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ORIGINAL PAPER

Identification and characterization of novel esterases from a deep-sea sediment metagenome

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Abstract A deep-sea sediment metagenomic library was constructed and screened for lipolytic enzymes by activity-based approach. Nine novel lipolytic enzymes were identified, and the amino acid sequences shared 56% to 84% identity to other lipolytic enzymes in the database. Phylogenetic analysis showed that these enzymes belonged to family IV lipolytic enzymes. One of the lipolytic enzymes, Est6, was successfully cloned and expressed in *Escherichia coli* Rosetta in a soluble form. The recombinant protein was purified by Ni-nitrilotriacetic affinity chromatography column and characterized using *p*-nitrophenyl esters with various chain lengths. The *est*6 gene consisted of 909 bp that encoded 302 amino acid residues. Est6 was most similar to a lipolytic enzyme from uncultured bacterium (ACL67845, 61% identity) isolated from the South China Sea marine sediment metagenome. The characterization of Est6 revealed that it was a cold-active esterase and exhibited the highest activity toward *p*-nitrophenyl butyrate (C4) at 20°C and pH 7.5.

Keywords Deep-sea sediment · Metagenomic library · Family IV lipolytic enzymes · Cold-active

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Introduction

Microorganisms play a surprisingly important role in life on earth. We depend on microbes in many ways than you can imagine. Understanding the microbe world could lead to practical applications in many areas, such as medicine, energy, agriculture, biotechnology, and the food industry. Yet, the majority of microbes on our planet have not been studied, largely because less than 1% of them can be cultivated by conventional methods (Amann et al. 1995; Ferrer et al. 2005). Fortunately, scientists have developed a culture-independent method to study unculturable microorganisms, called metagenomics. Metagenomics involves directly assessing the genomes of organisms in the environment (Handelsman et al. 1998). Thus, it is becoming one of the best approaches to mine novel enzymes from environment (Kennedy et al. 2008).

As we all know, approximately 71% of the earth's surface is covered by ocean, and the marine environment is extremely diverse, with microbes flourishing in cold polar and warm equatorial waters, in sunlit surface waters, in high-pressure deep-sea sediments, in hot acidic water near hydrothermal vents, and in association with various vertebrate and invertebrate animals (Kennedy et al. 2008). Therefore, the marine environment represents a vast pool of novel enzymes.

Lipolytic enzymes, including esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3), represent a group of hydrolases that catalyze the cleavage and formation of ester bonds. Many of them do not require cofactors and have wide substrate tolerance, high stereospecificity toward chemicals, and high stability in organic solvents (Bornscheuer 2001), which make lipolytic enzymes attractive biocatalysts for use in industry. Bacterial lipolytic enzymes have been classified into eight families (I–VIII) according to their

amino acid sequences (Arpigny and Jaeger 1999). Recently, using metagenomics, new families of lipolytic enzymes were identified, including LipG, from a Korean tidal flat sediments metagenomic library (Lee et al. 2006); EstA, from a South China Sea surface water metagenomic library (Chu et al. 2008); LipEH166, from an intertidal flat metagenomic library (Kim et al. 2009); EstD2, from a plant rhizosphere soil metagenomic library (Lee et al. 2010); EstGK1 and EstZ3, from a sheep rumen metagenomic library (Bayer et al. 2010), and EstF, from a South China Sea marine sediment metagenomic library (Fu et al. 2011).

In our study, we constructed a fosmid metagenomic library from deep-sea sediments and screened for lipolytic enzymes using an activity-based approach. We isolated 12 clones with lipolytic activity and obtained nine lipolytic encoding genes. We also reported the expression, purification, and characterization of one novel low-temperature-activated esterase that is a family IV lipolytic enzyme.

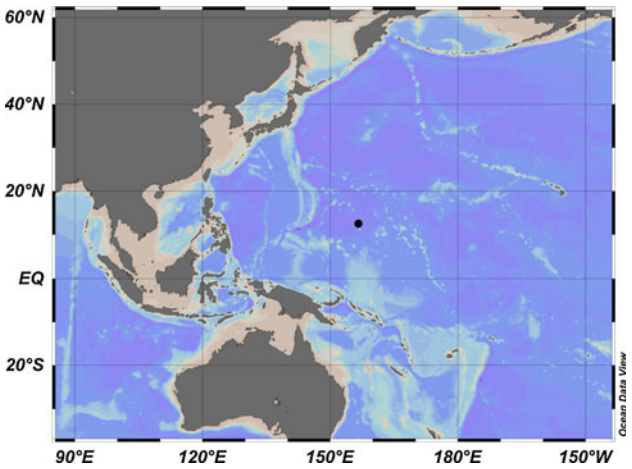

Fig. 1 Location of the deep-sea sediment sample used in the construction of the metagenomic library. The map was constructed with Ocean Data View software (http://odv.awi.de/). The sampling site is shown by a *black dot*

Materials and methods

Metagenomic library construction and screening for lipolytic positive clones

Deep-sea sediments were collected from the skirt of a seamount in the middle of the Pacific Ocean by using a multicorer during research vessel "DA YANG YI HAO" cruise DY115-20. The water depth of the sampling station was 5,886 m, and the temperature was 1.5°C (Fig. 1). DNA was extracted using a previously described method (Hu et al. 2010b). The metagenomic library was constructed using the CopyControl^{TM} HTP fosmid library production kit (Epicentre Technologies, Madison, WI, USA) according to the manufacturer's instructions, and the end-repaired to blunt, 5′-phosphorylated ends DNA with average length of 36 kb was ligated into the pCC2 FOS fosmid vector (Epicentre Technologies, Madison, WI, USA). Lambda-packaging extracts were added to ligates, and infection of a phage T1-resistant EPI300-T1^{R} *Escherichia coli* was performed according to the manufacturer's protocol. The resulting *E. coli* transformants were stored at −80°C. To screen for lipolytic clones, the fosmid clones were plated on Luria–Bertani (LB) agar medium supplemented with 1% tributyrin (Park et al. 2007) plus 12.5 μg/ml chloramphenicol and incubated at 30°C for 48 h. Clones with lipolytic activity were indicated by clear zones around the colonies.

Subcloning and sequence analysis

The fosmid DNA of lipolytic positive clones were induced to a high-copy-number using Copy-Control Fosmid Autoinduction Solution (Epicentre Technologies, Madison, WI, USA) and then extracted using the Plasmid Miniprep kit (Axygen, USA). The plasmids were partially digested with *Sau*3AI (Takara, Japan), and the 1.5- to 4.5-kb DNA fragments were ligated into a pUC19 vector (Novagen, Germany) that had been digested with *Bam*HI (Takara, Japan). The ligation products were transformed into *E. coli* DH5α (Novagen, Germany), and the transformants were spread onto LB agar medium supplemented with 100 μg/ml ampicillin and 1% tributyrin. The positive subclones were identified by clear zones around the colonies.

The insert sequences of the lipolytic positive subclones were determined by DNA sequencing. The open reading frames (ORFs) were identified via Open Reading Frame Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The amino acid sequences of the predicted proteins were compared with those of known lipolytic enzymes using BLASTp program (http://www.blast.ncbi.nlm.nih.gov/). The phylogenetic tree was constructed by neighbor-joining method (Saintou and Nei 1987) using Molecular Evolutionary Genetics Analysis 4.0 software (Tamura et al. 2007). Multiple alignments between amino acids sequences were performed using ClustalX program (Jeanmougin et al. 1998) and visually examined with BoxShade Server program (http://www.ch.embnet.org/software/BOX_form.html).

Overexpression and characterization of Est6

The *est*6 gene was amplified using fosmid Est6 as a template with the following primers: 5′-CGCACATATGGCC AGTCCACAGCTCC-3′ (*Nde*I site underlined) and 5′-AA TTAAGCTTCTAGCGTGCGGCGGCG-3′ (*Hin*dIII site underlined). The PCR product was digested by *Nde*I and *Hin*dIII (Takara, Japan) and cloned into a pET-28b (+)

expression vector (Novagen, Germany) that had been digested with the same enzymes. The recombinant plasmid was then transformed into *E. coli* Rosetta (DE3) (Novagen, Germany). When the OD_{600} reached 0.6, 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) was added, and after 7 h of cultivation at 25°C, the cells were harvested by centrifugation at 5,000×*g* for 5 min at 4°C. Then, the cells were resuspended in buffer A (500 mM NaCl, 10 mM imidazole, 20 mM Tris–HCl, pH 8.0) and disrupted by sonication. The cell debris was removed by centrifugation at 12,000×*g* for 20 min at 4°C. The resulting supernatant was purified on an Ni–NTA affinity chromatography column (Qiagen, Germany) according to the manufacturer's protocol. Denaturing discontinuous SDS–PAGE was conducted to determine the molecular weight of monomeric Est6. Native molecular weight was determined using a HPLC gel filtration column, Zorbax Bio-series GF-450 (9.4 × 250 mm; Agilent, USA). The protein was eluted with 100 mM sodium phosphate, pH 7.0, at a flow rate of 2 ml/min. The molecular mass of Est6 was compared with the retention time of lysozyme (14.3 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase from bovine erythrocytes (29.0 kDa), chicken ovalbumin (44.3 kDa), bovine serum albumin fraction V (66.4 kDa), and phosphorylase b from rabbit muscle (97.2 kDa). Protein concentration was determined by the method of Bradford (Bradford 1976), using bovine serum albumin as standard.

The standard reaction mixture contained 1 mM *p*-nitrophenyl butyrate (dissolved in acetonitrile), 100 mM Tris–HCl (pH 7.5) buffer, and 0.57 μg of purified enzyme in a final volume of 1 ml (Park et al. 2007). The activity of the enzyme was determined at 20°C, and the release of *p*-nitrophenol was measured at 405 nm using DU800 UV/Vis spectrophotometer (Beckman, USA). All values were determined in triplicate and corrected for autohydrolysis of the substrate.

The kinetic parameters (K_m and V_{max}) for Est6 were obtained using *p*-nitrophenyl butyrate as substrate at different concentrations ranging from 0.05 to 2 mM. The K_m and V_{max} values were determined by analyzing the slopes of the Michaelis–Menten equation using GraphPad Software (GraphPad Inc., USA).

To determine the substrate specificity, *p*-nitrophenyl esters with various chain lengths were added to the reaction mixture at a final concentration of 1 mM. The substrates used in the study were *p*-nitrophenyl acetate (C2) (Sigma), *p*-nitrophenyl butyrate (C4) (Sigma), *p*-nitrophenyl hexanoate (C6) (TCI, Japan), *p*-nitrophenyl octanoate (C8) (Sigma), *p*-nitrophenyl decanoate (C10) (Sigma), *p*-nitrophenyl laurate (C12) (Sigma), *p*-nitrophenyl myristate (C14) (Sigma), and *p*-nitrophenyl palmitate (C16) (Sigma).

The optimum temperature was examined at 10–50°C. The effect of pH on the activity of Est6 was measured over a pH range of 3.0–10.0. The buffers included 100 mM citrate buffer (pH 3.0-pH 6.5), 100 mM potassium phosphate buffer (pH 6.5–7.5), 100 mM Tris–HCl buffer (pH 7.5–9.0), and 50 mM CHES buffer (pH 9.0–10.0). Thermostability was analyzed by measuring the residual activity after incubating the enzyme at 10, 20, 30, 40, 50, and 60°C for 30 min, respectively.

The effect of metal ions (Co^{2+}, Cu^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, Sr^{2+}, Mn^{2+}, Ni^{2+}, and Ba^{2+}) and the chelating agent EDTA was examined at a final concentration of 10 mM. The effect of the detergents was determined using 1% Tween 20, Tween 80, Triton X-100, or SDS. The effects of organic solvents were examined using isopropanol, acetonitrile, alcohol, methanol, acetone, dimethyl sulfoxide (DMSO), or dimethylformamide (DMF) at a final concentration of 15%. All tests were performed in 100 mM Tris–HCl buffer (pH 7.5), and the activity of the enzyme, without additives, was defined as 100%.

Nucleotide sequence accession numbers

The nucleotide sequence of lipolytic genes *est*1, *est*2, *est*4, *est*6, *est*8, *est*21, *est*22, *est*23, and *est*24 has been submitted to GenBank with the accession numbers JF766281, JF766282, JF766284, JF766285, JF766286, JF766289, JF766290, JF766291, and JF766292, respectively.

Results

Screening of clones with esterase activity

The average insert length of the clones in our metagenomic library was 36 kb. We screened approximately 20,000 fosmid clones and found that 12 clones had clear zones around the colonies.

Subcloning and sequence analysis

Among the 12 clones that had clear zones on the plate, nine clones were successfully subcloned. Primer walking was used to sequence the inserted nucleotide sequences of the subclones. The ORF size of the putative lipolytic enzyme genes varied from 684 to 1,191 bp. BLASTp of the translated protein sequences found 56–84% sequence homology with the proteins from other bacterium (Table 1). Only one matched sequence was annotated from whole-genome sequences, and the other eight sequences were all derived from uncultured bacterium identified by metagenomic approaches. Comparison of the amino acid sequences of the nine putative lipolytic enzymes determined that Est2, Est4, and Est21 were highly similar, with an amino acid homology ranging from 96 to 97%.

Table 1 Putative lipolytic enzymes from the fosmid clones and similar enzymes

Gene name	ORF (bp)	Best match (accession no.)	Organism	Identities (%)	e-Value
Est1	684	Lipolytic enzyme (ACL67847)	Uncultured bacterium	84	2e-103
Est2	921	Lipolytic enzyme (ACL67849)	Uncultured bacterium	78	4e-133
Est4	921	Lipolytic enzyme (ACL67849)	Uncultured bacterium	77	2e-132
Est6	909	Lipolytic enzyme (ACL67845)	Uncultured bacterium	61	2e-94
Est8	939	Esterase (ABY61093)	Uncultured bacterium	66	9e-124
Est21	645	Lipolytic enzyme (ACL67845)	Uncultured bacterium	82	7e-90
Est22	1,035	Alpha/beta hydrolase fold domain protein (ZP_05096243)	Marine gamma proteobacterium HTCC2148	66	2e-135
Est23	1,191	Esterase (BAJ07043)	Uncultured bacterium	56	1e-98
Est24	948	Lipase/esterase (ACF04196)	Uncultured bacterium	60	5e-99

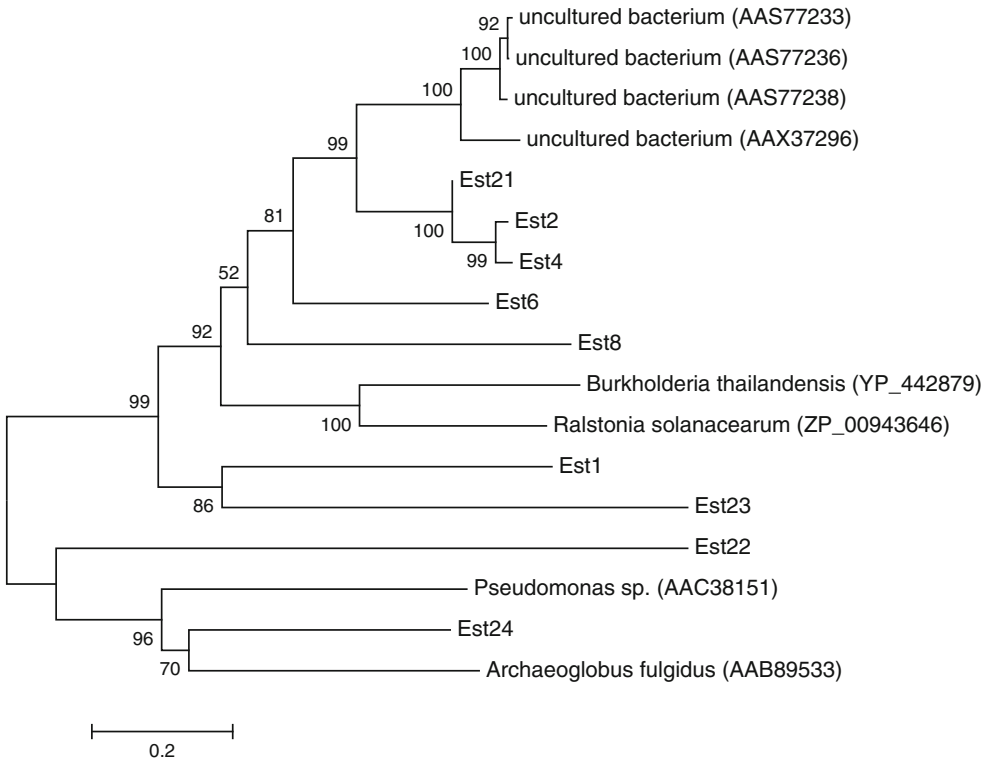

Fig. 2 Phylogenetic relationships between lipolytic enzymes in this study and other members of the family IV lipolytic enzymes. The protein sequences of the family IV lipolytic enzymes were retrieved from GenBank. The neighbor-joining tree was constructed using MAGA4.0 software. Bootstrap values were based on 1,000 replicates (values >50% were shown). *Scale bar* indicates 0.2 substitutions per amino acid position

Therefore, these three enzymes can be classified into one group. The other enzymes from the library had low similarity.

Phylogenetic tree and multiple-sequence alignment

We constructed a phylogenetic tree with the amino acid sequences of lipolytic enzymes from family IV (Arpigny and Jaeger 1999) (Fig. 2). All the nine enzymes grouped into family IV, and all of them contained the consensus pentapeptide G-X-S-X-G.

The analysis of the deduced amino acid sequence showed that Est6 was similar to several esterases from uncultured bacterium: FLS10 (ACL67845, 61% identity), FLS15 (ACL67849, 60% identity), EstMY (ADM67447, 60% identity), pELP11B (AAS77236, 55% identity), pELP45 (AAS77233, 55% identity), pELP141 (AAS77238, 55% identity), EstAT11 (ABY61093, 49% identity), and EstE5

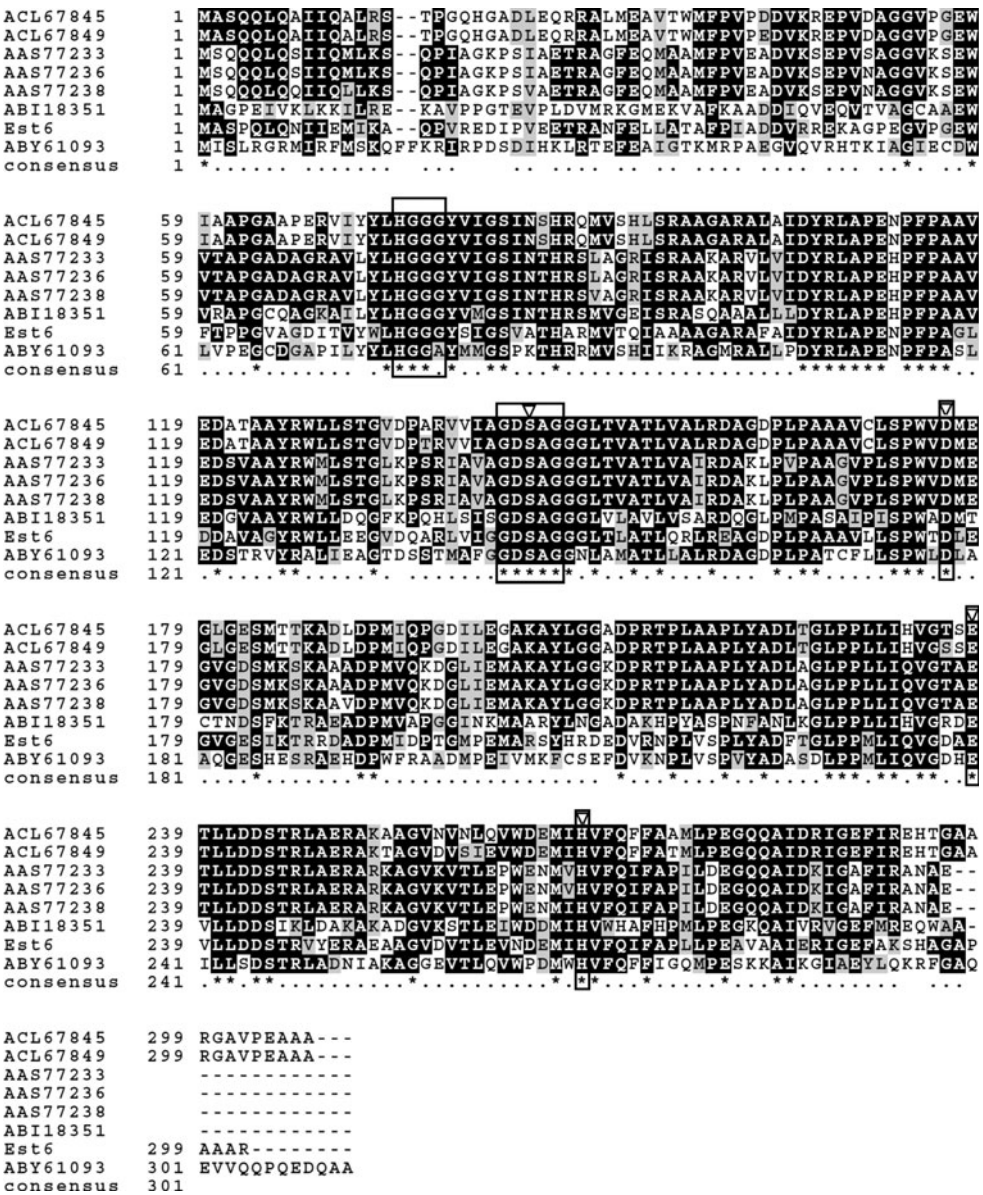

Fig. 3 Sequence alignment of Est6 and other similar esterases. The proteins used were all from uncultured bacterium: Est6 (in this study), FLS10 (ACL67845), FLS15 (ACL67849), pELP11B (AAS77236), pELP45 (AAS77233), pELP141 (AAS77238), EstAT11 (ABY61093), and EstE5 (ABI18351). *Open inverted triangles* indicate the conserved amino acid residues of the catalytic triad. The consensus blocks are *boxed*

(ABI18351, 46% identity). The pentapeptide, GDSAG, which includes the catalytic serine, is conserved at position 142–146, and the HGGG motif, which is the oxyanion hole of the family IV lipolytic enzymes, is conserved at position 74–77 (Fig. 3).

Expression and characterization of Est6

To characterize Est6, we constructed a recombinant plasmid, Est6-pET28b (+), and expressed it in *E. coli* Rosetta. The recombinant protein was soluble and was purified using an Ni–NTA affinity chromatography column. The calculated molecular weight of Est6 was 32.3 kDa, (http://www.expasy.org/tools/pi_tool.html), and it was confirmed by SDS–PAGE (Fig. 4). The native molecular weight of Est6 was 36.7 kDa by gel filter chromatography, which indicated that it was a monomer.

The activity of purified Est6 was examined using *p*-nitrophenyl butyrate as substrate, and Est6 had a specific activity of 104.41 U/mg under standard reaction conditions. The K_m and V_{max} values of Est6 for *p*-nitrophenyl butyrate were 0.89 mM and 66.7 μM/min, respectively.

The substrate specificity of Est6 was determined using *p*-nitrophenyl esters with various chain lengths (Fig. 5). The highest hydrolysis activity was obtained with *p*-nitrophenyl butyrate (C4). The enzyme preferred short- and middle-length *p*-NP esters but showed no activity toward *p*-nitrophenyl myristate (C14) and *p*-nitrophenyl palmitate (C16).

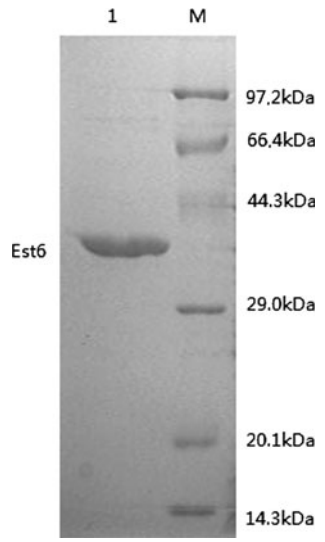

Fig. 4 SDS–PAGE of purified recombinant His-tagged Est6. Lane *M* marker; lane *1* purified protein. SDS–PAGE gel concentration was 12% and was stained by Coomassie R250. The amount of recombinant protein was 9.14 μg

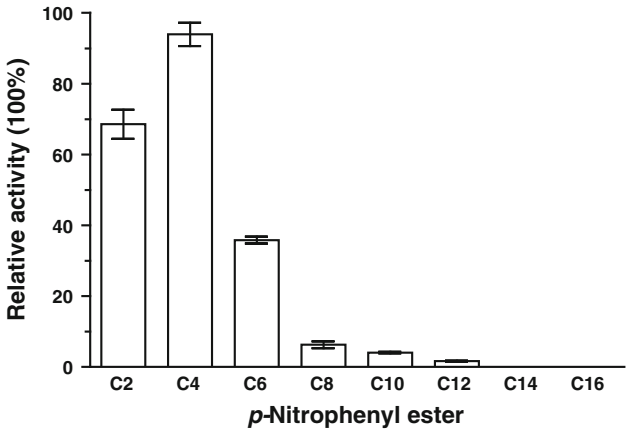

Fig. 5 Substrate specificity of Est6. The esterase activity of the purified recombinant enzyme, Est6, on *p*-NP esters with various chain lengths was assayed at 20°C, pH 7.5. The highest level of activity with the C4 substrate is shown as 100%

The optimum activity of Est6 was measured over a temperature range of 10–50°C and a pH range of 3.0–10.0 with *p*-nitrophenyl butyrate as the substrate. Est6 had the highest activity at 20°C (Fig. 6a) and pH 7.5 (Fig. 6b). Thermostability analysis showed that Est6 was stable at 10–20°C and was inactivated at 40°C (Fig. 6c). The activity of Est6 was strongly inhibited by Cu^{2+}, Zn^{2+}, and Ni^{2+} (Fig. 7a). The addition of 1% SDS inactivated Est6, while 1% Tween 20 or Tween 80 increased its activity. The activity of Est6 decreased in 15% isopropanol, acetonitrile, alcohol, methanol, acetone, dimethyl sulfoxide (DMSO), and dimethylformamide (DMF) (Fig. 7b).

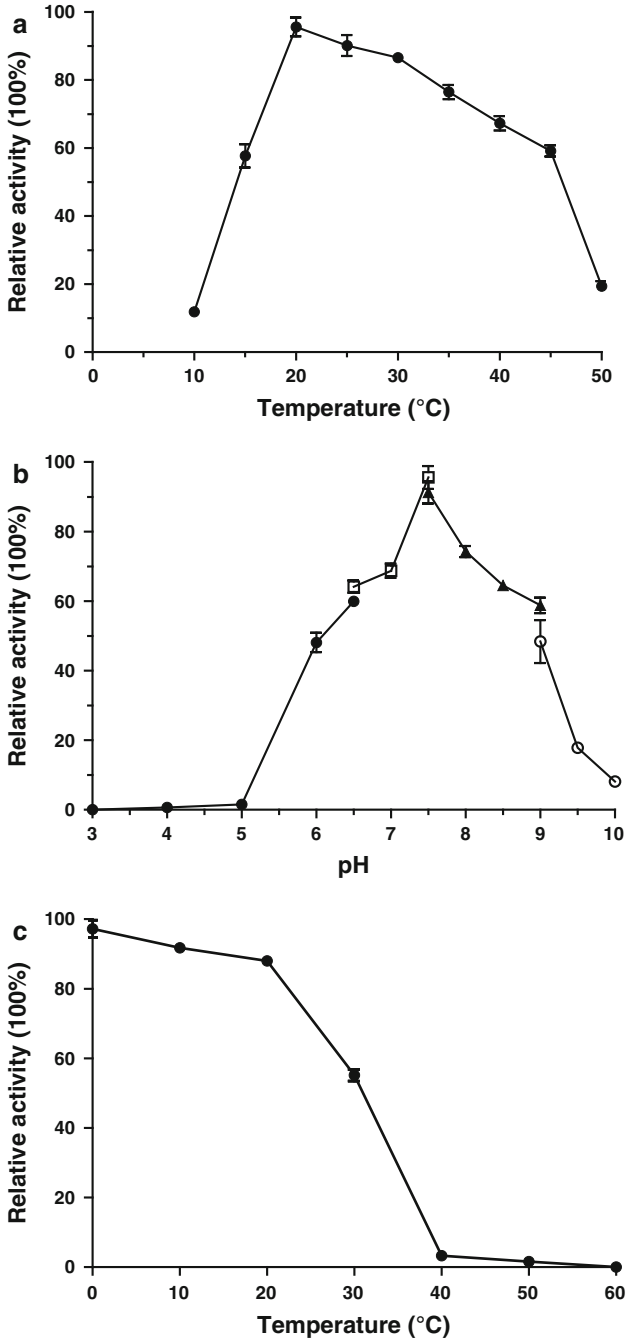

Fig. 6 **a** Effect of temperature on the activity of Est6. Enzyme activity was measured over various temperatures at pH 7.5 with *p*-nitrophenol butyrate as the substrate. The value obtained at 20°C is shown as 100%. **b** Effect of pH on the activity of Est6. The activity was measured with *p*-nitrophenol butyrate as the substrate. The assay was performed under 20°C in different buffers: 100 mM citrate buffer (pH 3.0–pH 6.5) (*filled circle*), 100 mM potassium phosphate buffer (pH 6.5–7.5) (*empty square*), 100 mM Tris–HCl buffer (pH 7.5–9.0) (*filled triangle*), and 50 mM CHES buffer (pH 9.0–10.0) (*empty circle*). **c** Thermostability of the recombinant Est6. Enzyme activity was measured under standard condition

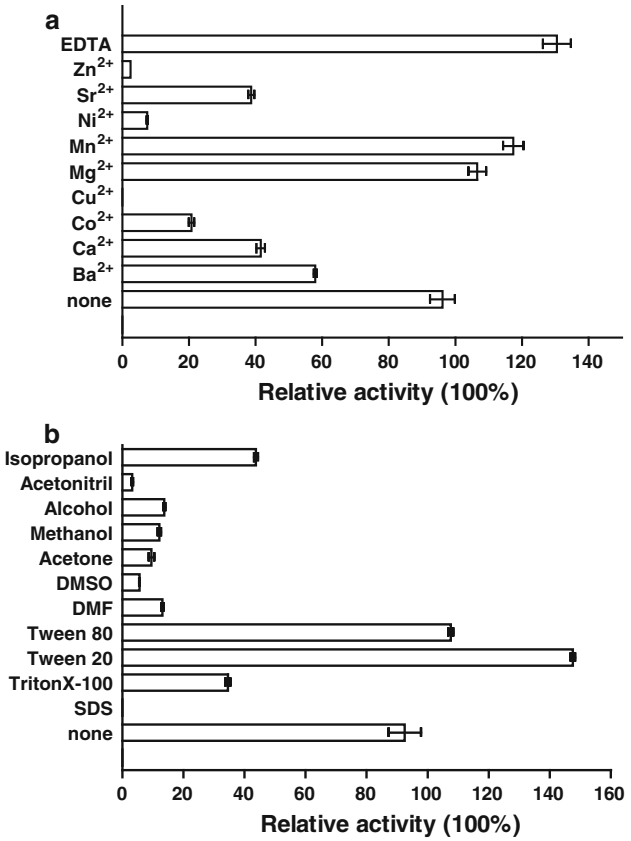

Fig. 7 Effects of metal ions, detergents, and organic solvents on the activity of Est6. An enzymatic assay was performed at 20°C in 100 mM Tris–HCl buffer (pH 7.5) with *p*-nitrophenol butyrate as the substrate. Metal ions (Co^{2+}, Cu^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, Sr^{2+}, Mn^{2+}, Ni^{2+}, and Ba^{2+}) and EDTA were added at the final concentration of 10 mM. Organic solvents were added at the concentration of 15%. And detergents (Tween 20, Tween 80, Triton X-100, and SDS) were added at the concentration of 1%. The value obtained with no additives in the reaction mixture is shown as 100%

Discussion

The marine environment contains a vast pool of novel enzymes and is receiving more and more interest. The oceans are full of mysteries, and we always wonder what is in the deep. Microbes reside everywhere and produce many enzymes with different properties. Using a metagenomic approach, we can discover various microbial enzymes from the deep-sea environment. The sample in our study was collected from a deep-sea sediment 5,000 m under water, and we used this sample to construct a fosmid library. An activity-based screening method was used to study this deep-sea sediment metagenome, and nine lipolytic genes were identified. To our knowledge, prior to this study, no lipolytic enzymes had been discovered from marine metagenomes that were constructed with samples collected deeper than ours.

We could not define the origin of the lipolytic genes using metagenomics because there was no 16S rRNA in the sequence of the inserts. We can ensure that it originated from deep-sea microorganisms because our metagenomic library originated from deep-sea sediment, and some of these genes were similar to the lipolytic genes from marine microbes.

Analysis of the amino acid sequences revealed that the lipolytic enzymes obtained in our study belong to family IV. This particular abundance has previously been found in marine sediment environment. For example, while screening lipolytic enzymes from the South China Sea marine sediment metagenomic library, researchers found 66.7% of the identified enzymes grouped into family IV (Hu et al. 2010a). Family IV lipolytic enzymes are significantly similar to mammalian hormone-sensitive lipases (HSL) (Langin et al. 1993), and most enzymes in this family are from psychrophilic and thermophilic bacteria (Jaeger et al. 1999). The deep sea is a cold environment with an average temperature of 4°C below 1,000 m (Jeon et al. 2009). Therefore, the abundance of family IV lipolytic enzymes in the deep sea may be linked to adaptation to the cold.

Est6 was assigned to family IV by the phylogenetic tree that was constructed using the amino acid sequences and was a new member of this family. It contained the active-site serine residue in the consensus pentapeptide, GDSAG, and an HGGG motif. The amino acid sequence of Est6 was most similar to the esterases FLS10 (61% identity) and FLS15 (60% identity), which were isolated from a deep-sea sediment (Qiongdongnan basin, South China Sea, water depth 778.5 m) metagenomic library. Both have an optimum temperature of 30°C. However, neither was further characterized for they were not overexpressed and purified (Hu et al. 2010a). Among the cultured microbial esterases, the esterase from *Synechococcus* sp. CC9311 was most similar to Est6 (45% identity). *Synechococcus* sp. CC9311 was isolated from the edge of the California Current (Toledo and Palenik 1997), and the genome sequence was reported (Palenik et al. 2006), but the esterase was not studied. Est6 was also similar to EstE5 (Nam et al. 2009), which was isolated from a metagenomic library constructed from soil samples. The functional analysis of EstE5 showed that it preferred short-chain ester compounds and that its optimal pH and temperature of hydrolysis were pH 9.0 and 35°C, respectively. Structural analysis showed that it belonged to the HSL family and that the residues Ser144, Glu238, and His268, which form the catalytic triad motif, were conserved in Est6 (in our study). These conserved residues indicate that Est6 is a member of the family IV (HSL family) lipolytic enzymes.

Est6 (in our study) was successfully expressed heterologously in *E. coli* Rosetta in soluble form and was purified and characterized. Est6 has activity at temperatures as low as 10°C and has an optimum temperature of 20°C, so it is a cold-active esterase. The substrate specificity experiment revealed that Est6 was an esterase that preferred short- and

mid-length acylglycerols and could not hydrolyze long-chain acylglycerols (C14 and C16). In addition, Est6 was activated by Tween 20 and Tween 80 and was stable in the presence of Mn^{2+}. With all these properties, we suggest that Est6 is a potential candidate for use in industry.

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