The Journal of Microbiology (2011) Vol. 49, No. 2, pp. 178-185
DOI 10.1007/s12275-011-1102-5

Isolation and Characterization of a Family VII Esterase Derived from Alluvial Soil Metagenomic Library

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(Received March 3, 2011 / Accepted April 6, 2011)

A novel esterase gene, *estDL30*, was isolated from an alluvial metagenomic library using function-driven screening. *estDL30* consisted of 1,524 nucleotides and encoded a 507-amino acid protein. Sequence analysis revealed that EstDL30 is similar to many type B carboxylesterases, containing a G-E-S-A-G pentapeptide with a catalytic Ser residue. Phylogenetic analysis suggested that EstDL30 belongs to the family VII lipases, together with esterases from *Bacillus subtilis* (P37967), *Streptomyces coelicolor* A3(2) (CAA22794), and *Arthrobacter oxydans* (Q01470). Purified EstDL30 showed its highest catalytic efficiency toward *p*-nitrophenyl butyrate, with a k_{cat} of 229.3 s^{-1} and k_{cat}/K_m of 176.4 $s^{-1}mM^{-1}$; however, little activity was detected when the acyl chain length exceeded C_8. Biochemical characterization of EstDL30 revealed that it is an alkaline esterase that possesses maximal activity at pH 8 and 40°C. The effects of denaturants and divalent cations were also investigated. EstDL30 tolerated well the presence of methanol and Tween 20. Its activity was strongly inhibited by 1 mM Cu^{2+} and Zn^{2+}, but stimulated by Fe^{2+}. The unique properties of EstDL30, its high activity under alkaline conditions and stability in the presence of organic solvents, may render it applicable to organic synthesis.

***Keywords*:** alluvial soil, esterase, family VII lipase, metagenome

Lipolytic enzymes catalyze the hydrolysis and synthesis of acylglycerides and other fatty acid esters, including carboxylesterases (EC 3.1.1.1) that preferentially hydrolyze short-chain triglycerides and lipases (EC 3.1.1.3) that hydrolyze water-insoluble long-chain fatty acids (Arpigny and Jaeger, 1999). Esterases and lipases are both important biocatalysts because of their broad substrate specificity, remarkable stability in organic solvents, enantioselectivity, and cofactor-independence. These properties make them useful for a variety of biotechnological applications (Gupta *et al.*, 2004).

Microbial lipolytic enzymes are particularly useful because their convenient expression in *Escherichia coli* has been reported (Kim *et al.*, 2006; Rashamuse *et al.*, 2009a), making the generation of recombinant proteins less problematic. Indeed, the potential biotechnological applications are currently being explored (Jaeger *et al.*, 1999; Bornscheuer, 2002). Moreover, natural environments harbor a great microbial resource of novel biocatalysts, most of which remains to be exploited.

Although great microbial diversity exists in natural environments, the overwhelming majority of microorganisms (>99%) are at present not culturable (Amann *et al.*, 1995), and cultivation-based approaches are not useful for investigating uncultured microorganisms. Metagenomics, a molecular approach that enables the study of all microorganisms in an environment, has recently been employed (Riesenfeld *et al.*, 2004; Venter *et al.*, 2004). Functional metagenomics is a particularly powerful strategy for the identification of novel biocatalysts in environmental microorganisms, including uncultured microorganisms (Handelsman, 2004; Wolfgang and Schmitz, 2004; Uchiyama and Miyazaki, 2009). To date, numerous novel biocatalysts have been identified by functional metagenomic analysis; these include lipolytic enzymes (Li *et al*., 2008; Jeon *et al.*, 2009; Rashamuse *et al*., 2009b), antimicrobial agents (Gillespie *et al.*, 2002; Chung *et al.*, 2008) and quorum-sensing interfering lactonases (Riaz *et al.*, 2008; Schipper *et al.*, 2009).

Since lipolytic enzymes are of importance in biotechnological applications, a large number of esterases have been isolated and characterized recently, based on functional screening of various microbial habitats, such as ponds (Ranjan *et al.*, 2005), hot springs (Bell *et al.*, 2002; Rhee *et al.*, 2005), and soils (Lee *et al.*, 2004; Heath *et al.*, 2009; Rashamuse *et al.*, 2009a). In our previous study, we demonstrated that plant rhizosphere soil is a good resource for screening for novel lipolytic enzymes (Lee *et al.*, 2010). In this study we screened an alluvial soil metagenomic library constructed from the fine-grained fertile soil of Eulsukdo, an alluvial island in the Nakdong River estuary, Busan, Republic of Korea, for novel lipolytic enzymes. A novel esterase, EstDL30, was isolated and identified as a new member of the family VII lipases, some of which have biotechnological potential (Prim *et al.*, 2001; Ewis *et al.*, 2004; Kim *et al.*, 2004). In the study, EstDL30 was cloned and expressed in *E. coli*, and the biochemical properties of the purified recombinant enzyme were investigated.

Materials and Methods

Strains and chemicals

E. coli strains were cultured on Luria-Bertani (LB) agar or broth supplemented with appropriate antibiotics at 37°C. The concentrations of antibiotics used were as follows: chloramphenicol, 50 μg/ml; ampicillin, 100 μg/ml; and kanamycin, 50 μg/ml. Chemicals, including all

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antimicrobial agents and *p*-nitrophenyl (*p*-NP) esters (acetate, C_2; butyrate, C_4; valerate, C_5; octanoate, C_8; decanoate, C_{10}; dodecanoate, C_{12}; and myristate, C_{14}), were purchased from Sigma- Aldrich (USA).

Lipolytic clone selection

A metagenomic library was constructed as previously described (Lee *et al*., 2004) using soil from Eulsukdo, an alluvial island in the Nakdong River estuary in Saha-Gu, Busan, Republic of Korea (unpublished). To identify lipolytic clones, metagenomic library pools were diluted and then incubated on tributyrin-containing LB agar. After 2-4 days of cultivation at 37℃, clones that showed a clear halo around colonies were selected. The uniqueness of selected clones was confirmed by *Bam*HI restriction digestion and agarose gel electrophoresis.

DNA manipulation

General DNA manipulation was performed as described previously (Sambrook *et al.*, 1989). To determine the corresponding open reading frame (ORF) for lipolytic activity in the selected clone pDL30, shotgun cloning with restriction enzymes *Eco*RI and *Sph*I was carried out. The resultant pUDL30ES contained a small DNA insert and exhibited lipolytic activity. The insert was sequenced using primer-walking (GenoTech Corp., Korea).

DNA sequence analysis

Complete ORFs contained in pUDL30ES were identified by ORF Finder and further examined by BLAST analysis (http://blast.ncbi.nlm.nih.gov). Multiple alignments of deduced amino acid sequences of EstDL30 with homologous proteins were performed using CLUSTAL X (Jeanmougin *et al.*, 1998) and were visually examined with the GENEDOC program (Nicholas and Nicholas, 1997). A phylogenetic tree of EstDL30 with other lipases and esterases was constructed using the neighbor-joining method (Saitou and Nei, 1987) with the MEGA 4.1 (Beta 3) software package (Tamura *et al.*, 2007). A bootstrap test (1,000 replicates) was performed to estimate the reliability of the phylogenetic analyses.

Heteroexpression and purification of EstDL30

The lipolytic gene *estDL30* was amplified using the primers E30F (5′-TGCCATGGCTATGAACGTTGTTGAGTCT-3′), and E30R (5′-CGGAATTCCTAAGGTCCGACAAACCG-3′). Restriction sites for *Nco*I and *Eco*RI were included in the primer sequences for the introduction of *estDL30* into expression vector pET-30b(+) (Novagen, Germany). The resultant pET30-*estDL30* was then transformed into *E. coli* BL21 (DE3) (Novagen).

E. coli BL21 (DE3) harboring pET30-*estDL30* was incubated in LB broth (50 μg/ml kanamycin) at 37℃. Protein induction was initiated by the addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when the OD_{600} had reached 0.8. The cultures were incubated for a further 4 h. The cells were then harvested and lysed in 20 mM Tris-HCl buffer (pH 8.0) using a sonic dismembrator (model 500; Fisher Scientific, USA). Protein purification was performed using an ÄKTA prime liquid chromatography system (GE Healthcare, UK), and a HisTrap™ HP affinity column (5-ml bed volume; GE Healthcare) was used for binding the 6× His-fusion protein. The eluted protein fractions were collected and the elution buffer containing imidazole was replaced with 20 mM Tris-Cl (pH 8.0) using Amicon® Ultra Centrifugal Filters (Millipore, Ireland). Purified fusion protein was analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and protein concentrations were assayed by the bicinchoninic acid method.

Biochemical characterization

Several *p*-NP esters (acetate, butyrate, valerate, octanoate, decanoate, dodecanoate, and myristate) were used to determine the substrate specificity of EstDL30. Enzyme activity was measured in 1 ml of 50 mM Tris-HCl buffer (100 mM NaCl, 0.3% Triton X-100, pH 8.0) containing 2.0 mM substrate at 25℃. The liberated *p*-nitrophenol was detected spectrophotometrically at 400 nm, and the extinction coefficient of *p*-nitrophenol was determined to be 16,642 $M^{-1}cm^{-1}$ under the above conditions. One unit of enzyme activity was defined as the amount of enzyme required for the production of 1 μmol of *p*-nitrophenol per min under the standard conditions.

The effect of temperature on enzyme activity was measured from 15 to 60℃ using 2.0 mM *p*-NP butyrate as a substrate. And thermostability of EstDL30 was determined as above after pre-incubation of the enzyme at 30 to 50℃ for the periods indicated.

The effect of pH on enzyme acidity was measured using *p*-NP butyrate in a series of pH buffers. Liberated *p*-nitrophenol was measured at 348 nm, which is the pH-independent isosbestic point of *p*-nitrophenol and the *p*-nitrophenoxide ion. The following pH buffers were used: 100 mM sodium citrate buffer (pH 4-6), 100 mM potassium dihydrogen phosphate (pH 6-8), 100 mM disodium hydrogen phosphate (pH 8-10), and 50 mM sodium bicarbonate (pH 10-11).

The stability of EstDL30 in the presence of denaturing agents and divalent cations was investigated. The enzyme was pre-incubated in the presence of the compounds of interest for 40 min at 25℃ in 50 mM Tris-Cl buffer (pH 8.0). The negative control was identical except that no denaturing agent or cation was included. The concentrations of the compounds tested were as follow: SDS, 0.1%; Tween 20, 1 and 5%; Triton X-100, 1 and 5%; organic solvents (methanol, ethanol, 2-propanol, acetone, acetonitrile, and dimethyl sulfoxide), 15 and 30%; ethylene diamine tetraacetic acid (EDTA), 10 mM; divalent cations (Ca^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, Ni^{2+}, and Co^{2+}), 1 mM.

Nucleotide sequence accession numbers

The nucleotide sequences of the pUDL30ES inserts have been deposited in GenBank under accession numbers JF427574.

Results

Selection and cloning of a novel lipolytic enzyme

Alluvial soil collected from Eulsukdo, Busan, Republic of Korea, was used to construct a metagenomic library. This was then investigated for the presence of lipolytic enzymes, and 50 unique lipolytic clones were identified (unpublished). Among these, pDL30 was selected for further investigation, as it exhibited exceptionally strong lipolytic activity compared with other library clones. pDL30 produced a clear halo surrounding the colony after 24 h of incubation on tributyrin-containing LB agar at 37℃, and its culture supernatant showed strong hydrolytic activity toward *p*-NP esters.

Shotgun cloning was carried out to identify the gene in pDL30 responsible for the hydrolytic activity. A small subclone was obtained by *Eco*RI and *Sph*I digestion; it carried a 4,515-bp DNA insert that encoded five ORFs and exhibited hydrolytic activity toward tributyrin and *p*-NP esters. The partial coding sequence (CDS) of ORF1 resembled that of the flavin-utilizing monooxygenase superfamily; ORF2 was similar to many alcohol dehydrogenases of the medium chain dehydrogenases/reductase (MDR) family; ORF3 showed similarity to cyclic nucleotide-

binding proteins; and the partial CDS of ORF5 encoded a hypothetical protein that resembled the glutathione S-transferase (GST) family. ORF4 (1524 nucleotides) encoded a protein of 507 amino acids that showed similarity to many carboxylesterases. Thus, ORF4 was designated EstDL30 and investigated further.

Phylogenetic analysis of EstDL30

The deduced amino acid sequence of EstDL30 showed only low similarity (≤42%) to known proteins, suggesting that EstDL30 was a novel enzyme. The carboxylesterases to which EstDL30 was most similar were esterase 54 from *Bacillus niacin* (accession no. AAQ03995; 40% identity), carboxylesterase from *Thermaerobacter subterraneus* DSM 13965 (ZP07836624; 41%), and thermostable carboxylesterase Est50 from *Geobacillus stearothermophilus* (AAN81910; 42%). Phylogenetic analysis revealed that EstDL30 falls into the family VII lipases, together with three other esterases (Arpigny and Jaeger, 1999), namely, *p*-nitrobenzyl esterase from *Bacillus subtilis* (P37967), phenmedipham hydrolase from *Arthrobacter oxydans* (Q01470), and putative carboxylesterase from *Streptomyces coelicolor* A3(2) (CAA22794) (Fig. 1). The multiple alignments suggested that EstDL30 shares several conserved motifs with homologous esterases and family VII lipases (Fig. 2). The conserved G-E-S-A-G pentapeptide containing the catalytic Ser residue, which is a consensus motif in hydrolytic enzymes (Arpigny

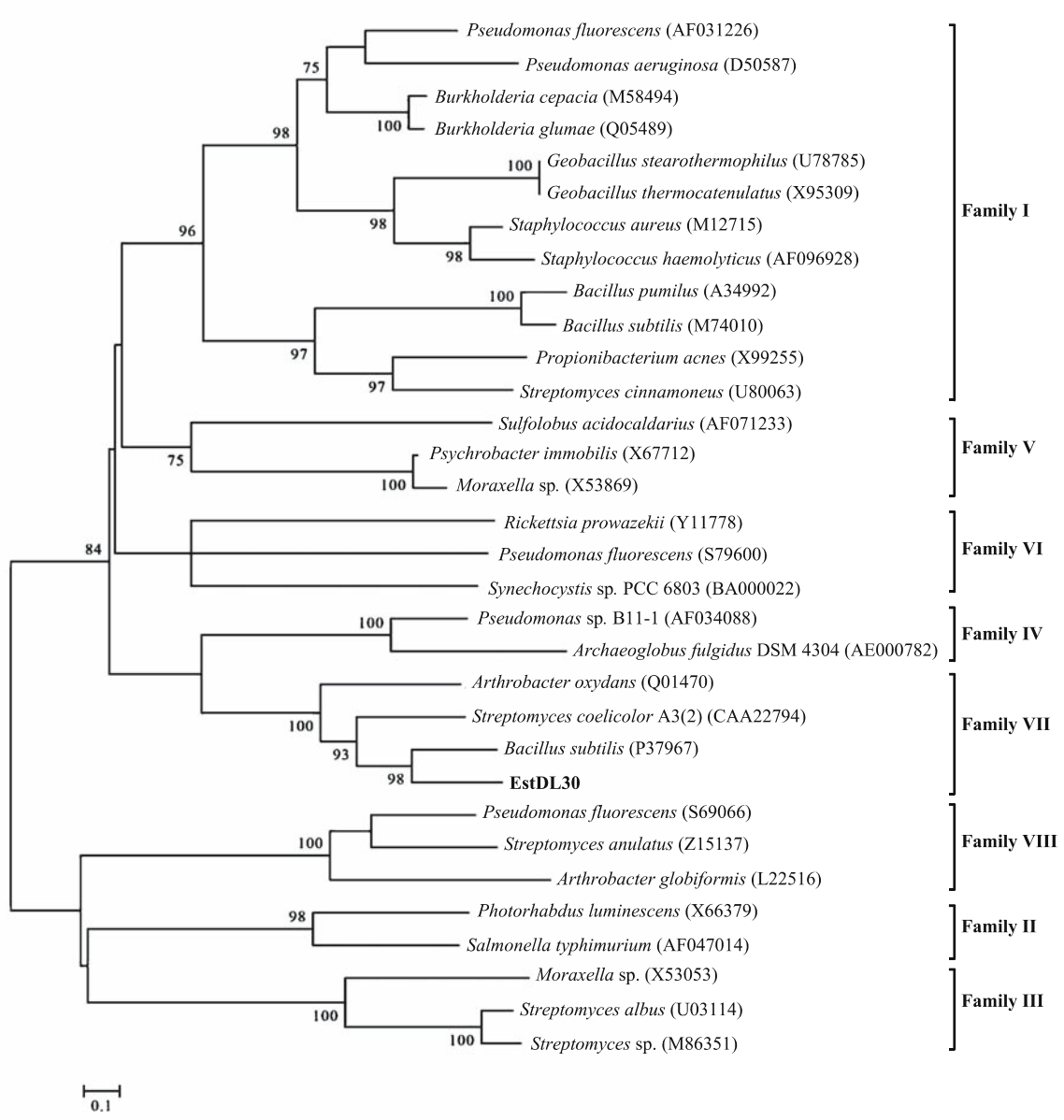

Fig. 1. Phylogenetic analysis of EstDL30 and other lipolytic enzymes. The phylogenetic tree was constructed by the neighbor-joining method using MEGA 4.1 (Beta 3), with a bootstrap test of 1,000 replicates. The scale indicates the number of amino acid substitutions per site.

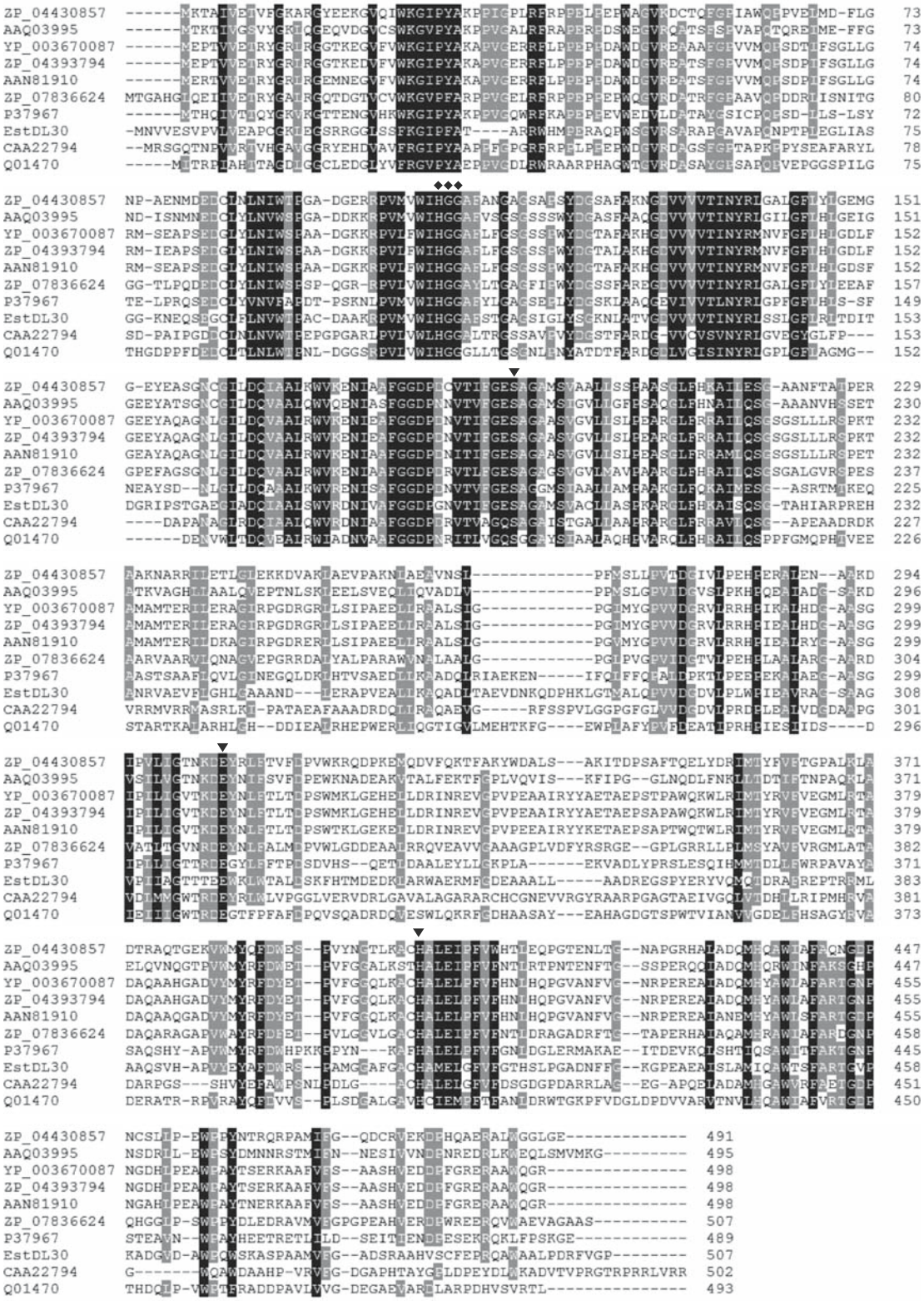

Fig. 2. Multiple alignments of EstDL30 with homologous proteins and family VII lipases. The amino acid sequences of the following bacterial lipolytic enzymes were retrieved from GenBank (http://www.ncbi.nlm.nih.gov): six carboxylesterases, from *Bacillus niacin* (AAQ03995), *Thermaerobacter subterraneus* DSM 13965 (ZP 07836624), *Geobacillus stearothermophilus* (AAN81910), *Geobacillus* sp. Y412MC52 (ZP 04393794), *Geobacillus* sp. C56-T3 (YP 003670087), and *Bacillus coagulans* 36D1 (ZP 04430857); and three previously identified esterases of family VII from *Bacillus subtilis* (P37967), *Arthrobacter oxydans* (Q01470), and *Streptomyces coelicolor* A3(2) (CAA22794). Filled diamonds indicate the conserved HGG motif, and filled triangles indicate the catalytic residues.

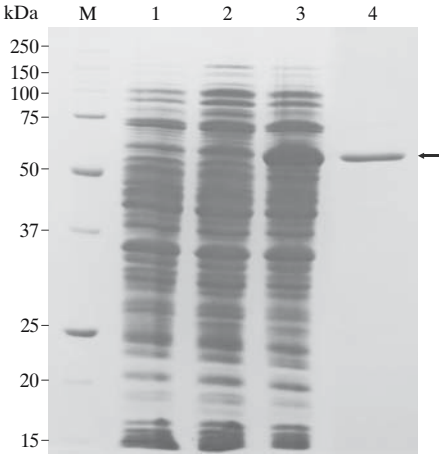

Fig. 3. SDS-PAGE (12%) analysis of the purified recombinant EstDL30. Lanes: M, molecular weight standards; 1 and 3, total lysate of *E. coli* BL21(DE3) carrying pET30b(+) and pET30-*estDL30* after IPTG induction, respectively; 2, total lysate of pET30-*estDL30* strain before IPTG induction; 4, purified EstDL30 (arrow) after Histrap HP column chromatography.

and Jaeger, 1999), was found at positions 193 to 197 of EstDL30. Additionally, catalytic residues Glu and His were found at positions 319 and 412, respectively (Liu et *al.*, 2007). Furthermore, a conserved H-G-G motif at positions 107 to 109 of EstDL30 was detected in all esterases analyzed. This may function as an oxyanion hole (Kim *et al.*, 2004).

Enzyme activity of EstDL30 toward *p*-nitrophenyl esters

EstDL30 was overexpressed and purified as an N-terminal His-tagged fusion protein. Recombinant EstDL30 was purified threefold over the crude extract, with 12% recovery. It showed specific activity of 161 U/mg toward *p*-NP butyrate after one-step affinity chromatography purification. The molecular mass of recombinant EstDL30 was approximately 59 kDa, as determined by SDS-PAGE (Fig 3). This was in accordance with the theoretical value.

The substrate specificity of EstDL30 was assessed using various *p*-NP esters with different acyl chain lengths (acetate, C_2; butyrate, C_4; valerate, C_5; octanoate, C_8; decanoate, C_{10}; dodecanoate, and C_{12}; and myristate, C_{14}). EstDL30 exhibited high catalytic efficiency toward *p*-NP esters with short acyl chains (acetate, butyrate, and valerate; Table 1). However, the catalytic efficiency was markedly lower when the acyl chain length exceeded C_5. For example, the catalytic efficiency toward octanoate was more than fivefold lower than that toward

Table 1. Kinetic parameters of recombinant EstDL30

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)
p-NP acetate (C_2)	0.8	90.6	113.2
p-NP butyrate (C_4)	1.3	229.3	176.4
p-NP valerate (C_5)	1.2	176.0	146.7
p-NP octanoate (C_8)	1.6	42.0	26.3
p-NP decanoate (C_{10})	2.0	1.1	0.6
p-NP dodecanoate (C_{12})	2.6	0.17	0.06
p-NP myristate (C_{14})	0.5	0.02	0.03

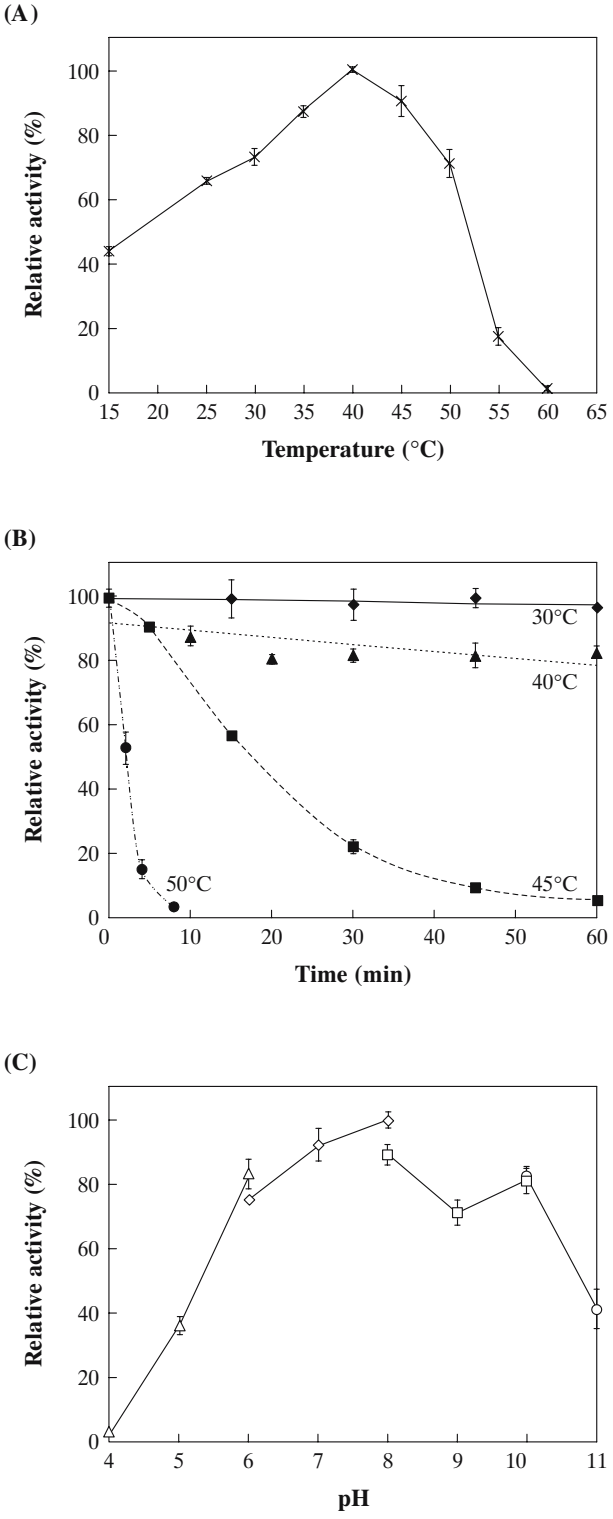

Fig. 4. Characterization of EstDL30. (A) Effect of temperature on enzyme activity. (B) Thermostability of EstDL30. (C) Effect of pH on enzyme activity. The pH buffers used were: (△) sodium citrate buffer (pH 4-6); (◇) potassium dihydrogen phosphate buffer (pH 6-8); (□) disodium hydrogen phosphate buffer (pH 8-10); and (○) sodium bicarbonate buffer (pH 10-11). Data are presented as the Means±SD from three independent determinations.

Table 2. Stability of EstDL30 in the presence of denaturing compounds

Compound[a]	Concentration (%)	% Relative activity
Organic solvents		
Methanol	15	107±12
	30	63±2
Ethanol	15	57±4
	30	18±5
2-Propanol	15	25±5
	30	3±0.4
Acetone	15	18±2
	30	4±1
Acetonitrile	15	22±3
	30	0.4±0.1
DMSO	15	58±7
	30	40±7
Detergents		
SDS	0.1	1±0.3
Tween 20	1	94±2
	5	47±4
Triton X-100	1	47±7
	5	21±3

[a] DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate.
Residual activity was measured by the standard *p*-NP butyrate assay. A control lacking test compounds was taken as 100% activity. Each value represents the Mean±SD from three replicate determinations.

valerate. EstDL30 showed the highest catalytic efficiency toward *p*-NP butyrate (Table 1). This preference for short-chain *p*-NP esters suggested that EstDL30 was a carboxylesterase.

Effect of temperature and pH on enzyme activity

The optimal temperature range for EstDL30 activity toward *p*-NP butyrate was 15 to 60°C. EstDL30 showed high activity at 25 to 50°C, with >60% of the maximum activity at 40°C (Fig. 4A). The enzyme was completely inactivated at temperatures ≥60°C. The thermostability of EstDL30 was determined by pre-incubation at various temperatures. EstDL30 was stable at 30 and 40°C; i.e., the activity did not change at 30°C, and >80% of the maximum activity was retained after pre-incubation at 40°C for 1 h (Fig. 4B). EstDL30 became unstable when pre-incubated at temperatures higher than 40°C; activity was markedly lower at 45 and 50°C, with half-lives of approximately 15 and 2 min, respectively.

The enzyme activity of EstDL30 at various pH values was determined. The data suggested that EstDL30 was an alkaline enzyme, as it was highly active at pH 6 to 10 and exhibited >70% of maximum activity at pH 8 (Fig. 4C).

Table 3. Stability of EstDL30 against divalent cations and EDTA

Cation	Concentration (mM)	% Relative activity
Ca^{2+}	1	104±6
Co^{2+}	1	89±5
Cu^{2+}	1	64±7
Fe^{2+}	1	152±6
Mg^{2+}	1	110±5
Mn^{2+}	1	101±6
Ni^{2+}	1	97±5
Zn^{2+}	1	47±3
EDTA	10	102±1

Residual activity was measured by the standard *p*-NP butyrate assay. A negative control lacking any cation was taken as 100% activity. Each value represents the Mean±SD from three replicate determinations.

Stability of EstDL30 against denaturing compounds and divalent cations

The effect of organic solvents on EstDL30 activity was examined by measuring residual activity after pre-incubation with organic solvents (15 and 30%, v/v; Table 2). At the lower concentration, EstDL30 was stable in the presence of methanol (residual activity, 107%), whereas enzyme activity was strongly inhibited by other organic solvents at the lower concentration: ethanol (57%), 2-propanol (25%), acetone (18%), acetonitrile (22%), and DMSO (58%). When the higher concentration of 30% organic solvent was used, enzyme activity was almost completely inhibited by ethanol (18%), 2-propanol (3%), acetone (4%), and acetonitrile (0.2%). However, it remained high in methanol and DMSO (63 and 40%, respectively).

The influence of a variety of detergents on EstDL30 activity was determined (Table 2). Enzyme activity was completely inhibited by the presence of 0.1% SDS (1% activity remaining after a 40-min pre-incubation). Likewise, EstDL30 was strongly inhibited by Triton X-100 (53 and 79% reduction at 1 and 5%, respectively). However, EstDL30 was stable in the presence of Tween 20 (94 and 47% remaining at 1 and 5%, respectively).

The influence of divalent cations (1 mM) on EstDL30 activity was also assessed (Table 3). EstDL30 was stable in the presence of most cations, as enzyme activity was slightly altered by the presence of Ca^{2+} (104%), Co^{2+} (89%), Mg^{2+} (110%), Mn^{2+} (101%), and Ni^{2+} (97%). EstDL30 was also stable in the presence of the chelating agent EDTA (102%). However, enzyme activity was strongly inhibited by the presence of Cu^{2+} (64%) and Zn^{2+} (47%). In contrast, Fe^{2+} greatly increased the enzyme activity (>150%).

Discussion

Lipolytic enzymes have attracted enormous attention for a variety of biotechnological applications, such as pure compound synthesis in the food, perfume, and pharmaceutical industries. For this reason, a large number of lipolytic enzymes have been isolated and characterized (Gupta *et al.*, 2004; Rhee *et al.*, 2005; Elend *et al.*, 2006; Jeon *et al.*, 2009), and several new families have recently been proposed based on their amino acid sequences or biochemical properties (Lee *et al.*, 2006; Chu *et al*., 2008; Kim *et al*., 2009; Hu *et al*., 2010; Lee *et al.*, 2010). In this study, we describe the cloning and biochemical characterization of a novel microbial esterase, EstDL30, with high activity toward short-chain *p*-NP esters.

The deduced amino acid sequence of EstDL30 showed similarity to those of several previously identified family VII members, with protein sequence identities of 39, 39, and 34% to esterases from *Bacillus subtilis* (P37967), *Streptomyces coelicolor* A3(2) (CAA22794), and *Arthrobacter oxydans* (Q01470), respectively. Phylogenetic analysis also suggested that EstDL30 clustered with other family VII lipases (Fig. 1). The molecular mass of EstDL30 was approximately 54 kDa, in agreement

with that of other bacterial family VII esterases (≈ 55 kDa; Arpigny and Jaeger, 1999). EstDL30 also showed sequence similarity to the type B carboxylesterases, such as esterase 54 (*Bacillus niacin*; 40% identity), carboxylesterase type B (*Geobacillus* sp. C56-T3; 41%), carboxylesterase type B (*Geobacillus* sp. Y412MC52; 41%), carboxylesterase type B (*Thermaerobacter subterraneus* DSM 13965; 41%), and carboxylesterase Est55 (*Geobacillus stearothermophilus*; 42%). Type B carboxylesterases are mostly higher eukaryotic esterases containing only a few prokaryotic origins, and the majority of which are evolutionarily related (Arpigny and Jaerer, 1999; Prim *et al.*, 2001). Moreover, when the EstDL30 sequence was used to search the Lipase Engineering Database (http://www.led.uni-stuttgart.de), the results indicated that EstDL30 belongs to a homologous family of *Bacillus* esterases (Fischer and Pleiss, 2003). The results stated above suggest that EstDL30 may be originated from a *Firmicutes*, although we have not investigated the origin of this enzyme.

Biochemical activity assays suggested that EstDL30 is highly active toward short acyl chain *p*-NP esters, which is similar to several type B carboxylesterases, such as type B carboxylesterase from *Bacillus* sp. BP-7 (Prim *et al.*, 2001), Est55 from *Geobacillus stearothermophilus* (Ewis *et al.*, 2004), and Est54 from *Bacillus niacini* EM00 (Kim *et al.*, 2004). EstDL30 showed the highest catalytic efficiency toward *p*-NP butyrate (k_{cat} of 229.3 s^{-1} and k_{cat}/K_m of 176.4 $s^{-1}mM^{-1}$); however, little activity was detected when the acyl chain length exceeded C_8 (Table 1). Remarkably, EstDL30 exhibited high activity in alkaline conditions (pH 8-11), with >80% of maximum activity at pH 10 (Fig. 4C). This suggests that EstDL30 may be of use in a variety of industrial applications requiring extreme pH conditions. And EstDL30 showed maximal activity at 40°C, at which temperature it was also stable (Figs. 4A and B).

Stability in organic solvents is desirable for many chemical applications. EstDL30 was stable in reaction buffers supplemented with methanol and DMSO, and retained high activity in the presence of Tween 20 (Table 2). These properties may make EstDL30 useful in organic synthesis applications in the presence of these solvents. The majority of divalent cations we tested only slightly affected the activity of EstDL30; however, Cu^{2+} and Zn^{2+} strongly inhibited the enzyme activity (Table 3). Similar results have been reported for EstCE1 from an uncultured microorganism (Elend *et al.*, 2006) and EstA1 from *Bacillus* (Prim *et al.*, 2001). Interestingly, Fe^{2+} strongly stimulated EstDL30 activity (>150%; Table 3). This appears to be unique to EstDL30, as ferrous ion has no stimulatory effect on lipolytic enzymes in general (Gupta *et al.*, 2004). Nonetheless, the chelating agent EDTA appears not to influence EstDL30 activity.

In conclusion, this study describes the successful isolation and characterization of the novel esterase EstDL30 from an alluvial soil metagenomic library. On the basis of its amino acid sequence, EstDL30 was classified into family VII of the bacterial lipases. Biochemical characterization revealed several fascinating properties of EstDL30 and suggested several possible biotechnological applications. However, its activity against additional substrates, for example, ofloxacin esters (Kim *et al.*, 2004) and *p*-nitrobenzyl esters (Zock *et al.*, 1994), should be investigated to determine the specific utility of EstDL30.

Acknowledgements

This research was supported by a grant (MG 08-0104-1-0) from the Microbial Genomics and Application Center of the 21st Century Frontier Research Program funded by the Ministry of Education, Science and Technology of the Republic of Korea.

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