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Thermostable Esterase from a Thermoacidophilic Archaeon: Purification and Characterization for Enzymatic Resolution of a Chiral Compound

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Homolog to lipolytic enzymes having the consensus sequence Gly–X–Ser–X–Gly, from the *Sulfolobus solfataricus* P2 genome, were identified by multiple sequence alignments. Among three potential candidate sequences, one (Est3), which displayed higher activity than the other enzymes on the indicate plates, was characterized. The gene (*est* 3) was expressed in *Escherichia coli*, and the recombinant protein (Est3) was purified by chromatographic separation. The enzyme is a trimeric protein and has a molecular weight of 32 kDa in monomer form in its native structure. The optimal pH and temperature of the esterase were 7.4 and 80 °C respectively. The enzyme showed broad substrate specificities toward various *p*-nitrophenyl esters ranging from C2 to C16. The catalytic activity of the Est3 esterase was strongly inhibited by phenylmethylsulfonyl fluoride (PMSF) and diethyl *p*-nitrophenyl phosphate. Based on substrate specificity and the action of inhibitors, the Est3 enzyme was estimated to be a carboxylesterase (EC 3.1.1.1). The enzyme with methyl (±)-2-(3-benzoylphenyl)propionate-hydrolyzing activity to (−)-2-(3-benzoylphenyl)propionic acid displayed a moderate degree of enantioselectivity. The product, (−)-2-(3-benzoylphenyl)propionic acid, rather than its methyl ester, was obtained in 80% enantiomeric excess ($e.e._p$) at 20% conversion at 60 °C after a 32-h reaction. This result indicates that *S. solfataricus* esterase can be used for application in the synthesis of chiral compounds.

Key words: esterase; thermostable; archaea; chiral compound; enzymatic resolution

The lipolytic enzymes such as esterase (EC 3.1.1.1) and lipase (EC 3.1.1.3) can hydrolyze fatty acid esters with various acyl chain lengths. These enzymes have been purified and characterized from many organisms, including microorganisms, plants, and animals. The physiological functions of these enzymes *in vivo* in organisms have not been made clear. As biocatalysts, however, they have been widely used in industrial processes to catalyze the stereospecific hydrolysis, transesterification, and synthesis of esters in addition to stereoselective conversion of a variety of amines, as well as primary and secondary alcohols.[1)] Esterase is especially used to catalyze the highly specific hydrolysis of short chain esters, such as for the resolution of α-aryl propionic acids in the anti-inflammatory agent, naproxen and for deblocking intermediates in the manufacture of beta-lactam antibiotics in aqueous solution.[2,3)]

An enzyme must be stable under harsh reaction conditions for application in industrial processes. Enzymes from mesophilic organisms are not often suitable due to their lack of high stability in extreme conditions. For this reason, novel enzymes from extremophilic microorganisms are more attractive than enzymes of mesophiles in the field of industrial enzymes.[4–6)] Extremophiles are organisms that thrive under extreme conditions of temperature, pH, pressure, or salt. Although most biomolecules unfold and break apart under these conditions, those from extremophiles are very stable due to being adapted to survive in extreme environments. Therefore, for several decades, much scientific interest in extremophiles has focused on these unique mechanisms. Thermostable enzymes among extremozymes are attractive sources for studying the mechanisms of biomolecules that are active and stable at temperature near, and even above, 100 °C. The enzymes from hyperthermophiles also have significant potential for industrial application as novel biocatalyst sources due to their extremely highly intrinsic stability at high temperatures and resistance to denaturing reagents, or organic solvents, and other deleterious influences.[7)] Recently, to mine novel enzymes in apply to industry, the whole genomes of various hyperthermophiles have

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been sequenced. In addition, their open reading frames and possible functions have been assigned according to their sequence similarity to functionally characterized genes. Although these genome sequences have been analyzed by sequence homology, it is necessary that the putative function of these gene products be confirmed by protein expression and characterization, such as substrate specificity, stability, and enantioselectivity for biotransformation and other applications.[7)]

It have reported only to apply to biotransformation that the lipolytic enzymes were purified and characterized from hyperthermophiles, *Archaeoglobus fulgidus*,[8)] *Pyrococcus furiosus*,[9)] and *Pyrobaculum calidifontis*.[10)] In the *Sulfolobus* species, up to now, two lipolytic enzymes from *Sulfolobus acidocdarius* have been partially purified and sequenced and one extracellular esterase from *Sulfolobus shibate* has been isolated.[11,12)] The carboxylesterases from *Sulfolobus solfataricus* P1 and MT4 were also cloned and characterized.[13,14)] But the lipolytic enzymes from *S. solfataricus* P2 have been not reported and their usability as biocatalysts is still unknown. In this paper, we report the characterization of the recombinant thermostable esterase (Est3) from *S. solfataricus* heterologously expressed in *E. coli*. We also discuss this enzyme as a biocatalyst from hyperthermophiles for catalyzing the kinetic resolution of the anti-inflammatory agent (ketoprofen) at high temperatures.

Materials and Methods

Bacteria, culture conditions, and plasmids. S. solfataricus P2 strain obtained from the Deutshe Sammlung von Mikroorganismen und Zellkultur GmbH (Braunschweig, Germany) was grown aerobically in a 2.5 liter jar fermenter at 400 rpm at 78 °C for 5 d in a modified Allen's basal medium (pH 3.0) containing, per liter: glucose, 3.0 g; yeast extract, 3.0 g; $(NH_4)_2SO_4$, 1.3 g; KH_2PO_4, 0.28 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $CaCl_2 \cdot H_2O$, 70 mg; trace metal solution (per liter of $FeCl_3 \cdot H_2O$ 20 mg; $Na_2B_4O_7 \cdot H_2O$ 4.5 mg; $MnCl_2 \cdot H_2O$ 1.8 mg; $ZnSO_4 \cdot H_2O$ 0.05 mg; $CuCl \cdot H_2O$ 0.05 mg; $VOSO_4 \cdot H_2O$ 0.04 mg; $Na_2MoO_4 \cdot H_2O$ 0.03 mg; $CoSO_4 \cdot H_2O$ 0.01 mg), 1 ml. OD_{600} before harvesting the cells was 1.25. *E. coli* DH5α was used as a host for cloning the DNA encoding esterases. *E. coli* BL21(DE3) was used as a host for the overexpression of an esterase gene from strain P2 with plasmid pRSET-a (Invitrogen, Groningen, Netherlands). *E. coli* strains were cultivated at 37 °C in Luria–Bertani (LB) medium with 100 μg/ml ampicillin.

Preparation of Total DNA from S. solfataricus. S. solfataricus genomic DNA was prepared as follows. Cells were harvested by centrifugation (6,000 × *g* for 30 min at 4 °C) and resuspended in 5 ml SET buffer (75 mM NaCl, 25 mM EDTA (pH 8.0), 20 mM Tris–HCl, pH 7.5). After 1 mg/ml of lysozyme was added, the mixture was incubated at 37 °C for 30–60 min. Proteinase K and sodium dodecyl sulfate (SDS) were added to final concentrations of 0.5 mg/ml and 1% respectively. The reaction mixture was incubated, with occasional inversion, at 55 °C for 2 h. NaCl was finally added into this reactant to a final concentration of 1.25 M. The mixture was allowed to cool to 37 °C and DNA was extracted twice with a phenol–chloroform–isoamyl alcohol (25:24:1) mixture, and twice with chloroform. Chromosomal DNA was then precipitated with isopropanol, washed once with 70% ethanol, dried, and redissolved in a TE buffer (pH 8.0).

Cloning of esterase gene and gene expression. Manipulations of plasmid DNAs from *E. coli* were carried out by standard methods.[15)] The putative genes encoding lipolytic enzymes were amplified by PCR reaction directly from the genomic DNA of *S. solfataricus*. Three pairs of oligonucleotides for cloning SSO2521 (*est*1), SSO2517 (*est*2), and SSO2493 (*est*3) were as follows: *est*1: 5′-CATATGATGAGAATACCATTAGTT-3′ and 5′-GAATCCTCAATAGAAACGTTAAACATTTT-3′, *est*2: 5′-GGATAAATGTATAAAGCTAGA-3′ and 5′-GAATTCCTAACGTTGTTGCGA-3′, and *est*3: 5′-GGATCCATGTCTATTTCAATTTT-3′ and 5′-GAATTCTTAACTTTTACTTTTATCATAAAATGCGC-3′. The PCR reaction conditions were as follows: 1 cycle (99.9 °C for 10 min), 30 cycles (94 °C, 45 s; 50 °C, 30 s; 72 °C, 50 s), and finally 1 cycle (72 °C, 7 min). The PCR reaction products were separated by electrophoresis in a 0.7% agarose gel and the fragments were purified using the gel purification kit. The purified DNA fragments were inserted into pGEM®-T easy (Promega, Madison, Wisconsin, U.S.A) (we designated these plasmids pGTE1, pGTE2, and pGTE3 respectively), then ligation mixtures were transformed into *E. coli* DH5α. The cells harboring the plasmids were re-streaked on a LB agar plate and incubated overnight. The plate was overlaid with 5 ml of soft agar (0.5% agar in distilled water) containing 80 μl of β-naphthylacetate (20 mg/ml in ethoxyethanol) and 80 μl of Fast Blue RR (80 mg/ml in dimethyl sulfoxide).[16)] Active clones showing esterase activity developed a brown color on the plate in a few minutes.

To overproduce the Est3 in *E. coli*, the esterase gene (*est*3) was amplified by PCR with *S. solfataricus* genomic DNA as the template, with *TaKaRa Taq*™ DNA polymerase (TakaRa, Otsu, Japan), and with primers Est31 (5′-GGATCCGCTAGCATGCCCCTAGATCGAATC-3′) and Est32 (5′-GAATTCTTAACTTTTACTTTTATCATAA AATGCGC-3′) as forward and reverse primer, respectively. The primers included the restriction enzyme sites (underlined) *Bam*HI and *Eco*RI upstream from the initiation site and downstream, respectively. The PCR reaction conditions were as follows: 1 cycle (99.9 °C for 10 min), 30 cycles (94 °C, 45 s; 50 °C, 30 s; 72 °C, 50 s) and finally 1 cycle (72 °C, 7 min). The purified PCR product was inserted into pGEM®-T easy and then the ligation product was

transformed into *E. coli* DH5α. The *est*3 gene cloned into the vector was digested with *Bam*HI and *Eco*RI and then inserted into pRSET-a (we designated this plasmid pEST3) and introduced into *E. coli* BL21 (DE3). The transformant was cultivated and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce gene expression when the optical density at 600 reached 0.4–0.6. After induction for 5 h, the cells were harvested.

Recombinant enzyme purification. The cells were suspended in 20 mM Tris–HCl buffer (pH 7.2). They were disrupted by sonication for 30 min at 50% output. Cell extracts were heated at 70 °C for 30 min and then centrifugated (at 50,000 × *g* for 30 min at 4 °C) to discard the denatured proteins. The supernatant was applied to a Q-Sepharose column (Amersham Pharmacia Biotech, Uppsala, Sweden) (5 × 12 cm) equilibrated with 20 mM Tri–HCl (pH 7.2) and eluted with a linear 0–0.5M NaCl gradient in the same buffer. Fractions containing esterase activity were collected and concentrated using a Vivaspin™ concentrator (Vivascience, Lincoln, U.K.). The sample was then applied to a Phenyl–Sepharose column (Amersham) (1 × 5 cm) equilibrated with 20 mM-Tris–HCl buffer (pH 7.2) containing 0.5 M NaCl. The column was then washed with the same buffer and eluted with a reverse 0.5–0 M NaCl gradient containing 50% ethylene glycol. The fractions containing esterase activity were collected and concentrated with a Vivaspin™ concentrator. The concentrated sample was applied to Superose 12 column (Amersham) (1 × 30 cm) equilibrated with 20 mM-Tri–HCl buffer (pH 7.2) containing 0.1 M NaCl. The fractions containing enzyme activity were collected and used as the purified enzyme.

Enzymatic assays. Esterase activity with *p*-nitrophenyl caprylate as a substrate was determined by measuring the amount of *p*-nitrophenol released by esterase catalyzed hydrolysis. The hydrolysis of the substrate was carried out at 70 °C for 5 min in 100 μl of 50 mM Tris–HCl buffer (pH 7.2) containing 0.4% Triton X-100 and 0.1% gum Arabic. Addition of SDS to a final concentration of 0.2% terminated the reaction. The amount of liberated *p*-nitrophenol was determined at 410 nm using a spectrophotometer ($\varepsilon = 1.034$ $mM^{-1}cm^{-1}$). One unit of esterase activity was defined as the amount of enzyme that produced 1 μmol of *p*-nitophenol per min at 70 °C. The background hydrolysis of the substrate was deduced by using a reference sample with an identical composition to the reaction mixture without the enzyme. The amount of protein was determined by the method of Bradford[17] with bovine serum albumin as the standard.

Esterase activity staining. Enzyme activity staining was performed by the modified staining technique of Sobeck *et al.*[11] The purified protein (25 μg) without heat treatment at 100 °C was loaded on SDS–PAGE. After SDS–PAGE, the gel was soaked three times for 30 min in 50 mM Tris–HCl (pH 7.2) containing 2.5% Triton X-100 and then incubated in a 50 ml solution of 50 mM Tris–HCl (pH 7.2) containing 5 mg of β-naphthylacetate dissolved in 0.5 ml of ethoxyethanol. After development by the dropwise addition of 0.5% Fast Blue RR solution at room temperature, the esterase active bands began to turn deep brown.

Characterization of the thermostable esterase. All biochemical and kinetic parameters for the enzyme were determined by the standard method described above. The temperature profile for activity of the esterase (5 μg/ml in 50 mM Tri–HCl buffer, pH 7.2) was determined between 40 and 100 °C. Enzyme thermostability was determined by residual activity assayed under the standard condition after incubating the enzyme solution at appropriate temperatures for 30 min. The effect of pH on enzymatic activity was studied between pH 3.6 and 10.0. The buffers used were a 50 mM acetic acid buffer (pH 3.6–5.0), a 50 mM sodium phosphate buffer, and a 50 mM boric acid buffer (pH 8.6–10.0).

Substrate specificity against *p*-nitrophenyl esters was determined using *p*-nitrophenyl (*p*-NP) esters with a chain length between C2 (*p*-nitrophenyl acetate) and C16 (*p*-nitrophenyl palmitate) as substrates in the standard assay condition. All experiments were performed in triplicate. Values for V_{max} and K_m were determined from Lineweaver–Burk plots.

The effect of detergents on esterase activity was determined by incubation of the enzyme for 30 min at 37 °C in 50 mM Tris–HCl buffer (pH 7.2) containing 1% (v/v) of detergent. Residual enzyme activity was measured by the standard assay described above. The inhibitory effects of esterase activity were examined using ethylenediaminetetra acetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), diethyl *p*-nitrophenyl phosphate (paraoxon), eserine, β-mercaptoethanol, diethyl pyrocarbonate, and mercuric chloride. The enzyme solutions were incubated for 1 h at 37 °C with 1 mM of each inhibitor and then the residual activities were assayed under the standard condition.

Enzymatic kinetic resolution of the chiral compound. To estimate the enzymatic hydrolysis resolution of the esterase toward a chiral compound, a methyl (±)-2-(3-benzoylphenyl)propionate (ketoprofen methylester) was synthesized chemically by esterification with a routinely acidic catalyst. 30 g of (±)-2-(3-benzoylphenyl)propionate was dissolved in 100 ml of methanol, and 2.5 ml sulfuric acid was added as a catalyst for esterification in the racemic ketoprofen solution. The solution was refluxed for 12 h. The reaction mixture was extracted and purified by repeated extraction with methylene chloride and 5% sodium bicarbonate solution. The structure of synthesized methyl ester was confirmed by ^{13}C-NMR (500 MHz, DMSO-d_6) and 1H-NMR

(500 MHz, DMSO-d_6). The racemic ketoprofen methyl ester was dissolved in methanol and added to 50 mM Tris–HCl, pH 7.0 (final substrate concentration 1 mM) containing the enzyme (0.234 mg). The reaction mixture was reacted at 60 °C in a water bath. Both the conversion and the enantiomeric excess of the products were determined by HPLC using the chiral column, Chirex™ Phase 3005 column (250 × 4.60 mm; Phenomenex, U.S.A) at 254 nm with a flow rate of 1.0 ml min^{-1} toward ketoprofen methyl ester and the mobile phase composition was 30 mM ammonium acetate in methanol. Under these conditions, racemic ketoprofen methyl ester, *R*-ketoprofen, and *S*-ketoprofen were detected at t_R 6.5, t_R 23.5, and t_R 26.3 respectively. The enantioselectivity of the enzyme was described as the enantiomeric ratio, *E*, which was obtained by the equation $E = \ln[1 - c(1 + ee_p)]/\ln[1 - c(1 - ee_p)]$, where c is the degree of conversion and ee_p the enantiomeric excess of the product.

Results

Identification of lipolytic enzymes in the genomic database

We identified five amino acid sequences (accession codes: SSO0102, SSO2493, SSO2517, SSO2518, and SSO2521) that have a typical motif, consensus sequence Gly–X–Ser–X–Gly, found at the active sites of serine protease (*e.g.* esterase, lipase, and protease) in the *S. solfataricus* P2 genomic database (http://www-archbac.u-psud.fr/Projects/sulfolobus/sulfolobus.html).[18] A comparison of the deduced amino acid sequences of these candidates with several esterases or lipases determined the residues forming the catalytic triads (His, Ser, and Asp) and the oxyanionic holes (His–Glu) (data not shown). An alignment of three amino acid sequences (SSO2493, SSO2517, and SSO2521) among them was found to be well conserved in the catalytic triads and oxyanionic holes (Fig. 1A).

A

```
                *         20        *         40        *         60        *         80        *        100
Est3 : MPLDPEVRKFLDYFYKANILDFTKVSLQEVREKLNKLLA-ETP-KDSVYKIEDRKIKCLETEIPIRIYYPDNKRLHPIILHFHGGAWILGSIETEDSVS :  98
Est2 : MPLDERIKELLESG---FIVFIGKASVDEVRKIFRQLAS--QAP-KVEVGKVEDIKIPGSEANINARVYLPKANGPYGVLIYLHGGGFVIGDVESYDPLC :  94
Est1 : MPLDPQVKELLTKIN--LLLETTPLTPQEFRKVRNELFIKMFNNEKVGLYNIRDVTIPVRNGDIKVRVYFPNQREKLPAVVYYHGGGFVYGNLDTHDSVC :  98
                                                                                                  HG

                *         120       *         140       *         160       *         180       *         200
Est3 : RILANSCNCIVISVNYRLAPEHKFPAAVTDCFDSIKWTYENAESLGCHPNRIAVFGISAGGNLAAATSILSRDQG-IKLRAQALVVPFVYLDLASTSMTE : 197
Est2 : RAITNACNCVVVSVDYRLAPEYKFPSAVIDSFDATNWVYNNLDKFDCKMG-VAIAGDSAGGNLAAVVALLSK--GKLNLKYQILIYPAVGFDSVSRSMIE : 191
Est1 : RLISKLSNTIIVSVDYRLAPEHKFPTQVYDAYDVVKWFANNGGKLSIDTSKIAVAGDSAGGNLSTVVSILDRDNGENVVKYQIMIYPVVNMLDSSPSMYN : 198
                                                                GXSXG

                *         220       *         240       *         260       *         280       *         300
Est3 : YRKGYFLDINVPLDYGIMMYIRDEKDLLNPMFVPLIAEDLSNLPQAIIVTAEYDPLRDQGEAYAKRLMEAGVLTLSPRVNGMVHGFLGS-PNIS--RLVS : 294
Est2 : YSDGFFLIREH-IEWFGSQYLRSPADLLDFRFSPILVQDLSGLPPALIITAEYDPLRDQGEAYANRLLQAGVPVTSVRFNNVIHGFLSFFPLIEQGRDAI : 290
Est1 : YGDGYFLIYER-ILWYNKQYVKEDSLYYNPLASPILAESHN-LPPALIITAEYDPLRDQGEMYAHKLKVSGVKTISIRYNGMIHGFVSFYEYLDTGKEAI : 296
                                                                D                   H

                *
Est3 : VMVGSALKDILMRG- : 308
Est2 : GLIGSVLRFAFYDKS : 305
Est1 : HHIASSIRKMFNVFY : 311
```

B

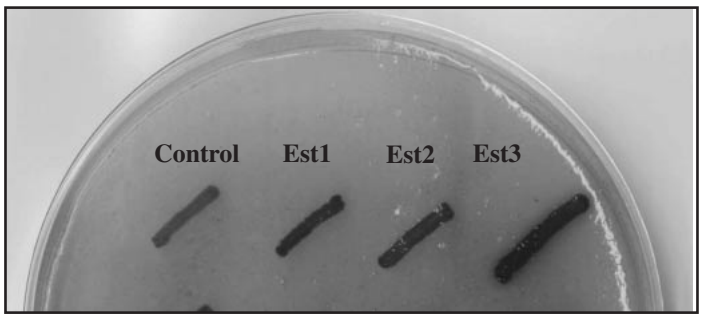

Fig. 1. Sequence Comparison of the Putative Lipolytic Enzyme Candidates and the Recombinant Enzyme Activities on an Indicator Plate.

(A) Est3, SSO2493; Est2, SSO2517 (this is the deduced amino acid sequence which extended to the 5′ direction of SSO2517 from the *S. solfataricus* P2 genomic database); Est1, SSO2521. The sequences were aligned using CLUSTAL W. Identical residues represent hypothetical active-site residues (catalytic triad) and an oxyanionic hole. Active site residues in the enzymes are underlined. (B) Lipolytic activities of recombinant enzymes on the Fast Blue RR indicator plate.

To identify the activities of the putative enzyme encoded by the three sequences, the genes containing their regulator regions were amplified directly from *S. solfataricus* genomic DNA. We constructed the recombinant plasmids (pGTE1: SSO2521, *est*1; pGTE2: SSO2517, *est*2; pGTE3: SSO2493, *est*3) through the process by which the PCR products were inserted into pGEM-T easy vector and then transformed into *E. coli* DH5α. These transformants were plated on replica plates and then, after the plates were overlaid with soft agar containing β-naphthylacetate as substrate and Fast Blue RR as an indicator, the enzyme activities produced by the recombinant strains were displayed as a brownish color on the indicator plates. The enzyme activities from all clones were detectable, but the color produced by the cell harboring pGTE3 was much deeper than that of any other clone (Fig. 1B). Based on the indicator plate experiments, Est3 was chosen for further study.

Expression and purification of a recombinant S. solfataricus esterase (Est3)

For identification and characterization of Est3, which had higher activity than the others, *est*3 was heterologously expressed in *E. coli*. The *est*3 gene encoding Est3 was amplified by PCR and then this PCR product was inserted into a pGEM®-T easy vector. The *est*3 from the cloned vector was subcloned into pRSET (we designated this vector pEST3) to overexpress this gene in *E. coli*, but the expression band of Est3 was not detectable on SDS–PAGE, although the recombinant strain harbored esterase activity. The protein expression level was very low, probably because the target protein was toxic toward the *E. coli* host system. Hence western blotting was performed with an antibody toward 6xHis-tag to identify the protein, Est3, expressed in *E. coli* BL21(DE3) (the pEST3 expression system has 6xHis tag residues torward N-terminal sequence of the recombinant Est3). No bands, however, were detected by western blotting with the anti-His-tag antibody (data not shown). It can be interpreted from these results that the N-terminal region of the recombinant Est3 fused with His-tag was cleaved by an unknown *in vivo* protease in *E. coli*, though the mechanisms are not clear. Another expression vector that has a C-terminal fused by the 6xHis-region was constructed. The esterase fused on the C-terminal with the 6xHis-tag was successfully overexpressed in *E. coli*, but the most expressed protein was produced as an inclusion body in non-active form (data not shown).

The recombinant esterase was purified from apparent homogeneity by heat treatment at 70 °C for 30 min followed by anion exchange and hydrophobic and gel filtration chromatography (Table 1, Fig. 2A.). The enzyme was purified 98.9-fold, with a yield of 2%. The enzymes purified from *E. coli* had a specific activity of 3,133 units/mg for Est3 against *p*-NPC. The molecular mass (34 kDa) of the recombinant Est3 was 34 kDa, in good agreement with the molecular mass (33,428 kDa) deduced from the amino acid sequence. In order to examine the subunit composition and activity of the protein, a Zymogram was performed under the conditions described above in the "Materials and Methods." Single protein bands of brown color of a multimer and a monomer were detected at locations of approximately 100 kDa and 34 kDa on SDS-PAGE respectively. Perhaps recombinant Est3 might be a trimer with subunits of 34 kDa (Fig. 2B.).

Table 1. Purification of Recombinant *S. solfataricus* Esterase (Est3) from *E. coli*

Step	Total protein (mg)	Total activity (Units)	Specific activity (Units/mg)	Purification (-fold)	Yield (%)
Crude extract	1482.0	46723	32	1.0	100
Heat treatment	150.0	27965	186	5.8	60
Q-Sepharose	57.5	22925	399	12.5	49
Phenyl Sepharose	6.2	5886	936	29.3	13
Superose 12	0.3	940	3133	98.9	2

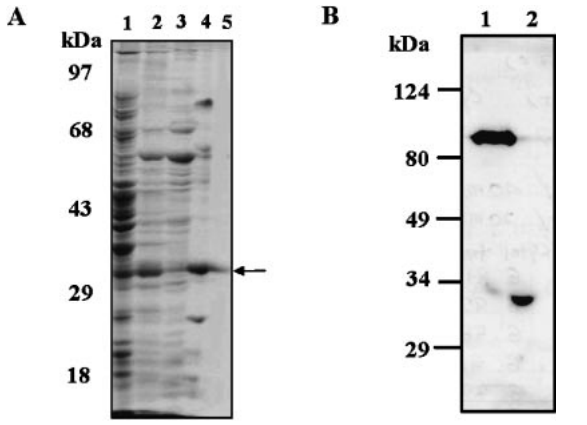

Fig. 2. SDS-PAGE and Zymogram of Purified Esterase from Recombinant *S. solfataricus* Est3.

(A) Protein staining. Lane 1, crude extract, lane 2, thermoprecipitation, lane 3, Q-Sepharose column, lane 4, Phenyl–Sepharose column, lane 5, Superose 12 column. (B) zymogram through activity staining using β-naphtyl acetate and Fast Blue RR. Lane 1, a multimer form of the purified protein, lane 2, a monomer form of the protein.

Effect of temperature and pH

The effect of temperature on esterase activity was studied using *p*-NPC (C8) as a substrate. The activity was measured in the range of 40 to 100 °C. The enzyme showed higher activity at higher temperatures, with an optimum temperature of 80 °C (Fig. 3A). The activation energy value was calculated from the Arrhenius plot to be 18.4 KJ/mol (4.3 Kcal/mol) in the range of 60 to 100 °C (Fig. 3B). The enzyme, however, was rapidly deactivated above 80 °C although *S. solfataricus* grows optimally at this temperature. The thermostability of *S. solfataricus* Est3 was also studied. The enzyme was

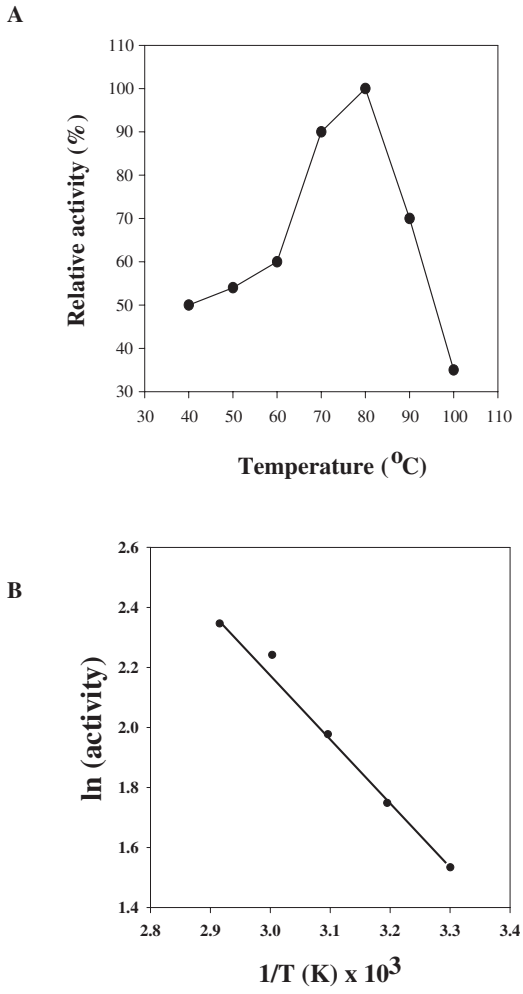

Fig. 3. Effect of Temperature on Recombinant Est3 Activity.
(A) Effect on Est3 activity. (B) Arrhenius plot of the data in A.

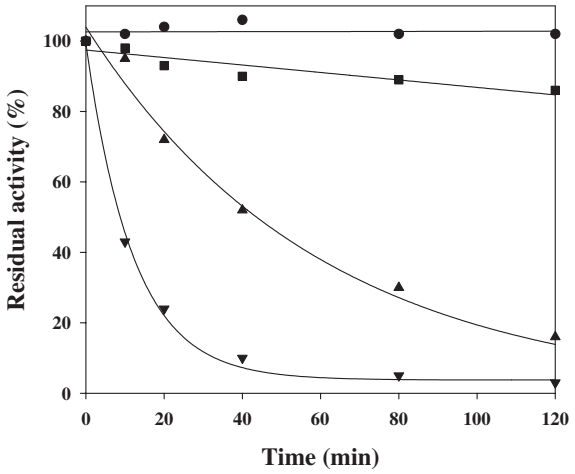

Fig. 4. Thermostability of Recombinant Est3 at Various Temperatures.
(●), 60 °C; (■), 70 °C; (▲), 80 °C; (▼), 90 °C.

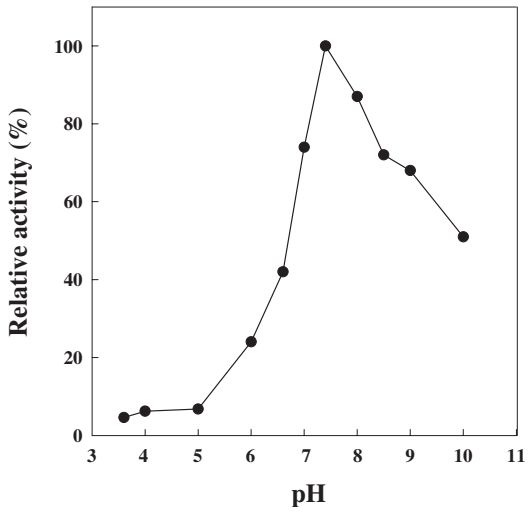

Fig. 5. The Effect of pH on Enzyme Activity.
The activity of esterase was obtained using *p*-nitrophenylester as a substrate at various pH levels.

incubated at 60, 70, 80, and 90 °C for various intervals of time. The Est3 enzyme was very stable for 2 h at 60 and 70 °C, with residual activity more than 90% of initial activity (Fig. 4). The stability of Est3 decreased rapidly at 80 °C, the optimum growth temperature of *S. solfataricus*. The half-life of the enzyme at 80 °C was 40 min.

The effect of pH on Est3 activity was investigated using *p*-NPC as a substrate. Activity was measured in a pH range of 3.6 to 10. The activity of the enzyme dramatically increased between pH 5.0 and 7.0 and then decreased to pH 10.0 (Fig. 5). Enzyme activity was 84% of maximum at pH 8.0, while no activity was observed below pH 5.0. Est3 displayed high activity at neutral pH, with an optimum pH of approximately 7.5. In the pH range from neutral to alkaline, the reaction rate of Est3 was higher than that for acidic pH. At high alkaline pH, the rate of chemical hydrolysis of the *p*-nitrophenyl caprylate was too fast to determine accurately the enzyme activity. Nevertheless, esterase activity was very high in the neutral pH range. This result means that the activity of intracellular enzymes from *S. solfataricus* is optimum in neutral pH ranges and that intracellular pH might be neutral, although *S. solfataricus*, a thermoacidophile, grows under extremely acidic conditions (pH < 3.0).

Substrate specificity and kinetic parameters

The hydrolysis ability of Est3 toward the length of the acyl chains from C2 to C16 was studied using various *p*-nitrophenyl (*p*-NP) esters. Higher activity of Est3 was shown toward *p*-NP-varelate (C5) as a substrate (Fig. 6). The activity of the enzyme decreased slowly from *p*-NP-caproate (C6) to *p*-NP-laurate (C12), and was very low for *p*-NP-myristate (C14) and *p*-NP-palmitate (C16). Enzyme activity against short and medium chains of substrates was above 80% of optimal activity between the C3 and the C8 acyl chains and below 50% of opti-

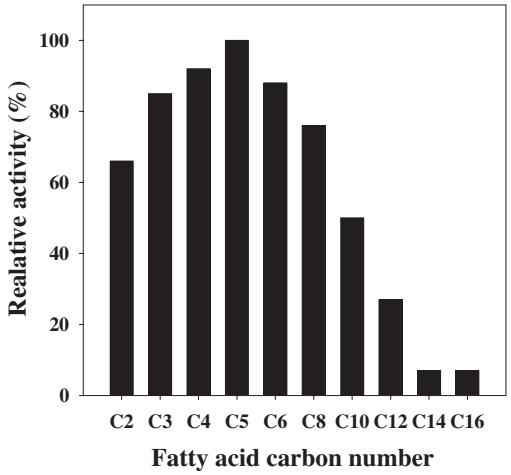

Fig. 6. Relative Activity toward *p*-Nitrophenyl Ester of Recombinant Est3.

Esterase activity was determined photometrically in a 50 mM sodium phosphate buffer (pH 7.0) using various *p*-nitrophenyl esters as substrates.

Table 2. Kinetic Parameters of Recombinant Est3 Esterase Activity toward Various *p*NP-Esters[a]

Substrates	K_m (mM)	V_{max} (μmol min^{-1}mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}mM^{-1})
*p*NP–acetate	2.5 ± 0.3	40.9 ± 1.2	45.3	16.2
*p*NP–valerate	2.1 ± 0.1	43.0 ± 0.1	46.3	21.1
*p*NP–caprylate	14.5 ± 1.0	70.4 ± 3.3	79.3	5.1
*p*NP–laurate	67.5 ± 0.2	72.3 ± 0.2	78.0	1.2
*p*NP–palmitate	4.8 ± 0.5	3.0 ± 0.5	3.8	0.7

[a]All substrates were dissolved in DMSO, which was dissolved under the standard assay condition. Each assay was done in triplicate. The enzyme concentration [E_0] was 4.5 nM.

mum between C10 to C12. It was less than 10% toward long-chain substrates (C14–C16). This indicates that *p*-nitrophenyl esters with acyl chains longer than C8 are not suitable against Est3 substrates. The enzyme can be classified as an esterase on the basis of these substrate profiles.

To determine kinetic parameters toward various *p*-nitrophenyl ester derivatives, the Est3 esterase was studied with each substrate range of 0.5 to 20 mM. The enzyme typically displayed a Michaelis–Menten kinetics pattern toward *p*-nitrophenyl ester derivatives. The kinetic parameters derived by a Lineweaver–Burk plot showed that the reaction velocity (V_m) has a tendency to increase with increases in the acyl chain length of substrates until the C12 substrate, *p*-NP-laurate (Table 2). The K_m value for *p*-NP-valerate (C5) was much lower than that for any other substrate, while the V_m value for *p*-NP-laurate (C12) was higher than for any other substrate tested. Activities toward longer ester chain substrates (*p*-NP-myristate (C14) and *p*-NP-palmitate (C16)) were very low due to their solubility in the reaction medium. Therefore, the k_{cat}/K_m ratio indicated that *p*-NP-valerate (C5) is the best substrate among the *p*-NP-esters examined. This result confirmed that the enzyme was specific for short-chain fatty acids rather than long-chain ones.

Effect of detergents, solvents, and inhibitors

To test the effect of detergents and solvents on esterase activity, various detergents and solvents were added in enzyme solutions with final concentrations of 5 and 10% (v/v) in total volume respectively. In the detergents test, any noticeable pattern of enzymatic activities toward effectors was not shown, but enzyme activities remained above 90% in 5% of Tween 80 or Triton X-100 in detergents, while Est3 activity decreased rapidly at 10% contents of the effectors in the solution after 1 h incubation. At 5% of water miscible solvents (polar solvents), esterase activity decreased below 10% of original activity except for ethanol and 2-butanol only (Table 3). The addition of 5 or 10% ethanol did not affect activity, whereas the addition of 5 or 10% methanol, 1-propanol, 1-butanol, or acetonitrile dramatically decreased enzyme activity. After incubation for 1 h at 37 °C, the relative activity of the enzyme decreased slightly with 10% acetonitrile or DMSO in the reaction mixtures.

Seven inhibitors were tested for their inhibition of esterase activity. The residual activities are shown in

Table 3. Effect of Detergents and Solvents on Est3 Esterase Activity[a]

Detergents & solvents	Concentration (%, w/v)	Relative activity (%)
Control	—	100.0
Tween 20	5	6.4
	10	8.2
Tween 60	5	161.0
	10	51.4
Tween 80	5	92.1
	10	98.7
Triton X-100	5	90.1
	10	7.9
Methanol	5	5.8
	10	6.9
Ethanol	5	100.0
	10	109.3
1-Propanol	5	8.9
	10	5.9
2-Propanol	5	41.5
	10	6.2
1-Butanol	5	4.8
	10	2.5
2-Butanol	5	70.6
	10	57.5
Acetonitrile	5	41.4
	10	39.4
DMSO	5	42.3
	10	34.3
Acetone	5	63.2
	10	12.3

[a]The enzyme was incubated for 1 h at 37 °C in 50 mM Tris-buffer (pH 7.0) with detergents or organic solvents.

Table 4. Effect of Inhibitors on Recombinant Esterase[a]

Inhibitors	Relative activity (%)
None	100
Mercury chloride	47
β-Mercaptoethanol	92
Phenylmethylsulfonyl fluroride	28
Diethyl *p*-nitrophenyl phosphate	30
Eserine	88
Diethyl pyrocarbonate	81
EDTA	102

[a]The enzyme was incubated in the presence of 1 mM inhibitors for 1 h at 37 °C. The reaction mixture was then assayed with *p*-nitrophenyl caprylate under the standard condition.

Table 4. PMSF and paraoxon strongly inhibited enzyme activity. This suggests the presence of a serine residue on the catalytic site of the enzyme. Activity was also strongly inhibited by mercury chloride, indicating that SH groups are essential for activity. Nevertheless, the eserine effect on enzyme activity was very low. Relatively, β-mercaptoethanol and diethyl pyrocarbonate did not damage enzymatic activity although they disrupted bonds for the multimer form in the enzyme and modified essential histidine residue for the catalytic reaction respectively. After treatment with EDTA, enzyme activity was almost unchanged.

Kinetic resolution of a chiral compound

The ability of the *S. solfataricus* esterase (Est3) to bring about kinetic resolution of a chiral compound was tested with the racemic ketoprofen methylester ((±)-2-(3-benzoylphenyl)propionic methylester) as a model compound. As shown in Fig. 7A, when biotransformation was performed in a 50 mM Tris–HCl buffer Est3 led to the production of the *R*-configured acid with an enantiomeric excess (*e.e.*) of 80% at a conversion rate of 20% in 32 h (Fig. 7B). The *E*-value was calculated as 10.5 at a conversion rate of 20%, as cording to Chen *et al.*[19]

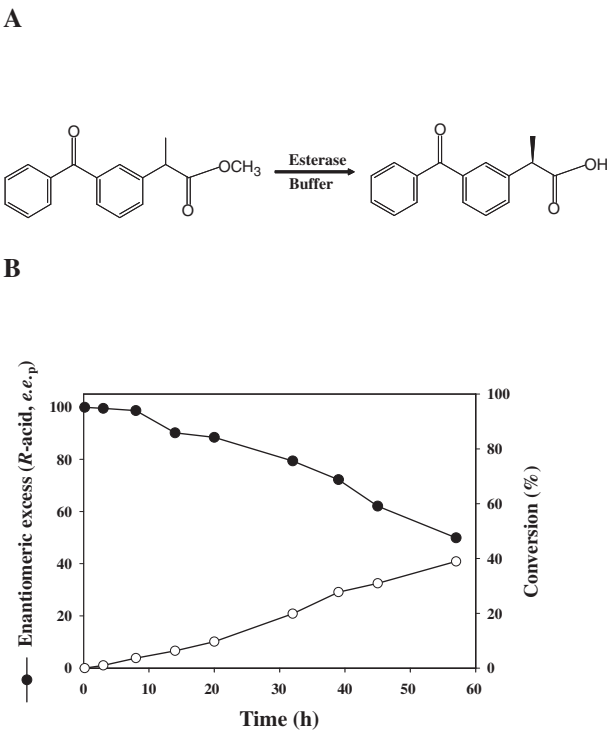

Fig. 7. Esterase-Catalyzed Kinetic Resolution of a Racemic Ketoprofen Methylester.

(A) Reaction scheme, (B) conversion rate (○) and enantiomeric excess (●) of racematic ketoprofen methylester.

Discussion

The *S. solfataricus* P2 genome showed several homologs to lipolytic enzymes having the consensus sequence Gly–X–Ser–X–Gly. Among these potential candidates, we cloned and expressed three putative genes (*est*1, *est*2, and *est*3) in *E. coli* and investigated their activities on the indicate plates. The cell harboring pGTE3 containing the *est*3 gene displayed higher activity than that of any other cell. When the Est3 (SSO2493) sequence was aligned with two candidates, the sequence appeared to be very similar to Est1 (51% similarity, 39% identity) and Est2 (59% similarity, 43% identity). Multiple sequence alignment allowed us to identify a highly conserved motif, Gly–X–Ser–X–Gly, at the active site and the catalytic triad, which can catalyze the ester hydrolysis reaction, formed by the amino acids Ser^{151}, Asp^{244}, and His^{274}. The alignment also indicated that the conserved His^{78} and Gly^{79} sequences are the oxyanion hole, in which the structure stabilizes the tetrahedral intermediate of the esterase reaction.

To study further Est3 based on the indicate plate experiments, the Est3 produced in *E. coli* BL21(pEST3) was characterized. Although the Est3 heterologously expressed in *E. coli* was obtained in a soluble form, a fusion partner, hexamer His-tag, in the N-terminus region of the enzyme was cleaved by an unknown proteolytic mechanism in the *E. coli* host. When that was fused at the C-terminus of the enzyme, Est3 was produced in an insoluble form, inclusion bodies. Due to these unfortunate results, we could not use the immobilized-metal affinity column. Therefore we tried to purify the enzyme using standard chromatographic techniques through ion-exchange, hydrophobic interaction, and gel-filtration column. Purification of the esterase, using the chromatography on Phenyl–Sepharose, yielded a highly active and concentrated esterase. It displayed a molecular weight of 32 kDa on SDS–PAGE. The native molecular weight of the enzyme was determined to be about 100 kDa by zymogram under non-denaturation conditions. These data indicate that the enzyme consists of three subunits with identical molecular masses.

The thermostability of Est3 was lower than that of the native esterase from the *Sulfolobus* species previously

reported. A similar phenomenon was also forward in the recombinant esterase from *S. acidocadarius*, reported by Arpigny *et al.*[20] The thermostability of the recombinant enzyme appears to be very different from that of the native esterase directly purified from *S. acidocadarius*.[11,20] The activity of a recombinant enzyme decreased to half at 80 °C after 45 min while the native enzyme did not lose activity for 1 h. The unstable characteristics of recombinant enzymes expressed in *E. coli* might be due to a lack of the unique mechanisms as compatible solutes, heat inducible molecular chaperones, and post-synthetic modifications for the thermostability of proteins discovered in hyperthermophiles. Further studies of the thermostability of Est3 are necessary to identify accurate mechanisms at high temperatures above 80 °C.

A substrate profile test of Est3 showed relative activity above 50% toward substrates with medium acyl chain lengths (C5–C10). This suggests that this enzyme is a typical esterase with broad substrate specificity. This characteristic of Est3, which has relatively high activity against medium chain ester substrates, is similar to those of esterases reported in prokaryotes, *Bacillus licheniformis*,[21] and *B. stearothermophilus*.[22] This feature of Est3 might be due to a hydrophobic binding pocket which might be suitable for binding the substrates of short or medium chains at the active site.

Esterases can be classified based on substrate specificity and sensitivity toward various inhibitors.[11,23,24] Although this categorization has limitations due to overlapping substrate specificities or inhibitor patterns, this classification is generally used to characterize the biochemical properties of esterases. Phenylmethylsulfonyl fluroride (PMSF) and diethyl *p*-nitrophenyl phosphate (paraoxon) inhibited Est3 activity in the above experiment. This suggests that serine residue might be involved at the catalytic site of the enzyme. Activity was also inhibited by mercuric chloride, indicating that sulfide (SH) groups are involved in the enzyme activity. Diethylpyrocarbonate or eserine, which modify histidine residue, did not significantly affect enzyme activity. EDTA also did not nearly inhibit the enzymatic activity, and hence non-metals might not be involved in the catalytic mechanism of Est3. On the basis of these inhibition patterns, the Est3 esterase from *S. solfataricus* can be classified as a B-type esterase, carboxylesterase (EC 3.1.1.1).

The Est3 esterase showed higher stability toward relatively high concentrations of solvents or detergents compared with other esterases. In particular, the resistance of Est3 to mild detergents or polar solvents is a very attractive property for use as a biocatalyst in organic media for the synthesis of chiral compounds. It suggests the possibility that the catalytic properties of Est3 might be improved by reaction media engineering.

In the above experimental data on kinetic resolution of α-arylpropionic acid, the enzyme hydrolyzed the (*R*)-ester of racemic ketoprofen methylester. These data led us to consider the enzyme from *S. solfataricus* as a novel biocatalyst for the kinetic resolution of racemic compounds. The carboxylesterase from *S. solfataricus* P1 (Sso Est1) showed a specific reaction toward the *S*-naproxen ester in co-solvent reaction conditions.[25] But Est3 displayed a different catalytic pattern toward an α-arylpropionic methylester substrate, ketoprofen methylester, containing the same chiral center. The selectivity toward substrates observed in previous literature might be elucidated by the fact that the change in the side-chain structure of the substrate leads to a drastic change in reaction rate and enantioselectivity.[26] On the basis of the data presented here, we are now investigating the enatioselectivity of this enzyme toward substrates and reaction media such as organic solvents.

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