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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Identification of a new subfamily of salt-tolerant esterases from a metagenomic library of tidal flat sediment

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Abstract To search for novel lipolytic enzymes, a metagenomic library was constructed from the tidal flat sediment of Ganghwa Island in South Korea. By functional screening using tributyrin agar plates, 3 clones were selected from among the 80,050 clones of the fosmid library. The sequence analysis revealed that those clones contained different open reading frames, which showed 50–57% amino acid identity with putative lipolytic enzymes in the database. Based on the phylogenetic analysis, they were identified to encode novel members, which form a distinct and new subfamily in the family IV of bacterial lipolytic enzymes. The consensus sequence, GT(S)SA(G)G, encompassing the active site serine of the enzymes was different from the GDSAG motif, conserved in the other subfamily. The genes were expressed in *Escherichia coli* and recombinant proteins were purified as active soluble forms. The enzymes showed the highest activity toward *p*-nitrophenyl valerate (C5) and exhibited optimum activities at mesophilic temperature ranges and slightly alkaline pH. In particular, the enzymes displayed salt tolerance with over 50% of the maximum activity remained in the presence of 3 M NaCl (or KCl). In this study, we demonstrated that the metagenomic approach using marine tidal flat sediment as a DNA source expanded the diversity of lipolytic enzyme-encoding genes.

Keywords Tidal flat sediment · Metagenome · Lipolytic enzymes · Family IV · Salt tolerance

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Introduction

Metagenomics involves the analysis of metagenomes, the collective genomes of all microorganisms present in a given habitat, to gain access to a wealth of information about novel genes and molecules for biotechnological and pharmaceutical applications (Handelsman 2004; Streit and Schmitz 2004). There are two strategies for the screening of novel genes from metagenomic libraries, which are based either on enzyme activity or sequence similarities. While the sequence-based screening has an advantage in that the time-consuming development of specialized screening systems is not required, activity-based analysis has the potential to identify entirely new classes of enzymes, as this approach disregards sequence information compared to the sequence-based screening, which reveals only homologues of known genes (Schloss and Handelsman 2003). Additionally, functional screening only results in positive signals for full-length genes and, therefore, functional gene products. Functional screens of metagenomic libraries have identified various industrial enzymes such as protease protease (Gupta et al. 2002), amylase (Yun et al. 2004), β-glucosidase (Gabor et al. 2003), nitrilase (Robertson et

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al. 2004), lyase (Solbak et al. 2005), and so on, even if the development has been relatively limited to enzymes whose activities could be screened easily.

Esterases and lipases belong to the class of carboxylic ester hydrolases that catalyze both the hydrolysis and synthesis of ester bonds. They are widespread in various organisms including bacteria, fungi, plants, and animals and have a wide range of biotechnological applications, such as organic chemical processing, detergent formulation, synthesis of biosurfactants, the oleochemical industry, the dairy industry, the agrochemical industry, paper manufacturing, nutrition, cosmetics, and pharmaceutical processing (Jaeger et al. 1999; Jaeger and Eggert 2002; Pandey et al. 1999; Sharma et al. 2001). Esterases hydrolyze water-soluble esters with short-chain carboxylic acids while lipases prefer long-chain acylglycerides. Esterases and lipases share a characteristic α/β hydrolase fold including consensus sequence GXSXG (X represents any amino acid) around the active site serine in the three-dimensional structure, but show differences in substrate preferences (Nardini and Dijkstra 1999). They have been classified into eight families based on the conserved sequence motifs and biological properties (Arpigny and Jaeger 1999). A number of bacterial enzymes belonging to family IV showed striking similarities to the mammalian hormone-sensitive lipase (HSL) (Hemila et al. 1994). Recently, new family IV enzymes have been identified in metagenomic libraries prepared from various environmental samples such as hot springs (Rhee et al. 2005), deep sea (Ferrer et al. 2005), the Arctic sediments (Jeon et al. 2009), marine sediment (Hu et al. 2010), and soil (Bunterngsook et al. 2010).

In this study, a tidal flat sediment sample was used to search for novel esterases. Tidal flat sediments have been known to possess a unique microbial diversity including various unculturable microorganisms (Kim et al. 2004,2005). Therefore, they could be good reservoirs to find novel enzymes. In previous reports, two novel lipases, LipG and LipEH166, were identified from metagenomic libraries constructed from tidal flat sediments and belong to a new family of bacterial lipolytic enzymes (Kim et al. 2009; Lee et al. 2006). Here, we present that three esterases, which were isolated from a tidal flat metagenomic library, are grouped together within family IV, forming a new subfamily separately from two other subfamilies. We investigated the biochemical characteristics of the recombinant enzymes including salt tolerance by expressing the esterase genes in *Escherichia coli*.

Materials and methods

Strains and vectors

E. coli DH5α (Stratagene, LaJolla, CA, USA), EPI300-T1R (Epicentre, Madison, WI, USA), and BL21(DE3) (Novagen, Madison, WI, USA) were used as host strains for cloning and expression. pBluescript SK- (Stratagene), pET-24a(+), pET-24d(+) (Novagen), and pCC1FOS (Epicentre) were used as vectors.

Metagenomic library construction and screening for lipolytic clones

A sediment core (approximately 50 cm depth) was taken within an undisturbed tidal flat (37°36′ N/126°22′ E) in Ganghwa Island in South Korea. DNA from the sediment sample was extracted based on a previously described method (Jeon et al. 2009). After extraction, the DNA was further purified by gel electrophoresis in a 1% low melting temperature agarose gel (FMC Bioproducts, Rockland, ME, USA) containing 1% polyvinyl polypyrrolidone (Sigma, St. Louis, MO, USA). Gel electrophoresis was performed at 35 V for 13 h and DNA fragments of approximately 40 to 50 kb were then isolated from the gel. The isolated DNA was end-repaired with End-It DNA End-Repair kit (Epicentre), which caused the DNA to be blunt-ended and 5′-phosphorylated. The blunt-ended DNA was ligated into a pCC1FOS vector (Epicentre). Lambda packaging extracts were added to ligations, and infection of phage T1-resistant EPI300-T1^R cells was performed according to the manufacturer's instructions. The *E. coli* transformants were transferred to 96-well microtiter plates and stored at −80°C. To screen for esterase activity, the transformants were plated on Luria–Bertani (LB) agar plates containing 12.5 μg/ml of chloramphenicol and 1% tributyrin as a substrate. Colonies were incubated for 1 day at 37°C, and subsequently incubated for a week at 4°C. Candidates surrounded by a clear halo on the plate were selected. The positive clones were reconfirmed and subcloned.

Subcloning and sequence analysis

Fosmid clones showing lipolytic activity on the tributyrin agar plate were inoculated into 200 ml of LB broth containing 12.5 μg/ml of chloramphenicol. After overnight incubation at 37°C, the cells were harvested by centrifugation at 5,000×*g* for 15 min and washed twice with distilled water. The fosmid DNA was purified using the modified alkaline lysis method as previously described (Jeon et al. 2009) and was randomly sheared by nebulization according to the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). After nebulization, DNA fragments of 2 to 4 kb were isolated from a 0.6% low melting temperature agarose (FMC Bioproducts) gel and end-repaired to generate blunt ends. The blunt-ended DNA was ligated into the *Eco*RV site of pBluescript SK(−) (Stratagene), and the ligations were introduced into *E. coli* DH5α cells. The *E. coli* transformants were plated onto LB agar plates containing

100 μg/ml of ampicillin and 1% tributyrin. After incubation at 37°C for 24 h, colonies surrounded by a clear halo were selected.

Nucleotide sequencing was performed with the automated sequencer (ABI3100) using the BigDye terminator kit (PE Applied Biosystems, Foster City, CA, USA). The DNA sequence was determined by primer walking in both directions and assembled using the ContigExpress program of the Vector NTI suite 7 software package (InforMax, North Bethesda, MD, USA). The open reading frame (ORF) was detected using the ORF search tool provided by the National Center for Biotechnology Information. Sequence homology searches were performed with the basic local alignment search tool program (Altschul et al. 1997). Signal sequence search was performed with the SignalP 3.0 program (Emanuelsson et al. 2007). Multiple alignments between protein sequences were performed with the ClustalW program (Thompson et al. 1994). The phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei 1987) using the Molecular Evolutionary Genetics Analysis 4.1 software (MEGA, version 4.1) (Tamura et al. 2007).

Overexpression of esterase-encoding genes and purification of recombinant proteins

The *estKT4* gene was amplified without its signal sequence using the pBluKT4 as a template using the following primers: 5′-GAAGGCTTCATATGAATTCACGACTCGCGATCATTCT-3′ and 5′-CGTTGCGGCCGCCTTTAGATGCTTGTCAAAGAATC-3′ (*Nde*I and *Not*I restriction enzyme sites are underlined). The *estKT7* gene was amplified using the pBluKT7 as a template using the following primers: 5′-CTTGACCCTTCAGCCATGGATTATGTTCCTGCCAC-3′ and 5′-TGCTTTGCGGCCGCGTATCCAACGGACTTTG-3′ (*Nco*I and *Not*I restriction enzyme sites are underlined). The *estKT9* gene was amplified without its signal sequence using the pBluKT9 as a template using the following primers: 5′-ACGCATATGAAATCAAATCTCATCAGAATACAGGCTTTG-3′ and 5′-CAG AAACCCTCGAGTTTCGGGACCTCAATATCCTTC-3′ (*Nde*I and *Xho*I restriction enzyme sites are underlined). The amplified DNA fragments were inserted into the pET-24a(+) or pET-24d(+) (*estKT7*) expression vectors, and the recombinant plasmids were transformed into *E. coli* BL21 (DE3) cells.

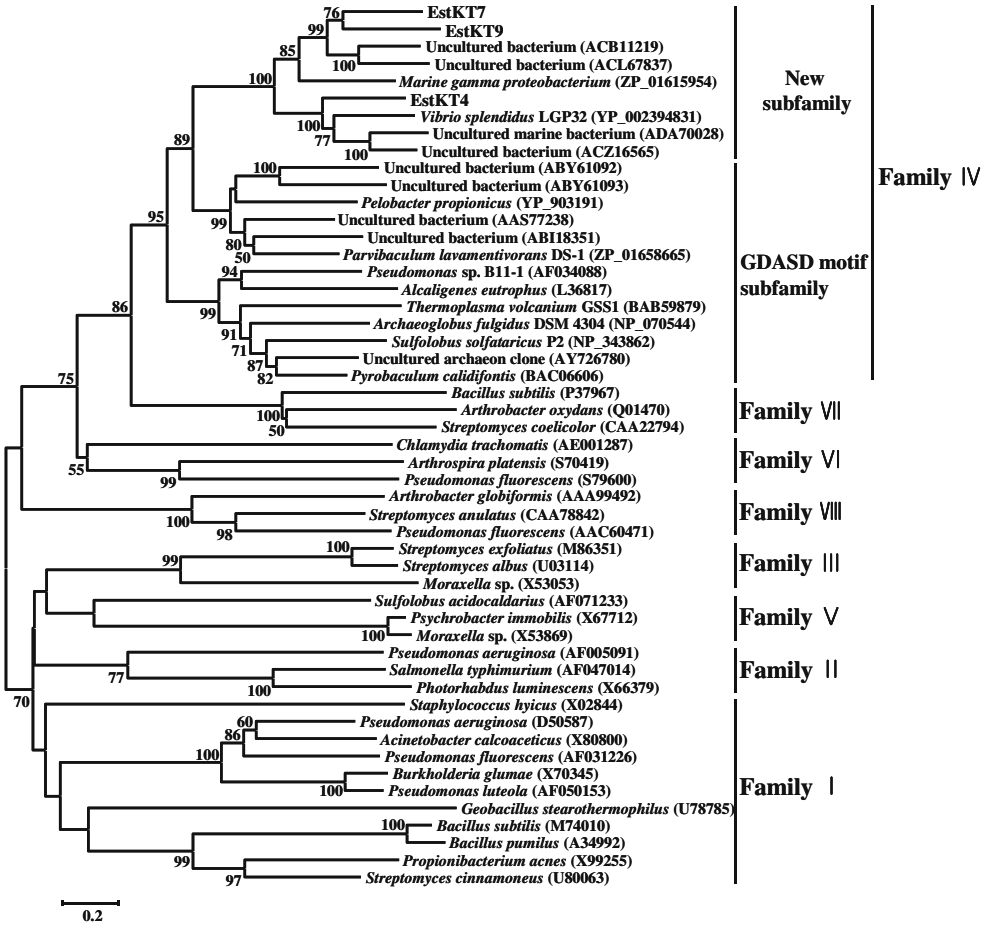

Fig. 1 Phylogenetic tree of the lipolytic enzymes. The tree was constructed using the MEGA 3.1 program with the neighbor-joining algorithm. Only bootstrap values greater than 50% are shown. *Bar*—0.2 substitutions per amino acid site

As cell density reached a turbidity of about 0.6 at 600 nm, 1-mM isopropyl β-D-1-thiogalactopyranoside was added to the culture to induce the protein expression. Three hours later, the cells were harvested by centrifugation (6,000×*g*, 20 min, 4°C) and resuspended in 50 mM Tris–HCl buffer (pH 8.0) containing 0.1 M KCl and 10% glycerol. The cells were disrupted by sonication and centrifuged (20,000×*g*, 1 h, 4°C). The resulting supernatants were applied to a column of TALON metal-affinity resin (BD Biosciences Clontech, Palo Alto, CA, USA) and washed with 10 mM imidazole (Sigma) in 50 mM Tris–HCl buffer (pH 8.0) containing 0.1 M KCl and 10% glycerol, and the esterases were eluted with 300 mM imidazole in the buffer. The protein concentration was measured by the method of Bradford using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard (Bradford 1976). The purity of the protein was examined by sodium dodecyl sulfate-polyacrylamide

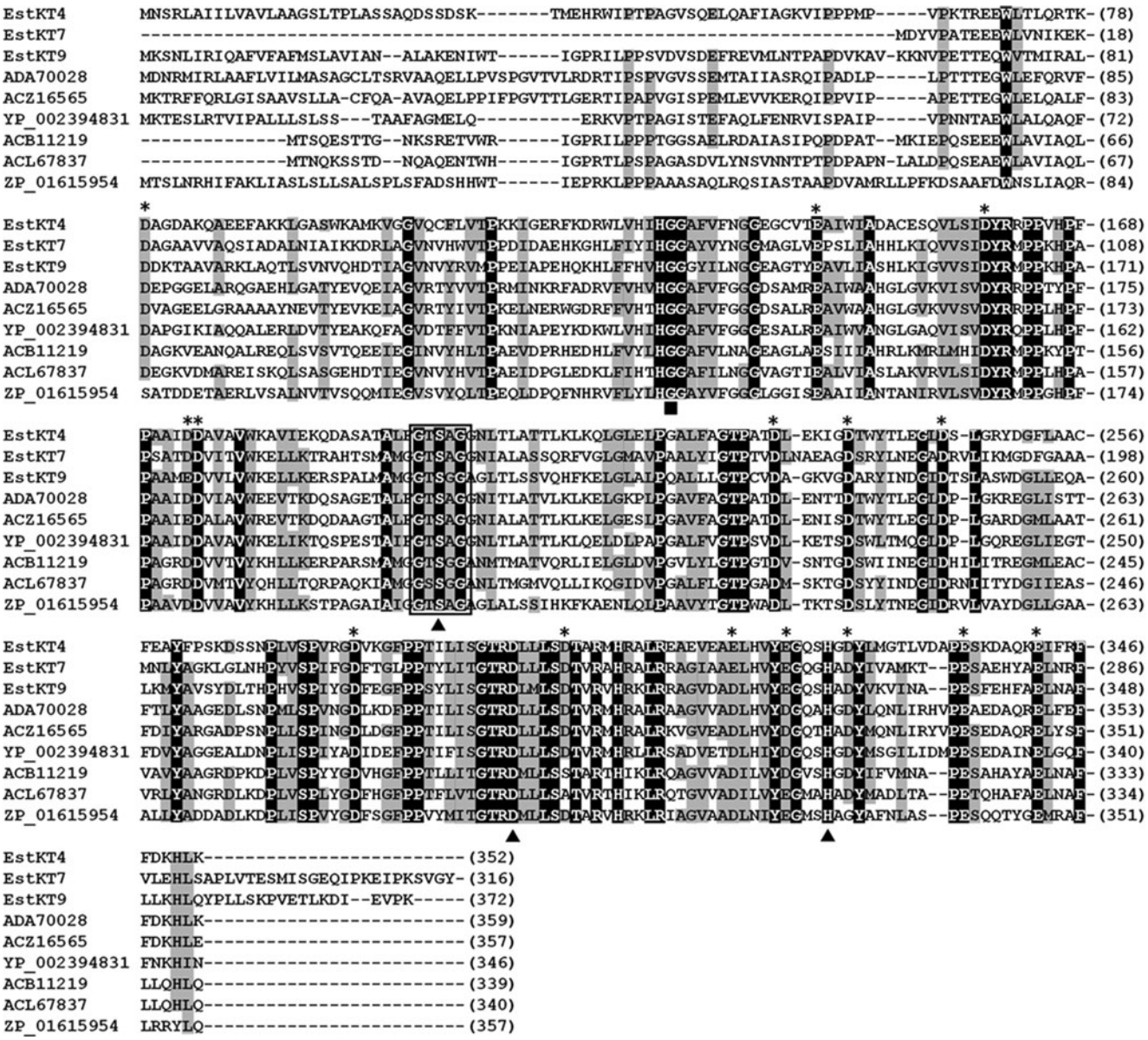

Fig. 2 Multiple sequence alignment of EstKT4, EstKT7, and EstKT7 with other related proteins: ADA70028, a lipolytic enzyme precursor from uncultured marine bacterium; ACZ16565, a putative esterase from uncultured bacterium; YP_002394831, a putative esterase from *Vibrio splendidus* LGP32; ACB11219, an esterase from uncultured bacterium; ACL67837, a lipolytic enzyme from uncultured bacterium; ZP_01615954, an esterase from *marine gamma proteobacterium* HTCC2143. *Triangles* and *squares* represent the residues involved in the formation of the catalytic triad and the oxyanion hole, respectively, and the conserved pentapeptide motifs are *boxed*. *Asterisk* indicates negatively charged amino acids. *Black* and *gray boxes* indicate regions with identical or similar amino acids residues, respectively

gel electrophoresis (SDS-PAGE) under denaturing conditions as described by Laemmli (1970).

Esterase assay

Enzyme activity was measured by a spectrophotometric method using *p*-nitrophenyl esters (Sigma) as substrate. After incubation at each optimum temperature for 5 min, the absorbance at 405 nm was measured to detect the released *p*-nitrophenol. One unit of esterase activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol from *p*-nitrophenyl esters per minute.

Biochemical properties of esterases

The substrate specificity was determined by using *p*-nitrophenyl esters with different aliphatic side chains, C2 (acetate), C4 (butyrate), C5 (valerate), C8 (octanoate), C10 (decanoate), C12 (laurate), C14 (myristate), and C16 (palmitate). Kinetic parameters, k_{cat} and K_M, were determined by analyzing initial hydrolysis rates of enzymes towards the *p*-nitrophenyl valerate of 10–1,000 μM by fitting the data to the Michaelis–Menten equation. The optimum temperature of the enzyme reaction was determined in the same substrate solution described above at various temperatures of 5°C to 65°C. The optimum pH was determined over a pH range of 4.0 to 10.0, using the following buffer systems: 50 mM sodium acetate (pH 4.0 to 6.0), 50 mM sodium phosphate (pH 6.0 to 7.5), 50 mM Tris–HCl (pH 7.5 to 8.5), and 50 mM CHES (pH 8.5 to 10.0). Various metal ions ($MnCl_2$, $MgCl_2$, $CaCl_2$, $CuCl_2$, $ZnSO_4$, $FeSO_4$, $CoSO_4$, and $NiSO_4$) and enzyme inhibitors (phenymethylsulfonyl fluoride [PMSF] and ethylenediaminetetraacetic acid [EDTA]) at a final concentration of 1 mM were incubated with enzyme in 50 mM Tris–HCl buffer (pH 8.0 or pH 8.5) at 35°C for 1 h and then assayed for enzyme activity. The effect of detergents on enzyme activity was determined by incubating the enzyme in 50 mM Tris–HCl buffer (pH 8.0 or pH 8.5) with 1% (*w*/*v*) of SDS, Triton X-100, and Tween-20, Tween-40, Tween-60, and Tween-80 at 35°C for 1 h. The effect of salt concentration on esterase activity was investigated by adding 0.5 to 4 M of NaCl or KCl into enzyme solution and measuring the enzyme activity after preincubation at 35°C for 30 min.

Nucleotide sequence accession numbers

The obtained nucleotide sequences have been deposited in the GenBank database under the accession numbers EstKT4 (GQ340931), EstKT7 (GQ340932), and EstKT9 (GQ340933).

Results

Construction of metagenomic library and screening for lipolytic clones

A fosmid metagenomic library consisting of 80,050 clones was constructed using high molecular weight DNA isolated from the tidal flat sediment sample of Ganghwa Island in South Korea. This region is expected to possess remarkable bacterial diversities mainly caused by the dynamic physicochemical conditions, such as periodic flood tides, a high degree of change in salinity, and fluctuations in the water temperature (Kim et al. 2004). Moreover, the location of the site in the estuary region of the Han River and the presence of four distinct seasons in the Korean peninsular may expose the bacterial community to the great climate change (Kim et al. 2007, 2008). The library was screened for clones displaying lipolytic activity using tributyrin agar plates, and five positive clones were detected by zones of clearance around the colonies. Among them, three clones with bigger halos in the plate were selected.

Primary sequence analysis of lipolytic enzyme-encoding genes

Three fosmid clones were first subjected to subcloning as described in "Materials and methods". The deduced amino acid sequences of three ORFs, *estKT4*, *estKT7*, and *estKT9*, showed the greatest extent of similarity to a lipolytic enzyme precursor from an uncultured bacterium (57% identity), an esterase from an uncultured bacterium (50% identity), and a lipolytic enzyme (53% identity) from an uncultured bacterium, respectively. To see how the ORFs were related to known esterases/lipases, the phylogenetic relationship was analyzed based on the esterase/lipase

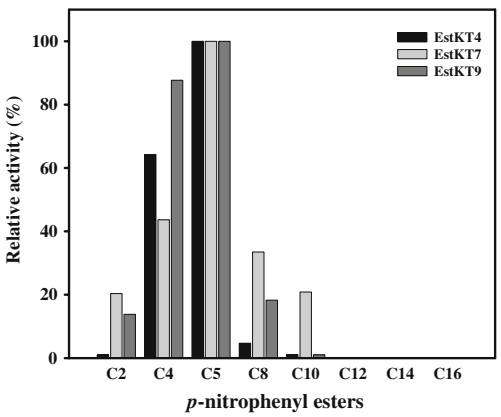

Fig. 3 Substrate specificity of EstKT4, EstKT7, and EstKT9 toward various *p*-nitrophenyl esters. The specific activity of 237.9 U/mg of EstKT4, 0.3 U/mg of EstKT7, and 426.8 U/mg of EstKT9, respectively, was defined as 100%

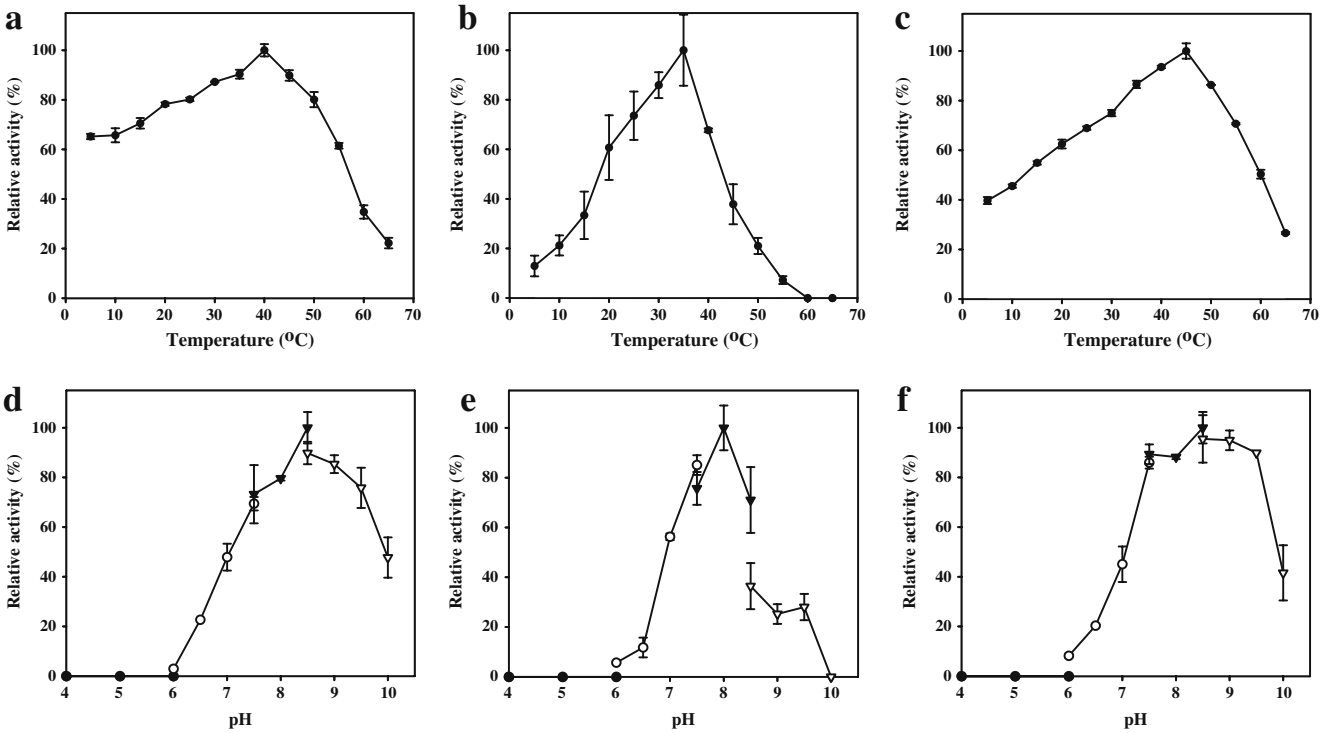

Fig. 4 Effects of temperature (**a**–**c**) and pH (**d**–**f**) on the esterase activity of EstKT4, EstKT7, and EstKT9. The enzyme activity of EstKT4, EstKT7, and EstKT9 was determined at various temperatures or pH values using *p*-nitrophenyl valerate as a substrate. Buffers used were 50 mM sodium acetate buffer (*closed circles*; pH 4.0 to 6.0), 50 mM sodium phosphate buffer (*open circles*; pH 6.0 to 7.5), 50 mM Tris–HCl buffer (*closed triangles*; pH 7.5 to 8.5), and 50 mM CHES buffer (*open triangles*; pH 8.5 to 10.0). The value obtained at each optimal condition was taken as 100%

classification proposed by Arpigny and Jaeger (1999). In the phylogenetic tree, EstKT4, EstKT7, and EstKT9 were assigned to family IV (HSL) (Fig. 1). Interestingly, EstKT4, EstKT7, and EstKT9 branched out to a subfamily within family IV. The in-depth sequence analysis indicated that the active site Ser residue in the EstKT4, EstKT7, and EstKT9 is encompassed by a unique pentapeptide motif, GT(S)SA(G)G, which is slightly different from the conserved motif, GDSAG, among the esterases/lipases in family IV (Fig. 2). The lipolytic enzymes belonging to this new subfamily have never been purified or characterized to date.

Purification and characterization of the recombinant proteins

To express three genes coding for EstKT4, EstKT7, and EstKT9, we investigated the presence of a signal sequence in the genes using the SignalP 3.0 program and found that *EstKT4* and *EstKT9* retained putative signal sequence of 24 and 25 amino acids long, respectively. *EstKT4* and *EstKT9* genes were amplified without signal sequences and the EstKT7 gene was amplified from the first ATG of the ORF, and the resulting expression constructs were expressed in *E. coli*. SDS-PAGE analysis of each purified proteins showed a single band corresponding to its predicted size of 36, 34, and 38 kDa, respectively (Supplementary Fig. S1).

The purified proteins could hydrolyze a wide range of substrates (C2 to C10) with the highest activity toward *p*-nitrophenyl valerate (C5) while no enzyme activity was detected toward longer *p*-nitrophenyl esters (C12 to C16)

Table 1 Effect of various metals and inhibitors on the enzyme activity of EstKT4, EstKT7, and EstKT9

Metals or inhibitors (1 mM)	Relative activity (%)		
	EstKT4	EstKT7	EstKT9
None	100	100	100
$MnCl_2$	27	65	92
$MgCl_2$	81	75	96
$CaCl_2$	83	68	90
$CuCl_2$	68	25	77
$ZnSO_4$	38	22	26
$FeSO_4$	93	60	109
$CoSO_4$	57	115	78
$NiSO_4$	43	0	86
PMSF	0	1	6
EDTA	71	64	64

Table 2 Effect of various detergents on the enzyme activity of EstKT4, EstKT7, and EstKT9

Detergent (1%)	Relative activity (%)		
	EstKT4	EstKT7	EstKT9
None	100	100	100
Tween-20	120	14	74
Tween-40	111	28	56
Tween-60	98	28	57
Tween-80	97	30	43
Triton X-100	85	6	20
SDS	0	0	0

(Fig. 3), implicating that they are esterases. The *p*-nitrophenyl valerate was used for further work since the catalytic activities of EstKT4, EstKT7, and EstKT9 were the highest towards the substrate. The *p*-nitrophenyl valerate was used for further work since the catalytic activities of EstKT4, EstKT7, and EstKT9 were highest towards the substrate. The K_M values of EstKT4, EstKT7, and EstKT9 were determined to be 71.3, 85.1, and 60.8 μM, respectively, and the k_{cat} values of those were determined to be 141.4, 0.2, and 272.2/s, respectively. The K_M values were determined to be within a similar range; however, the k_{cat} value of EstKT7 was approximately 3 orders of magnitude lower than those of EstKT4 and EstKT9. When compared with an *E. coli* esterase, a member of family IV, with the K_M value of 260 μM and the k_{cat} value of 20.5/s, EstKT4 and EstKT9 showed higher hydrolytic activities toward *p*-nitrophenyl valerate (Kanaya et al. 1998).

The optimum activities of EstKT4, EstKT7, and EstKT9 were monitored at a temperature range of 5°C to 65°C and at a pH range of 4.0–10.0 using *p*-nitrophenyl valerate as substrate. The optimum activities of enzymes occurred at mesophilic temperature ranges (40°C for EstKT4, 35°C for EstKT7, and 45°C for EstKT9) (Fig. 4a–c) and at slightly alkaline pH (8.0 for EstKT7 and 8.5 for EstKT4 and EstKT9) (Fig. 4d–f). It is worthy to note that three enzymes showed different activity patterns at low temperature ranges. Whereas EstKT4 and EstKT9 could sustain over 60% and 40% of maximum activities, respectively, EstKT7 showed rapid decrease of activities and even lower than 40% below 15°C.

The effects of various chemicals on the enzyme activity were evaluated by measuring residual activity after incubation. Metal ions differently affected enzyme activity of EstKT4, EstKT7, and EstKT9, that is, Zn^{2+} decreased the activity of all three enzymes by more than 50%, Ni^{2+} for EstKT4 and EstKT7, Mn^{2+} for EstKT4, and Cu^{2+} for EstKT7. All three enzymes were inhibited by PMSF, confirming they are serine esterases as predicted by the presence of Ser as a catalytic residue in Fig. 2. The enzyme inhibition caused by metal ions and EDTA suggests that metal ions may be required for optimum esterase activity (Table 1). Whereas EstKT7 and EstKT9 were significantly inhibited by non-ionic and ionic detergents, EstKT4 was positively affected by Tween-20 and Tween-40 and was very slightly inhibited by other non-ionic detergents as shown in Table 2.

Since the enzymes were prepared from marine resources enzyme, the effect of salinity (NaCl and KCl) on the enzyme activity was investigated by measuring the residual activity after incubation in solution containing 0.5 to 4 M NaCl or KCl for 30 min. As shown in Fig. 5, both NaCl and KCl increased the activity of three enzymes within a concentration of 0 to 1.0 M. In particular, EstKT7 activity was increased 1.7- and 1.3-fold in the presence of 0.5 M concentration of NaCl and KCl, respectively. Furthermore, all three enzymes retained more than 50% of their initial activities by the addition of two salts up to 3 M, indicating their good tolerance to high salinity.

Discussion

In this study, we present that three esterases, which were isolated from a tidal flat metagenomic library, are grouped together within family IV, forming a new subfamily

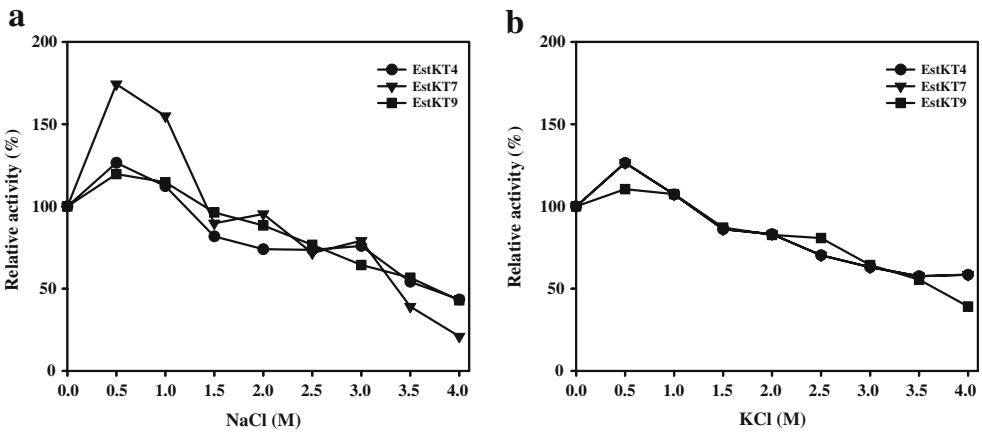

Fig. 5 Effects of NaCl (**a**) and KCl (**b**) on the esterase activity of EstKT4, EstKT7, and EstKT9. The enzyme activity of EstKT4, EstKT7, and EstKT9 was determined at each optimum temperature and pH value using *p*-nitrophenyl valerate as a substrate. The value obtained in the absence of salts was taken as 100%

separately from two other subfamilies. It is intriguing that all the genes in the new subfamily of family IV (included in this study) originated from marine environments (Fig. 1). For instance, four genes originated from metagenomic libraries derived from tidal flat sediment (GenBank accession no. ACB11219), neritic sediment (ACZ16565), and deep-sea sediment (ACL67837, ADA70028) (Hu et al. 2010), and two genes were identified from genome sequences of marine bacteria, *gamma proteobacterium* HTCC2143 (ZP_01615954), and *Vibrio splendidus* LGP32 (YP_002394831). The lipolytic enzyme (ACL67837), which originated from deep-sea sediment at a water depth of 778.5 m, showed optimum temperature for hydrolysis of *p*-nitrophenol butyrate at 45°C, similar to those of EstKT4, EstKT7, and EstKT9 (Hu et al. 2010); however, it is not clear whether the mesophilic temperature range optimal for the enzymes could define the subfamily at this moment.

It has been known that halophilic proteins maintained their activity and stability in the presence of the high concentration of salts and possessed high fraction of negatively charged residues, which are assigned to locate on the surface of proteins (Zaccai and Eisenberg 1990; Madern et al. 2000). The halophilic esterase, recently identified from *Haloarcula marismortui* ATCC 43049, possesses 16.8% of negatively charged amino acids (Rao et al. 2009). EstKT4, EstKT7, and EstKT9 esterases showed salt tolerance with over 50% residual activity in the presence of 3 M NaCl (or KCl). The primary sequence analysis revealed that the enzymes contain 11–13.4% of negatively charged amino acids, and the residues are conserved among the members belonging to the new subfamily (Fig. 2). The modeling of the 3-D structure of EstKT4, EstKT7, and EstKT9, performed by an automated homology modeling program (SWISS-MODEL) (Arnold et al. 2006; Kiefer et al. 2009), showed that the conserved negatively charged amino acids are situated on the surface of the proteins (data not shown).

In conclusion, a metagenomic library constructed using tidal flat sediment DNA was screened for lipolytic activity, and three novel genes were identified to have a motif unique to the new subfamily of family IV of bacterial lipolytic enzymes. All of them showed esterase activity toward water-soluble esters with short-chain carboxylic acids and exhibited distinct biochemical features such as salt tolerance and optimum temperature in the mesophilic range. This study expands the diversity of lipolytic enzyme-encoding genes and demonstrates that marine tidal flat sediments are a good source for screening novel lipolytic enzymes, especially those from metagenomes. Further studies are required to investigate biotechnological usefulness of the enzymes as biocatalysts by examining the enzyme activity and stability in non-aqueous solvent and enantioselectivity toward substrates.

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