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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Molecular cloning and characterization of a new cold-active esterase from a deep-sea metagenomic library

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Abstract A clone which conferred lipolytic activity at low temperature was identified from a fosmid library constructed from a South China Sea marine sediment sample. The gene responsible, *estF*, consisted of 1,080 bp that encoded 359 amino acid residues, with a typical N-terminal signal peptide of 28 amino acid residues. A phylogenetic analysis of amino acid sequence with other lipolytic enzymes revealed that EstF and seven closely related putative lipolytic enzymes comprised a unique clade in the phylogenetic tree. Moreover, these hypothetic esterases showed unique conservative sites in the amino acid sequence. The recombinant EstF was overexpressed and purified, and its biochemical properties were partially characterized. The optimal substrate for EstF to hydrolyze among a panel of *p*-nitrophenyl esters (C2 to C16) was *p*-nitrophenyl butyrate (C4), with a K_m of 0.46 mM. Activity quickly decreased with substrates containing an acyl chain length longer than 10 carbons. We found that EstF was active in the temperature range of 0–60°C, showed the best activity at 50°C, but was unstable at 60°C. It exhibited a high level of activity in the pH range of 7.0–10.0 showing the highest activity at pH 9.0.

Keywords Marine sediment · Esterase · Metagenome · Cold active

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Introduction

Many organisms produce a variety of lipolytic enzymes required for lipid metabolism. The majority of lipolytic enzymes found in bacteria are carboxylesterases (EC 3.1.1.1) and true lipases (EC 3.1.1.3) that are classified into eight families (I to VIII) (Arpigny and Jaeger 1999). True lipases hydrolyze ester bonds of water-insoluble lipid substrates which have long acyl chains (>10 carbon atoms), while esterases display preferred hydrolytic activity against ester bonds in small ester containing molecules (<10 carbon atoms) (Verger 1997). Most bacterial lipolytic enzymes belong to the α/β-hydrolases structural superfamily and

share a highly conserved catalytic Ser-Asp-His triad (Arpigny and Jaeger 1999). In recent years, several novel bacterial lipolytic enzymes such as LipG (Lee et al. 2006), EstA (Chu et al. 2008), and LipEH166 (Kim et al. 2009) have been identified and proposed as new families, respectively.

Owing to their useful and unique catalytic properties in industrial applications, there has been considerable effort invested in discovering more novel lipolytic enzymes. For example, lipases and esterases potentially catalyze regio- and stereo-selective hydrolysis reactions and can exhibit organic solvent-stable catalytic activities (Jensen and Hamosh 1996; Klibanov 2001; Lang and Dijkstra 1998). Furthermore, microbial enzymes proved especially useful during the outbreak of bovine spongiform encephalopathy when certain animal-derived products, such as pig liver esterase, were banned for pharmaceutical use. However, as it has been estimated that more than 99% of microorganisms cannot be cultured (Schloss and Handelsman 2003), and the full potential of this vast gene pool is still under-explored. This was highlighted in studies by the BASF (Ludwigshafen, Germany) and Chirotech (Cambridge, UK) in the exploration of nitrilase and γ-lactamase producing microorganisms. Despite access to a large collection of microbial species and efficient de-replicated methods, molecular ecological studies revealed that coverage of the diversity was still extremely low, less than 1% (Lorenz et al. 2002). Metagenomics, a culture-independent approach, may be an appropriate solution to circumvent the problem of utilizing untapped gene resources from uncultured microorganisms (Voget et al. 2003). Using this method, many novel biocatalysts have been screened for and identified, such as chitinase (LeCleir et al. 2007), protease (Waschkowitz et al. 2009), oxidase (Ye et al. 2010), oxygenase (Singh et al. 2010), amylase (Sharma et al. 2010), xylanase (Hu et al. 2008), lipase/esterase (Rhee et al. 2005), lysine racemase (Chen et al. 2009), and polyketide synthase (Chung et al. 2008).

Despite the fact that humans have landed on the moon and begun to explore outer space, we still know very little about the microbial life inhabiting the oceans in our own planet. The environments within the oceans, especially the deep sea, are unique and extremely diverse. These extreme environmental conditions differ from terrestrial habitats with respect to salinity, pressure, oxygen distribution, and temperature, thus resulting in unique biodiversity. The accordant novelties in the biocatalyst gene pool, for habitat adapted metabolic flux, are waiting to be exploited (Steele et al. 2009; Streit et al. 2004; Streit and Schmitz 2004). In this study, we explored a metagenomic library previously constructed from a South China Sea sediment sample, for novel lipases/esterases. Fifteen lipases/esterases have already been identified with this approach (Hu et al. 2010a). In this study, we investigate another esterase, EstF, which probably comprises a new family with six putative esterases previously identified in bacterial genomes and uncultured bacterium sequences. Subsequent subcloning and overexpression in *Escherichia coli* enabled us to partially characterize the biochemical properties of the purified recombinant esterase.

Materials and methods

Screening of esterase gene

A metagenomic fosmid library, previously constructed from a South China Sea sediment core, was employed to screen for clones exhibiting lipolytic activity using the tributyrin agar diffusion assay (Hu et al. 2010a, b). The clones were spread on Luria–Bertani (LB) agar medium supplemented with 1% tributyrin, 1% arabic gum, and 12.5 μg/ml chloramphenicol (Chung et al. 1991). After an overnight incubation at 37°C and then 24–48 h at 30°C, clones with lipolytic activity were identified by degradation of the adjacent substrate forming a clear halo zone around the clone (Lee et al. 2004).

Subcloning and sequence analysis of the esterase gene

Positive clones were cultured at 37°C in LB media supplemented with 12.5 μg/ml chloramphenicol and auto-induction buffer (Epicentre Technologies, Madison, WI, USA). The fosmid was extracted and purified using Omega Plasmid Mini Kit II (Doraville, GA, USA) and partially digested with the restriction enzyme *Sau*3AI. The 2–3-kb digested DNA fragments were subcloned in pUC18 digested with *Bam*HI and introduced into *E. coli* DH5α. The transformants were again subjected to the tributyrin agar diffusion assay to determine lipolytic activity. The desired clone was picked out and the plasmid it carried was extracted and sequenced at Beijing Genomics Institute with forward and reverse universal primers. The sequence was analyzed by SignalP 3.0 server (Nielsen et al. 1997) and AnthePro software (version 5.0) to identify the signal peptide. The sequence similarity comparison was performed with the BLAST 2.2 program (Altschul et al. 1997). Alignments of the amino acid sequences of EstF and homologous proteins were performed with the AlignX module of the Vector NTI suite (InforMax, North Bethesda, MD, USA) using the blosum scoring matrix (Henikoff and Henikoff 1992). The phylogenetic tree was constructed using the neighbor-joining method with 1,000 bootstrap replicates in Molecular Evolutionary Genetics Analysis software (MEGA version 4.1) (Kumar et al. 2008).

Gene cloning and expression

Oligonucleotides EstF-f (5′-GTCCATATGCAAGAGCTTCTTCCGGT-3′) and EstF-r (5′-CATGGATCCTTTGAGGTGCTTGTC-3′) were designed, with restriction enzyme sites for *Nde*I and *Bam*HI, respectively (underlined). This *estF* gene was amplified by PCR, minus the sequence encoding its signal peptide, using the plasmids from a subclone transformant carrying the *estF* gene as template. The PCR products were digested with *Nde*I and *Bam*HI and then ligated into the expression vector pET28b treated with *Nde*I and *Bam*HI, resulting in the plasmid pET28b::*estF*. For overexpression of the recombinant EstF protein in *E. coli*, a freshly streaked kanamycin selection plate of *E. coli* BL21 (DE3) harboring the plasmid pET28b::*estF* was prepared after overnight incubation at 37°C. A single colony was picked and inoculated into 5 ml LB medium for another overnight incubation at 37°C with 200 rpm of agitation. The full 5-ml overnight culture was inoculated into 500 ml LB medium supplemented with 50 μg/ml kanamycin and incubated under the same conditions. When the optical density at 600 nm reached 0.6, and isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 0.5 mM. After an additional 4-h incubation, the cell mass was harvested by centrifugation at 6,000×*g* for 30 min and washed twice with phosphate-buffered saline (pH 7.4).

Protein purification

The washed cell mass was resuspended in 100 ml binding buffer (20 mM sodium dihydrogen phosphate, 500 mM sodium chloride, 20 mM imidazole, pH 7.4). The cells were then sonicated on ice and the cell lysate was collected after centrifugation at 20,000×*g* for 30 min. The supernatant was filtered with a Millipore 0.22-μm filter to remove small particles and applied to a HisTrap FF affinity column (GE Healthcare) for purification according to the manufacturer's instructions. The protein concentration was measured using the Bradford protein assay (Bradford 1976).

Enzyme assay

To measure enzyme activity, *p*-nitrophenyl esters were employed as substrates. The amount of *p*-nitrophenol released by esterase-catalyzed hydrolysis was used to determine the catalytic activity. Unless otherwise described, esterase activity was measured at 45°C for 5 min with 1 mM *p*-nitrophenyl butyrate as a substrate and by monitoring the amount of liberated *p*-nitrophenol at 410 nm using a spectrometric method. The standard reaction conditions for testing esterase activity contained 10 μl of 100 mM substrate in acetonitrile and 1 μg of esterase made up to 1 ml using Tris–HCl (50 mM, pH 8.0) buffer. Blank reactions (no enzyme added) were performed for each reaction to allow the background absorbance caused by nonenzymatic substrate hydrolysis to be subtracted. All measurements were performed at least in triplicate. One unit (U) of enzyme activity was defined as the amount of activity required to release 1 μM of *p*-nitrophenol/min from *p*-nitrophenyl ester under the assay conditions.

Characterization of EstF

Substrate specificity towards *p*-nitrophenyl esters with different chain lengths was determined at 45°C and pH 8.0. The substrates tested were *p*-nitrophenyl acetate (C2) (Alfar Aesar), *p*-nitrophenyl butyrate (C4) (Sigma), *p*-nitrophenyl caproate (C6) (TCI, Japan), *p*-nitrophenyl octanoate (C8) (Sigma), *p*-nitrophenyl caprate (C10) (Sigma), *p*-nitrophenyl laurate (C12) (Alfar Aesar), *p*-nitrophenyl myristate (C14) (Sigma), and *p*-nitrophenyl palmitate (C16) (Sigma). All *p*-nitrophenyl esters were dissolved in acetonitrile to a final concentration of 100 mM. Initial reaction velocities measured at various concentrations (0.1–10 mM) of the substrates were fitted to the Lineweaver–Burk transformation of the Michaelis–Menten equation allowing the calculation of K_m.

The optimum pH for esterase activity was determined by measuring the *p*-nitrophenol released by the enzyme hydrolysis in buffers with different pH. A series of buffers with pH ranging from 5.0 to 11.0 were tested for 20 min at 45°C. The buffers used were 50 mM citrate buffer (pH 5.0 to 6.0), 50 mM sodium phosphate (pH 6.0 to 8.0), 50 mM Tris–HCl (pH 8.0 to 9.0), and 50 mM sodium hydrogen carbonate (pH 9.0 to 11.0).

The optimum temperature for esterase activity was determined by measuring the *p*-nitrophenol released by the enzyme hydrolysis at various temperatures (0–65°C) under standard assay conditions. The thermostability was determined by measuring the residual activity of purified enzyme towards the *p*-nitrophenyl butyrate every 10 min during a 1-h incubation in Tris–HCl buffer (50 mM, pH 8.0) at 40°C, 50°C, and 60°C.

The effects of various additives on esterase activity were tested. The effects of metal ions ($CaCl_2$, $FeCl_2$, $NiCl_2$, $CoCl_2$, $ZnCl_2$, $MgCl_2$, $CuCl_2$, and $MnCl_2$) on the esterase activity were determined at a final concentration of 5 mM. The effects of detergents, sodium dodecyl sulfate (SDS), Tween 20, Tween 40, Tween 80, Triton X-100, Nonidet P-40 (NP-40), and hexadecyl trimethyl ammonium bromide (CTAB), on the esterase activity were determined at a concentration range of 0.2–5%. The effects of inhibitors, phenylmethanesulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid

(EGTA), 2-mercaptoethanol (2-ME), and dithiothreitol (DTT), on the esterase activity were determined at a final concentration of 5 mM. The effects of organic solvents, DMSO and DMF (at a concentration range of 1%, 5%, and 10%), methanol, ethanol, and acetone (at a concentration range of 10%, 20%, and 40%) on the esterase activity were determined. All the experiments were performed under the standard assay conditions. The activity of esterase measured under the same conditions without additives was defined as 100%.

Nucleotide sequence accession number

The nucleotide sequence reported in this study has been deposited in GenBank, under accession number GU226858.

Results

Screening and subcloning

Hydrolytic activity-based screening of the fosmid library was performed on LB agar supplemented with tributyrin. One positive clone, carrying the *estF* gene and expressing lipolytic activity, was isolated. In order to identify the gene responsible for the hydrolytic activity, this clone containing a 36-kb insert fragment was subjected to further investigation. A subclone library containing >10^3 clones with insert fragments of 2~3 kb was produced and positive clones were picked out. The sequencing of the plasmids extracted from the positive subclones produced the esterase gene.

Fig. 2 Phylogenetic tree of EstF and related proteins. The phylogenetic tree was constructed by the neighbor-joining method with 1,000 bootstrap replicates (values >50% were shown at the nodes). The closely related protein sequences and sequences from other families retrieved from GenBank were aligned using ClustalW. *Scale bar*, 0.05 change per nucleotide position

Sequence analysis of EstF

A deduced signal peptide region of 28 amino acids was found at the N-terminal region of one 1,080-bp ORF. Removal of this signal peptide yielded a putative esterase/lipase (EstF) of 331 amino acids, with deduced molecular mass of 35,928 Da and isoelectric point (p*I*) of 5.02. The nucleotide and amino acid sequences of this esterase were novel (Fig. 1). Seven related potential lipases/esterases have been deposited in GenBank from genome sequencing projects or metagenomic libraries, but none have been characterized. Most of these potential isozymes belong to Gammaproteobacteria, which may indicate the origin of EstF.

Based on the amino acid sequence comparisons and phylogenetic analysis, we propose that EstF and its related isozymes may comprise a new family of lipolytic enzymes. In the phylogenetic analysis of EstF and the seven closely related putative lipolytic enzymes, a distinctive clade was observed that did not group with any of the known lipase families (Arpigny and Jaeger 1999; Chu et al. 2008; Kim et al. 2009; Lee et al. 2006). Family IV lipases are the most related clade with EstF (Fig. 2). The classical pentapeptide (GDSAG) and the dipeptide (HG) motifs present in family IV lipases play a critical role in the formation of the

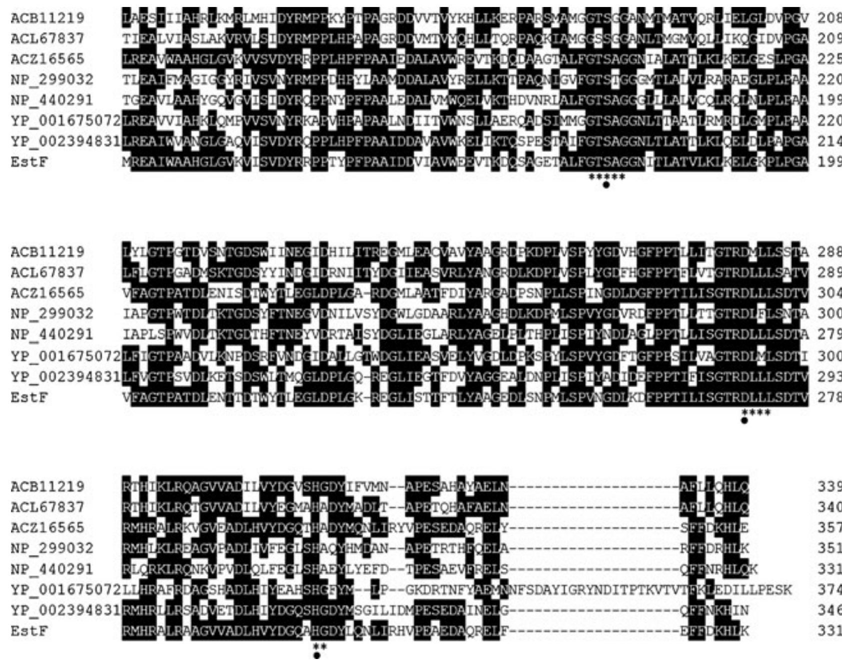
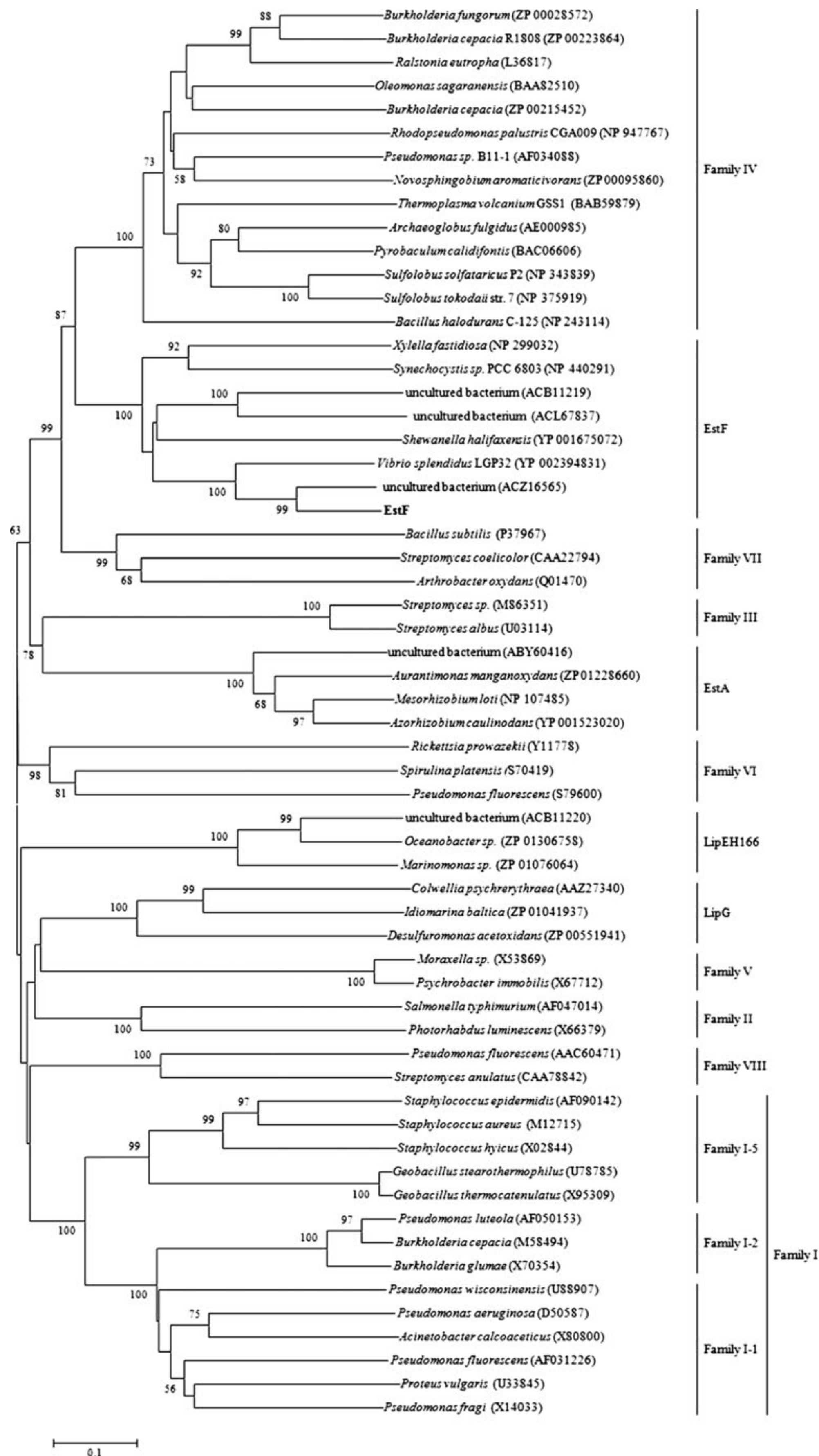


Fig. 1 Amino acid sequence alignment of esterase EstF with the most closely related proteins in GenBank: esterase from tidal flat sediments (ACB11219), esterase from South China Sea marine sediment (ACL67837), putative esterase from neritic sediment (ACZ16565), putative esterase from *Xylella fastidiosa* 9a5c (NP_299032), putative esterase from *Synechocystis* sp. PCC 6803 (NP_440291), putative esterase from *Shewanella halifaxensis* HAW-EB4 (YP_001675072), and putative esterase from *Vibrio splendidus* LGP32 (YP_002394831). Identical residues have a black background and the conserved motifs –GTSXG–, –DLLL–, and –HG– are indicated by *asterisks*. Those amino acids forming the catalytic triad are indicated by *filled circles*


Burkholderia fungorum (ZP 00028572)
Burkholderia cepacia R1808 (ZP 00223864)
Ralstonia eutropha (L36817)
Oleomonas sagaranensis (BAA82510)
Burkholderia cepacia (ZP 00215452)
Rhodopseudomonas palustris CGA009 (NP 947767)
Pseudomonas sp. B11-1 (AF034088)
Novosphingobium aromaticivorans (ZP 00095860)
Thermoplasma volcanium GSS1 (BAB59879)
Archaeoglobus fulgidus (AE000985)
Pyrobaculum calidifontis (BAC06606)
Sulfolobus solfataricus P2 (NP 343839)
Sulfolobus tokodaii str. 7 (NP 375919)
Bacillus halodurans C-125 (NP 243114)
Family IV
Xylella fastidiosa (NP 299032)
Synechocystis sp. PCC 6803 (NP 440291)
uncultured bacterium (ACB11219)
uncultured bacterium (ACL67837)
Shewanella halifaxensis (YP 001675072)
Vibrio splendidus LGP32 (YP 002394831)
uncultured bacterium (ACZ16565)
EstF
EstF
Bacillus subtilis (P37967)
Streptomyces coelicolor (CAA22794)
Arthrobacter oxydans (Q01470)
Family VII
Streptomyces sp. (M86351)
Streptomyces albus (U03114)
Family III
uncultured bacterium (ABY60416)
Aurantimonas manganoxydans (ZP 01228660)
Mesorhizobium loti (NP 107485)
Azorhizobium caulinodans (YP 001523020)
EstA
Rickettsia prowazekii (Y11778)
Spirulina platensis (S70419)
Pseudomonas fluorescens (S79600)
Family VI
uncultured bacterium (ACB11220)
Oceanobacter sp. (ZP 01306758)
Marinomonas sp. (ZP 01076064)
LipEH166
Colwellia psychrerythraea (AAZ27340)
Idiomarina baltica (ZP 01041937)
Desulfuromonas acetoxidans (ZP 00551941)
LipG
Moraxella sp. (X53869)
Psychrobacter immobilis (X67712)
Family V
Salmonella typhimurium (AF047014)
Photorhabdus luminescens (X66379)
Family II
Pseudomonas fluorescens (AAC60471)
Streptomyces anulatus (CAA78842)
Family VIII
Staphylococcus epidermidis (AF090142)
Staphylococcus aureus (M12715)
Staphylococcus hyicus (X02844)
Geobacillus stearothermophilus (U78785)
Geobacillus thermocatenulatus (X95309)
Family I-5
Pseudomonas luteola (AF050153)
Burkholderia cepacia (M58494)
Burkholderia glumae (X70354)
Family I-2
Pseudomonas wisconsinensis (U88907)
Pseudomonas aeruginosa (D50587)
Acinetobacter calcoaceticus (X80800)
Pseudomonas fluorescens (AF031226)
Proteus vulgaris (U33845)
Pseudomonas fragi (X14033)
Family I-1
Family I
0.1

oxyanion hole which is important for catalytic activity (Arpigny and Jaeger 1999; Langin and Holm 1993; Wei et al. 1999). However, in EstF, the classical pentapeptide motif is altered to –GTSXG– (174–178). The –HG– dipeptide is present in both EstF and family IV, but differs in the flanking sequences. Moreover, the two C-terminus conserved motifs, –DPLR– and –HGF–, are changed to –DLLL– (271–274) and –SHG/A– (300–302), respectively, in EstF. EstF and related sequences also contain a conserved FPPXXLITGTRDLLLS (260–275) sequence, overlapping with the –DLLL– region (Fig. 1). Generally, EstF and related sequences are conservative and have unique conserved sequence motifs. Therefore, these data indicate that they comprise a new family of bacterial lipase/esterase.

Overexpression and purification of EstF

To characterize the biochemical properties of EstF, the gene sequence encoding the mature form of EstF, without the signal peptide, was amplified and cloned into pET28b. The resulting expression plasmid pET28b::*estF* contained six histidine tags at both the C-terminus and N-terminus of EstF, thereby enabling rapid isolation of enzymatically active protein. The dual histidine tags facilitated stringent binding and washing conditions during chromatography resulting in enzyme preparations that were >95% pure, as identified by SDS-PAGE (Fig. 3). Here, a single species was observed with a molecular weight corresponding to calculated size of 38 kDa.

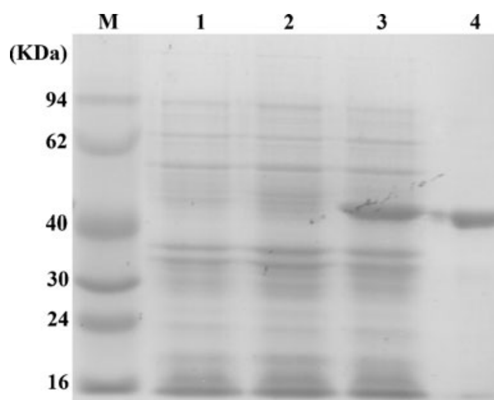


Fig. 3 Expression and purification of recombinant EstF. Proteins from various steps during the expression and purification were separated by 12.5% SDS-PAGE gel. The gel was stained by the Coomassie brilliant blue. *Lane M*, protein molecular weight marker; *lane 1*, total lysate of *E. coli* BL21 (DE3) transformed with an empty pET28b vector; *lane 2*, total lysate of *E. coli* BL21 (DE3) transformed with pET28b::*estF*; *lane 3*, total lysate of *E. coli* BL21 (DE3) transformed with pET28b::*estF* induced by IPTG; *lane 4*, purified EstF from Ni-NTA column

Biochemical properties of EstF

The substrate specificity of the esterase was tested utilizing *p*-nitrophenyl ester with acyl chains of different lengths. The results showed that EstF had a strong preference for the hydrolysis of the ester bonds of short acyl chain esters, such as *p*-nitrophenyl acetate (C2) and *p*-nitrophenyl butyrate (C4). The *p*-nitrophenyl butyrate was the optimal substrate with K_m=0.46 mM and k_{cat}=1,200 s^{-1}, so the k_{cat}/K_m was approximately 2,600 s^{-1} mM^{-1}. Moreover, EstF exhibited a very low level of hydrolytic activity against *p*-nitrophenyl esters with long acyl chains (C>10) (Fig. 4a). The preference of the substrate with short acyl chain (C<10) of EstF indicates that EstF is a "true" esterase but not a lipase (Arpigny and Jaeger 1999).

The optimum temperature for EstF was 50°C. EstF still exhibited good activity against *p*-nitrophenyl butyrate at very low temperatures (0°C, 5°C, and 10°C). However, the activity of EstF reduced quickly above 60°C (Fig. 4b). Thermostability analysis showed that EstF was unstable and lost activity in less than 10 min at 60°C. Although EstF was most active after 5 min at 50°C, the enzyme lost over 30% of its activity after half an hour of incubation at the same temperature. The EstF maintained hydrolytic activity at 40°C even after 1 h of incubation (Fig. 4c).

The activity of EstF was also tested at various pH values (5–11). The optimum was found to be pH 9 at 45°C using *p*-nitrophenyl butyrate as substrate. In the range of pH 7–10, the esterase showed >80% hydrolytic activity of its own maximum (Fig. 4d).

Various additives were tested for interference against EstF activity. In the case of metal ions, most had no significant influence on the hydrolytic activity of EstF under standard assay conditions. The exceptions were 5 mM Cu^{2+} and Zn^{2+} that inhibited 60% and 70% of the activity, respectively, after a 5-min reaction time. As observed with other esterases in the family, PMSF at a concentration of 5 mM inhibited 90% of the enzymatic activity, suggesting that EstF catalyses a reaction conserved with serine proteases. EGTA and 2-ME at a concentration of 5 mM inhibited 40% and 60% of the activity (Table 1). Consistent with the observation that the mature enzyme contained no cysteine residues required for disulfide bond formation, DTT displayed no effect on EstF activity. The ionic surfactants SDS and CTAB strongly inhibited enzymatic activity of EstF even at low concentration of 0.2%, whereas higher concentrations of nonionic surfactants NP-40, Tween 20, 40, and 80, and Triton X-100 were required to reduce EstF activity by >50% (Table 2). Organic solvents DMSO and methanol enhanced enzymatic activity at concentrations below 10% and 20%, respectively, whereas ethanol, acetone, and DMF were all inhibitory at 10% final concentration (Table 3).

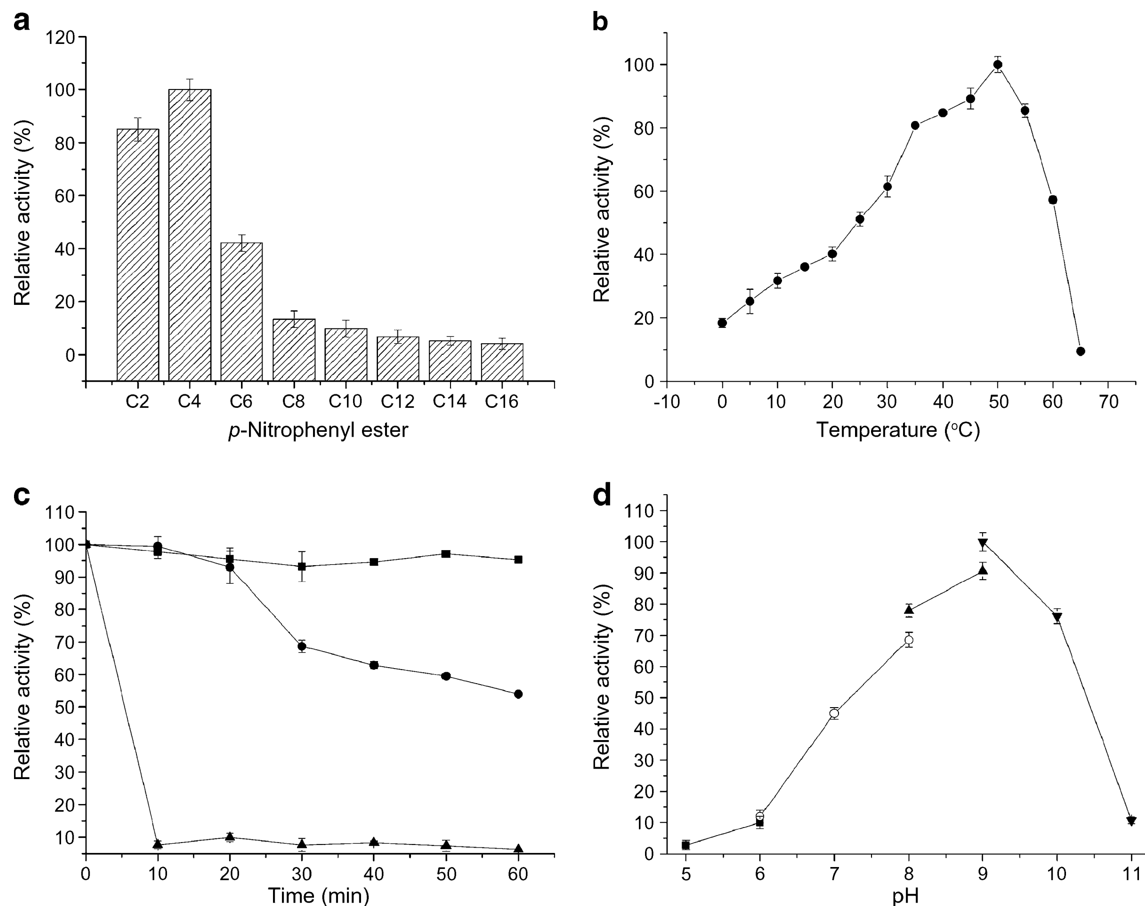


Fig. 4 Biochemical properties of recombinant EstF. **a** Determination of the substrate specificity of purified recombinant EstF. The *p*-nitrophenyl acetate (C2), *p*-nitrophenyl butyrate (C4), *p*-nitrophenyl caproate (C6), *p*-nitrophenyl octanoate (C8), *p*-nitrophenyl caprate (C10), *p*-nitrophenyl laurate (C12), *p*-nitrophenyl myristate (C14), and *p*-nitrophenyl palmitate (C16) were taken as substrates. **b** Optimum temperature of recombinant EstF. The *p*-nitrophenyl butyrate (C4) was selected as substrate. The relative activity of EstF was measured after 5 min pre-incubation at different temperatures from 0°C to 65°C with intervals of 5°C. **c** Thermostability of recombinant EstF. Residual specific activity of the esterase EstF after incubation at different temperatures: 40°C (*filled square*), 50°C (*filled circle*), and 60°C (*filled triangle*). **d** Optimum pH of recombinant EstF. The *p*-nitrophenyl butyrate (C4) was selected as substrate. The relative activity of EstF was measured at different pH. The buffers used were: citrate buffer (*filled square*) (pH 5.0 to 6.0), sodium phosphate (*empty circle*) (pH 6.0 to 8.0), Tris–HCl (*filled triangle*) (pH 8.0 to 9.0), and sodium hydrogen carbonate (*filled inverted triangle*) (pH 9.0 to 11.0)

Discussion

While microorganisms in various environments are a treasure trove of novel genes and enzymes, most of these microbes and their products were not be available to us until the metagenomic approach emerged (Steele et al. 2009; Streit et al. 2004; Streit and Schmitz 2004). Using these approaches, microbes with unique physicochemical

Table 1 Effects of metal ions and inhibitors on the lipolytic activity of EstF

Control	Relative activity (%)	Cation (5 mM)	Relative activity (%)	Inhibitor (5 mM)	Relative activity (%)
	100.0	Fe^{2+}	90±4	PMSF	9±1
		Ca^{2+}	103±4	EDTA	79±5
		Zn^{2+}	32±8	EGTA	56±3
		Ni^{2+}	88±4	2-ME	37±2
		Mg^{2+}	56±3	DTT	100±3
		Cu^{2+}	37±5		
		Mn^{2+}	104±2		
		Co^{2+}	66±3		

Table 2 Effects of detergents on the lipolytic activity of EstF

Detergents	Relative activity (%) at concentration (*w*/*v*) of (%):		
	0.2	1	5
Ionic			
CTAB	3±0	1±0	0
SDS	1±0	0	0
Nonionic			
NP 40	63±7	54±2	16±2
Tween 20	105±3	82±2	27±2
Tween 40	174±2	60±2	23±3
Tween 80	216±5	54±4	13±6
Triton X-100	57±2	80±8	19±4

properties from a variety of environments, such as the deep sea, are receiving increasing interest (Blunt et al. 2010; Debashish et al. 2005). The ocean environments are rich in various lipids since great amounts of living things are found therein (Harvey 1989). It is worth considering that lipids from phytoplankton are understood to be one of the major sources of nutrition in marine food chains (Berge and Barnathan 2005). These lipids from the surface layers are removed by microbial activity both during the fall through the water column and when resting on the benthos (Kiriakoulakis et al. 2001). Lipids are utilized by marine bacteria in determining the biophysical properties of bacterial membranes, which are of great importance under environmental stresses of extreme temperature and pressure (Russell and Nichols 1999). Hence, marine habitats are really vast riches for novel gene resources of lipolytic enzymes because of the numerous microorganisms residing there. Many novel lipolytic enzymes have already been isolated from marine environments as described, and we ourselves have isolated many novel esterases from a metagenomic library from the South China Sea (Hu et al. 2010a).

EstF, which was recombinant cloned, expressed, and purified, was subjected to molecular and biochemical experiments in this study. Based on the phylogenetic, molecular, and biochemical analysis results, we propose that EstF and its related hypothetic proteins from sequenced microbial genomic or metagenomic data could be classified as a novel lipolytic enzyme family. In the amino acid sequence of EstF, the conserved catalytic triad (Ser-Asp-His) of α/β-hydrolases is still the same. However, the classical pentapeptide (containing serine residue of catalytic triad) and two C-terminus conserved motifs (containing aspartic acid residue and histidine residue of catalytic triad respectively) are all different from the adjacent clade, namely, family IV according to the phylogenetic analysis. The flanking conserved sequences of these three main conserved motifs and dipeptide (amino acids involved in oxyanion hole formation) are also different. These evidences from phylogenetic analysis suggested that EstF and its related sequences could form a novel family of lipase/esterase.

EstF shows unique characteristics not only in sequence, but also in biochemical properties. According to the results of biochemical experiments, EstF could exhibit relatively high catalytic activity under low temperature, which suggests cold-active property. Even at 0°C, EstF could maintain almost 20% relative activity. The low temperature of the deep-sea sampling site may contribute to this cold-active property. The highest relative activity under pH 9.0 and a quick loss of activity at pH<7 suggest that EstF is an alkaline esterase. It is noticeable that the activity of EstF against *p*-nitrophenyl butyrate (C4) could be enhanced in 10% and 20% methanol and low concentration of DMSO (<10%). These properties should be of great use when considering organic synthesis (Klibanov 2001). The chelating reagents (EDTA and EGTA) were tested against EstF, and the results showed that the lipolytic activity of EstF declined more in the presence of EGTA. It is suggested that Ca^{2+} is more important than Mg^{2+} for enzymatic activity maintenance of EstF since EGTA has a much higher affinity for Ca^{2+} than for Mg^{2+}. The investigation of divalent cation effects on EstF also proved this since only Ca^{2+} could maintain almost 100% relative enzymatic activity of EstF. The activity inhibition of EstF by PMSF demonstrates that serine is involved in the catalytic center, since PMSF could mimic the first transition state in ester bond hydrolysis presumably by linking to the hydroxyl

Table 3 Effects of organic solvents on the lipolytic activity of EstF

Organic solvent	Relative activity (%) at concentration (*w*/*v*) of (%):			Organic solvent	Relative activity (%) at concentration (*w*/*v*) of (%):		
	1	5	10		10	20	40
DMSO	163±9	111±7	102±8	Methanol	96±11	128±8	58±5
DMF	88±11	57±6	47±2	Ethanol	70±5	53±6	23±2
				Acetone	65±5	46±6	41±3

group of serine in the active site covalently (Das et al. 2000). The enzymatic activity of EstF is totally inhibited by ionic detergent and enhanced by a low concentration of Tween, and the probable reasons for these are ionic detergents such as SDS inhibit both inter- and intra-molecular protein–protein interaction and low concentrations of a nonionic detergent such as Tween could help the substrate interact with the catalytic triad.

In summary, functional screening for lipolytic enzymes from a metagenomic library is a remarkably effective way to exploit the great gene pool in marine environments. In this study, a novel alkaline esterase, EstF, was isolated from a deep-sea sediment sample from the South China Sea. It is proposed that EstF and its related sequences could be classified as a novel family of lipase/esterase. The stereoselectivity study of EstF and a structure elucidation through X-ray crystallography are in progress. In future work, finding some unique properties of this new family and protein structure in comparison with the known three-dimensional structures will be meaningful and significant.

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