Characterization of an alkaline esterase from an enriched metagenomic library derived from an oil-spill area

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Abstract A novel esterase gene (*est7S*) was cloned from an enriched metagenomic library derived from an oil-spill area. The gene encoded a protein of 505 amino acids, and the molecular mass of the Est7S was estimated to be 54,512 Da with no signal peptide. Est7S showed the highest identity of 40% to an esterase from a sludge metagenome compared to the characterized enzymes with their properties, although it showed 99% identity to a carboxylesterase in the genome sequence of *Alcanivorax borkumensis* SK2. Est7S had catalytic triad residues, Ser183, Glu312, and His420, and the GESAG motif in most family VII lipolytic enzymes. Est7S was purified from the crude extract of clone SM7 using Sephacryl S-200 HR and HiTrap Q column chromatographies. The purified Est7S was optimally active at 50 °C and pH 10.0. Est7S showed a high specific activity of 366.7 U/mg protein. It preferred short length esters, particularly *p*-nitrophenyl acetate, efficiently hydrolyzed *R*- and *S*-enantiomers of methyl-3-hydroxy-2-methylpropionate, and glyceryl tributyrate. These properties of Est7S may provide potential merits in biotechnological applications such as detergent and paper processing under alkaline conditions.

Keywords Alkaline family VII esterase · Enriched metagenomic library · Purification · Short length *p*-nitrophenyl esters

Introduction

Esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) are generally categorized based on the chain length preference of fatty acid esters to be hydrolyzed. Esterases and lipases have been used in a variety of biological applications for degradation processes, such as detergent, food, animal feed, textile, and pulping, as well as for synthesis of pharmaceuticals, agrochemicals, biopolymers, and flavor compounds [1,2]. Recently, the lipolytic enzymes have been attracted as fused forms for the improvement of their thermostability, catalytic activity, substrate specificity, and regio- or enantio-selectivity, and also for facilitation of their purification and yield [3].

Bacterial lipolytic enzymes were originally classified into eight families based their sequences and biological properties [4], and expanded to fifteen families [5]. Besides microorganisms, biocatalysts have been explored using a metagenomic approach without culturing of microorganisms, due to the limitation of culture [6]. Marine microorganisms can serve as an untapped source of industrial biocatalysts, which are different from terrestrial microorganisms. We isolated two novel strains from an oil-contaminated seashore to degrade the contaminants [7,8]. We also have previously reported two novel enzymes, a family IV esterase and a family I.3 lipase, from an oil-polluted mud flat metagenome [9]. When the sample was enriched with specific substrates, microbial diversity could hugely increase.

In this study, we isolated a novel alkaline family VII esterase gene (*est7S*) with high specific activity from a metagenomic library derived from an enriched marine sediments contaminated with crude oil, and examined enzymatic properties of Est7S.

Materials and Methods

Materials

Substrates, such as *p*-nitrophenyl esters, enantiomers [(*R*)- and

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(*S*)-methyl-3-hydroxy-2-methylpropionate], glyceryl esters (glyceryl tributyrates and glyceryl trioctanoate), and oils (fish oil and olive oil), along with other chemicals, were purchased from Sigma (St. Louis, MO, USA). The compounds 5-Bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) and isopropylthio- β -D-galactoside (IPTG) were obtained from Bioneer (Daejeon, Korea). Sephacryl S-200 HR and HiTrap Q HP columns were from GE Healthcare (Uppsala, Sweden).

Selection of esterase-positive clones from enriched and mixed culture

Oil-spilled fine gravels and sands were sampled, as described previously [9]. The sample was enriched by adding 0.5% crude oil into BM medium [2% sea salt, 10 mM phosphate buffer, 0.25 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.02 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2.38 g $(\text{NH}_4)_2\text{SO}_4$ per L] and grown three times at 25 °C for 10 days. Enriched metagenome was then extracted and partially digested with *Sau3A*I by the method previously described [10]. After the digested 3–8 kb fragments were inserted into pUC19 vector, transformants were grown on LB agar plates supplemented by X-gal and IPTG, and then positive clones were selected by using LB plates that contained 1% glyceryl tributyrates [11]. Recombinant plasmids of the clones were analyzed.

Sequence analysis of the gene

The nucleotide sequence of the insert DNA was determined by SolGent (Daejeon, Korea). The conserved regions were detected using BLAST at the NCBI (<http://www.ncbi.nlm.nih.gov>). Putative signal peptide was predicted by SignalP 4.1 in CBS (<http://www.cbs.dtu.dk/services/SignalP/>) [12]. The molecular mass estimation and multiple alignment were analyzed using DNA/MAN (Lynnon Biosoft, version 4.11, Quebec, Canada).

Esterase assay

Esterase activity was assayed by measuring the amount of *p*-nitrophenol generated from *p*-nitrophenyl butyrate (*p*NPB), as described previously [10]. Under the standard conditions, the reaction was carried out for 2 min at 25 °C in the presence of 1 mM *p*NPB in 50 mM Tris/HCl (pH 8.0) and continuously measured at 400 nm using a spectrophotometer (Mecasys, Model OPTIZEN, Korea). Enzyme activities were calculated using molar-extinction coefficients for *p*-nitrophenol (16,520/M/cm at pH 8.0) [10]. One unit of esterase activity was defined as the amount of enzyme that generated 1 μmol of *p*-nitrophenol in 1 min under the condition.

Purification of the enzyme

Crude enzyme preparation was performed as previously described [13]. The crude enzyme was loaded onto a Sephacryl S-200 HR column (HiPrep 16/60) after dialysis against 50 mM sodium phosphate buffer (pH 7.2) containing 150 mM NaCl [14]. The protein was eluted at a flow rate of 0.5 mL/min. The enzyme was

purified by HiTrap Q HP (5 mL) column chromatography. Protein concentration was determined using the Bradford method [15] and SDS-PAGE on an 11.5% polyacrylamide gel [16].

Characterization of the enzyme

Optimum temperature, thermostability, optimum pH, cation effect, and substrate specificity of the enzyme were determined, as described previously [10]. Hydrolyzing activities for *p*-nitrophenol derivatives were analyzed using the following *p*-nitrophenyl esters: *p*-nitrophenyl acetate (C2), *p*-nitrophenyl butyrate (C4), octanoate *p*-nitrophenyl octanoate (C8), *p*-nitrophenyl caprate (C10), *p*-nitrophenyl laurate (C12), *p*-nitrophenyl myristate (C14), and *p*-nitrophenyl palmitate (C16). Enantioselectivity was analyzed using 300 mM (*R*)- or (*S*)-methyl-3-hydroxy-2-methylpropionate in 20 mM Tris/HCl, (pH 8.0) containing phenol red (0.01 g/L) with modifications [11]. The reaction mixtures (0.6 mL) containing 2.0 U/mL of Est7S were reacted for 1 h at 25 °C and the absorbance spectra were recorded at 560 nm. Hydrolysis of glyceryl esters, such as glyceryl butyrate and glyceryl trioleate, and oils, such as fish and olive oil, were analyzed using 1% substrates.

Nucleotide sequence accession number

The nucleotide sequence of the esterase gene *est7S* has been deposited in the GenBank under accession number MK290416.

Results and Discussion

Characterization of an esterase gene *est7S* and Est7S

Among 1,500 transformants, eight clones were screened as positive clones and confirmed on an LB plate containing 1% glyceryl tributyrates as a substrate (Fig. 1). After analyzing the plasmids by four restriction enzymes such as *Eco*RI, *Bam*HI, *Hind*III, and *Pst*I, and comparing esterase activities on the plate, clone SM7 was selected for further study. The size of the insert fragment of SM7 was about 3.0 kb, containing an open reading

Fig. 1 Detection of esterase-positive clones. C, control

(A)

(B)

Fig. 2 Multiple alignment of the amino acid sequences (A), putative conserved domains (B), and phylogenetic tree of Est7S (C). The GxSxG and HGG motifs are boxed in orange. Red circles represent catalytic triad residues. In (A): Est-XG2, from *Thermaerobacter marianensis* DSM 12885 (AGS38342); PNBCE, *Bacillus subtilis* (P37967); Est-B, *Bacillus* sp. Za (AGY14298); EstDL30, an uncultured organism of soil (AEK77432); Est50, *Geobacillus stearothermophilus* (AAN81910); Est-Ts, *Thermaerobacter subterraneus* DSM 13965 (ZP_11320927); Est-Tm, *Thermaerobacter marianensis* (WP_013495056); SCO6127, *Streptomyces coelicolor* A3(2) (CAA22794); PMPH, *Arthrobacter oxydans* (Q01470). In (B): Est-Hc, from *Hahella chejuensis* KCTC2396 (ABC27396); AES, *Escherichia coli* str. K-12 substr. MG1655 (AAC73578); EstA, *Archaeoglobus fulgidus* DSM 4304 (AAB89533); LipP, *Pseudomonas* sp. strain B11-1 (AAC38151); SAL, *Sulfolobus acidocaldarius* DSM 639 (AAC67392); Lip3, *Moraxella* sp. TA144 (CAA37863); Lip1, *Psychrobacter immobilis* B10 (CAA47949).

Table 1 Purification of an esterase Est7S from the clone SM7

Procedure	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	5.0	20.1	151.8	7.6	1.0	100
Sephacryl S-200 chromatography	22.4	0.19	47.8	251.6	33.1	31.4
HiTrap Q chromatography	1.0	0.012	4.4	366.7	48.3	2.9

frame of 1,518 bp, which was expected to encode a protein of 505 amino acid residues with no signal peptide (Fig. 2A). The gene was matched to a putative α/β hydrolase superfamily and a carboxylesterase/lipase family, and was named *est7S*. Est7S was calculated to be 54,512 Da.

The amino acid sequence of Est7S showed the highest identity of 40% to an esterase Est-XG2 from an activated sludge metagenome [17], followed by 36% to a *p*-nitrobenzyl esterase of *Bacillus subtilis* [18] and an esterase EstDL30 from a soil metagenome [19], and 35% to an esterase Est55 of *Geobacillus stearothermophilus* [20], by comparison enzymes with the reported properties. In comparison with the amino acid sequences from the genome sequences, Est7S showed 99% identity to a carboxylesterase of *Alcanivorax borkumensis* SK2 (CAL16699), followed by 83% identity to that of *Alcanivorax* sp. DG881 (EDX91080), 76% identity to that of *Alcanivorax* sp. MD8A (WP_102791219), and 72% identity to that of *A. nanhaiticus* (WP_035231190). These sequences were annotated from genome sequences and their enzymatic properties have not yet been characterized. Analysis of the amino acid sequence and multiple alignment revealed that Est7S contained a catalytic triad, such as Ser183, Glu312, and His420; a GxSxG motif as GESAG in the 181st-185th residues; and an HGG motif at the N-terminus, found in most family IV, V, and VII. In the phylogenetic tree, Est7S was clustered with family VII esterases (Fig. 2B).

The highest similarity of Est7S was matched to the esterase of *A. borkumensis*. Though the production of hydrocarbon degrading enzymes (e.g. lipase and esterase) was recently studied from *A. borkumensis*, the enzymes were not cloned and characterized [21]. An esterase EstB was cloned and characterized from *A. dieselolei*, but the enzyme was different from Est7S, in that it was 45.1 kDa, cold-active, and *p*NP-caproate (C6) preferred [22].

The α/β -hydrolase superfamily, including esterases, contains Ser, Asp, and His, to form a catalytic triad [4]. Est7S contained Glu instead of Asp in the catalytic triad, like an acetylcholinesterase from *Torpedo californica* [23], a *p*-nitrobenzyl esterase of *Bacillus subtilis* [24], family VII Est-XG2 [17], and two family IV esterases (DMW18-543 and DMWf18-538) from a deep-sea sediment metagenome [25].

The typical pentamotif GxSxG has been reported to contain a serine residue that has a catalytic function found in most esterase families and in Est7S [26]. The pentamotif sequence was partially conserved in family VIII esterases, such as GMS₃₇₂RG of Est2K, GMS₃₇₃EG of EstC, and GLS₃₂₁VG of LipBL, but the Ser in the

Fig. 3 SDS-PAGE of the Est7S during purification. M, molecular weight markers; lane 1, crude extract of the clone; lane 2, pooled from Sephadex S-200 chromatography; lanes 3-8, fractions 68-78 at even numbers from HiTrap Q

motifs was not essential for the catalytic activity [10,27,28]. Instead, the family VIII enzymes have the SxxK motif, while family II (GDSL family) contain the GDSL motif.

Purification of Est7S

The crude extract of the clone SM7 showed 30.4 U/mL of esterase activity (Table 1). Est7S was purified from the crude extract using Sephadex S-200 HR and HiTrap Q columns. Esterase activity was observed at late fractions from 96 to 120 in Sephadex S-200 chromatography. In HiTrap Q chromatography, the activity was eluted from 69 to 77 after binding to the resin. Fraction 72 showed a thick protein band and fractions 71 and 72 were pooled. The recovery rate and purification fold were 2.9% and 48.3-fold, respectively (Table 1). Purified Est7S showed a single band and had a molecular mass of 51.0 kDa, slightly smaller than the theoretical value, on an SDS-PAGE gel (Fig. 3). The smallest lipolytic enzymes belonged to family I.4, likely Lip7-3 (19 kDa) of marine bacterium *Bacillus* sp. W130-35 [11]. The large esterases were found in family I.3, likely Lip3K (64 kDa) from the oil-polluted mud flat metagenome [9], or in most of family VII (~55 kDa) [1]. Based on the results, Est7S was a large-sized enzyme with 55 kDa.

Specific activity of the purified enzyme was 366.7 U/mg protein. The specific activity of Est7S was in the upper range among family VII esterases such as Est-XG2 (17.71 U/mg protein) [17], EstDL30 (161 U/mg protein) [19], and an esterase NaM1 (488.9 U/mg protein) from a desert metagenome [29]. Compared to others, Est7S was in the middle range; an esterase Est2K (family VIII, 17.1 U/mg protein) from a compost metagenome [10], Lip7-

Fig. 4 Effect of temperature and pH on the enzyme activity of Est7S. (A), Optimum temperature; (B), Optimum pH; (C) Thermostability. In the thermostability experiments, enzyme was pre-incubated without a substrate, and then the residual activity was measured under the standard assay condition

3 (family I.4, 81.3 U/mg protein) [11], EstN7 of *B. cohnii* strain N1 (family IV, 336.89 U/mg protein) [30], EstSP (family IV, 745.14 U/mg protein) from a soil metagenome [31], and Est7K (family VIII, 790.2 U/mg protein) from the compost metagenome [32].

Properties of Est7S

Est7S showed its maximal activity at 50 °C, and greatly decreased at 40 or 60 °C (Fig. 4A). Est7S also showed its maximal activity at pH 10.0 (Fig. 4B). At pH 9.0, the activity was decreased to 58.4%; however, it increased to 78.0% at pH 8.0. The value of Est7S was higher than that of EstDL30 (pH 8.0) [19] and that of

Fig. 5 Effect of metal ions on Est7S

Fig. 6 Substrate specificity of Est7S for pNP-esters. C2, *p*-nitrophenyl acetate; C4, *p*-nitrophenyl butyrate; C8, octanoate *p*-nitrophenyl octanoate; C10, *p*-nitrophenyl caprate; C12, *p*-nitrophenyl laurate; C14, *p*-nitrophenyl myristate; and C16, *p*-nitrophenyl palmitate

Est-XG2 (pH 8.5) [17]. The results suggested that Est7S had a broad alkaline range. In thermostability experiments, half-life of Est7S at 30 °C was 29.7 min, but those were less than 5 min at 40 or 50 °C (Fig. 4C). The optimum temperature (50 °C) of Est7S was in the middle range compared to those of other enzymes (20–80 °C).

Cations testing showed no significant effects on the enzyme activity at 2 mM, except that Fe²⁺ and Zn²⁺ inhibited to 34.1 and 34.5% of the activity (Fig. 5). At 5 mM, Mg²⁺ increased the activity to 126.0%, but Cu²⁺ and Zn²⁺ inhibited to 29.0 and 24.0%, respectively.

Purified Est7S hydrolyzed the ester bond of C2 with maximal activity, and then followed by C4 and C8 with 81.7 and 38.4%, respectively (Fig. 6). Est7S preferred to short-chain fatty acid esters but hardly hydrolyzed the long-chain fatty acids, such as C14 and C16. The activity ratio for C10 to C4 was 0.13, between Est7K (0.035) [32] and Est2K (0.4) [10]. Est7S preferred a short-chain fatty acid (C2 or C4) as the substrate, likely many esterases, such as EstSP (C2) [31], EstN7 (C2) [30], *B*/Est1 (C2) of *B. licheniformis* [33], EstSP2 (C2) of *Sphingomonas glacialis* [34],

Fig. 7 Analysis of enantioselectivity and glyceryl substrate hydrolysis of Est7S. (A) Enantioselectivity analysis with (*R*)- or (*S*)-methyl-3-hydroxy-2-methylpropionate containing phenol red; (B) Hydrolysis of glyceryl esters and oils

and an esterase (C2) of *Xanthomonas oryzae* [35], but contrary to Lpc53E1 (C16) from the metagenome of the marine sponge *Haliclona simulans* [36] and KM12 lipase (C12) of *B. licheniformis* KM12 [37]. Based on the substrate preference, Est7S is a typical carboxylesterase rather than a lipase [4].

Est7S hydrolyzed an *R*-enantiomer, (*R*)-methyl-3-hydroxy-2-methylpropionate, and its *S*-enantiomer. The half-lives of *R* and *S* isomer were 24.4 and 21.0 min, respectively, under the experimental condition by measuring A_{560} (Fig. 7A). Est7S showed a lower enantioselectivity level for (*S*)-enantiomer of methyl-3-hydroxy-2-methylpropionate than Est7K, which showed *S*-enantiomer preference [32]. Est7S efficiently hydrolyzed glyceryl tributyrate with a half-life of 15.0 min, but hardly hydrolyzed glyceryl trioleate, fish oil, and olive oil (Fig. 7B). The results suggested that Est7S was specific to short glyceryl esters.

In this study, we cloned a new alkaline esterase Est7S from an enriched metagenomic library. Est7S showed a high specific activity and a specificity for short-chain fatty acid esters and activity including glyceryl tributyrate and the enantiomers. These properties of Est7S may provide potentials in biotechnological applications under alkaline condition and for short glyceryl esters.

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