

# Characterization of a thermostable carboxylesterase from the hyperthermophilic bacterium *Thermotoga maritima*

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**Abstract** The gene encoding carboxylesterase from the hyperthermophilic bacterium *Thermotoga maritima* (*tm0053*) was cloned. The recombinant protein (EST53) was overexpressed in *Escherichia coli* without its NH<sub>2</sub>-terminal hydrophobic region, and with a C-terminal hexahistidine sequence. The enzyme was purified to homogeneity by heat treatment, followed by Ni<sup>2+</sup> affinity chromatography, and then characterized. Among the *p*-nitrophenyl esters tested, the best substrate was *p*-nitrophenyl decanoate with  $K_m$  and  $k_{cat}$  values of 3.1  $\mu$ M and 10.8 s<sup>-1</sup>, respectively, at 60°C and pH 7.5. The addition of *O,O'*-bis(2-aminoethyl)ethyleneglycol-*N,N,N',N'*-tetraacetic acid decreased the esterase activity, indicating that EST53 is dependent on the presence of Ca<sup>2+</sup> ion. In addition, its activity was inhibited by the addition of phenylmethylsulfonyl fluoride and diethyl pyrocarbonate, indicating that it contains serine and histidine residues, which play key roles in the catalytic mechanism. EST53 shows a relatively high degree of similarity to *Burkholderia* lipases that belong to family I.2 of the lipolytic enzymes, whereas the local sequence around the pentapeptide of EST53 is most similar to those of *Bacillus* lipases belonging to family I.4.

**Keywords** Esterase · Carboxylesterase · Hyperthermophilic enzyme · Lipase · Alpha/beta-Hydrolase · Lipolytic enzyme

## Introduction

Various organisms, including animals, plants, and microorganisms, produce lipolytic enzymes that catalyze the cleavage of ester bonds. These enzymes are classified into two major families: the carboxylesterases (EC 3.1.1.1) and the lipases (EC 3.1.1.3). While similar in molecular structure and catalytic mechanisms, carboxylesterases are distinguishable from lipases in that they lack the requirement of interfacial activation and show preferential activity toward acylglycerols with short chains (<10 carbon atoms) as substrates (Jaeger et al. 1999). Lipolytic enzymes do not require cofactors and are usually rather stable and active in organic solvents, and also show high regio- and stereospecificity. These properties make them attractive biocatalysts for the production of optically pure compounds in fine chemical synthesis (Jaeger and Reetz 1998). There is a great deal of interest regarding these enzymes from thermophilic microorganisms because of their stability at high temperatures and in organic solvent. Analysis of these enzymes may also provide new insights into the molecular basis of protein thermostability.

The lipolytic enzymes have a highly conserved catalytic triad, which is mostly composed of Ser, Asp, and His residues. In addition, they have a pentapeptide sequence motif, GX SXG, around the active site serine. The enzymes display the common  $\alpha/\beta$  hydrolase fold (Ollis et al. 1992), which is commonly found in other hydrolases, such as haloalkane dehalogenase (Franken et al. 1991), *meta*-cleavage product hydrolase (Fushinobu et al. 2005),

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acetylcholinesterase (Sussman et al. 1991), dienelactone hydrolase (Pathak and Ollis 1990), and serine carboxypeptidase (Liao and Remington 1990).

Bacterial lipolytic enzymes were recently classified into eight different families according to their amino acid sequence (Arpigny and Jaeger 1999). The largest family, family I, consists of six subfamilies, including the best-studied “true lipases,” such as *Pseudomonas* lipases (Nardini et al. 2000), *Bacillus* lipases, and *Staphylococcus* lipases. Family I.2 includes lipases from *Chromobacterium viscosum* (Lang et al. 1996), *Burkholderiagluma* (Noble et al. 1993), and *Burkholderiacepacia* (Schrag et al. 1997). Family I.4 contains lipases from *Bacillus* species, whose characteristic pentapeptide sequence, GX SXG, around the catalytic serine residue is changed to AX SXG. Family IV is referred to as the hormone-sensitive lipase (HSL) family, and many carboxylesterases from hyperthermophilic archaea belong to this family. Although there have been many reports on the cloning and expression of thermostable carboxylesterases from archaea (Hotta et al. 2002; Manco et al. 2000; Morana et al. 2002), there have been no previous reports regarding hyperthermophilic bacteria as enzyme sources.

In this study, we report the isolation of the carboxylesterase gene (*tm0053*) from the hyperthermophilic bacterium *Thermotoga maritima*, which has a growth temperature of around 80°C, and characterization of the recombinant enzyme (EST53). EST53 was purified and demonstrated to be a thermophilic and thermostable carboxylesterase.

## Materials and methods

### Cloning and overexpression

*T. maritima* MSB8 (JCM 10099) was obtained from the Japan Collection of Microorganisms. Genomic DNA from *T. maritima* was isolated using a DNA purification kit (Promega). The *tm0053* gene was amplified by PCR using Easy-A *Taq* polymerase (Stratagene) and oligonucleotides 5'-CAT ATG TTT GGC TTC AAC ATC ATT CTG GAA TC-3' and 5'-GCG GCC GCT TGC GAT CCC CCC TTC TTT AAA ATC-3', which were designed to introduce *NdeI* and *NotI* restriction sites (underlined), respectively. This construct was designed for expression of the esterase gene lacking the putative transmembrane region encoding the NH<sub>2</sub>-terminal 14 amino acid residues, deleting the hydrophobic helix region predicted by the SignalP server (Bendtsen et al. 2004). The PCR product (1.1 kb), which was extracted from an agarose gel, was ligated into the linear vector pT7blue-2 (Novagen), and the resulting plasmids were transformed into *Escherichia coli* JM109. The transformants were subsequently plated onto Luria–Bertani (LB) agar plates containing 100 µg/ml of ampicillin, 40 µg/ml of

5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). White colonies were picked and their plasmids were purified using a Plasmid DNA purification kit. The DNA sequencing was performed by CEQ DTCS Quick Start kit for Dye Terminator Cycle Sequencing (Beckman Coulter) with a CEQ 8000 Genetic Analysis System (Beckman Coulter). Sequence primers were designed to cover the whole open reading frame region of the gene to confirm the sequence is identical to the published genome sequence of *T. maritima* MSB8. The plasmid was then digested with *NdeI* and *NotI*, inserted into pET-21a (+), and introduced into *E. coli* BL21-CodonPlus(DE3) RIL (Novagen). The transformants were cultivated at 37°C in 400 ml of LB broth containing 100 µg/ml of ampicillin and 35 µg/ml of chloramphenicol, and 0.1 mM IPTG was added to induce gene expression when optical density at 600 nm (OD<sub>600</sub>) reached 0.5. Culture was continued for a further 3 h and the cells were collected by centrifugation.

### Protein purification

Cells were suspended in 50 mM of Tris–HCl buffer (pH 7.5). Cell extracts were obtained by sonication and incubation at 60°C for 30 min, followed by centrifugation (8,000×g for 30 min at 4°C) to remove the denatured proteins. The supernatant was then applied to a HiTrap Chelating HP column (GE Healthcare) equilibrated with 50 mM of Tris–HCl buffer (pH 7.5) containing 0.5 M NaCl and eluted with a linear gradient of 0 to 0.5 M of imidazole in 50 mM of Tris–HCl buffer containing 0.5 M of NaCl. The fractions containing esterase activity were collected and concentrated using an Amicon Ultra centrifugal filter unit (10 kDa cut-off) (Millipore). The protein concentration was determined with bicinchoninic acid protein assay system (Pierce) with bovine serum albumin as a standard.

### Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE, 12%) was performed to determine the purity and apparent molecular weight of the esterase by the method of Laemmli (1970). The molecular mass was calibrated using a low molecular weight calibration kit (GE Healthcare) containing phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). Gels were stained with Coomassie Brilliant Blue R-250. The molecular mass of the denatured enzyme was calculated by interpolation on a plot of log of molecular mass against relative migration.

## Enzyme assays

The esterase activity against *p*-nitrophenyl esters was determined by measuring the amount of *p*-nitrophenol released by esterase-catalyzed hydrolysis. The production of *p*-nitrophenol was monitored continuously at 405 nm using a JASCO V-550 spectrophotometer with a thermal control unit. Unless otherwise noted, in the standard assay esterase activity was measured using a substrate mixture consisting of 100 mM *p*-nitrophenyl caprylate in acetonitrile, isopropanol, and 50 mM of Tris–HCl buffer (pH 7.5) at a ratio of 1:4:94 (vol/vol/vol) at 60°C. The reaction mixture, containing 990 µl of substrate mixture and 5 µl of 1 M of CaCl<sub>2</sub>, was preincubated for 2 min and then the reaction was initiated by adding 5 µl of enzyme solution to the reaction mixture. Thus, the final concentrations of *p*-nitrophenyl caprylate and calcium ions were 1 and 5 mM, respectively. One unit of enzymatic activity was defined as the amount of esterase capable of releasing 1 µmol of *p*-nitrophenol/min. The background hydrolysis of the substrate was deduced using a reference sample of identical composition to the reaction mixture without enzymes. Initial velocity of the enzymatic reaction was measured within 1 min in which the inactivation does not appear. Measurements were carried out at least three times, and the absorption coefficients were measured under all conditions. The activity was determined from the rate of the hydrolysis reaction.

The effect of pH on esterase activity was examined over the range of pH 4.0 to 9.0. The buffers used were 50 mM of citrate–NaOH (pH 4.0 to 6.0), MES–NaOH (pH 6.0 to 7.0), HEPES–NaOH (pH 7.0 to 7.5), and Tris–HCl (pH 7.5 to 9.0). The effects of temperature on esterase activity were examined in the range of 30 to 100°C in 50 mM of Tris–HCl buffer (pH 7.5) with *p*-nitrophenyl caprylate as a substrate. The thermostability of the esterase was examined by incubating the enzyme in 50 mM of Tris–HCl buffer (pH 7.5) at 60, 70, and 80°C, and the residual activities were measured by the standard assay as described above.

Substrate specificity was determined using *p*-nitrophenyl acetate (0.2 to 8.0 mM), *p*-nitrophenyl butyrate (0.02 to 1.0 mM), *p*-nitrophenyl caprylate (0.002 to 0.2 mM), *p*-nitrophenyl decanoate (0.001 to 0.1 mM), and *p*-nitrophenyl myristate (0.001 to 0.1 mM) as substrates in 50 mM of Tris–HCl buffer (pH 7.5) containing 1% acetonitrile and 4% isopropanol at 60°C.

The effects of metal ions on esterase activity were determined using various metal salts (CaCl<sub>2</sub>, FeCl<sub>2</sub>, KCl, NiCl<sub>2</sub>, MgCl<sub>2</sub>, MnCl<sub>2</sub>, and ZnCl<sub>2</sub>) at final concentrations of 5 mM in 50 mM of Tris–HCl (pH 7.5) at 60°C. The esterase activity of the enzyme without addition of metal ions was defined as 100%. The effects of inhibitors on esterase activity were examined using ethylenediaminetetraacetic acid (EDTA), *O,O'*-bis(2-aminoethyl)ethyleneglycol-*N,N,N',N'*-

tetraacetic acid (EGTA), phenylmethylsulfonyl fluoride (PMSF), diethyl pyrocarbonate (DEPC), dithiothreitol (DTT), and 2-mercaptoethanol (2-ME) at final concentrations of 5 mM. The enzyme was incubated with each inhibitor at 37°C for 10 min in 50 mM of Tris–HCl buffer (pH 7.5). The effects of organic solvents on esterase activity were examined using dimethylformamide (DMF), dimethyl sulfoxide (DMSO), and 1,4-dioxane at final concentrations of 1 to 10%. The reactions were stopped by cooling the samples on ice and aliquots were assayed to measure the residual activity by the standard assay described above. Reaction mixture without additives was used as a reference.

## Results

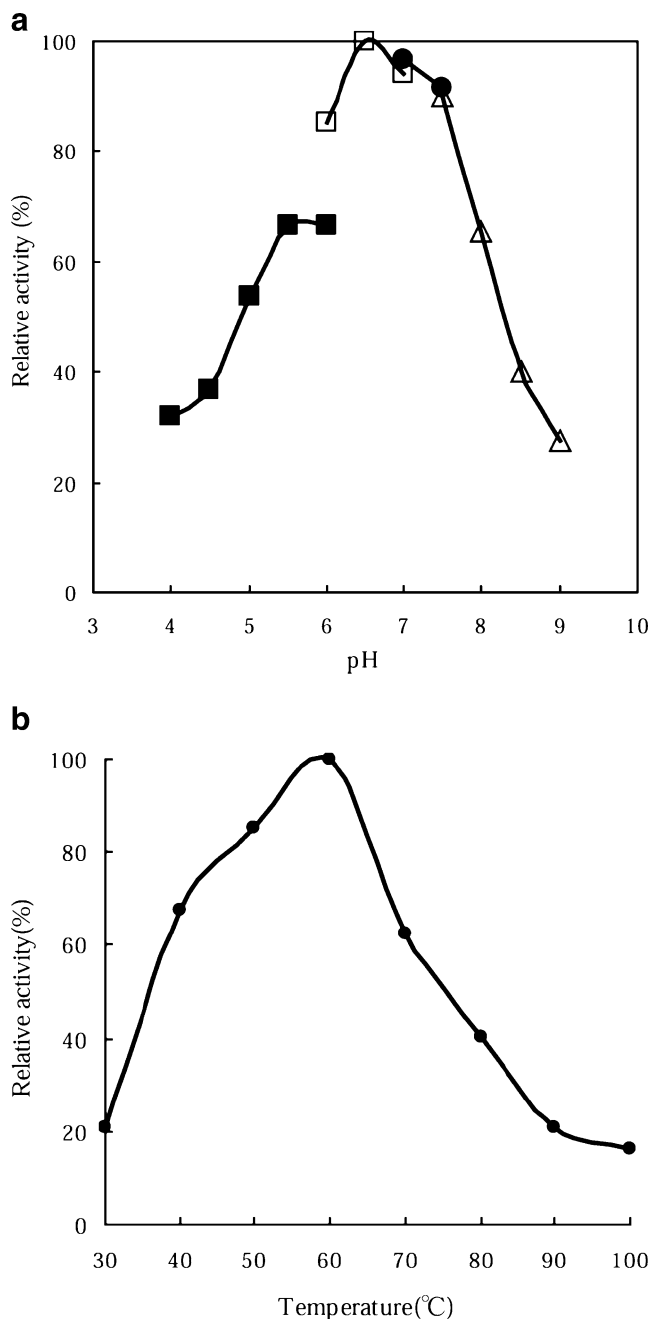
### Cloning of the esterase gene

The esterase gene (*tm0053*) without a part of its putative N-terminal hydrophobic signal region was amplified by PCR from *T. maritima* genomic DNA and cloned into the pT7blue-2 cloning vector. Analysis of the deduced amino acid sequence showed that EST53 has the esterase/lipase catalytic triad, Ser162, Asp323, and His344 (Fig. 1). BLAST search revealed significant similarity between EST53 and *Burkholderia* lipases, including those from *B. glumae* (Noble et al. 1993), *B. cepacia* (Schrag et al. 1997), and *Burkholderia* sp. 99-2-1 (Park and Lee 2005), with about 24% similarity in the 125 amino acids around the catalytic serine residue. Multiple sequence alignment with lipolytic enzymes from *Burkholderia*, *Bacillus*, and *Pseudomonas* indicated that EST53 is most similar to the *Burkholderia* lipases (family I.2) (Fig. 1), while it shows little similarity to archaeal carboxylesterases (family IV/HSL and V; data not shown). Most of the proteins belonging to family I.2, which have an NH<sub>2</sub>-terminal transmembrane region, are lipases that catalyze the hydrolysis of triacylglyceride into free fatty acids and glycerol. However, EST53 has no lipase activity. The activity toward *p*-nitrophenyl paltimate (C16) and *p*-nitrophenyl stearate (C18) at 100 µM were in about 50 and 25%, respectively, in comparison with that toward *p*-nitrophenyl decanoate (C10). We assayed the activity toward olive oil, but pH change was not detected (data not shown). EST53 shows especially high levels of similarity to *Bacillus* enzymes (family I.4) in only the short region around the catalytic serine residue including the AXSXG motif, although there is little similarity over the entire sequence.

### Overexpression and purification of recombinant EST53

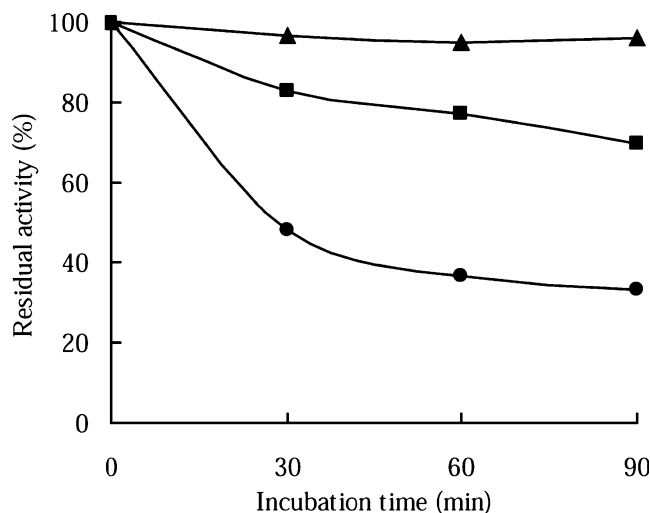
For expression in *E. coli* and subsequent purification of EST53, the cloned gene was subcloned into the expression





**Fig. 2** Effects of **a** pH and **b** temperature on the esterase activity of EST53. **a** The activity of the enzyme at different pH values was measured at 60°C. Buffers used were citrate–NaOH (closed boxes; pH 4.0 to 6.0), MES–NaOH (open boxes; pH 6.0 to 7.0), HEPES–NaOH (closed circles; pH 7.0 to 7.5), and Tris–HCl (open triangles; pH 7.5 to 9.0). **b** The activity of the enzyme at different temperatures was measured using the standard assay described in the “Materials and methods”

increase with decreasing acyl chain length of the substrate. In contrast, the  $K_m$  value decreased with increasing substrate acyl chain length. Therefore, the value of  $k_{cat}/K_m$  indicated that *p*-nitrophenyl decanoate was the best substrate for EST53 among the *p*-nitrophenol esters examined ( $3.5 \text{ s}^{-1} \mu\text{M}^{-1}$ ).



**Fig. 3** Thermostability of EST53. The enzyme was incubated at 60°C (triangles), 70°C (boxes), or 80°C (circles). Residual activity was measured using the standard assay. Activity before incubation was defined as 100%

The effects of metal ions on esterase activity were determined using various metal ions:  $\text{Ca}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Ni}^{2+}$ , and  $\text{Zn}^{2+}$  at concentrations of 5 mM (Table 2).  $\text{Ca}^{2+}$ ,  $\text{Fe}^{2+}$ , and  $\text{Mg}^{2+}$  significantly increased the esterase activity.  $\text{Ca}^{2+}$  was especially effective as an activator, increasing the activity by more than twofold. On the other hand,  $\text{Zn}^{2+}$ ,  $\text{Ni}^{2+}$ , and  $\text{Mn}^{2+}$  reduced the esterase activity to 41, 67, and 62%, respectively. The effects of various inhibitors on esterase activity were examined using EDTA, EGTA, PMSF, DEPC, DTT, and 2-ME (Table 3). Significant inhibition was observed in the presence of chelating agents, especially the  $\text{Ca}^{2+}$  chelator EGTA. This observation also indicated that the esterase activity of EST53 is dependent on calcium ions. Furthermore, PMSF and DEPC markedly inhibited the esterase activity. Neither DTT nor 2-ME led to remarkable reductions in the esterase activity.

The effects of organic solvents on esterase activity were determined using three organic solvents: DMF, DMSO, and 1,4-dioxane at concentrations of 1 to 10% (Table 4). Addition of DMF did not significantly affect the esterase activity of EST53, whereas DMSO and 1,4-dioxane increased the activity.

**Table 1** Kinetic parameters for hydrolysis of various *p*-nitrophenyl esters

<i>p</i> -Nitrophenyl esters	$K_m$ ( $\mu\text{M}$ )	$k_{cat}$ ( $\text{s}^{-1}$ )	$k_{cat}/K_m$ ( $\text{s}^{-1} \mu\text{M}^{-1}$ )
Acetate ( $\text{C}_2$ )	$1,150 \pm 135$	$50 \pm 2$	0.04
Butyrate ( $\text{C}_4$ )	$243 \pm 43$	$14 \pm 0.8$	0.06
Caprylate ( $\text{C}_8$ )	$4.7 \pm 0.4$	$13 \pm 2$	2.7
Decanoate ( $\text{C}_{10}$ )	$3.1 \pm 0.6$	$11 \pm 0.4$	3.5
Myristate ( $\text{C}_{14}$ )	$2.1 \pm 0.3$	$4.9 \pm 0.2$	2.3

**Table 2** Effects of metal ions on the esterase activity of EST53

Metal ions	Relative activity (%)
Ca <sup>2+</sup>	236
Fe <sup>2+</sup>	196
K <sup>+</sup>	137
Mg <sup>2+</sup>	214
Mn <sup>2+</sup>	62
Ni <sup>2+</sup>	67
Zn <sup>2+</sup>	41

## Discussion

This paper described the cloning and characterization of a thermophilic carboxylesterase from *T. maritima*. The expression level of the recombinant enzyme in *E. coli* was fairly high and allowed the production of sufficient material for successful purification in only a few steps (heat treatment and HiTrap Chelating column) with subsequent characterization. The catalytic efficiency ( $k_{cat}/K_m$ ) was greatest with *p*-nitrophenyl decanoate (C<sub>10</sub>) among the *p*-nitrophenyl esters with acyl chains of various lengths, but no lipase activity was detected. These results indicate that EST53 is not a true lipase but a short-length acyl esterase. The  $K_m$  value of EST53 was less than 5  $\mu$ M when substrates with acyl chains of C<sub>8</sub>–C<sub>14</sub> were used (Table 1). The  $K_m$  value of EST53 is the lowest among the thermophilic carboxylesterases tested to date, including esterases from *Archaeoglobus fulgidus* (11  $\mu$ M) (Manco et al. 2000) and *Pyrobaculum calidifontis* (44  $\mu$ M) (Hotta et al. 2002). This low  $K_m$  value of EST53 makes it an attractive enzyme for industrial application.

Experiments on the effect of temperature and organic solvents revealed that EST53 is a thermophilic, thermostable, and tolerant enzyme for organic solvents. The optimal temperature was, however, 60°C, which is different from the optimal growth temperature of *T. maritima* (80°C). Recently, the NH<sub>2</sub>-terminal region was reported to contribute to enzyme activity and stability in esterases of the HSL family (Mandrich et al. 2005). The relatively low thermophilicity of EST53 may be due to deletion of a part of the

**Table 3** Effects of inhibitors on the esterase activity of EST53

Inhibitors	Remaining activity (%)
EDTA	81
EGTA	39
PMSF	11
DEPC	15
DTT	119
2-ME	94

**Table 4** Effects of organic solvents on the esterase activity of EST53

Organic solvents	Concentration (%)	Relative activity (%)
DMF	1	105
	5	99
	10	87
DMSO	1	114
	5	146
	10	137
1-Dioxiane	1	154
	5	141
	10	151

NH<sub>2</sub>-terminal transmembrane region. However, the expression of the whole esterase gene was unsuccessful, the gene product being found in the insoluble fraction (data not shown). Therefore, the role of this region remains to be elucidated. Homologous gene to the lipB accessory protein (Frenken et al. 1993a,b), which is required for secretion and correct folding of *B. glumae* lipase, is not found in the *T. maritima* genome. However, two genes immediately downstream of the *tm0053* gene, *tm0054* and *tm0055*, encode possible extracellular proteins or enzymes; *tm0055* encodes a putative alpha-glucuronidase and *tm0054* gene product contains a domain similar to those that bind to polysaccharides (carbohydrate-binding module). Therefore, the gene product of *tm0053* gene is likely to be an extracellular enzyme, and its N-terminal region seems to be a signal sequence as predicted by the SignalP server (Bendtsen et al. 2004).

Some esterases have a Ca<sup>2+</sup>-binding GXXGXD motif, which plays an important role in enzyme activity and thermostability (Rashid et al. 2001; Simons et al. 1999). Analysis of the effects of Ca<sup>2+</sup> and EGTA on the esterase activity indicated that EST53 showed Ca<sup>2+</sup>-dependent activation and suggested the presence of a Ca<sup>2+</sup>-binding site in the esterase. However, we could not find any known Ca<sup>2+</sup>-binding motifs in the sequence of EST53. EST53 was inhibited by PMSF or DEPC, specific modifiers of serine proteases and histidine residues, respectively. These observations indicate that the active site of EST53 consists of Ser and His.

Analysis of the primary structure of EST53 indicated a relatively high degree of similarity to family I.2 enzymes. Sequence analysis suggested that TM0053 has a signal sequence region in its NH<sub>2</sub> terminus, which is occasionally observed in proteins belonging to family I.2. However, EST53 showed some differences, no lipase activity, and relatively low levels of similarity in the short but critical region around the catalytic serine residue. In EST53, the characteristic pentapeptide sequence GX SXG around the catalytic serine residue is changed into an AX SXG sequence, which is often seen in *Bacillus* lipases belonging to family

I.4. However, the amino acid sequences around the catalytic Asp and His are not similar to those of *Bacillus* lipases. EST53 also has little similarity to archaeal esterases, which belong to subfamilies IV and V, and displays activity at the highest temperature among bacterial esterases.

The genome of *T. maritima* includes several carboxylesterase homologs ( $\alpha/\beta$  hydrolases; TM0033, TM0053, TM0336, TM0435, TM1160, and TM1350), the functions of which have not been determined. This study represents the first characterization of one of these putative enzymes of this organism. Further studies, including site-directed mutagenesis and X-ray crystallographic analysis of EST53, are required to elucidate the details of the  $\text{Ca}^{2+}$ -binding site and its contribution to the thermostability of the enzyme.

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