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Characterization of a novel esterase isolated from intertidal flat metagenome and its tertiary alcohols synthesis

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ABSTRACT

A gene coding for an esterase (EstEH112) was isolated from metagenome originated from Korean intertidal flat sediment. The putative esterase gene encoded a 340 amino acids protein with characteristic residues of lipolytic enzymes such as a conserved pentapeptide (GXSXG), the typical catalytic S-D-H triad, and a GGG(A)X-motif in the oxyanion hole near the active site similar to the hormone sensitive lipase (HSL) family. *p*-Nitrophenyl butyrate was the best substrate for the enzyme among the other *p*-nitrophenyl esters investigated. The apparent optimal temperature and pH for EstEH112 was 35 °C and at pH 8.0, respectively. EstEH112 efficiently catalyzed the hydrolysis of various large tertiary alcohol esters. These characteristics of EstEH112 make it a potential candidate for application in biocatalysis.

1. Introduction

The metagenomic approach is based on the construction of a genetic material library directly obtained from environmental sources and is one of the simplest ways of exploring the enormous natural genetic space for a better understanding of the microbial community in various environments and for finding new biocatalysts or compounds useful for the biochemical and pharmaceutical industries. Through screening based on functions or sequence homologies, many unique enzymes have been isolated from metagenome originated from diverse environments such as soil, water, sediment, and extreme habitats [1–3]. Intertidal flat sediments are affected by dynamic physicochemical conditions such as periodic floodtides, a higher degree of change in the salinity, and water temperature. These additional variables in the environment cause a remarkable and unique bacterial diversity in these types of sediments compared to other marine sources [4]. Therefore, metagenome libraries from intertidal flat sediments include diverse and valuable genetic materials encoding biocatalysts including lipases and esterases [5,6].

Esterases (EC 3.1.1.1), as well as lipases (EC 3.1.1.3), are a group of carboxyesterases catalyzing the cleavage and formation of ester bonds. Esterases prefer to hydrolyze the ester bond of short chain triglyceride or ester whereas lipases catalyze the hydrolysis of triglyceride bearing long-chain fatty acids. These enzymes are α/β-hydrolase fold enzymes and contain the catalytic triad (Ser-Asp(Glu)-His) and an oxyanion hole. The serine residue is generally located in a conserved Gly-X-Ser-X-Gly pentapeptide. Microbial lipolytic enzymes have been divided into eight families (Family I–VIII) [7]. Recently further subgroups were suggested based on the lipolytic enzymes isolated from metagenomic libraries from marine environment including intertidal flat sediment [5,6], surface sea water [8], and deep sea sediment [9].

Since many of lipases and esterases exhibit broad substrate specificity, position selectivity, and stereoselectivity, they serve as useful biocatalysts in order to obtain especially optically pure secondary alcohol synthesis and primary alcohol synthesis [10,11]. Similarly, optically pure tertiary alcohols (TAs) are also widely used as building blocks for synthesis of various organic chemicals and valuable pharmaceuticals. For instance, linalyl alcohol, which is found in many flowers and spices, is one of the most important terpene alcohols in fragrance and flavor industry. The tertiary α-acetylenic alcohols 2-phenylbut-2-yn-2-ol and 1,1,1-trifluoro-2-phenylbut-3-yn-2-ol were intermediates for the synthesis of A_{2A} receptor antagonists that were orally active in a mouse catalepsy model [12] or the acetylene moiety of 2-pyridylbut-3-yn-2-ol can be used to synthesize useful heterocyclic structures [13]. Chemical synthesis of optically pure TAs generally requires harsh conditions

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[14]. On the other hands, the synthesis using enzymatic biotransformation gives an opportunity to make more environmentally friendly synthetic routes. Esterases [15] as well as lipases [16], proteases [17], halohydrin dehalogenases [18], or epoxide hydrolases [19] were reported for the synthesis of TAs through kinetic resolution of racemates. However, tertiary alcohol esters are not suitable substrates for most of esterases and lipases. Recently, the GGG(A)X-motif in oxyanion hole was found important for the activity toward tertiary alcohols, though a GX-type lipase from *Burkholderia cepacia* was able to catalyze the enantioselective hydrolysis of tertiary cyanohydrins [17,20]. Lipase from *Candida rugosa*, pig liver esterase, acetylcholine esterases, recombinant esterase from *Bacillus subtilis*, and some esterases from metagenome possessing the GGG(A)X-motif catalyzed the reaction toward tertiary alcohol with various enantioselectivity [21–23].

In this study, we explored a metagenomic library constructed from intertidal flat sediment at Saemankum in the west coastal region of Korea to isolate a new hormone sensitive lipase like esterase from the family IV using a catalytic activity based screening. The cloning, expression, and determination of biochemical properties of this recombinant esterase and its activity toward tertiary alcohols are reported in this paper.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Fluka (Buchs, Switzerland), Sigma (Steinheim, Germany) and Merck (Darmstadt, Germany), unless stated otherwise. The synthesis of tertiary alcohol esters (TAEs) **1a**–**1h** was performed as described [22,24,25] (Fig. 1).

2.2. Methods

2.2.1. DNA extraction from environmental samples and construction of the metagenomic library

A metagenome sample from the intertidal flat sediments of the Korean western coastal region in Saemankum was extracted as previously described [26]. The environmental genomic DNAs were resolved in 1% (w/v) agarose by pulsed-field gel electrophoresis with a CHEF-DRIII system (Bio-Rad, USA) in order to fractionate the approximately 35-kb DNA. A metagenomic library was constructed using a CopyControl™ fosmid library production kit (Epicentre, USA) according to the manufacturer's instructions.

2.2.2. Screening of a lipase/esterase gene and subcloning of positive clone

Activity-based esterase/lipase screening was performed by plating the transformants onto Luria-Bertani (LB) agar plates containing chloramphenicol (12.5 μg/mL) and 1% (v/v) emulsified tributyrin. The plasmid designated pFosLip was isolated from the *Escherichia coli* transformant that showed the largest halo. The pFosLip was partially digested with *Sau3A*I, ligated with a pUC19 vector, and transformed into *E. coli* DH5α. The colony with the halo was selected and sequenced. The open reading frame of lipase/esterase was defined using the Lasergene 6.0 (DNASTAR Inc., USA).

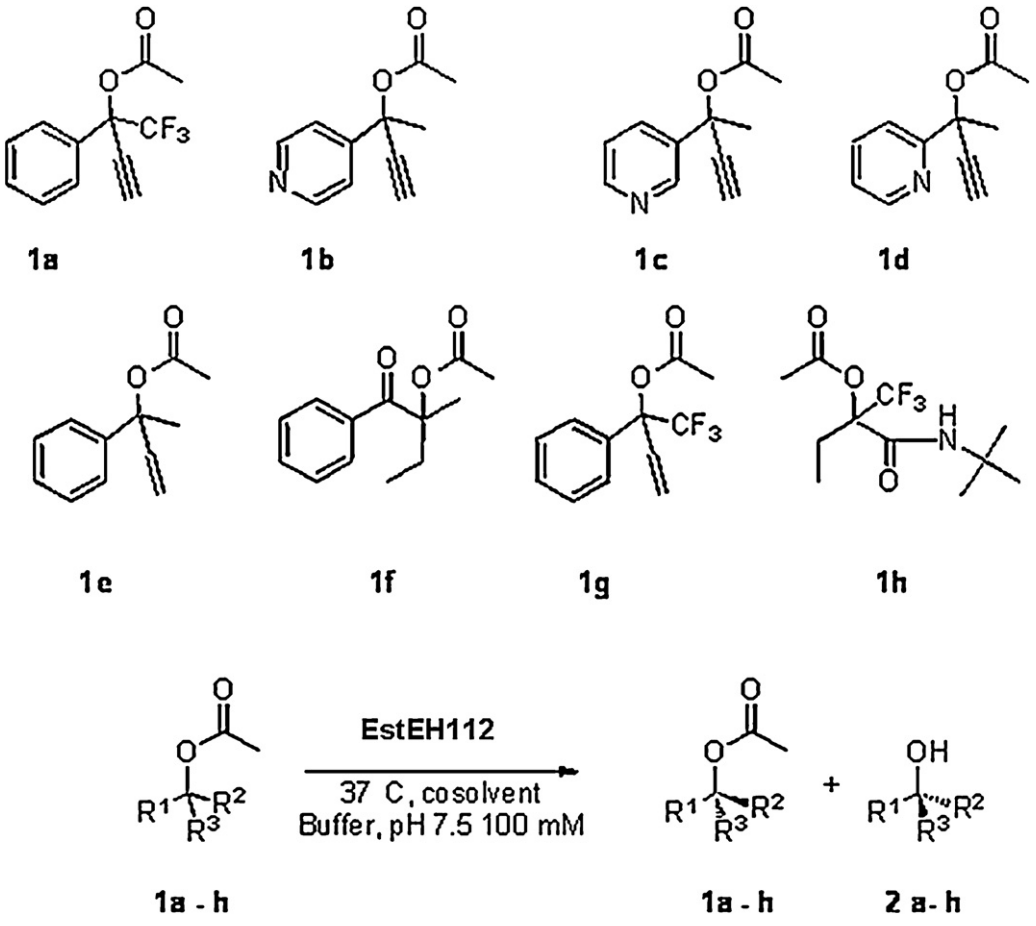

Fig. 1. Kinetic resolution of tertiary alcohol acetates with EstEH112.

2.2.3. Expression and purification of the esterase

The full length of the putative esterase gene was amplified by PCR with two primers (5′-GGAATTCCATATG-AGGTGAATCTATGACAAGC-3′ and 5′-CCGCTCGAGAATTTATTG-TAGGTGCTGCA-3′; underlined letters indicate the *Nde*I and *Xho*I recognition sites, respectively). The amplified DNA fragment was digested with *Nde*I and *Xho*I, and was then ligated with plasmid pET-22b(+) (Novagen, Germany). The resulting recombinant plasmid, designated as pET-estEH112, was used to transform *E. coli* DH5α. Once the sequence had been confirmed, the expression was done by introducing the recombinant plasmid into *E. coli* C43(DE3). The recombinant, *E. coli* EstEH112, was cultured in an LB medium containing ampicillin (50 μg/mL) at 37 °C. The EstEH112 expression was induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at an absorbance of ~0.6 at 600 nm before cultivation for 12 h at 21 °C. Cells were harvested by centrifugation and resuspended in 50 mM Tris–HCl buffer (pH 8.0) containing 300 mM NaCl. Cells were then disrupted by sonication and were centrifuged in 28,000 × *g* at 4 °C for 30 min. The supernatant was applied to a nickel–nitrilotriacetic acid (Ni–NTA) column (QIAGEN GmbH, Germany). After washing with a buffer (5 mM imidazole; 300 mM NaCl; 50 mM Tris–HCl buffer, pH 8.0) the bound esterase was eluted using 250 mM imidazole in the same buffer. The collected proteins were loaded onto a Superdex200 Gel Filtration Column (GE Healthcare, USA) equilibrated with 20 mM Tris–HCl buffer (pH 8.0) containing 150 mM NaCl. The column was eluted with 0.5 mL/min of an equilibrium buffer using a BioLogic DuoFlow Chromatography System (Bio-Rad Laboratories, USA). Fractions exhibiting esterase activity were analyzed using SDS-PAGE.

2.2.4. Biochemical characterization of the EstEH112

The enzyme concentration was determined by the Bradford assay (BioRad, USA) using bovine serum albumin as standard. The standard assay was carried out for 4 min at 35 °C using reaction mixture consisted of 20 μL of 100 mM *p*-nitrophenyl butyrate (pNPB) in ethanol, 40 μL of ethanol, and 0.94 mL of 100 mM Tris–HCl buffer (pH 8.0) containing an appropriate amount of the enzyme, unless otherwise specified. The amount of *p*-nitrophenol liberated during the reaction was kinetically measured by its absorbance at 405 nm using a DU800 spectrophotometer (Beckman, USA) and using a molar extinction coefficient of 17,000 M^{-1} cm^{-1}. One unit of enzyme activity was defined as the amount of enzyme needed to release 1 μmol of *p*-nitrophenol per min at 35 °C. Several *p*-nitrophenyl esters (acetate, propionate, butyrate, caproate, caprylate, caprate, and laurate) were used for the determination of substrate specificity. Kinetic constant of EstEH112 was determined using non-linear regression analysis in Origin 8.5 (Microcal, USA). The optimum pH for enzyme activity was determined at 35 °C from pH 4.0 to 11.0 in 100 mM GTA buffer by measuring liberated *p*-nitrophenol at 348 nm (isosbestic point of *p*-nitrophenol). pH stability of esterase was determined by incubating the enzyme in 100 mM GTA buffer (pH 4–11) for 1 h at 25 °C, and relative activity was estimated at 35 °C. To determine optimum temperature, the reaction mixture was incubated for 10 min at various temperatures (10–55 °C). Thermostability of esterase was determined by preincubating for 1 h at 10–60 °C in 20 mM Tris–HCl buffer, pH 8.0 with 150 mM NaCl. Subsequently, the residual activity was measured at 35 °C.

Thermal denaturation curve of EstEH112 were obtained from molar ellipticity change of 0.16 mg/mL of EstEH112 in 20 mM Tris–HCl buffer, pH 8.0 at 222 nm using a JASCO J-815 (Jasco, Japan) with 1 cm of pathlength cuvette. The temperature was gradually increased by 1 °C/min and signal changes were measured and averaged from three independent experiments. Molar ellipticity per mean residue, [θ], was calculated from the equation: $[\theta] = [\theta]_{obs} \times mrw/10lC$, where $[\theta]_{obs}$ is the ellipticity measured in degrees, mrw is the mean residue molecular mass, *C* is the protein concentration in g/L, and *l* is the optical pathlength of the cell (in cm). The thermal denaturation curve was fitted as two-state transition model [27].

2.2.5. Effect of detergents, metal ions, and organic solvents on the activity of EstEH112

The effects of detergents on the esterase activity were analyzed by measuring the remaining activities through additions of 0.1% and 1% (v/v) of various detergents in 100 mM Tris–HCl buffer (pH 8.0) into the enzyme solution. The effect of divalent metal ions on the activity of EstEH112 was compared by adding 1, 5, and 10 mM of $CaCl_2$, $CuSO_4$, $MgSO_4$, $FeSO_4$, $ZnSO_4$, $NiSO_4$, $MnSO_4$, or $CoCl_2$.

In order to estimate the organic solvent tolerance of EstEH112, 1 mg/mL of enzyme solutions were incubated in 10, 30, and 50% (v/v) of water-miscible organic solvents such as dimethylsulfoxide (DMSO), dimethylformamid (DMF), acetonitrile, ethanol, acetone, and isopropanol, and water-immiscible organic solvents such as ethyl acetate, *n*-butanol, chloroform, toluene, xylene, and *n*-hexane for 1 h at 35 °C with shaking at 1400 rpm using a Thermoshaker (Eppendorf, Germany). The treated enzyme solutions were 50-fold diluted in 100 mM Tris–HCl, pH 8.0. The residual activity of EstEH112 relative to non-solvent containing enzyme solution was measured. All assays were performed in three times independently under standard assay condition.

2.2.6. General procedure for esterase-catalyzed kinetic resolution

100 μg of the enzyme with 13.5 mg of racemic linalyl acetate in 1 mL of 100 mM Tris–HCl buffer, pH 8.0 was mixed on a shaking incubator (rpm 200) at 35 °C for 12 h. The reaction mixture was diluted by four times volume of ethanol and analyzed by thin layer chromatography using Merck silica gel 60 F_{254} (Merck, Germany) plates and petroleum ether/ethyl acetate (5:1) as eluent. Compounds were visualized by spraying with vanillin/concentrated sulfuric acid (5 g/L) followed by heat treatment [28].

In order to determine the catalytic activity of EstEH112 on the synthetic tertiary alcohol esters by **1a**–**i**, 12 μmol of each ester was dissolved in 100 μL co-solvent and 900 μL esterase solution [with a crude esterase concentration of 1–3 mg/mL dissolved in phosphate buffer (100 mM, pH 7.5)] was added to form a total volume of 1 mL. The reaction mixtures were shaken in a Thermoshaker (Eppendorf, Germany) at 37 °C for a certain time (0.5, 1, 4 and 24 h), after which a 300 μL sample was taken. The sample was extracted twice with 400 μL dichloromethane. The combined organic layers were dried over anhydrous Na_2SO_4, filtered and transferred to a GC-vial. Enantioselectivity and conversion were calculated according to Chen et al. [29].

2.2.7. Chiral gas chromatography analysis

Chiral GC-analyses were performed by using a Hydrodex-β-3B (Column A), Hydrodex-β-TBDAc (Column B) and Hydrodex-γ-TBDAc (Column C) from Machrey-Nagel (Düren, Germany) on a GC-2010 gas chromatograph (Shimadzu, Tokyo, Japan). Chiral analysis of **1a**–**h** was performed as described [22,24,25].

2.2.8. Nucleotide sequence accession number

The nucleotide sequences obtained were deposited in the GenBank database under accession number EU515238.

3. Results

3.1. Screening and analysis of putative esterase sequence

A DNA library from a sediment sample collected from an intertidal flat sediment was constructed using a fosmid vector,

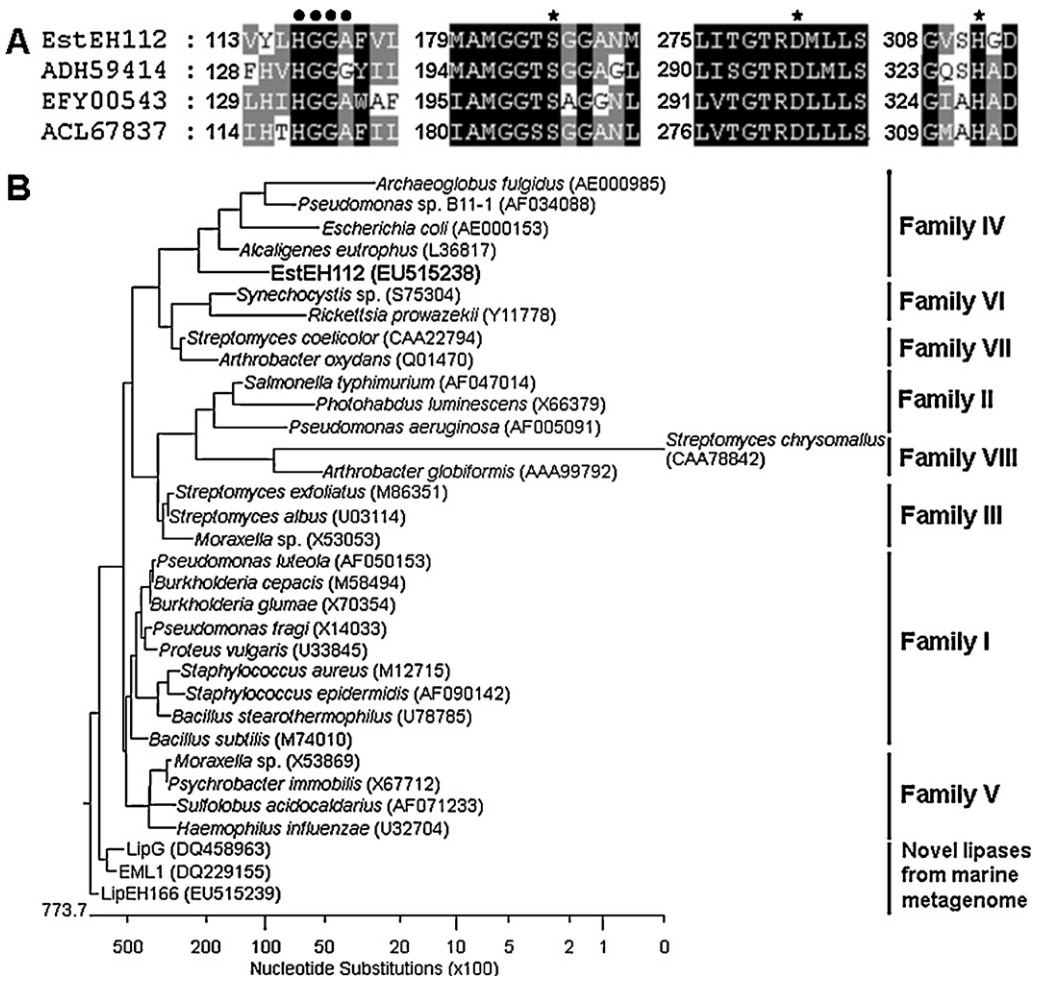

Fig. 2. Conserved amino acids sequence and phylogenetic analysis of EstEH112. (A) Multiple alignment of the partial amino acid sequences containing the conserved motifs of the pentapeptide GXSXG and the putative catalytic triad residues of the esterases from the metagenome (EU515238), *fls*1 gene from South China Sea Marine Sediment belonging to Hormone Sensitive Lipase (HSL) family (ACL67387) [9], esterase from marine sediment (ADH59414), and esterase from *Marine gamma proteobacterium* HTCC2143 (EAW33037). Symbols: (●) GGG(A)X-motif in oxyanion hole; (*) amino acid residues belonging to the catalytic triad. (B) Phylogenetic tree of EstEH112 and closely related proteins. Phylogenetic analysis was performed using the Megalign in Lasergene 6.0 (DNASTAR Inc., USA). The alignment of amino acids sequence was carried out by Clustal V. Except for EstEH112 and related proteins, the protein sequences for families of bacterial lipolytic enzymes previously classified by Arpigny et al. [7] and novel lipases from marine environmental metagenomes (Lee et al. [5], Kim et al. [6], Jeon et al. [31]) were retrieved from GenBank (http://www.ncbi.nlm.nih.gov).

pCC1FOS. This was followed by screening for lipolytic activity on a tributyrin agar plate. As a result, a positive clone showing the highest lipolytic activity from approximately 6000 colonies was selected and sub-cloned. The sequence analysis of the insert DNA of the selected sub-clone, which gave halo formation showed the presence of one open reading frame (ORF) consisting of 1020 nucleotides that encode for a protein (EstEH112) of 340 amino acids with a molecular mass of 37 kDa. The BLAST search against the NCBI non-redundant protein database (http://www.ncbi.nlm.nih.gov/BLAST/) revealed that the deduced amino acid sequence of the ORF was similar to several predicted lipolytic enzymes annotated as esterase from various cultured or uncultured bacteria such as the *fls*1 gene from South China Sea Marine Sediment belonging to the hormone sensitive lipase (HSL) family (GenBank accession no. ACL67387, identity 62%) [9], an esterase from marine sediment (GenBank accession no. ADH59414, identity 50%), and an esterase from *Marine gamma proteobacterium* HTCC2143 (GenBank accession no. EAW33037, identity 48%). In the amino acid sequence of EstEH112, the catalytic triad, Asp281, His311, and catalytic nucleophile serine at position 185 in the consensus GXSXG pentapeptide, and GGG(A)X motif at the positions 117–120 in the oxyanion hole which are characteristic for the HSL family could be found (Fig. 2A). EstEH112 was also clustered in family IV through phylogenetic analysis (Fig. 2B). These results show that EstEH112 belongs to the HSL family.

3.2. Biochemical characteristics of the recombinant EstEH112

In order to investigate the biochemical properties, EstEH112 was over-expressed with a six-histidine tag fused at its C terminus in *E. coli* and purified to homogeneity. Specificity of the enzyme was investigated using *p*-nitrophenyl esters (pNPEs) of varying chain-length as substrates. The esterase showed hydrolyzing activity toward a wide range of pNPEs (C2–C12) and the highest activity was found for *p*-nitrophenyl butyrate (pNPB) (Fig. 3). These results indicated that EstEH112 is an esterase not a lipase. The kinetic parameters, Km and k_{cat}, for EstEH112 were determined using pNPB as a substrate over a concentration range of 0.05–3 mM by non-linear regression analysis using the Michaelis–Menten equation (Fig. S1). The Km, k_{cat}, and k_{cat}/Km values of EstEH112 for pNPB were 0.92 ± 0.07 mM, $23.0 \pm 1.0\ s^{-1}$, and $25.1\ s^{-1}\ mM^{-1}$, respectively.

The optimal conditions for the activity of the enzyme were measured at a temperature range of 10–60 °C and at a pH range of 6–11 using *p*-nitrobutyrate as substrate. EstEH112 showed maximal activity at 35 °C and pH 8.0, respectively (Fig. 4A and B).

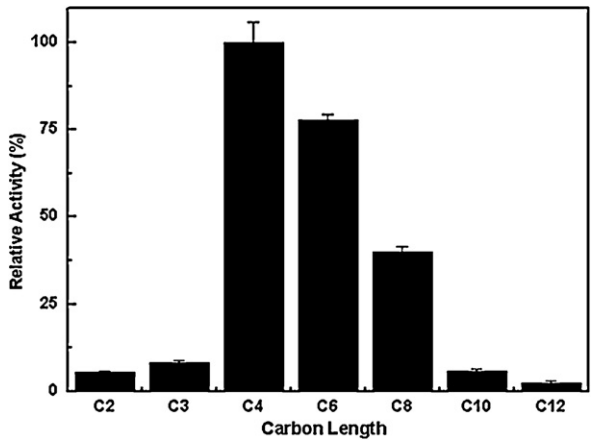

Fig. 3. Substrate specificity and specific activity toward pNPEs and natural substrate of the EstEH112. The esterase activity of the purified EstEH112 enzyme was assayed toward various pNPEs at 25 °C and pH 8.0. Substrates used were *p*-nitrophenyl acetate (C_2), *p*-nitrophenyl propionate (C_3), *p*-nitrophenyl butyrate (C_4), *p*-nitrophenyl caproate (C_6), *p*-nitrophenyl caprylate (C_8), *p*-nitrophenyl caprate (C_{10}), and *p*-nitrophenyl laurate (C_{12}). The error bars represents means ± SD (n = 3).

EstEH112 retained the 30% of its maximum activity at 10 °C. The enzyme was stable up to 40 °C after incubation at each temperature for 1 h (data not shown). Also, the thermal denaturation curve showed that EstEH112 started to unfold around 40 °C and was fully denatured above 60 °C (Fig. 4C). The melting temperature of this enzyme was determined to 54.8 ± 0.2 °C.

3.3. Effects of various detergents, metal ions, and organic solvents on the activity of EstEH112

The effects of various detergents and divalent metal ions on the EstEH112 were also examined. 1% detergent already severely inhibited the hydrolysis of pNPB by the enzyme (Table S1). Most divalent metal ions had little effect on the enzyme activity except ferric ions which inhibited the activity to about 40% in 5 mM $FeSO_4$ (Table S2). These results indicate that divalent metal ions are unnecessary for the catalytic activity of EstEH112.

In order to test the organic solvent tolerance of EstEH112, various organic solvents added in enzyme solution at concentration of 10, 30, and 50% (v/v). In 10% water-miscible organic solvents, the enzyme activity retained or was only slightly decreased. However, higher concentration of organic solvents caused dramatic deactivation of the enzyme with the exception using DMSO. The addition of 10% *n*-butanol, toluene, and xylene caused a rapid decrease of the enzyme activity (Table 1).

Table 1
Effects of various organic solvent on EstEH112 activity.

Organic solvents	log *P*[a]	Residual activity (%)[b] at concentration (%, v/v) of		
		10%	30%	50%
Water-miscible organic solvents				
DMSO	−1.35	95.8 ± 5.0	105.8 ± 2.0	22.7 ± 0.5
DMF	−1.01	88.6 ± 4.4	30.3 ± 2.6	1.6 ± 0.7
Acetonitrile	−0.34	71.0 ± 4.3	5.9 ± 0.9	5.7 ± 2.3
Ethanol	−0.31	87.8 ± 6.3	58.5 ± 4.0	2.5 ± 1.1
Acetone	−0.24	99.0 ± 2.7	2.4 ± 0.9	0.5 ± 0.8
Isopropanol	0.05	90.4 ± 3.5	5.9 ± 0.7	4.8 ± 1.4
Water-immiscible organic solvents				
Ethyl acetate	0.73	64.5 ± 5.9	38.7 ± 2.2	3.4 ± 2.0
n-Butanol	0.88	3.7 ± 2.4	5.0 ± 2.1	3.4 ± 2.0
Chloroform	1.97	76.2 ± 4.4	6.3 ± 2.2	5.2 ± 1.6
Toluene	2.73	16.0 ± 0.3	15.9 ± 1.4	3.0 ± 0.1
Xylene	3.12	5.9 ± 1.7	6.3 ± 3.2	5.0 ± 0.1

[a] log *P* value is the partition coefficient of an organic solvent between water and *n*-octanol phases.

[b] The enzyme solutions were incubated with organic solvents at various concentrations for 1 h at 35 °C with shaking at 1400 rpm. The residual activity of EstEH112 relative to non-solvent containing enzyme solution was measured. All assays were performed three times under standard assay condition.

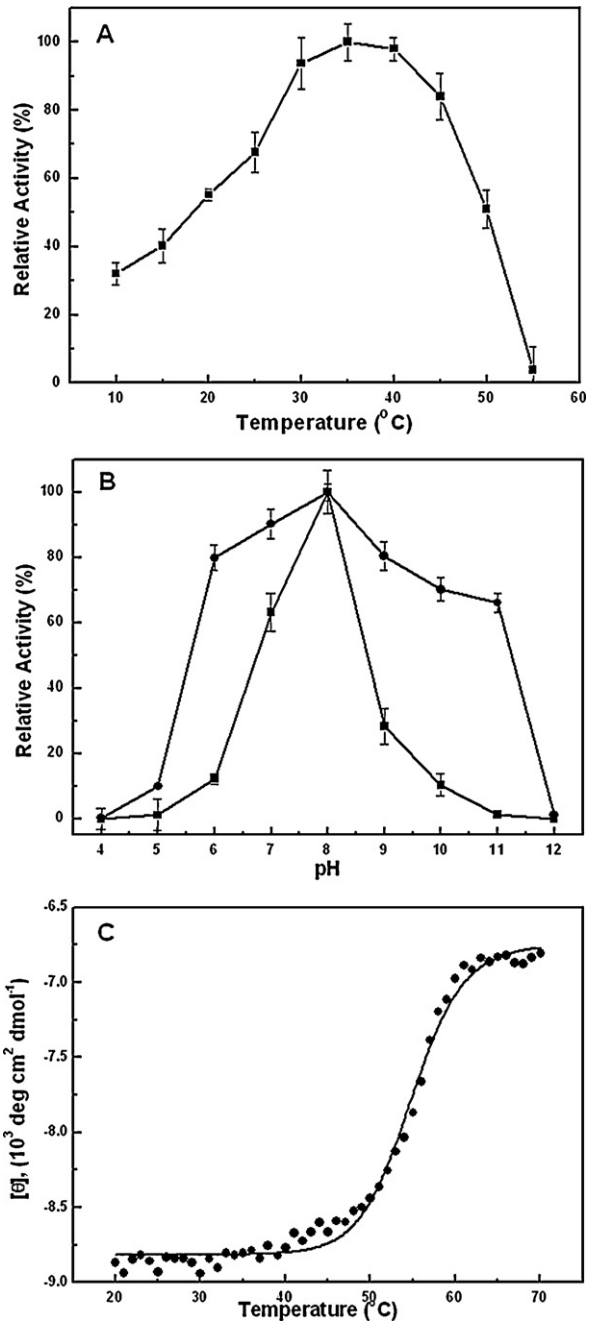

Fig. 4. Properties of EstEH112. (A) Effect of temperature on enzyme activity in pH 8.0. (B) pH effect on enzyme activity (■) and stability (●). (C) Thermal denaturation curve of EstEH112. The error bars represents means ± SD (n = 3).

3.4. The activity of EstEH112 toward tertiary alcohols

For the activity toward tertiary alcohols, linalyl acetate was initially selected as a model substrate. After the reaction, thin layer chromatography analysis showed that linalyl acetate converted to linalool by EstEH112 (Fig. S2). Therefore, this enzyme can efficiently catalyze the hydrolysis of tertiary alcohol esters to tertiary alcohol. Because many valuable tertiary alcohols contain of bulky hydrophobic residues, the efficient and enantioselective hydrolysis

Table 2
Activity and enantioselectivity of EstEH112 toward tertiary alcohol acetates.[a]

Compound	a	b	c	d	e	f	g[b]	h
Conversion (%)	59	55	53	50	47	n.c	41	n.c
Enantioselectivity	3	1	4	3	3		4	

n.c, no conversion. The enantioselectivity of the enzyme in the resolution of each substrate was calculated by the formula: $E = [\ln[1-c](1+ee_p)]/[\ln[1-c](1-ee_p)]$.
[a] 4-h samples.
[b] 24-h sample.

of these ester forms is important. EstEH112 showed the catalytic activity toward a wide range of bulky synthetic tertiary alcohol acetates (Table 2). Most of tested TAEs were efficiently hydrolyzed in 4 h, but enantioselectivities were low. Compounds **f** and **h**, however, are not converted by EstEH112. In previous studies, these two compounds were also converted by only a limited number of esterases [24,25].

4. Discussion

The metagenomic library from intertidal flat sediment was screened for lipolytic enzymes based on functional screening. Amino acid sequence analysis exhibited that the isolated EstEH112 contained the GGG(A)X-motif in the oxyanion hole, the typical conserved sequence motifs of esterase/lipase, GXSXG, and a putative catalytic triad composed of Ser_{185}, Asp_{281}, His_{311}. EstEH112 preferred short-chain *p*-nitrophenylesters as substrate and showed optimal activity at pH 8.0 and 35 °C. EstEH112 was stable up to 40 °C though it became inactivated rapidly at 55 °C. It displayed approximately 30% of its activity at 10 °C compared to the level of activity at its optimal temperature. These characteristics are similar to other lipolytic enzymes isolated from metagenomic library of marine environments.

Industrial bioconversion by enzyme in water-organic solvent mixture has several advantages over aqueous media especially to make valuable products from water-insoluble compounds. Therefore, it is important to find out naturally stable enzyme against organic solvents or to improve organic solvent stability [30]. EstEH112 showed around 80–100% of residual activity in 10% of water-miscible organic solvents whereas higher concentration caused a dramatic decrease of activity except DMSO. In addition, water-immiscible organic solvents caused quick denaturation of protein folds severely. These features generally followed log *P* trends.

EstEH112 belongs to the family IV (HSL family) by Arpigny and Jaeger based on the phylogenetic analysis (Fig. 2). Interestingly, it was reported that a lot of esterases from metagenomic library from marine environment belongs to family IV [9,31]. However, the reason of this abundance of this family in marine environment is not clear. A marine environment is generally cold and the intertidal flat region is a very dynamic environment. Some esterases of HSL family like *Moraxella* sp. and *Psychrobacter immobilis* are psychrophilic enzyme and others are from mesophilic and thermophilic environments. Therefore, conserved amino acids within this family are not simply linked to thermal adaptation.

The esterases of the family IV are related to hormone sensitive lipase-like enzymes and the family VII is similar to acetylcholine esterases, which all have the GGG(A)X-motif but most of the other family members have a GX motif in the oxyanion hole [7]. By molecular modeling, the carbonyl group of the backbone in the oxyanion hole of esterases possessing GX motif prevents the binding of tertiary alcohol ester into the substrate binding pocket [23]. On the other hand, the oxyanion hole of esterases possessing the GGG(A)X-motif provide more space for the binding of TAs [32]. Therefore, the esterases of this family are suitable for the production of tertiary alcohols. However, in most cases, GGG(A)X-motif hydrolases do not show high enantioselectivity [21,22]. It have been reported that exchange of glycine in this motif to alanine and structure based mutations of other amino acid positions in substrate binding pocket influenced the activity and enantioselectivity of esterase toward TAEs [32,33]. Therefore, although the enantioselectivity of EstEH112 toward the investigated compounds is not satisfying, this can be a good starting point for further protein engineering studies of this esterase, especially when the structure of EstEH112 is resolved, to extend the application of EstEH112 for the synthesis of enantio-pure tertiary alcohols through structure based protein engineering.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2012.04.015.

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