New Thermophilic and Thermostable Esterase with Sequence
Similarity to the Hormone-Sensitive Lipase Family,
Cloned from a Metagenomic Library
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A gene coding for a thermostable esterase was isolated by functional screening of Escherichia coli cells that
had been transformed with fosmid environmental DNA libraries constructed with metagenomes from thermal
environmental samples. The gene conferring esterase activity on E. coli grown on tributyrin agar was composed
of 936 bp, corresponding to 311 amino acid residues with a molecular mass of 34 kDa. The enzyme showed
significant amino acid similarity (64%) to the enzyme from a hyperthermophilic archaeon, Pyrobaculum calidifontis.
An amino acid sequence comparison with other esterases and lipases revealed that the enzyme
should be classified as a new member of the hormone-sensitive lipase family. The recombinant esterase that
was overexpressed and purified from E. coli was active above 30°C up to 95°C and had a high thermal stability.
It displayed a high degree of activity in a pH range of 5.5 to 7.5, with an optimal pH of approximately 6.0. The
best substrate for the enzyme among the p-nitrophenyl esters (C4 to C16) examined was p-nitrophenyl caproate
(C6), and no lipolytic activity was observed with esters containing an acyl chain length of longer than 10 carbon
atoms, indicating that the enzyme is an esterase and not a lipase.

Many lipolytic enzymes, including lipases (EC 3.1.1.3), es-
terases or carboxylesterases (EC 3.1.1.1), and various types of
phospholipases, have been found in a wide range of organisms
from bacteria to humans (22). True lipases can be defined as
carboxylesterases that catalyze the hydrolysis and synthesis
of relatively long-chain acylglycerols with acyl chain lengths of
>10 carbon atoms, with trioleoylglycerol as the standard sub-
strate. In contrast, esterases catalyze the hydrolysis of glycer-
ol esters with acyl chain lengths of <10 carbon atoms, with trioleoylglycerol as the standard sub-
strate. Lipases from prokaryotes share a group of conserved
amino acids, including a serine in a highly conserved Gly-X-
Ser-X-Gly pentapeptide and an aspartate or glutamate residue
that is hydrogen bonded to a histidine to form a catalytic triad.
Based on comparisons of amino acid sequences and biological
properties, prokaryote-derived lipases have been classified into
eight different families (2).

In an effort to isolate novel genes from enormous and largely
unexploited gene pools in uncultured microorganisms and/or
those that are difficult to culture, the metagenomic library
approach has recently been used successfully (14, 25, 29, 36, 38,
41, 43). This type of approach does not require the cultivation
of diverse microorganisms from environmental samples, which
is often difficult or impossible and can result in an enrichment
of dominant strains under a specific selective condition. Thus,
more global microbial genetic information can be provided
from total microorganisms than from culturable subpopula-
tions or enrichment cultures (10). Two different approaches
have been previously used to isolate novel lipase genes. Hennen
et al. isolated novel genes conferring lipolytic activity in Escherichia coli transformed with metagenomic libraries con-
structed with temperate environmental soil samples (17). As an
alternative approach, Bell et al. used a PCR method for the
direct isolation of novel lipase genes from metagenomes to
avoid potential difficulties in achieving the expression of a
lipase in a heterologous host (5).

Thermophiles are a valuable source of thermostable en-
zymes that are often associated with stability in
solvents and detergents, giving these enzymes considerable
potential for many biotechnological and industrial applications
(7, 13, 27). One of these enzymes is a thermophilic and ther-
mostable lipolytic enzyme that has been applied to the synthe-
sis of biopolymers and biodiesel and used for the production of
pharmaceuticals, agrochemicals, cosmetics, and flavors (13,
23). To date, fewer than one dozen thermostable lipases/ester-
ases have been isolated from thermophiles and hyperthermo-
philes (13). In particular, lipases and esterases, which are func-
tional at temperatures over 80°C, have been isolated mainly
from hyperthermophilic archaea. Currently, three esterases
with thermophilic archaeon origins and known amino acid
sequences have been biochemically characterized, and all of
them belong to family IV. These include esterases from Ar-
chaeoglobus fulgidus (31), Sulfolobus solfataricus (33), and Py-
robaculum calidifontis (18). Besides these enzymes, esterases
from Sulfolobus acidocaldarius (39, 40), Pyrocococcus furiosus
(21), and Sulfolobus shibatae (19) have been purified and char-
acterized, but their cognate genes have not been reported.

Recent direct genome shotgun sequencing and molecular
phylogenetic studies using metagenomes have indicated sub-
stantial microbial diversity even in high-temperature environ-
ments (3, 4, 20, 34). Therefore, metagenomic libraries of ther-
mal environments should be useful for screening novel thermostable enzymes, including lipases, but no such libraries

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have been reported yet. For this work, we have isolated a gene encoding a new esterase with sequence similarity to the hormone-sensitive lipase (HSL) family (family IV) by a functional screening of metagenomic libraries derived from thermal environmental samples from Indonesia. The cloned esterase gene was overexpressed in E. coli. The recombinant esterase was purified to homogeneity, and its biochemical properties were partially characterized.

MATERIALS AND METHODS

DNA extraction from environmental samples. For the construction of metagenomic libraries, environmental samples (mixtures of mud and sediment-rich water) were collected from hot springs and mud holes in solfatary fields in the Tangkuban Perahu (85 to 92°C, pH 3 to 4), Cisolok (95°C, pH 8), Sileri (80 to 95°C, pH 4 to 6), and Likupang (65 to 80°C, pH 4.5 to 8) regions of Indonesia. After draining of the excess water, the metagenomes from these thermal environmental samples were extracted as described previously (45), with slight modifications. Environmental samples (100 g of wet weight) were mixed well with an equal volume of DNA extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM EDTA, 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, 1% hexadecyl methylglammonium bromide), to which sodium dodecyl sulfate (SDS) and N-lauroyl sarcosine were each added to a final concentration of 1.5%. After the mixtures were incubated in a 65°C water bath for 1 h with gentle inversion every 15 to 20 min, an equal volume of chloroform-isooamyl alcohol (24:1) was added and gently mixed, followed by incubation for 1 h at 4°C. The aqueous phase was recovered by centrifugation at 12,000 × g for 20 min. DNAs were precipitated with a 0.6 volume of isopropanol and recovered by centrifugation at 12,000 × g for 20 min at room temperature. Recovered DNAs were extracted once more with phenol-chloroform (1:1). DNAs precipitated with isopropanol were air dried and resuspended in an appropriate volume of distilled water.

Construction of metagenomic libraries. The environmental genomic DNAs were digested in 1% agarose (pulse field-certified agarose; Bio-Rad) containing 1% polyvinylpyrrolidone (Sigma) by pulse-field gel electrophoresis with a CHEF-DRIII system (Bio-Rad) in order to fractionate the isolated DNAs by size. Pulsed-field gel electrophoresis gels were run at 4.5 V/cm with an angle of 120° and with ramping from an initial switch time of 10 s to a final switch time of 100 s at 14°C in 0.5 × Tris-borate-EDTA buffer for 18 h. DNAs with approximate lengths of 40 to 50 kbp were recovered by electroelution. A metagenomic library for each sample was constructed by use of a CopyControl fosmid library production kit (Epitc) according to the manufacturer’s instructions. Both ends of the size-fractionated DNA were repaired to create blunt 5′-phosphorylated ends and were ligated into the pCC1FOS fosmid vector (Epicentre). Li-gated DNA mixtures were then packaged by use of the supplied lambda packaging extracts and were transformed into an EPI300-T1R phage T1-resistant E. coli host.

Screening of esterase gene. E. coli BL21(DE3) (Novagen) cells were transformed with each library. An activity-based esterase/lipase screening was performed in LB medium containing 100 μM of ampicillin/ml at 37°C, and protein expression was induced at 25°C by the addition of 1 mM IPTG when the optical density at 660 nm reached 0.8. After induction for 12 h, the cells were harvested, washed twice with phosphate-buffered saline (pH 7.4), resuspended in 10 ml of binding buffer A (50 mM sodium phosphate [pH 8.0], 300 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol, 10% glycerol, and 1% Nonidet P-40), and frozen at −80°C. After thawing, the cells were sonicated on ice and crude cell extracts were centrifuged at 20,000 × g for 30 min to obtain cleared lysates. The supernatant was incubated at 80°C for 10 min and then centrifuged for 30 min at 4°C to discard denatured proteins. The supernatant was then incubated for 2 h on ice with Ni-nitrotriacetic acid (NTA)-agarose resin (QIAGEN) that had been pre-equilibrated with binding buffer. After being washed with 20 column volumes of binding buffer, bound esterase proteins were eluted with binding buffer containing 50 to 450 mM imidazole. Esterase-containing fractions were collected, dialyzed against gel filtration column buffer (50 mM Tris-HCl [pH 7.8], 150 mM NaCl, 1 mM dithiothreitol, 0.5 mM EDTA), and then run at a flow rate of 0.5 ml/min on a Sephacryl S-200HR column (Amersham Pharmacia Biotech) (9). A Prime instrument (Amersham Pharmacia Biotech) was used for fractionation. The recombinant esterase was recovered and purified to homogeneity by passage through a Ni-NTA column (Amersham Pharmacia Biotech). Adsorbed proteins were then eluted with a linear gradient of 50 to 450 mM imidazole in binding buffer A and dialyzed against 50 mM potassium phosphate buffer (pH 7.0) containing 10% glycerol. The protein concentration was determined by use of a Bio-Rad protein assay kit, with bovine serum albumin as a standard.

Western blotting assay. After SDS-polyacrylamide gel electrophoresis, the proteins were transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech). The membranes were blocked with 2% nonfat milk in phosphate-buffered saline, incubated with an anti-penta-His antibody (QIAGEN) for 2 h at room temperature, washed, and incubated with the appropriate secondary antibody coupled to horseradish peroxidase (Amersham Pharmacia Biotech). Immunoblots were developed with an ECL detection kit (Amersham Pharmacia Biotech).

Enzyme assays. Esterase activities against p-nitrophenyl esters were determined by measuring the amount of p-nitrophenol released by esterase-catalyzed hydrolysis (28). The production of p-nitrophenol was continuously monitored at 405 nm by use of a DU-650 spectrophotometer with a thermal controller (Beckman). Unless otherwise described, esterase activity was measured by a standard assay at 70°C, with 1 mM p-nitrophenyl caprate as substrate in 50 mM morpholineethanesulfonic acid (MES; pH 6.0) with 1% acetonitrile at 70°C. The esterase was affinity-purified from E. coli BL21(DE3) as described above for nonenzymatic hydrolysis of substrates from the results. The extinction coefficients of p-nitrophenol were determined under each reaction condition prior to the measurements. The activity was determined by measuring the initial rate of hydrolysis of p-nitrophenyl ester. One unit of enzymatic activity was defined as the amount of activity required to release 1 μmol of p-nitrophenol/min from p-nitrophenyl ester.

Modification of enzymes. The inhibitory effects of modifying reagents for Ser and His were examined by using phenylmethylsulfonyl fluoride (PMSF; Sigma) and diethyl pyrocarbonate (DEPC; Sigma), respectively. The enzyme (0.6 μM), in 50 mM MES (pH 6.0), was incubated with various concentrations of PMSF or DEPC at 37°C for 10 min. The modification reactions were stopped by cooling samples in ice water, and the residual activities were measured by the standard assay described above.

Substrate specificity. Substrate specificities for p-nitrophenyl esters were determined by using p-nitrophenyl butyrate (0.2 to 3 mM) (Sigma), p-nitrophenyl valerate (0.2 to 3 mM) (Sigma), p-nitrophenyl caproate (0.4 to 3 mM) (Fluka), and p-nitrophenyl caprate (0.2 to 3 mM) (Sigma) as substrates in 50 mM MES (pH 6.0) containing 1% acetonitrile at 70°C. The p-nitrophenyl ester substrates with C2 to C4 acyl chains were dissolved in acetonitrile at a final concentration of 100 mM, p-nitrophenyl palmitate (Sigma) was dissolved in a mixture of acetonitrile and 2-propanol (1:4) in order to solubilize the substrate, and reactions were performed in a final concentration of 1% acetonitrile and 4% 2-propanol. Initial reaction velocities measured at various concentrations of substrates were fitted to the Lineweaver-Burk transformation of the Michaelis-Menten equation. Kinetic
analyses by curve fitting were performed with the SigmaPlot program (SPSS
Science, Chicago, Ill.).

Effect of pH on enzyme activity. For determination of the optimum pH of the
enzyme, esterase activities were measured for a pH range of 3.0 to 9.5 under
standard assay conditions. The buffers used were 50 mM sodium acetate (pH 3.0
to 5.5), 50 mM MES (pH 5.5 to 7.0), 50 mM HEPES (pH 7.0 to 7.5), and 50 mM
glycine (pH 7.5 to 9.5). The production of p-nitrophenol and p-nitrophenol
from p-nitrophenyl caprate was monitored at 405 nm (the pH-independent
isosbestic wavelength of p-nitrophenol and p-nitrophenol).

Effect of temperature on enzyme activity and thermostability. The optimal
temperature for enzyme activity was determined for a temperature range of 30
to 95°C under standard assay conditions. Thermostability was analyzed by mea-
suring the residual activity after incubating the enzyme (6.0 to 95°C under standard assay conditions. Thermostability was analyzed by mea-
suring the residual activity after incubating the enzyme (6.0 μM
potassium phosphate buffer, pH 7.0) at 80, 85, 90, or 95°C for various times in
Eppendorf tubes with mineral oil on top to prevent evaporation. For calculation
of the half-life of the enzyme, the experimental data were fitted to a single
exponential decay curve (y = ae−kt) by a nonlinear regression procedure based
on the Marquardt-Levenberg algorithm in SigmaPlot software.

Nucleotide sequence accession number. The estE1 nucleotide sequence re-
ported here is available in the GenBank database under accession number
AY720780.

RESULTS

Screening for thermostable esterases from metagenomic li-
braries. We constructed a total of four independent fosmid
metagenomic libraries from thermal environmental samples
(ca. 300 to 500 g of wet weight) from the Cislock, Likupang,
Tangkuban Perahu, and Sileri areas of Indonesia. Environ-
mmental DNAs in the size range of 30 to 50 kbp were routinely
obtained, with yields of 1 to 4 μg per 100 g of sample. To-
gether, these libraries consisted of approximately 5,000 inde-
dependent recombinant fosmids with inserts in the range of 20
to 40 kbp (data not shown).

In order to screen for esterase/lipase genes, we individually
introduced these metagenomic libraries into E. coli BL21
(DE3) cells, which were then plated onto LB medium contain-
ing tributyrin and IPTG to induce gene expression from the T7
RNA polymerase promoter located at either the S' or the 3’
end of the insert. IPTG was added in order to induce the
transcription of genes that lacked a promoter or did not con-
tain a promoter that was functional in a heterologous host.
After the growth of E. coli cells transformed with each library
at 37°C, followed by a further incubation at 50°C, a total of four
esterase/lipase-positive transformants which formed clear ha-
os on tributyrin plates were isolated from a library constructed
with an environmental sample from the Sileri hot spring area.
This metagenomic library consisted of approximately 2,000
fosmids with various sizes of metagenomic DNA inserts of
over 20 kbp.

The four fosmids, named Mge1, -2, -3, and -4, contained
metagenomes of 33, 32, 38, and 30 kbp, respectively. All E. coli
cells that were transformed with these fosmids, except for
those transformed with Mge1, were dependent on IPTG for
halo formation on plates with the tributyrin substrate, indicat-
ing that transcription in these fosmids relied on the T7 RNA
polymerase promoter in the fosmid. However, all four fosmids
did not confer on E. coli transformants a lipolytic activity on
the agar plate containing triolein (C18:1), indicating that there
was no lipase activity.

EstE1 is a new member of the hormone-sensitive lipase
family. We used Mge4, which had the smallest insert size, for
subcloning of the minimal esterase gene in an E. coli expres-
sion vector. We initially attempted, without success, to clone
the gene by subcloning of either EcoRI- or BamHI-digested
DNA fragments into a T7 RNA polymerase-driven E. coli
expression vector and screening for tributyrin-digesting trans-
formants. Therefore, we sequenced both the 5’ and 3’ ends of
each EcoRI-digested DNA fragment of Mge4 that was cloned
in the expression vector. BLAST searches for the deduced
amino acid sequence revealed two DNA fragments encoding
the N- and C-terminal portions of EstE1 that were most similar
to Pyrobaculum caldifontis esterase (18). The esterase gene,
named estE1, contained an open reading frame of 936 bp that
was capable of encoding a protein with a predicted molecular
mass of 34 kDa and an isoelectric point of 5.82. PCRs with
estE1 gene-specific primers and with Mge1-3 as a substrate,
followed by restriction enzyme digestion, indicated that all of
these fosmids had the same esterase gene (data not shown).

The deduced amino acid sequence of EstE1 was used to
perform a BLAST search of the National Center for Biotechnol-
ogy Information and SwissProt databases. This search re-
vealed a relatively high similarity (over 50%) between EstE1 and
other thermostable esterases/lipases from archaea, includ-
ing an esterase (BAC06606) from Pyrobaculum caldifontis
(64%), the lipase Lipp-P-2 (NP_343862) from S. solfataricus
(63%), a carboxylesterase (NP_070544) from Archaeoglobus
fulgidus DSM 4304 (57%), an esterase (NP_375919) from Sul-
folobus tokodaii (57%), the lipase Lipp-P-1 (NP_343839) from S.
solfataricus (57%), a carboxylesterase (BAB59879) from Ther-
mosphaera volcanium (53%), and an esterase (NP_111246)
from Thermoplasma volcanium (53%). A high similarity was
also observed for esterases/lipases from therophilic bacteria,
including the carboxylesterase Est2 (IEVQ_A) from Alcyocl-
cellularis acidocaldarius (51%) and an esterase/lipase (ZP_00057664)
from Thermotoga maritima (52%). EstE1 was also relatively
similar to human HSL (NP_005348) (28% identity and 43% sim-
ilarity). Multiple alignments of the entire 313 amino acids of
EstE1 with the most closely related sequences of lipases/ester-
ases (>40% similarity) in the National Center for Biotechnol-
yogy Information database are presented in Fig. 1A.

EstE1 contains the lipase-conserved catalytic triad residues
Asp251 and His281 and the catalytic nucleophile Ser154 in the
hydrogen bonding interactions for stabilization of the oxyanion
hole and plays a role in catalysis (44), was found upstream of
the active-site conserved motif (Fig. 1A). These amino acid
sequence comparisons indicated that EstE1 should be classi-
fied as a new member of the hormone-sensitive lipase (HSL)
family. The phylogenetic tree shown in Fig. 2 indicates that
EstE1 is most closely related to the esterase of Pyrobaculum
caldifontis and clusters with family IV esterases from several
species of Sulfolobus and from Archaeoglobus fulgidus.

A sequence alignment of EstE1 with open reading frames
from metagenomes which were recently identified by shotgun
sequencing of seawater environmental DNA samples from the
Sargasso Sea near Bermuda (41) revealed 20 homologous
HSL-like esterase/lipase genes with approximately 50% simi-
larity in their amino acid sequences. The presence of HSL
family conserved motifs in these genes with hitherto unknown
functions indicates that these EstE1 homologs are all novel
HSL family esterases or lipases (Fig. 1B).
FIG. 1. Amino acid sequence blocks conserved in the deduced amino acid sequences of EstE1 and homologous lipases and esterases. (A) Multiple amino acid sequence alignments of EstE1 and its homologs. The accession numbers of the aligned sequences are for the following organisms: 1EVQ_A, carboxylesterase Est2 from Alcylobacillus acidocaldarius; ZP_00095860, esterase/lipase from Novosphingobium aromaticivorans; AAC38151, lipase from Pseudomonas sp. strain B11-1; ZP_00170489, hypothetical protein from Balstonia eutropha JMP134; ZP_00215452, hypothetical protein from Burkholderia cepacia R18194; ZP_00057664, esterase/lipase from Thermobifida fusca; ZP_00215124, hypothetical protein from Burkholderia cepacia R18194; NP_343839, lipase (LipP-1) from Sulfolobus tokodaii; NP_343862, lipase (LipP-2) from Sulfolobus solfataricus; NP_005348, hormone-sensitive lipase from Homo sapiens (human); BAB59879, carboxylesterase from Thermoplasma volcanium; BAC06606, esterase from Pyrobaculum calidifontis; NP_343862, lipase (LipP-2) from S. solfataricus; NP_070544, carboxylesterase (EstA) from Archaeoglobus fulgidus DSM 4304; NP_343839, lipase (LipP-1) from S. solfataricus; NP_375919, 303-amino-acid hypothetical esterase from Sulfolobus tokodaii; NP_243114, esterase (esterase) from Bacillus halodurans C-125; NP_947767, putative lipase/esterase from Rhodopseudomonas palustris CGA009; ZP_00057664, esterase/lipase from Thermobifida fusca; ZP_00215124, hypothetical protein from Burkholderia cepacia R18194; NP_960379, hypothetical protein from Mycobacterium avium subsp. paratuberculosis strain k10; NP_124276, hypothetical protein from Helicobacter pylori 26695; NP_181814, lipase from Bacillus subtilis; ZP_00028572, hypothetical protein from Burkholderia cepacia R18194; ZP_00223864, hypothetical protein from Burkholderia cepacia R1808; AAO17429, unknown protein from Pseudomonas aeruginosa; BAA82510, esterase HDE from Oleomonas sagaranensis. (B) Amino acid sequence alignment of EstE1 with putative esterases identified from Sagasso Sea environmental genomes. The accession numbers are indicated to the left of the amino acid sequences. Identical residues have a gray background. Symbols: ◆, amino acids forming a catalytic triad; ○, amino acids involved in oxyanion hole formation.
Overexpression and purification of recombinant EstE1. In order to investigate the biochemical properties of EstE1, we expressed the EstE1 protein with a six-histidine tag at its C terminus in E. coli and purified the protein to homogeneity. Bacteria transformed with the expression vector and induced with IPTG abundantly expressed the histidine-tagged protein, as observed by the appearance of an extra protein band migrating at 34 kDa in a Coomassie-stained gel (Fig. 3A, compare lane 2 with lane 1). A heat treatment of the bacterial lysates harboring recombinant EstE1 at 80°C resulted in an enrichment of EstE1 (lane 3), which was then further purified by Ni-NTA-agarose chromatography. Additional bands representing degraded and/or nonspecifically bound proteins that appeared in the eluates from Ni-NTA-agarose chromatography (lane 4) were removed by subsequent gel filtration chromatography (GFC). Substantial purification of the protein was achieved by GFC, which yielded the recombinant EstE1 protein purified to homogeneity (lane 5). The fractions from GFC were combined, purified, and concentrated by a second round of Ni-NTA chromatography, resulting in homogenous EstE1, as shown by silver staining (Fig. 3C). This purification protocol routinely yielded >4 mg of homogeneous EstE1 from 1 liter of bacterial culture. In order to verify the purification protocol for recombinant EstE1, we performed a Western blot analysis of the various purification steps by using an anti-penta-His antibody. A protein band of approximately 34 kDa was identified as EstE1 (Fig. 3B).

Since the esterase catalytic triad Asp, His, and Ser residues were found in EstE1 (Fig. 1A), we attempted to confirm the function of Ser and His residues in the enzyme’s activity by chemical modification with various concentrations (0.01 to 10 mM) of either PMSF or DEPC. Modification of the enzyme with either 1 mM PMSF or 1 mM DEPC reduced the esterase activity by 88 and 63%, respectively. Less than 5% of the
residual activity was observed with each inhibitor at a concentration of 10 mM (data not shown), indicating that these amino acids are probably essential for the enzyme’s function. However, we could not completely rule out the possibility that a modification of amino acids other than those in the lipase catalytic triad may affect the protein structure and/or activity of the enzyme.

**Substrate specificity of EstE1.** To examine substrate specificity and to find the best substrate for an enzyme activity assay, we tested various p-nitrophenyl esters with acyl chain lengths of different lengths (butyrate, C4; valerate, C5; caproate, C6; caprate, C8; and palmitate, C16). Under our standard assay conditions of pH 6.0 and 70°C, both the $K_m$ and $k_{cat}$ values of purified EstE1 decreased with increases in the acyl chain length up to C6 (Table 1). The catalytic efficiency represented by the value of $k_{cat}/K_m$ slightly increased with an increase in the acyl chain length. p-Nitrophenyl caproate produced the highest value. No detectable activity was observed at pH 6.0 with p-nitrophenyl caprate or palmitate. The $k_{cat}$ value of EstE1 for the best substrate among the p-nitrophenyl esters tested for this work, which was C6, was approximately 1,600 s$^{-1}$, with a 1.54- and a 1.77-fold increase compared to the C4 and C5 substrates, respectively. These values are within the range reported for other thermostable esterases, including the esterase from *Pyrobaculum caldus*, *Pyrobaculum aerophilum*, and *Pyrococcus furiosus*.

![FIG. 3. Expression and purification of recombinant EstE1.](image)

**TABLE 1. Kinetic parameters for recombinant EstE1**

<table>
<thead>
<tr>
<th>Substrate (p-nitrophenyl ester)</th>
<th>$K_m$ (mM)$^a$</th>
<th>$k_{cat}$ (s$^{-1})^a$</th>
<th>$k_{cat}/K_m$ (s$^{-1}$mM$^{-1})^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butyrate (C4)</td>
<td>2.3</td>
<td>2,950</td>
<td>1,280</td>
</tr>
<tr>
<td>Valerate (C5)</td>
<td>0.6</td>
<td>1,300</td>
<td>2,170</td>
</tr>
<tr>
<td>Caproate (C6)</td>
<td>0.7</td>
<td>1,600</td>
<td>2,290</td>
</tr>
<tr>
<td>Caprate (C8)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Palmitate (C16)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^a$ ND, not detectable.

The effect of temperature on enzyme activity and thermal stability of EstE1. The effect of temperature on esterase activity was determined by using p-nitrophenyl caproate as a substrate at pH 6.0 in the temperature range of 30 to 95°C. The esterase activity increased with increases in temperature up to 95°C in a reproducible manner. At lower temperatures, the enzyme still showed activity, exhibiting 21 and 30% of the maximal activity at 30 and 40°C, respectively. An Arrhenius plot analysis revealed that the activity was maintained in the temperature range of 30 to 95°C as a linear relationship with a constant activation energy of 20.1 kJ/mol (Fig. 5A). To examine the thermal stability of this esterase, we incubated the enzyme (6.0 µM) for 30 to 120 min and measured its residual activity under standard assay conditions. EstE1 displayed a high thermal stability at 80°C in the absence of any stabilizer. It lost only approximately 20% of its activity, even after incubation for 120 min at 85°C (Fig. 5B). The stability of the enzyme decreased at 90 and 95°C, with half-lives of approximately 20 and 2 min, respectively, but the esterase activity did not decrease at these temperatures (Fig. 5A). These results indicate that even though the apparent optimum activity for the enzyme occurs at
95°C, the purified enzyme in the absence of substrate is not stable at temperatures over 90°C.

**DISCUSSION**

We isolated a gene (estE1) encoding a thermostable esterase from a fosmid metagenomic DNA library constructed from a thermal environmental sample from Indonesia. Both the nucleotide and amino acid sequences of the EstE1 enzyme were novel. The thermophilic and thermostable properties of EstE1 and its remarkable amino acid sequence similarity to other esterases from thermophilic archaea (Fig. 1A) indicate that EstE1 is likely derived from a hyperthermophilic archaean.

Furthermore, partial sequencing of the Mge4 fosmid revealed that it contains open reading frames that are similar to those of enzymes of hyperthermophilic archaea, including *S. solfataricus* inorganic pyrophosphatase (63% identity) and a *Pyrobaculum aerophilum* DNA endonuclease rad2 homolog (70% identity) and acylamino acid-releasing enzyme (48% identity). Therefore, both phylogenetic analysis of EstE1 and comparative sequence analyses of other genes in the Mge4 fosmid strongly supported the idea that the origin of the EstE1 enzyme is a hyperthermophilic archaean.

Family IV lipases show significant similarity to human HSL, which is involved in lipid metabolism by controlling the release of fatty acids from stored triacylglycerols in adipose tissue. Human HSL contains a catalytic domain and a regulatory module, which is unique to this enzyme, at the N terminus (15, 35). The catalytic domain displays similarity to prokaryote-derived family IV esterases/lipases, indicating that mammalian HSLs probably evolved from prokaryotic family IV enzymes. A homology search of the EstE1 sequence revealed that family IV prokaryote-derived esterases/lipases include the enzymes from *Moraxella* sp. (11), *E. coli* (24), *Pseudomonas* sp. strain B11-1 (6), *Alicyclobacillus acidocaldarius* (30), *Archaeoglobus fulgidus* (31), and *Pyrobaculum calidifontis* (18) (Fig. 1A). EstE1 was strikingly similar to the estPc-encoded carboxylesterase of *Pyrobaculum calidifontis* (18) (52 and 64% amino acid identity and similarity, respectively). These two proteins are more closely related to each other than to any other members of the HSL family and also show a functional relationship in that both enzymes exhibit only esterase activity with no lipase activity.

EstE1 exhibited thermophilic and thermostable properties. It was active above 30°C up to 95°C (Fig. 5A), while its activity decreased above 95°C. The enzyme was almost 100% stable at 80°C, even after 120 min of incubation (Fig. 5B). However,
EstE1 had half-lives of approximately 20 and 2 min at 90 and 95°C, respectively. Thus, the optimal temperature (95°C) may be an artifact of the initial reaction rate measurement of the enzyme activity prior to complete thermal inactivation. Alternatively, this discrepancy may be due to an enhanced stability of the enzyme in the presence of substrate. The activation energy of EstE1 was unchanged between 30 and 95°C and was estimated to be 20.1 kJ/mol from an Arrhenius plot (Fig. 5A). This value is comparable to those for esterases from Pyrobaculum calidifontis (26.4 kJ/mol) (18), Archaeoglobus fulgidus (26 kJ/mol) (31), and Alcylcobacteriaci acidocaldarius (32 kJ/mol) (30) and is approximately one-half of the value for a cold-adapted lipase from Pseudomonas sp. strain B11-1 measured with p-nitrophenyl butyrate (47 kJ/mol) (6). Thus, EstE1 and the esterases of Pyrobaculum calidifontis and Archaeoglobus fulgidus seem to have comparable catalytic activities considering that the activities were all measured with the same substrate, p-nitrophenyl caproate. In addition, the esterase of Archaeoglobus fulgidus and EstE1 display similar stabilities, with approximately 20% of the residual activity present after incubation for 1 h at 90°C (Fig. 5B) (31). They are both, however, less stable than the esterases of Pyrobaculum calidifontis (18) and Pyrococcus furiosus (21), both of which show no activity change after incubation for 2 h, even at 100°C. Even though EstE1 and the esterases of Pyrobaculum calidifontis and Archaeoglobus fulgidus displayed optimal activities above 80°C, these enzymes still exhibited detectable activities at 30°C that were approximately 22, 16, and 25%, respectively, of their activities at their optimal temperatures (Fig. 5A) (18, 31). This is one of the unique properties of extremely thermostable esterases in the HSL family that is not observed for several other thermostable enzymes, including the esterase from Pyrococcus furiosus (21) and the less thermostable HSL family esterase from Alcylcobacteriaci acidocaldarius (30, 31). This is likely due to their ability to maintain a very stable conformation even at low temperatures. The high degree of thermostability of EstE1 and its functionality at lower temperatures are potentially applicable to biotechnological procedures.

The metagenomes of natural microbial communities contain an immense pool of genes, most of which are not represented by pure and enrichment cultures established under certain selective conditions. Our finding of a new thermostable and versatile carboxylesterase from a hyperthermophilic archaeon. Appl. Environ. Microbiol. 67:103–109.

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