of the vicinity of the cholesterol esterase gene (data not shown).

Chromosomal DNA of ST-200 digested with *Bam*HI and *Sac*I showed a single hybridization band of 6 kb. DNA fragments of 5–7 kb in size were recovered from the *Bam*HI–*Sac*I digest of the chromosomal DNA and were ligated into the same sites of pBS KS+. *E. coli* XL1-Blue was transformed with the resulting plasmids. Among about 3,000 transformants, one showed a positive signal in reaction with the probe. It was shown that this clone contained a recombinant plasmid with a 6-kbp *Bam*HI–*Sac*I insert. The clone grown in LB/Amp liquid medium showed cholesterol esterase activity, whereas *E. coli* XL1-Blue (pBS KS+) did not show the activity. The plasmid recovered from the clone was designated pBS6000.

Nucleotide sequence of ST-200 cholesterol esterase gene

The sequence contains two open reading frames (Fig. 4). The former open reading frame was 1,323 bp within a TAG at nucleotide position 52 and a TAA codon at nucleotide position 1,375. We found a structural gene of 1,095 bp, coding for a polypeptide consisting of 364 amino acid residues, with the ATG initiation codon at nucleotide position 283 and the TAA termination codon at position 1,375. A potential ribosome-binding site (GGAG) was observed eight bases upstream of this ATG. The nucleotide sequence of the structural gene coded for the N-terminal amino acid sequence

determined for the cholesterol esterase purified from ST-200. The mature polypeptide consisted of 324 amino acid residues, with a molecular mass of 37,589 Da. This is nearly 37 kDa, the mass of the enzyme as estimated by SDS-PAGE (Fig. 1). It was concluded that this structural gene coded for the cholesterol esterase, and the gene was named *cheS*.

We found several amino acid sequences showing high similarities with CheS in the databases. The amino acid sequence of CheS (between positions 1 and 364) showed the highest similarity (87%) to the LipA lipase (accession no. M58494-1) from *B. cepacia* DSM3959 (Jorgensen et al. 1991). The second amino acid sequence showing significant identity (86%) with the CheS sequence was a lipase (S77842-1) from *B. cepacia* KWI-56 (Izumi et al. 1991).

The other open reading frame was 1,038 bp within a TAA at nucleotide position 1,375 and a TGA codon at nucleotide position 2,413. There was a structural gene of 1,032 bp, coding for a polypeptide consisting of 344 amino acid residues, with the ATG initiation codon at nucleotide position 1,381 and the TAA termination codon at position 2,413. This structural gene was named *limS*. The amino acid sequence of LimS (between positions 1 and 364) showed the highest similarity (75%) to a lipase chaperone (accession no. AJ295615-2) from *B. cepacia* KWI-56 (Izumi et al. 1991) and showed significant similarity (74%) to a LimA chaperone (accession no. M58494-2) from *B. cepacia* DSM3959 (Jorgensen et al. 1991).

We constructed a *limS* deletion plasmid to examine the activation of cholesterol esterase by the *limS* gene as reported previously (Jorgensen et al. 1991). pBS6000, digested with *Sac*I and *Sac*II, was blunt-ended with T4 DNA polymerase and then self-ligated to create *limS* deletion plasmid pBS4000. *E. coli* XL1-Blue (pBS 4000) did not show cholesterol esterase activity (data not shown). It seemed that LimS is required for the activation of ST-200 cholesterol esterase as reported previously.

Table 2 Effects of various chemicals on the stability of ST-200 cholesterol esterase

Chemical	Relative activity (%)[a]
None	100
$CaCl_2$	111
$MgCl_2$	97
$FeCl_3$	103
$BaCl_2$	116
$NiCl_2$	91
$MnCl_2$	92
$ZnCl_2$	104
$AgNO_3$	72
$CuSO_4$	61
$HgCl_2$	11
EDTA	92

[a]The enzyme was incubated at 30°C for 1 h with each chemical (1 mM). Residual activity was measured by Allain's method. The relative residual activity shows the activity compared to that observed in the enzyme solution without chemicals

Table 3 Substrate specificity of ST-200 cholesterol esterase

Substrate[a]	Relative activity (%)[b,c]
Cholesterol esters[b]	
Linoleate (18:2)	100
Oleate (18:1)	53
Palmitate (16:0)	5
Decylate (10:0)	34
Capronate (6:0)	29
Butyrate (4:0)	18
Acetate (2:0)	3
p-Nitrophenyl esters[c]	
Acetate (2:0)	5
Propionate (3:0)	17
Capronate (6:0)	53
Caprylate (8:0)	79
Laurate (12:0)	92
Myristate (14:0)	100
Palmitate (16:0)	68
Stearate (18:0)	38

[a]Number of carbon atoms to number of double bonds is given in parentheses

[b]Enzyme activity was measured by Allain's method. Activity is represented as a percentage of that obtained with cholesterol linoleate as substrate

[c]Assay solution contained 180 mM potassium phosphate buffer (pH 7.0), 0.8 mM *p*-nitrophenyl ester, 10% (v/v) 2-propanol, 0.45% Triton X-100, and ST-200 cholesterol esterase (0.2 U/ml). The assay solution was incubated at 37°C. After 6 min, the amount of *p*-nitrophenol was estimated by measuring the absorbance at 410 nm. Activity is represented as a percentage of that obtained with *p*-nitrophenyl myristate

Discussion

Cholesterol esterase from ST-200 was purified from the culture supernatant. The enzyme exists in the form of aggregates, such as mammalian (Durham and Grogan 1984; Hyun et al. 1971) and some microbial cholesterol esterases (Okawa and Yamaguchi 1977; Taketani et al. 1981). The use of DEAE cellulose and gel filtration with 2% sodium cholate led to successful purification. The enzyme was most active at pH 5.5–6. Most cholesterol esterases showed optima around pH 6.0–7.0 (Kamei et al. 1977; Okawa and Yamaguchi 1977; Uwajima and Terada 1976). The enzyme from ST-200 was stable in the wide pH range 5.5–12. The enzymes of *F. oxysporum* IGH-2 (Okawa and Yamaguchi 1977), *Streptomyces lavendulae* H646-SY2 (Kamei et al. 1977) and, *P. aeruginosa* (Sugihara et al. 2002) were remarkably inactivated at pH above 11.0. The enzyme from ST-200 showed an optimal temperature at 45°C and was relatively stable up to 65°C. Although cholesterol esterases of *F. oxysporum* IGH-2 (Okawa and Yamaguchi 1977) and *P. aeruginosa* (Sugihara et al. 2002) lost most of their activity at 60°C, 95% of the activity of ST-200 cholesterol esterase remained at 60°C. The high thermal stability of ST-200 enzyme is similar to that reported for cholesterol esterase from *P. fluorescens* (Uwajima and Terada 1976). ST-200 cholesterol esterase hydrolyzed

Table 4 Effects of organic solvents on the stability of cholesterol esterases

Solvent	Log P_{ow}	Relative activity[a]				
		ST-200	Sch	Pse	Por	Bov
None	–	1	1	1	1	1
DMSO[b]	−1.38	1.1	0.84	0.45	< 0.01	< 0.01
DMF[b]	−1.04	0.98	0.03	0.07	0.11	< 0.01
Methanol	−0.76	0.58	0.06	0.05	0.11	0.17
Ethanol	−0.24	0.98	0.03	0.04	0.11	0.17
Acetone	−0.21	0.53	0.03	0.33	0.16	< 0.01
2-Propanol	0.28	1.1	0.03	0.04	0.21	0.17

Cholesterol esterases other than that of ST-200 were obtained commercially
[a]Each organic solvent (0.5 ml) was added to 1 ml of cholesterol esterase solution [0.2 U/ml in 10 mM Tris–HCl buffer (pH 8.0)]. This mixture was incubated at 30°C for 24 h. Residual activity of the cholesterol esterase was measured by Allain's method. The relative activity shows the activity compared to that found in the enzyme solution not exposed to organic solvent
[b]Dimethylsulfoxide and dimethylformamide are abbreviated as DMSO and DMF, respectively

Table 5 Effects of organic solvents on the activity of ST-200 cholesterol esterases

Concentration [%(v/v)]	Relative activity[a]					
	DMSO	DMF	Methanol	Ethanol	2-Propanol	Acetone
0	1	1	1	1	1	1
5	4.8	4.9	6.5	5.4	5.8	3.5
10	5.0	5.4	6.8	5.8	5.7	4.6
20	5.6	6.0	7.2	4.6	5.1	3.8
50	4.3	3.1	2.8	0.43	0.23	0.21

[a]The assay solution [180 mM potassium phosphate buffer (pH 7.0), 0.8 mM *p*-nitrophenyl caprylate, 0–50% (v/v) each solvent, and 0.2 U/ml cholesterol esterase] was incubated at 37°C. After 6 min, the amount of *p*-nitrophenol was estimated by measuring the absorbance at 410 nm. Relative activity shows the activity compared to that found in the enzyme solution not exposed to any organic solvent

most cholesterol esters of different fatty acids. The enzyme showed remarkably low activity for cholesteryl palmitate. This substrate specificity was similar to that reported for the cholesterol esterase of *F. oxysporum* IGH-2 (Okawa and Yamaguchi 1977), but was different from those of the cholesterol esterases from *P. aeruginosa* (Sugihara et al. 2002) and *P. fluorescens* ATCC21156 (Uwajima and Terada 1976).

A microbial cholesterol esterase gene has been cloned and sequenced only from *S. lavendulae* H646-SY2 (Nishimura and Sugiyama 1994). The cloning of a microbial cholesterol esterase gene is reported for the second time in this paper. The amino acid sequence of ST-200 cholesterol esterase showed the highest similarity to lipase LipA (87%) from *B. cepacia* DSM3959 (Jorgensen et al. 1991) and to a lipase (86%) from *B. cepacia* KWI-56 (Izumi et al. 1991). It has been reported that a lipase of *P. cepacia* (reclassified *B. cepacia*) DSM3401 possessed cholesterol esterase activity (Svendsen et al. 1995). However, little has been known about its properties as a cholesterol esterase. We investigated the relative rates of hydrolysis of different *p*-nitrophenyl esters by ST-200 cholesterol esterase. The enzyme exhibited lipolytic activity toward various *p*-nitrophenyl esters. In this study, relative activities to *p*-nitrophenyl caprylate and *p*-nitrophenyl palmitate by ST-200 enzyme were similar to one another. However, in the case of *B. cepacia* KWI-56 lipase, the activity relative to *p*-nitrophenyl caprylate was about two-fold higher than that relative to *p*-nitrophenyl palmitate (Yang et al. 2002). The effect of several organic solvents on the activity of KWI-56 lipase has been reported (Izumi et al. 1990). Although KWI-56 lipase was partially inactivated by the addition of 50% (vol/vol) methanol, ST-200 cholesterol esterase was activated in the presence of 50% (vol/vol) methanol. ST-200 cholesterol esterase was activated by the addition of 5–20% (vol/vol) water-miscible organic solvents. It was reported that several lipases showed the enhanced activity in the presence of organic solvents (Tsuzuki et al. 2003). However, the activation of the cholesterol esterase by the addition of organic solvents was not reported.

In recent years, a variety of *Pseudomonas* lipase genes have been cloned, sequenced, and characterized (Chihara-Siomi et al. 1992; Chung et al. 1991; Frenken et al. 1992). These lipases can be divided into three classes (designated classes I–III) depending on their amino acid sequence homology (Quyen et al. 1999). The lipases of classes I and II, including the broadly used lipases of *P. cepacia* (reclassified *B. cepacia*) and *P. glumae* strains (class II) as well as those of *P. aeruginosa* strains (class I), have been found to need a chaperone, whose gene is located downstream of the lipase gene, for efficient secretion and folding of active lipase (Aamand et al.

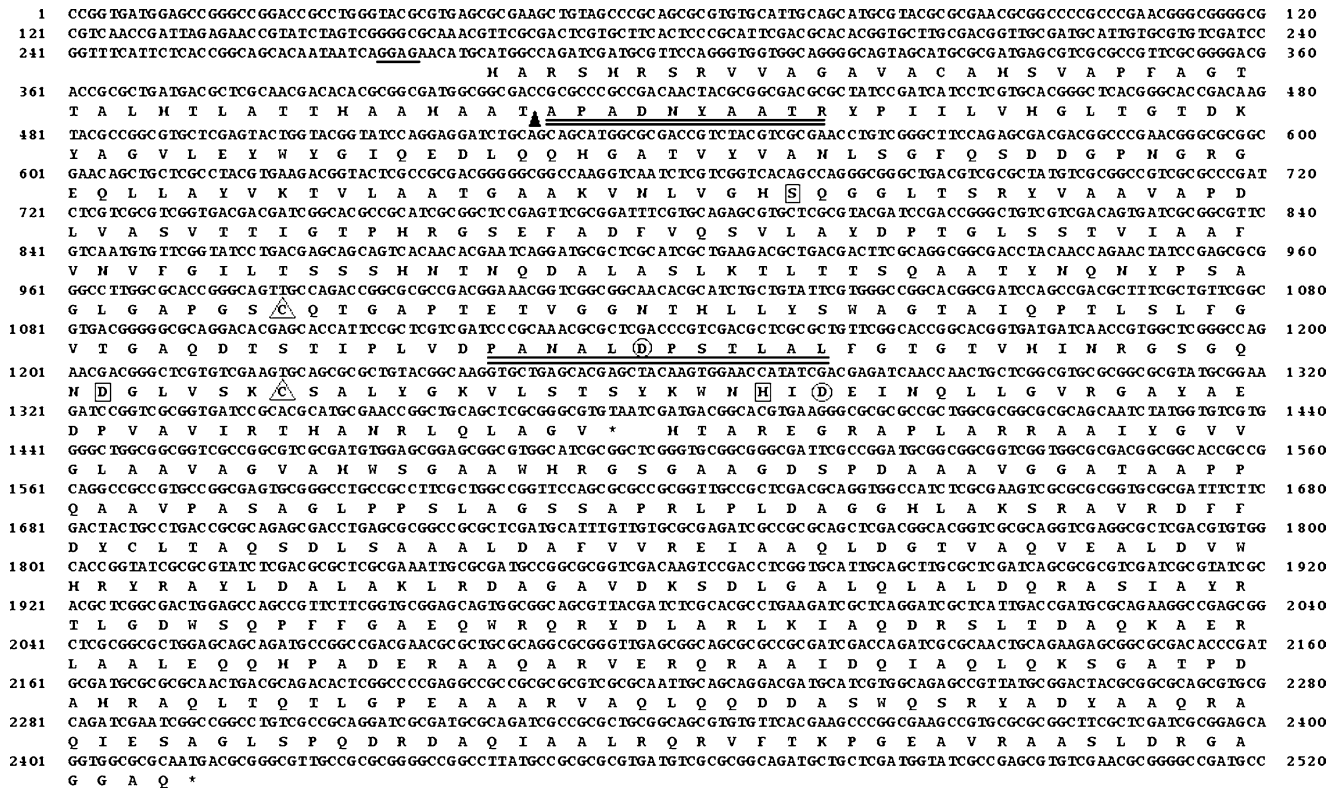

Fig. 4 Nucleotide sequence of the *cheS* gene from strain ST-200 and the deduced amino acid sequence. *Numbers* on the right denote nucleotide positions. The N-terminal amino acid sequence determined through analysis of the mature CheS from strain ST-200 is doubly underlined. The *vertical arrowheads* indicate the cleavage sites of CheS in strain ST-200. The Shine-Dalgarno sequence (S.D.) is *underlined*. Amino acid residues belonging to a putative catalytic triad (Ser131, Asp308, and His330), disulfide bonds (Cys234 and Cys314), and Ca^{2+} binding sites (Asp286 and Asp332) were enclosed in a *box*, *triangle*, and *circle*, respectively

1994; Chihara-Siomi et al. 1992; Hobson et al. 1993). The amino acid sequences of CheS and LimS showed significant similarity to a lipase and its chaperone from *B. cepacia*, respectively. From the results obtained with the *lim*S deletion plasmid, the *lim*S gene seemed to be required for the activation of ST-200 cholesterol esterase. The crystal structure of the lipase from *B. cepacia* was determined previously (Kim et al. 1997). It was found that a catalytic triad, a disulfide bond, and a calcium-binding site existed in the tertiary structures of the lipase. By a comparative amino acid sequence analysis, amino acid residues belonging to the catalytic triad (Ser131, Asp308, and His330) were conserved (Fig. 4). Moreover, it was found that Cys234 and Cys314 might form a sole putative disulfide bond and that Asp286 and Asp332 were deduced to belong to a Ca^{2+} binding site. The disulfide bond and the calcium-binding site are thought to be important for the stability of lipase. Further study of the expression and purification of the recombinant ST-200 cholesterol esterase is in progress.

Cholesterol esterase is an enzyme of great commercial value. It is widely employed by laboratories routinely devoted to the determination of cholesterol concentrations in food, serum, and other clinical samples in the presence of organic solvents, because organic solvents are usually employed to solubilize the steroids. This paper has made the first report of an organic-solvent-tolerant cholesterol esterase. The cholesterol esterase of strain ST-200 was active and highly stable in the presence of organic solvents. Therefore, this cholesterol esterase might improve the usability of cholesterol esterase for reactions containing various organic solvents.

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