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# Characterization of a novel thermophilic pyrethroid-hydrolyzing carboxylesterase from *Sulfolobus tokodaii* into a new family

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ABSTRACT

A novel gene ST2026 encoding a putative carboxylesterase from the thermophilic crenarchaeota *Sulfolobus tokodaii* (named EstSt7) was cloned and functionally overexpressed in *Escherichia coli*. The recombinant enzyme was purified to homogeneity after heat treatment, Ni-NTA affinity and Superdex-200 gel filtration chromatography. EstSt7 showed maximum activity at 80 °C over 30 min and had a half-life of 180 min at 90 °C. Its enzymatic activity was stable in the pH range of 8.0–10.0 with an optimum at 9.0. The enzyme exhibited significant esterase activity toward various *p*-nitrophenyl esters and the most preferable substrate was *p*-nitrophenyl butyrate ($k_{cat}/K_m$ of 246.3 $s^{-1}$ $mM^{-1}$). In addition, EstSt7 showed high activity and stability against organic solvents (20% and 50% v/v) and detergents (1% and 5% v/v). Furthermore, EstSt7 could efficiently hydrolyze a wide range of synthetic pyrethroids including fenpropathrin, permethrin, cypermethrin, cyhalothrin, deltamethrin and bifenthrin, which makes it a potential candidate for the detoxification of pyrethroids for the purpose of biodegradation. Sequence alignment, phylogenetic analysis and comparison of the conserved motif reveal that this novel carboxylesterase EstSt7 should be grouped into a new bacterial lipase and esterase family.



## 1. Introduction

Carboxylesterase (EC3.1.1.1, carboxylester hydrolases) and lipase (EC3.1.1.3, triacylglycerol hydrolases) belong to a family of carboxylic ester hydrolases that catalyze the hydrolysis and synthesis of ester bonds. These enzymes are found throughout the three phylogenetic domains of life, but the physiological role of carboxylic ester hydrolase is still not clear [1,2]. Many carboxylesterases/lipases have been used in biotechnological applications such as medical biotechnology, detergent manufacturing, organic chemical synthesis, paper manufacturing, biodiesel production and bioplastics food technology [3,4]. They function via a catalytic triad formed from a nucleophilic serine in a conserved pentapeptide GXSXG motif, and an acidic residue (aspartic acid or glutamic acid) that is hydrogen-bonded to a histidine residue [5,6]. Carboxylesterases differ from lipases in that they show a preference toward water-soluble short-chain acylglycerols (≤10 carbon atoms) and lack the interfacial activation. Carboxylesterases and lipases have been classified into eight families (I–VIII) based on conserved sequence motifs and fundamental biological properties. Enzymes within family I are true lipases and are further classified into six subfamilies (I.1–I.6), while enzymes belonging to families II–VIII are carboxylesterases [3,7].

Pyrethroids are synthetic analogs of pyrethrins, which are natural chemicals with insecticidal activity derived from Chrysanthemum flowers [8]. Synthetic pyrethroids are used for insect control in agriculture, public health, and households as replacements for more toxic organochlorine and organophosphorus pesticides. Although pyrethroid pesticides generally have lower mammalian toxicity than organochlorine or organophosphate insecticides, they may cause endocrine disruption, spleen damage, allergic skin reactions and carcinogenesis [9,10]. In addition, extensive applications of this kind of pesticide have caused pest resistance, soil and/or water contamination, and high amounts of residues in agricultural products. Carboxylesterases from microorganisms are known to have an important role in degradation of pyrethroids in the natural environment [11,12]. Several carboxylesterases from *Klebsiella* sp. strain ZD112, *Aspergillus niger* ZD11, *Nephotettix cincticeps*, *Ochrobactrum anthropi* YZ-1, *Sphingobium* sp. strain JZ-1 and

*Abbreviations:* SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; EDTA, ethylene diamine tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; DEPC, diethyl pyrocarbonate; DTT, dithiothreitol; DMF, dimethylformamide; IPTG, isopropyl-β-D-thiogalactopyranoside; *p*NP-esters, *p*-nitrophenyl esters.

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metagenomic libraries capable of hydrolyzing pyrethroids have been identified and characterized [12–18].

*Sulfolobus tokodaii* strain 7, originally isolated from an acidic hot spring in Beppu, Kyushu, Japan in the early 1980s, grows optimally at 75–80 °C [19]. To date, only one thermostable carboxylesterase ST0071 from *S. tokodaii* strain 7 has been identified and characterized [20]. In this study, we report the identification, cloning, expression and biochemical characterization of a novel thermophilic and pyrethroid-hydrolyzing carboxylesterase EstSt7 from *S. tokodaii* strain 7. The recombinant EstSt7 is identified as a member of a new family of carboxylesterases which diverge from the eight families classified by Arpigny and Jaeger [3].

## 2. Materials and methods

### 2.1. Chemicals, strains and plasmids

*Sulfolobus tokodaii* strain 7 was purchased from the Japan Collection of Microorganisms and cultured in a modified Allen mineral medium [21]. The genomic DNA was extracted as described previously [22]. Restriction enzymes, Pyrobest DNA polymerase, and the DNA ligation kit were purchased from Takara Biotechnology (Dalian, China). All the substrates (*p*-nitrophenyl esters) were obtained from Sigma (St. Louis, MO, USA). Fenpropathrin, permethrin, cypermethrin, cyhalothrin, deltamethrin and bifenthrin were obtained from Yangnong Chemical Group Co., Ltd., Jiangsu, China. All the other chemicals were of the highest reagent grade and obtained from Sangon (Shanghai, China). Nickel columns and Superdex 200 gel filtration columns were from GE Healthcare (Buckinghamshire, UK). The plasmid pET15b (Novagen, Madison, USA) was modified with *Bam*HI, *Nsi*I, *Sal*I, and *Sac*II to introduce multiple cloning sites for the expression of His-tagged protein [23].

### 2.2. Sequence analysis

The homologous sequence and conserved domain were identified through Blastp [24] provided by the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignment and prediction of secondary structure were carried out by CLUSTALW and Phyre 2.0 [25,26]. The phylogenetic tree was built by the neighbor-joining analysis method using Molecular Evolutionary Genetics Analysis 4.1 software (MEGA, version 4.1) [27].

### 2.3. Cloning of the gene and construction of expression vector

The gene (ST2026) encoding for a putative carboxylesterase from *S. tokodaii* strain 7 was amplified using PCR with the upstream primer 5′-GCCCATATGTTAATCAAAAGCTTTGC-3′ and the downstream primer 5′-GGCGTCGACTTAACTATTGTCACCATTAAAC-3′ (the underlined regions in the upper-stream and down-stream primer indicate the *Nde*I and *Sal*I sites, respectively). The amplified fragment was digested with *Nde*I and *Sal*I and cloned into pET15b and the resulting plasmid was designated as pET15b/EstSt7. DNA sequencing was performed to confirm that no unintended mutation had occurred.

### 2.4. Expression and purification of the recombinant enzymes

The constructed pET15b/EstSt7 was transformed into the host *Escherichia coli* BL21-CodonPlus (DE3)-RIL to express the recombinant enzyme. About 2% of overnight culture of the transformed cells was inoculated into 1000 ml of LB medium containing 100 mg/L ampicillin and 34 mg/L chloramphenicol. The cells were grown at 37 °C with shaking. When the cultures reached an $OD_{600}$ of 0.4–0.6, IPTG was added to a final concentration of 0.5 mM to induce gene expression. After being cultured for 4 h at 37 °C, the cells were harvested by centrifugation, resuspended in buffer A (50 mM Tris–HCl, pH 8.0, 50 mM NaCl) and then disrupted by sonication. The disrupted cells were heated at 75 °C for 30 min and then centrifuged at 12,000 g for 30 min to remove the cell debris and denatured proteins. The soluble fraction was loaded onto a nickel column (GE Healthcare, Buckinghamshire, UK). After washing with buffer containing 20 mM imidazole, the enzymes were eluted with buffer containing 300 mM imidazole. For further purification, the samples were dialyzed overnight in buffer B (50 mM Tris–HCl, pH 8.0, 200 mM NaCl), concentrated by ultrafiltration using a 10-kDa membrane (Stirred Cell Model 8050, Millipore) and loaded onto a Hiload Superdex 200 (16/60) gel filtration column (GE Healthcare, Buckinghamshire, UK). The column was pre-equilibrated with buffer B at a flow rate of 0.5 ml/min. The peak fractions were determined by the analysis of enzymatic activity under the standard conditions. The fractions containing esterase activity were collected and analyzed by SDS-PAGE. The protein concentration was determined according to the Bradford method.

### 2.5. Enzymatic activity assay

Esterase activity was determined spectrophotometrically by hydrolysis of *p*-nitrophenyl esters of various chain lengths as described previously [28]. The standard reaction mixture (1 ml), consisting of 10 μl of 20 mM *p*NP-butyrate (*p*NP-C4) as a substrate and 0.98 ml of 50 mM Tris–HCl buffer (pH 9.0), was preincubated for 2 min and then the reaction was started by adding 23 μg of purified enzyme (10 μl). The esterase activity was measured at 80 °C for 30 min and the extinction coefficient ($\varepsilon_{405}$) for *p*NP-C4 was 16,460 $M^{-1}$ $cm^{-1}$. One unit of esterase activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenyl per min under the standard conditions. Measurements were corrected for background hydrolysis in the absence of enzyme.

The hydrolytic activity of EstSt7 toward various triacylglycerols was determined spectrophotometrically by measuring the free fatty acids in the form of copper soaps [29]. The reaction mixture was incubated with shaking for 14 h at 80 °C. Absorption at 430 nm was measured immediately after the start of the reaction. One unit of enzymatic activity was defined as the amount of enzyme releasing 1 μmol of free fatty acid per minute under standard conditions.

### 2.6. Hydrolysis of the pyrethroids

Various pyrethroids including fenpropathrin, permethrin, cypermethrin, deltamethrin, cyhalothrin and bifenthrin were used to test the hydrolytic activity of the esterase EstSt7 according to the method of Stock et al. with slight modifications [30]. A total of 1 μl of substrate (25 mM in ethanol) was added to 0.5 ml of the preincubated enzyme (20–30 μg/ml), and then all the treatments were carried out in 50 mM Tris–HCl buffer (pH 9.0) at 80 °C. The enzyme mixture was incubated from 1 to 10 min depending on the substrate. Then the sample was extracted with an equal volume of *n*-hexane twice, and the organic layer was dried and redissolved in *n*-hexane. The pyrethroid residue was quantified by GC analysis (GC-2010, Shimadzu Corporation, Japan). The analysis conditions were as follows: ECD detector with RestekRTX-5 column (30 m × 0.25 mm × 0.25 μm), $N_2$ carrier gas at 1 ml/min; inlet temperature of 260 °C, oven temperature of 240 °C, ECD detector temperature of 300 °C; 1 μl sample with split ratio of 49:1. One activity unit was defined as the amount of enzyme releasing 1 μmol product or hydrolyzing 1 μmol substrate per min. A control without enzyme was used to eliminate the autohydrolysis of pyrethroids at high temperatures and alkaline pHs. All enzyme activity assays were carried out at least three times and the data were averaged.

### 2.7. Substrate specificity

Substrate specificity was determined by hydrolysis of different *p*NP-esters including *p*NP-acetate (C2), *p*NP-C4, *p*NP-octanoate (C8), *p*NP-decanoate (C10), *p*NP-laurate (C12), *p*NP-myristate (C14), and *p*NP-palmitate (C16). The $K_m$ and $V_{max}$ values were determined by measuring the initial velocity of hydrolysis at different substrate concentrations (0.001–1.0 mM) in three independent trials. The corresponding $K_m$ and $V_{max}$ values were computed using Hanes–Wolff plots and the Michaelis–Menten equation.

### 2.8. Effect of temperature and pH on enzyme activity and stability

The effects of temperature and pH on the esterase activity were examined using *p*NP-C4 as the substrate. The optimum pH was studied at 80 °C in the pH range of 5.0–11.0. The following buffers (50 mM) were used: sodium acetate (pH 5.0–6.0), sodium phosphate (pH 6.0–7.5), Tris–HCl (pH 7.5–9.5), and *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS, pH 9.5–11.0). The pHs of the different buffers were adjusted at 80 °C. The pH stability was measured after incubation of the purified enzyme (23 μg/ml) in the reaction mixture at 80 °C for 60 min, and then the residual enzyme activity was measured by the standard method. The optimum temperature was determined at temperatures ranging from 30 °C to 100 °C in 50 mM of Tris–HCl buffer (pH 9.0). The buffer solutions were adjusted to pH 9.0 at each assayed temperature. In addition, the thermostability of the purified esterase (23 μg/ml) was examined in 50 mM Tris–HCl buffer (pH 9.0) at three different temperatures (70 °C, 80 °C, and 90 °C). Each sample (50 μl) was assayed after incubation for 60, 120, 180 or 240 min. The residual activities were assayed under the standard conditions.

### 2.9. Effect of metal ions, organic solvents, inhibitors and detergents on enzyme activity

The effects of metal ions on the esterase activity were investigated by adding 5 mM of various metal salts ($CaCl_2$, $CoCl_2$, $CuCl_2$, $FeSO_4$, NaCl, KCl, $NiSO_4$, $MgCl_2$, $MnCl_2$, and $ZnCl_2$) directly to the assay solution containing the purified enzyme (23 μg/ml) and 50 mM Tris–HCl buffer (pH 9.0) for 60 min at room temperature. The stability of the esterase against organic solvents was determined using methanol, ethanol, acetone, propanol, chloroform, DMF, *n*-hexane, heptane, formaldehyde and toluene at final concentrations of 20% or 50% (v/v) in 50 mM Tris–HCl buffer (pH 9.0). The mixture containing the purified enzyme (23 μg/ml) and each organic solvent of interest was incubated at 30 °C with constant shaking at 160 rpm for 12 h. Potential inhibitors (EDTA, PMSF, DEPC, and DTT) at a final concentration of 5 mM were incubated with the enzyme (23 μg/ml) in 50 mM Tris–HCl buffer (pH 9.0), and then the solutions were assayed for enzyme activity. The effect of detergents on enzyme activity was determined by incubating the enzyme (23 μg/ml) in 50 mM Tris–HCl buffer (pH 9.0) with 1% or 5% (w/v) of Tween 20, Tween 80, Triton X-100 and SDS at room temperature for 60 min. In all of these assays, the residual enzyme activity was measured under the standard assay conditions and was initiated by addition of the substrate *p*NP-C4.

## 3. Results and discussion

### 3.1. Amino acid sequence comparison

An ORF (ST2026) of 969 bp encoding a hypothetical protein (named EstSt7) of 322 amino acids was identified from the genomic DNA of *S. tokodaii* strain 7. The nucleotide sequence reported herein was submitted to the GenBank database with the accession number NP_378015. Sequence alignment demonstrated that EstSt7 shares only 11–17% sequence identity with the characterized thermophilic carboxylesterases (data not shown). BLAST-P analysis revealed the highest similarity to other hypothetical proteins and a putative esterase. The deduced amino acid sequence of ST2026 showed 58, 58, 59, 47, 43, and 42% sequence identity with the putative esterases from *Sulfolobus acidocaldarius* DSM 639 (Saci_0120), *Sulfolobus solfataricus* (SSO2979), *Acidianus hospitalis* W1 (Ahos_0898), *Metallosphaera cuprina* Ar-4 (Mcup_0788), *Thermaerobacter marianensis* DSM 12885 (Tmar_0396), and *Thermobaculum terrenum* ATCC BAA-798 (Tter_0425). In addition, the multiple sequence alignment revealed that EstSt7 contains a typical catalytic triad composed of Ser131-Asp263-His299 and the conserved motif Gly-X-Ser-Ser-Gly-Gly-Trp-Gly containing the active site residue. Based on the results of the secondary structure prediction and conserved domain search, EstSt7 consists of a large domain with a classical α/β hydrolase core including seven α-helices (α1–α4 and α8–α10) and seven-stranded β-sheets (β1–β7), and a smaller domain comprising three α-helices (α5–α7) (Fig. 1). The hypothetical model of EstSt7 was constructed using the 3D-structural threading program Phyre. The Esta protein from *Streptococcus pneumonia* (PDB: 2uz0) was used to build the model of EstSt7 (Supplementary Fig. 1). The overall hypothetical structure covered the α/β hydrolase core of EstSt7 (amino acid residues 1–162 and 220–332), but the smaller domain has not been modeled.

### 3.2. Phylogenetic tree

To clarify the possible evolutionary position of EstSt7 and its homologs, a phylogenetic tree was constructed based on the classification of bacterial esterases and lipases proposed by Arpigny and Jaeger [3]. Phylogenetic tree analysis showed that EstSt7 and its homologs, Saci_0120 from *S. acidocaldarius* DSM 639 and SSO2979 from *S. solfataricus* are not grouped in any of the eight families and instead formed a distinct group (Fig. 2). This divergence from the current families can be verified by aligning the conserved motif (Supplementary Fig. 2). A highly conserved motif GXSSGGYG (X indicating any amino acid), which is different from the consensus of the current esterase and lipase families, was identified in EstSt7 and its homologs. Taken together, these data suggest that EstSt7 and its homologs should form a new bacterial esterase and lipase family that diverges from the eight families proposed by Arpigny and Jaeger. The enzymes belonging to this new family have not been identified or characterized to date. Several carboxylesterases from other thermophilic bacteria have been grouped into new carboxylesterase families including PhaZ7 from *Paucimonas lemoignei*, EstD from *Thermotoga maritima*, Est30 from *Geobacillus stearothermophilus*, and EstGtA2 from *Geobacillus thermodenitrificans*, rather than one of the eight families [31–34].

### 3.3. Expression and purification of EstSt7

The recombinant plasmid pET15b/EstSt7 was constructed to determine the biochemical properties of EstSt7. EstSt7 was expressed in *E. coli* BL21-CodenPlus (DE3) and purified to homogeneity after heat treatment, Ni-NTA affinity and Superdex-200 gel filtration chromatography. The purified recombinant protein as a His6-EstSt7 fusion had a molecular weight of about 37 kDa by SDS-PAGE, which is consistent with its calculated mass of 37.4 kDa (Fig. 3). The specific activity of the purified EstSt7 reached 354 U/mg using *p*NP-C4 as a substrate in Tris–HCl buffer at pH 9.0 and 80 °C. The enzyme was finally purified 11.7-fold with a yield of 43.2% (Table 1). The SDS-PAGE analysis of the EstSt7 is shown in Fig. 3.

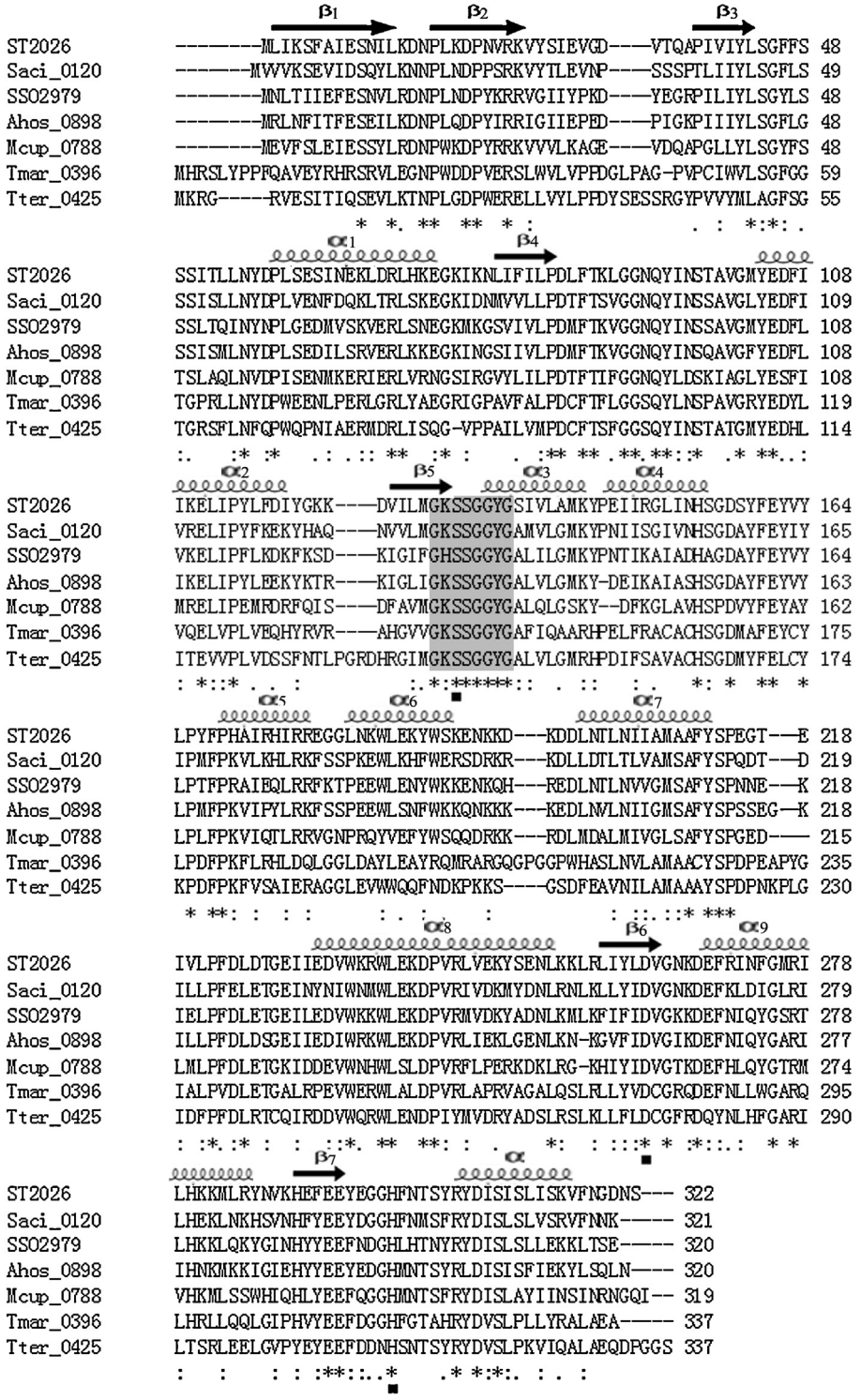


**Fig. 1.** Multiple sequence alignment for EstSt7 and the related sequences using Clustal W program with manual adjusting: EstSt7 from *Sulfolobus tokodaii* Strain 7 (NP_378015); Saci_0120 from *Sulfolobus acidocaldarius* DSM 639 (YP_254837); SSO2979 from *Sulfolobus solfataricus* P2 (NP_344293); Ahos_0898 from *Acidianus hospitalis* W1(YP_004458082); Mcup_0788 from *Metallosphaera cuprina* Ar-4 (YP_004409377); Tmar_0396 from *Thermaerobacter marianensis* DSM 12885 (YP_004101244); Tter_0425 from *Thermobaculum terrenum* ATCC BAA-798 (YP_003322168). The conserved motif GXSSGGYG is in *shallow*. Symbols above blocks of sequence represent the predicted secondary structure, springs represent helices, and arrows represent β-strands. The closed black squares represent the amino acids of the catalytic triad Ser131-Asp263-His299.

### 3.4. Substrate specificity and kinetics

The substrate specificity of EstSt7 was determined using *p*NP-esters with acyl chains of different lengths. The purified enzyme showed the highest activity with *p*NP-C4 (354 U/mg) among the *p*NP-esters examined (Table 2). The $K_m$ and $k_{cat}$ values of the enzyme were calculated from Hanes–Wolff plots and the Michaelis–Menten equation. The $k_{cat}$ and $K_m$ values decreased with increasing acyl chain length of the substrates. The $k_{cat}/K_m$ value for EstSt7 toward different *p*NP-esters followed the order *p*NP-C4 ($246.3\ s^{-1}\ mM^{-1}$) > *p*NP-C2 ($160.7\ s^{-1}\ mM^{-1}$) > *p*NP-C8 ($138.0\ s^{-1}\ mM^{-1}$) > *p*NP-C10 ($45.7\ s^{-1}\ mM^{-1}$) > *p*NP-C12

**Table 1**
Purification of the carboxylesterase EstSt7 from *S. tokodaii* strain 7.

| Step | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Purification fold | Yield (%) |
|---|---|---|---|---|---|
| Crude cell extract | 32.5 | 9845 | 303 | 1 | 100 |
| Heat treatment | 15.3 | 7481 | 489 | 1.6 | 76.0 |
| Ni-NTA affinity | 2.8 | 6023 | 2151 | 7.1 | 61.2 |
| Superdex-200 gel filtration | 1.2 | 4250 | 3542 | 11.7 | 43.2 |

**Table 2**
Kinetic parameters of the purified esterase EstSt7 for hydrolysis of various *p*NP-esters.[a]

| Substrate | Specific activity (U/mg) | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($s^{-1}$ $mM^{-1}$) |
|---|---|---|---|---|
| *p*NP-acetate (C2) | 213 ± 22 | 464.2 ± 54.2 | 74.6 ± 12.5 | 160.7 |
| *p*NP-butyrate (C4) | 354 ± 16 | 239.1 ± 31.2 | 58.9 ± 6.0 | 246.3 |
| *p*NP-caprylate (C8) | 189 ± 28 | 152.9 ± 7.3 | 21.1 ± 5.8 | 138.0 |
| *p*NP-decanoate (C10) | 56 ± 13 | 32.8 ± 3 | 1.5 ± 1.2 | 45.7 |
| *p*NP-laurate (C12) | 37 ± 4 | 14.8 ± 2 | 0.2 ± 0.2 | 13.5 |
| *p*NP-myristate (C14) | 19 ± 3 | 9.4 ± 0.1 | 0.11 ± 0.2 | 11.7 |
| *p*NP-palmitate (C16) | 13 ± 2 | 5.1 ± 0.3 | 0.03 ± 0.001 | 5.9 |

[a] The values are means of three independent experiments.

($13.5\ s^{-1}\ mM^{-1}$) > *p*NP-C14 ($11.7\ s^{-1}\ mM^{-1}$) > *p*NP-C16 ($5.9\ s^{-1}\ mM^{-1}$) (Table 2). Therefore, the $k_{cat}/K_m$ values demonstrated that *p*NP-C4 was the best substrate for EstSt7 among the substrates tested. Similar results have been found for esterases from the hyperthermophilic bacterium *T. maritima* and esterases from *Thermoanaerobater tengcongensis* and *Thermus scotoductus* SA-01, which all comparatively have high activity toward *p*NP-C4 [32,35–37]. The results were not surprising because esterases use short chain fatty acids as substrates for their catalytic activity.

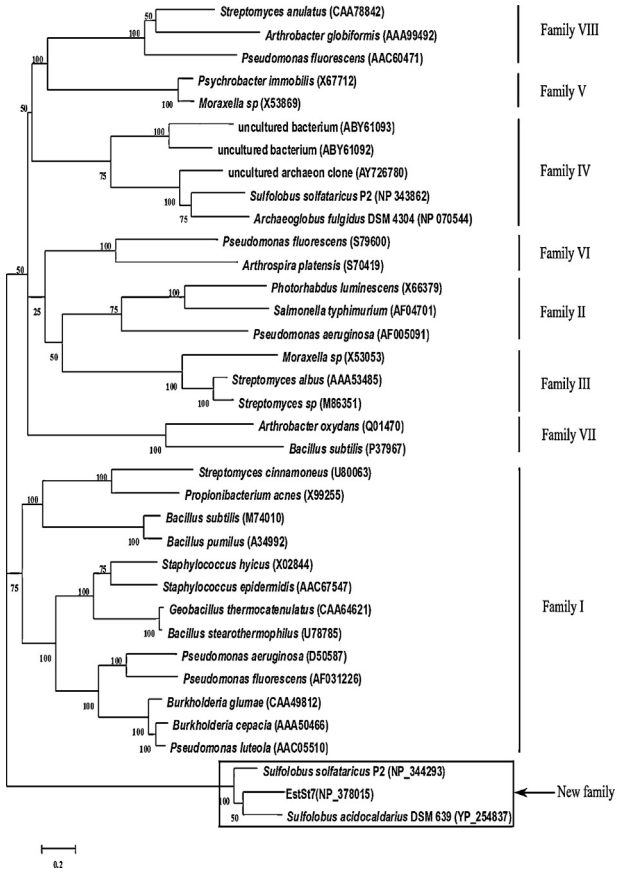


**Fig. 2.** Phylogenetic tree of EstSt7 and related lipolytic enzymes. The tree was constructed using the MEGA 4.1 program with the neighbor-joining method. *Bar*-0.2 substitutions per amino acid site. Sequences were obtained from Genbank and sequenced genomes.

The hydrolytic activity of EstSt7 toward triglycerols (triacetin, tributyrin, tricaproin, tricaprylin, tricaprin and triolein) was tested, but no enzyme activity was determined (data not shown). These results indicate that the enzyme could be classified as a true esterase based on this demonstrated substrate specificity [3].

### 3.5. Effects of temperature and pH on enzyme activity

The effects of temperature and pH on the esterase activity were studied from 30 °C to 100 °C and from pH 5.0–11.0 using *p*NP-C4 as a substrate. EstSt7 has an optimum temperature at 80 °C and an optimum pH at 9.0. The pH optimum was higher than that of most reported thermostable esterases except the esterase from *Archaeoglobus fulgidus*, which has an optimum pH of 10–11 and esterases from *T. tengcongensis* and a metagenomic library, both of which have optimum pHs of 9.0 [36,38,39]. The thermostability of EstSt7 was examined at three different temperatures (70 °C, 80 °C, and 90 °C) with increasing incubation times up to 240 min. Most of the enzyme activity was maintained after incubation at 70 °C for at least 240 min, indicating the enzyme to be highly thermostable, with a half-life of 180 min at 90 °C. The results of the pH stability assay indicated that EstSt7 maintained over 80% of its maximal activity in the pH range of 8.0–10.0, even after incubation at 80 °C for 60 min (Fig. 4). These results indicated that EstSt7 possesses

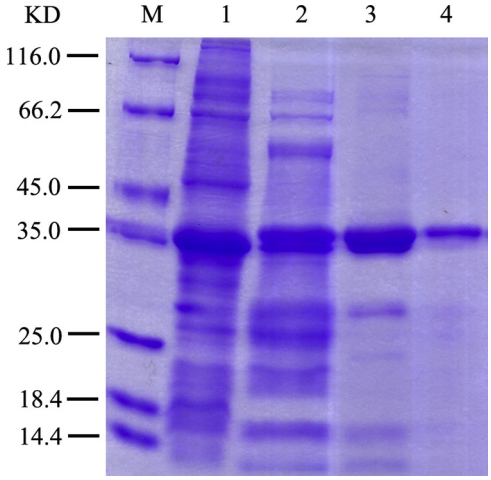


**Fig. 3.** SDS-PAGE of the recombinant esterase EstSt7. Lanes 1, crude cell extract; 2, supernatant after heat treatment; 3, the EstSt7 after nickel affinity chromatography; 4, the purified EstSt7 after Superdex-200 gel filtration chromatography.

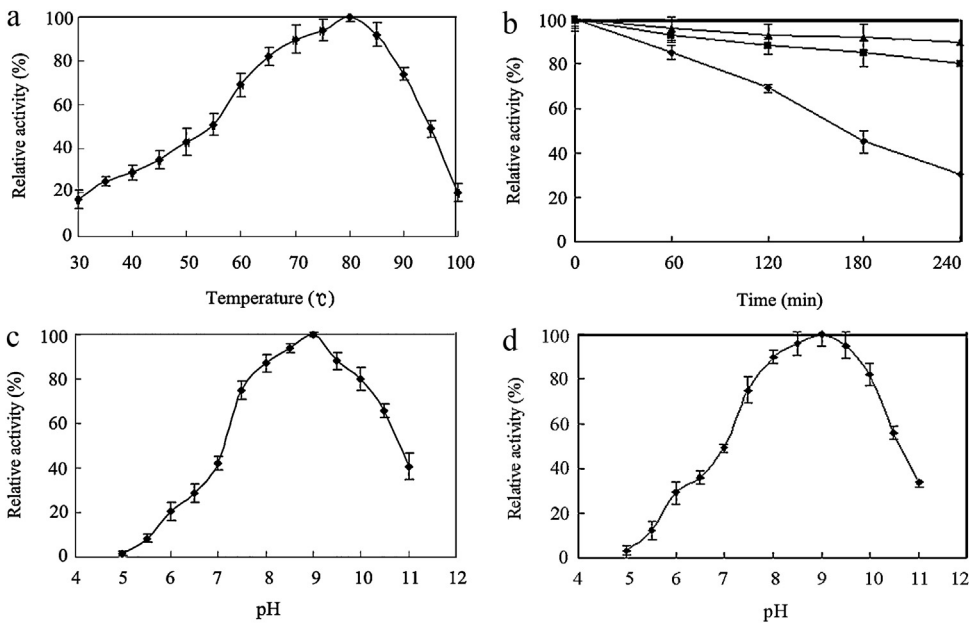


**Fig. 4.** The effects of temperature (a) and pH (b) on the enzymatic activity, and results of thermostablility (c) and pH stability (d) tests. (a) Optimal temperature of EstSt7 was determined with *p*NP-C4 as substrates in 50 mM Tris–HCl buffer (pH 9.0) at different temperatures ranging from 30 °C to 100 °C. (b) Thermostability of EstSt7. The residual enzyme activity was measured after incubation of the purified enzyme at 70 °C (*triangles*), 80 °C (*boxes*), and 90 °C (*diamonds*), respectively. (c and d) Optimal pH and pH stability of the enzyme at pHs ranging from 5.0 to 11.0 was measured using *p*NP-C4 as substrates for 60 min at 80 °C. The buffers used were 50 mM of sodium acetate (pH 5.0–6.0), sodium phosphate (pH 6.0–7.5), Tris–HCl (pH 7.5–9.5), and CAPS buffer (pH 9.5–11.0). The values are means of three independent experiments.

high stability at high temperatures and at alkaline pHs and is a thermophilic and alkaliphilic esterase.

### 3.6. Tolerance of the esterase EstSt7 against organic solvents

Stability and activity in organic solvents are important characteristics of esterases used as many industrial biocatalysts [34]. The effect of the organic solvent depends on the nature of both the enzyme and solvent. The tolerance of EstSt7 was studied for some polar solvents (methanol, ethanol, acetone, propanol, chloroform, and DMF) and non-polar solvents (*n*-hexane, heptane, formaldehyde and toluene) after incubation for 2 h. Addition of polar solvents did not cause a drastic loss of enzyme activity at 20% (v/v) organic solvent while the enzymatic activity was reduced below 50% at 50% (v/v) solvent (Table 3). In contrast, less than 25% of the activity of EstSt7 was maintained at 20% (v/v) concentrations of non-polar solvents. This result demonstrated that esterase EstSt7 is relative stable in the presence of various organic solvents.

**Table 3**
Effect of organic solvents on the esterase activity of EstSt7.[a]

| Organic solvents or detergents | Concentration (%) | Relative activity (%) |
|---|---|---|
| None | – | 100 |
| Methanol | 20 (v/v) | 74 ± 2 |
| | 50 (v/v) | 42 ± 3 |
| Ethanol | 20 (v/v) | 81 ± 2 |
| | 50 (v/v) | 33 ± 4 |
| Acetone | 20 (v/v) | 85 ± 1 |
| | 50 (v/v) | 40 ± 3 |
| Propanol | 20 (v/v) | 92 ± 5 |
| | 50 (v/v) | 41 ± 3 |
| Chloroform | 20 (v/v) | 80 ± 2 |
| | 50 (v/v) | 34 ± 2 |
| DMF | 20 (v/v) | 92 ± 4 |
| | 50 (v/v) | 28 ± 6 |
| *n*-hexane | 20 (v/v) | 23 ± 42 |
| Heptane | 20 (v/v) | 12 ± 3 |
| Formaldehyde | 20 (v/v) | 19 ± 2 |
| Toluene | 20 (v/v) | 14 ± 5 |

[a] The values are means of three independent experiments.

### 3.7. Effect of metal ions, inhibitors and detergents on the enzymatic activity

The effects of metal ions, inhibitors and detergents on the enzymatic activity were determined by the standard esterase assay. Addition of 5 mM of various metal ions ($Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Na^{+}$, and $K^{+}$) did not significantly affect the enzymatic activity after incubation for 60 min at room temperature. On the other hand, $Cu^{2+}$, $Fe^{2+}$ and $Zn^{2+}$ reduced the esterase activity to 43, 19, and 13%, respectively. The effects of non-ionic detergents (Tween 20, Tween 80, Triton X-100) and the ionic detergent SDS on the esterase activity were also investigated. Incubation with Tween 20, Tween 80, or Triton X-100 at 1% and 5% reduced the esterase activity by less than 50% (Table 4). However, SDS strongly inhibited its activity at both concentrations tested (1% and 5%).

To identify the amino acids involved in the catalytic mechanism, the inhibitory effects of various chemical modifiers including EDTA, PMSF, DEPC and DTT on the esterase EstSt7 were determined. The enzymatic activity was not significantly affected by EDTA (5 mM), indicating the enzyme was not a metalloprotein. EstSt7 was completely inhibited by PMSF at 5 mM and the enzymatic activity was reduced to 35% by the addition of DEPC (5 mM). This result demonstrated that serine and histidine might be involved at the esterase catalytic site, which is in agreement with the anticipated catalytic triad (Ser131-Asp263-His299). The enzyme activity was activated by 5 mM DTT (120%) (Table 4).

### 3.8. Hydrolytic activity toward the pyrethroids

Hydrolysis of the pyrethroids for EstSt7 was determined using fenpropathrin, permethrin, cypermethrin, deltamethrin, cyhalothrin and bifenthrin as the substrates. As shown in Fig. 5, purified EstSt7 was able to hydrolyze all the pyrethroids tested. The relative hydrolytic activity of EstSt7 toward various pyrethroids

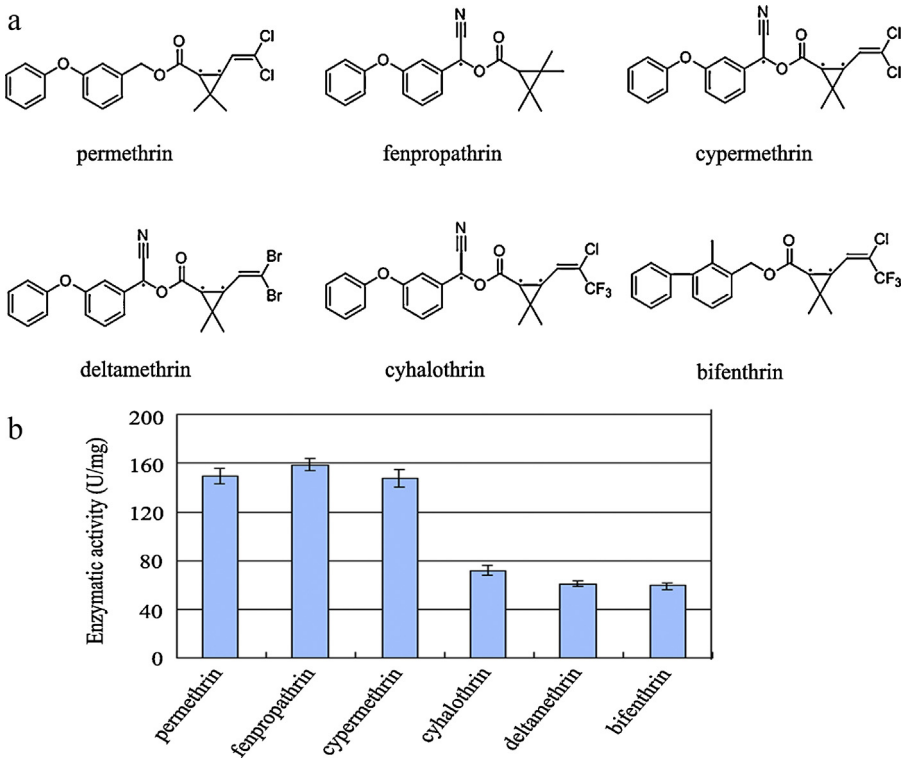


**Fig. 5.** (a) Molecular structures of pyrethroids used in this test. Stereogenic centers are indicated by black dots. (b) Hydrolytic activity of EstSt7 to various pyrethroids (fenpropathrin, permethrin, cypermethrin, cyhalothrin, deltamethrin and bifenthrin). The values are means of three independent experiments.

was as follows: fenpropathrin (159 U/mg) > permethrin (150 U/mg) > cypermethrin (148 U/mg) > cyhalothrin (72 U/mg) > deltamethrin (61 U/mg) > bifenthrin (59 U/mg). The hydrolysis activities of EstSt7 toward fenpropathrin, permethrin, cypermethrin, cyhalothrin and deltamethrin were higher than that of most reported pyrethroid-hydrolyzing esterases including the esterases from *Klebsiella* sp. strain ZD112, *A. niger* ZD11, *O. anthropi* YZ-1 and *Sphingobium* sp. strain JZ-1 [12–14,16].

**Table 4**
Effect of various metals, inhibitors and detergents on the esterase activity of EstSt7 [a]

| Metals or inhibitors | Concentration | Relative activity (%) |
|---|---|---|
| None | – | 100 |
| $Ca^{2+}$ | 5 mM | 81 ± 3 |
| $Mg^{2+}$ | 5 mM | 89 ± 4 |
| $Mn^{2+}$ | 5 mM | 73 ± 2 |
| $Cu^{2+}$ | 5 mM | 43 ± 3 |
| $Zn^{2+}$ | 5 mM | 13 ± 5 |
| $Fe^{2+}$ | 5 mM | 19 ± 5 |
| $Co^{2+}$ | 5 mM | 62 ± 4 |
| $Ni^{2+}$ | 5 mM | 78 ± 1 |
| $Na^{+}$ | 5 mM | 98 ± 3 |
| $K^{+}$ | 5 mM | 102 ± 4 |
| EDTA | 5 mM | 83 ± 5 |
| PMSF | 5 mM | 0 |
| DEPC | 5 mM | 35 ± 4 |
| DTT | 5 mM | 120 ± 3 |
| Tween 20 | 1% (w/v) | 88 ± 3 |
| | 5% (w/v) | 72 ± 2 |
| Tween 80 | 1% (w/v) | 80 ± 5 |
| | 5% (w/v) | 62 ± 3 |
| Triton X-100 | 1% (w/v) | 93 ± 2 |
| | 5% (w/v) | 58 ± 4 |
| SDS | 1% (w/v) | 28 ± 3 |
| | 5% (w/v) | 10 ± 2 |

[a] The values are means of three independent experiments.

The hydrolysis activities of EstSt7 toward fenpropathrin, permethrin and cypermethrin were found to not be significantly distinguishable, suggesting that the replacement of the hydrogen atom with a cyano group on the methylene carbon of the 3-phenoxybenzyl alcohol moiety and the replacement of the methyl with dichloroethylene on the chrysanthemic acid only had a slight influence on the enzyme activity. Cyhalothrin, deltamethrin and bifenthrin were degraded much more slowly than cypermethrin, suggesting that the substitution of chloroyl with fluoro or bromovinyl on the chrysanthemic acid or replacement of the 3-phenoxybenzyl with a biphenyl group greatly reduced the enzyme activity. This result indicated that EstSt7 was a broad-spectrum pyrethroid-hydrolyzing enzyme and might have applications in biodegradation. Although many thermophilic carboxylesterases have been identified and characterized, EstSt7 is the first reported thermophilic carboxylesterase with high pyrethroid-hydrolyzing activity from archaea. These results supported the idea that carboxylesterase EstSt7 possesses relatively broad pyrethroid substrate specificities and has practical applications in the field of biodegradation.

## 4. Conclusions

In this study, a novel thermophilic carboxylesterase EstSt7 displaying pyrethroid-hydrolyzing activity from *S. tokodaii* strain 7 was purified and characterized for the first time. Based on the sequence alignment, phylogenetic analysis and comparison of the conserved motif, it should be grouped into a new family of bacterial lipases and esterases. The purified recombinant EstSt7 possesses high activity and extreme stability at high temperatures and alkaline pHs, and displays high hydrolysis activities toward pyrethroids. To our knowledge, EstSt7 is the first characterized thermophilic and alkaliphilic carboxylesterase with high pyrethroid-hydrolyzing activity from archaea. These results indicate that the carboxylesterase EstSt7 could be a potential candidate

for the detoxification of pyrethroids in biotechnological applications.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2013.07.022.

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