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Molecular Cloning and Characterization of a Novel Cold-Adapted Family VIII Esterase from a Biogas Slurry Metagenomic Library

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A novel esterase gene, *est01*, was successfully unearthed from a biogas digester microbiota metagenomic library. The 1,194 bp *est01* gene encodes a protein of 44,804 Da (designated Est01). The amino acid sequence of Est01 shows only moderate (33%) identity to a lipase/esterase. Phylogenetic analysis and biochemical characterization confirmed that Est01 is a new member of family VIII esterases. The purified Est01 from recombinant *Escherichia coli* BL21 (DE3) showed high hydrolytic activity against short-chain fatty acid esters, suggesting that it is a typical carboxylesterase rather than a lipase. Furthermore, the Est01 was even active at 10°C (43% activity remained), with the optimal temperature at 20°C, and had a broad pH range from 5.0 to 10.0, with the optimal pH of 8.0. These properties suggest that Est01 is a cold-adaptive esterase and could have good potential for low-temperature hydrolysis application.

Keywords: Lipolytic enzyme, family VIII esterase, cold-adapted esterase, metagenomic library, biogas digester

Introduction

The lipolytic enzymes, comprising esterase (E.C. 3.1.1.1) and lipase (E.C. 3.1.1.3), are usually grouped into eight families (I–VIII) according to their amino acid sequences [1]. Esterases and lipases typically differ in their ability to hydrolyze substrates of variable chain length. Typically, lipases act on water-insoluble (chain length of >10 carbon atoms) triglycerides, whereas esterases hydrolyze short-chain (chain length of <10 carbon atoms) esters [19]. Both esterases and lipases are important biocatalysts for various industrial applications, for the production of fine chemicals in detergent and food industries, as well as in bioremediation processing [2]. The traditional way to finding useful lipolytic enzymes is culture-dependent screening of a wide variety of microorganisms for the desired lipolytic activity, which is cumbersome and time-consuming. Recently, the metagenomic approach, which requires no cultivating of any particular microorganisms, has become a powerful and advantageous technique that enables the discovery of novel biocatalysts with ease [9, 21]. Nowadays, energy crisis and global warming concerns require the saving of energy and reducing pollution. In this point of view, cold-adapted lipolytic enzymes could be of great value, with respect to possible savings in energy input, as compared with their mesophilic counterparts [11]. Although many microbial esterases have been isolated from the metagenomic libraries derived from various environments [13, 18, 23],

among of them only a few cold-adapted enzymes were documented.

It seemed reasonable that some cold-active enzymes could be fished from various low-temperature hydrolytic environments, such as activated sludge, antarctic desert soil, and biogas digester. In this study, a microbiota metagenomic library was constructed using a biogas slurry sample collected from a well-operated biogas digester in the winter with the average temperature nearly 15°C, to find some novel genes coding for cold-adapted lipolytic enzymes.

Materials and Methods

Sample Collection, Metagenomic DNA Extraction and Purification

Biogas slurry was collected from an efficient 8 m^3 biogas-producing digester under low temperature (15°C), which fed mainly on liquid pig manure and domestic wastewater in the countryside of Jiangyou, Sichuan Province, China. Methods used for metagenomic DNA extraction and purification were the same as reported previously [5].

Construction and Screening of the Metagenomic Library

The purified DNA was partially digested with *Sau*3AI to obtain 3–10-kb-size DNA, and ligated into a purified *Bam*HI /SAP pBluescript SK (+) vector from Novagen. Ligation products were transformed into *E. coli* DH5α cells (TaKaRa) to construct the metagenomic library. Lipolytic activity clones were screened from the metagenomic library by replica plating transformants onto LB (ampicillin, 100 μg/ml) plates containing 1% (v/v) emulsified tributyrin as the indicator substrate [20]. Clones with a surrounding clear halo were selected for further study after incubation for 2 days at 37°C.

DNA Sequencing and Sequence Analysis

DNA sequencing was conducted with the methods reported [5]. Open reading frames (ORFs) were identified by using ORF Finder of NCBI. Multiple sequence alignment was performed by using Clustal W [16]. The active site was predicted on pfam [8]. A phylogenetic tree was constructed the same way as documented [5].

Heterologous Expression and Purification of Est01

For the overexpression of Est01 , the full length of the *est01* gene was amplified using primers *est01*-F 5′CGGGGTACCATGAAT AATTCAATAACGATTG-3′ and *est01*-R 5′-CCGGAATTCTCA AATAATTGAAGAAAAAATC-3′ containing restriction enzyme sites (underlined) for *Kpn*I/*Eco*RI. The integrity of the nucleotide sequence of all newly constructed plasmids was confirmed by DNA sequencing. The *est01* gene was cloned into a pET-30a(+) vector to obtain expression plasmid. The expression plasmid was transformed into *E. coli* BL21 (DE3) cells to obtain the expression strain. The Est01 was expressed after induced with isopropyl-β-D-thiogalactoside. The recombinant Est01 was purified using nickel-iminodiacetic acid (Ni-IDA) resin per the manufacturer's instructions. The purified Est01 was dialyzed against storage buffer (20 mM Tris-HCl, 200 mM NaCl, 10% glycerol, pH 8.0) at 4°C overnight, and then was flash-frozen in liquid nitrogen before storing at −80°C. To estimate the molecular mass and homogeneity of Est01, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 15% running gels [15]. The protein concentration was determined according to the Bradford method [3], using bovine serum albumin as a standard.

Characterization of the Enzyme Est01

The substrate specificity of the purified Est01 was analyzed by using the following substrates of *p*-nitrophenyl esters: acetate (C2), butyrate (C4), caprylate (C8), decanonate (C10), laurate (C12), myristate (C14), and palmitate (C16), respectively, under the same conditions as reported earlier [23]. One unit of esterase is defined as the amount needed to release 1 μmol *p*-nitrophenol per minute from the *p*-nitrophenyl ester under the conditions specified. The highest enzyme activity on a substrate was defined as 100%. The standard curve was established *via* serial dilutions of *p*-nitrophenol. Using *p*-nitrophenyl butyrate as the substrate, the optimal temperature and pH of purified Est01 were determined, by measuring the enzyme activity after incubation at various temperatures (10–60°C) in 50 mM Tris-HCl buffer (pH 8.0) or after incubation at 20°C for 10 min in the following buffers [23]: 50 mM phosphate buffer (pH 5.0–7.0), 50 mM Tris-HCl (pH 8.0–10.0). The thermostability of the enzyme was determined by incubating the enzyme for up to 60 min at various temperatures (30°C, 40°C, and 50°C) before the activity assay. β-Lactamase activity against ampicillin was measured according to the established method [6].

Nucleotide Sequence Accession Number

The nucleotide sequence of the family VIII esterase gene (*est01*) has been deposited into the GenBank database under the accession number HQ444406.

Results and Discussion

Construction of Biogas Slurry Metagenomic Library and Enzymatic Screening for Lipolytic Activities

A 9,600 clones library was constructed. To estimate the quality of the library, plasmids of 21 randomly selected clones were prepared and subjected to restriction analysis. All 21 clones contained insert fragments. The average insert size was about 5 kb with a range from 3 to 9 kb. The library was estimated to contain approximately 48 Mb of metagenomic DNA. By functional screening of the library clones on LB agar plate containing 1% emulsified tributyrin, one clone with a clear halo, indicating lipolytic activity, was identified.

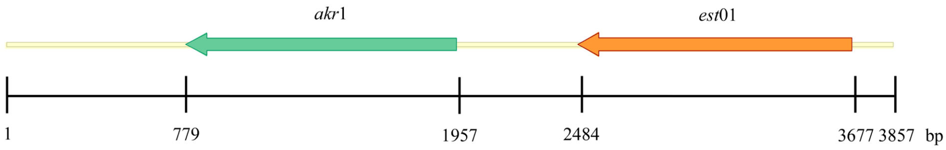

Fig. 1. Schematic diagram of the DNA insert of the plasmid isolated from the positive clone.
*akr*1 is a putative aldo-keto reductase gene, and *est*01 is a putative lipolytic enzyme gene encoding Est01.

Analyses of Nucleotide and Amino Acid Sequences of Est01

Two complete ORFs were revealed in the 3,857 bp insert DNA within the plasmid of the positive clone (Fig. 1). At the nucleotide level, both genes showed no significant similarity against the non-redundant nucleotide database of NCBI. One of the genes designated *est01* (1,194 bp) encoded a 397-amino-acid protein (Fig. 1). Amino acid sequence alignment indicated that this Est01 showed only moderate identity with other esterases/lipases, being highest with the lipase/esterase of an uncultured bacterium [17] (GenBank Accession No. AAZ32715, 33% identity). However, it showed higher identity (45%) with β-lactamase from *Paenibacillus* sp. JDR-2 (GenBank Accession No. YP_003012288). Another gene, designated *akr1*, encodes a putative aldo-keto reductase (Akr1) upstream the *est01* gene within the 3,857 bp insert fragment. Aldo-keto reductase is known to catalyze the biotransformation from sugar to alcohol. Hence, it is very likely that the putative functions of these two genes are related to the production of small molecules such as acetic acid, propanoic acid, methanol, and ethanol from fatty acids and sugars in the process of biogas fermentation [7]. The predicted protein (Est01) molecular mass is 44,804 Da, based on the deduced amino acids. Multiple sequence alignment of Est01 and other closely related proteins revealed that amino acid residues S-M-S-K (Fig. 2) from positions 68th to 71st represent a conserved catalytic S-X-X-K motif found in family VIII carboxylesterases [1, 13, 18], class C β-lactamases [14], and penicillin-binding proteins [12]. Additionally, Tyr184 (Y for abbreviation) of Est01 was conserved in all the proteins used for alignment (Fig. 2 black box). It is also reported that any substitution of these three sites will likely lead to most or complete loss of enzyme activity [18]. Hence, these conserved sites were considered to be signatures of these families.

To better understand the phylogenetic relationship of Est01 with other related proteins, a neighbor-joining tree was constructed by applying the deduced amino acid sequences. As shown in Fig. 3, unlike other members of family VIII esterases, Est01 is most closely related to β-lactamase of *Paenibacillus* sp. JDR-2 and then to the esterases LR1, EstC, and Est2k, which were derived from an uncultured bacterium of pond water [17], soil [18] and

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AAA99492       1  ......MDAQTIAPGFESVAELFGRFLSEDREYSAQLAAYHRGVKVLDISGGPHRRPDSVTGVFSCSKGV
CAD61039       1  ......MHSQVIAPGFEPVAELFGVFLEQDPDYSAQVAAYHRGVKVLDLSGGPHIRPDSVTGVFSCSKGM
ACH88047      39  AAGFSSAGVQALADGMRAAVDEGNLSGIVSALLRDGKLVHMDAYGYQDVENQIPVSEDTLFRIYSMTKPV
GQ426329      39  RVGLSADGIEDLKEGMRKAVDDGNLAGIVSALVRDNKLVFLDAYGYQDMENEVAMSEDSIFRIFSMTKPI
YP826528      38  SLGFSSERLGRLHDAMQRPVDEKALAGVVTLLMRHGKLVEQRSYGVKDMASGAPMTNDTIFRIYSMTKPV
YP002130700   71  AVGFDSARLARLDAYMAKVVESGRVAGMTTFLARHGKVVSFKTYGKKSLATGEPMTPDTIFRIYSMTKPV
AAZ32715       8  DIDLHPQRTQNIVHLLQERVAQQHIPGAVVLVARRGRIGLYEAIGQQDPAAGTPMRTDSIFRIYSMTKPL
YP003012288    1  ..MLDQTAVSQIKATLRKSIDNNEVAGASFMVIKDGEEIFYHEDGLADREAGRPVERNTIFRLYSMTKPV
Est01          4  SITIDINRLNRIQNQLNEAVESKTVAGCSCLVVRSGEELGYYEAGLRDIDKNLPITRDTIFRMFSMSKPI
P15555        31  AADLPAPDDTGLQAVLHTALSQG.APGAMVRVDDNGTIHQLSE.GVADRATGRAITTTDRFRVGSVTKSF
CAA09676      42  LQTNTQRDRTSVKQAMRDTLQLG.YPGILAKTSEGGKTWGYAA.GIADLRTKKPMKTDFRFRIGSVTKTF

AAA99492     118  LTLAEYNNSELAAAKLAQMRPLWKPGTAFGYHALTIGVFMEELCRRITGSTLQEIYEQRIRSVTGAHFFL
CAD61039     118  LTLHEVNNSELAAAKLAELPPLWKPGTAFGYHALTIGIFMEELCRRITGSTLQEVFEQRIRAVTGANFYL
ACH88047     179  SQVDSMYVAANILDANGTLKDMIDKLARIPLRQQPGTLWHYSVSVDVQ.GYLVEVLSGQTFEEFLQERLF
GQ426329     179  SQVDSQYVAANVLDATGTLKNMIDKLARIPLRQQPGTQWHYSVSVDVQ.GYLVEVLSGMPFDEFLETRLF
YP826528     178  TSIDKQYLARNCFGAANL.HAMTQCFAGIPLLFQPGSKWVYSVSMDIQ.GYIIEKLSGKPLAEFLEERIF
YP002130700  211  HPVDKLYREKQVLSASST.HEMIQRTAQIPLAFQPGTQWRYSSSVDIQ.GYIVEKLTGQRLGDFMAERIF
AAZ32715     142  SPVQRQYAEANIASRLRSNSDFSQTLAGIPLQFQPGSVWAYSRATDVL.GRLVEVVSGKTLGQFLHSEIF
YP003012288  138  EALFREIGERLLGESPMGTVEAMNKLGEGPLAFQPGTSWTYGTSADVL.GAVVEVVSGMRFGEFLQKEIF
Est01        144  AVFFDEAANKMHTENEMTTYELCNEIGKLPLAFNPGDKWNYGFSADVL.GAIVEVVSGMKYSEYLSANIF
P15555       150  MFAQTVPGFESVRNKVFSYQDLITLSLKHGVTNAPGAAYSYSNTNFVVAGMLIEKLTGHSVATEYQNRIF
CAA09676     166  KDVDFTD.....TKKSYTAEELVKMGISFPPDFAPGKGWSYSNTGYVLLGILIEKVTGNSYAEEVENRII
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Fig. 2. Multiple sequence alignment of the deduced amino acid sequences of Est01 and other related proteins.

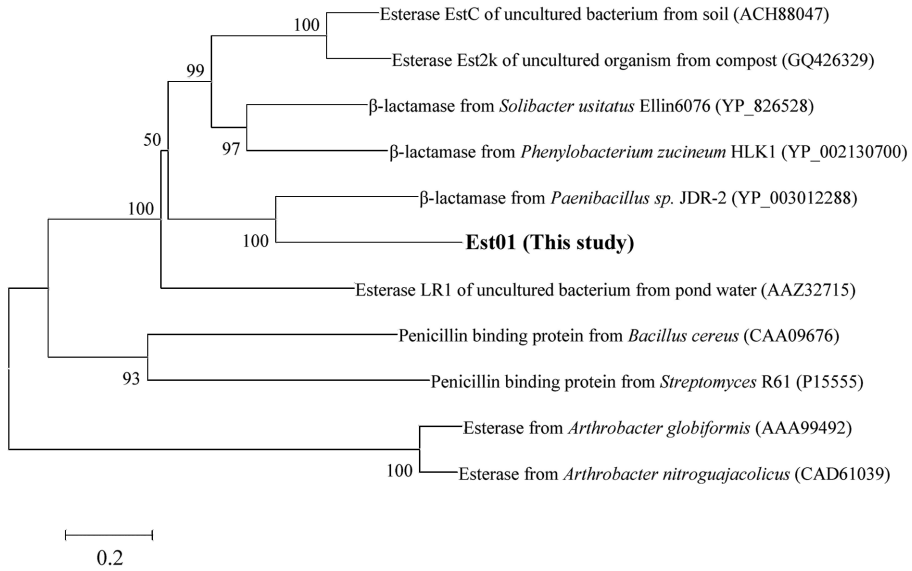

Fig. 3. Neighbor-joining tree showing the phylogenetic relationship of Est01 and closely related proteins from family VIII carboxylesterases, class C β-lactamases, and penicillin-binding proteins.

compost [13], respectively. This result coincides with the amino acid sequence similarity search result that strongly suggests Est01 as a new member of family VIII esterases.

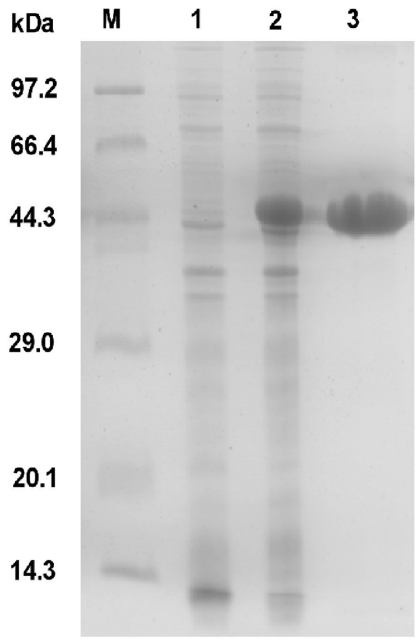

Fig. 4. SDS-PAGE (15%) of overexpressed recombinant Est01 in *E. coli* BL21(DE3).

Lane M, molecular weight protein marker; lane 1, total protein extract of *E. coli* harboring pET-30a(+), as negative control; lane 2, crude extract of *E. coli* harboring pET-30a(+) with the *est*01 gene; lane 3, purified Est01.

Expression and Purification of Est01

To investigate the hydrolyzing activity and biochemical properties of Est01, the *est01* gene was overexpressed in *E. coli* BL21 (DE3) using the pET-30a(+) expression system. The molecular mass of Est01 was about 44.3 kDa as analyzed by SDS-PAGE (Fig. 4), matching the molecular mass predicted from the amino acid sequence. After purification by Ni-IDA affinity chromatography, SDS-PAGE analysis revealed a single band, indicating that the recombinant Est01 was purified to homogeneity (Fig. 4).

Table 1. Substrate specificity of Est01.

Substrates	Specific activity[a] (U/mg)	Relative activity (%)
p-Nitrophenyl acetate (C2)	9.8 ± 0.6	35.9
p-Nitrophenyl butyrate (C4)	27.3 ± 0.5	100.0
p-Nitrophenyl octanoate (C8)	13.0 ± 0.3	47.6
p-Nitrophenyl caprate (C10)	1.2 ± 0.1	4.4
p-Nitrophenyl laurate (C12)	UD	
p-Nitrophenyl myristate (C14)	UD	
p-Nitrophenyl palmitate (C16)	UD	

UD, undetectable.

[a]The values shown are mean values ± standard deviation, derived from three independent experiments.

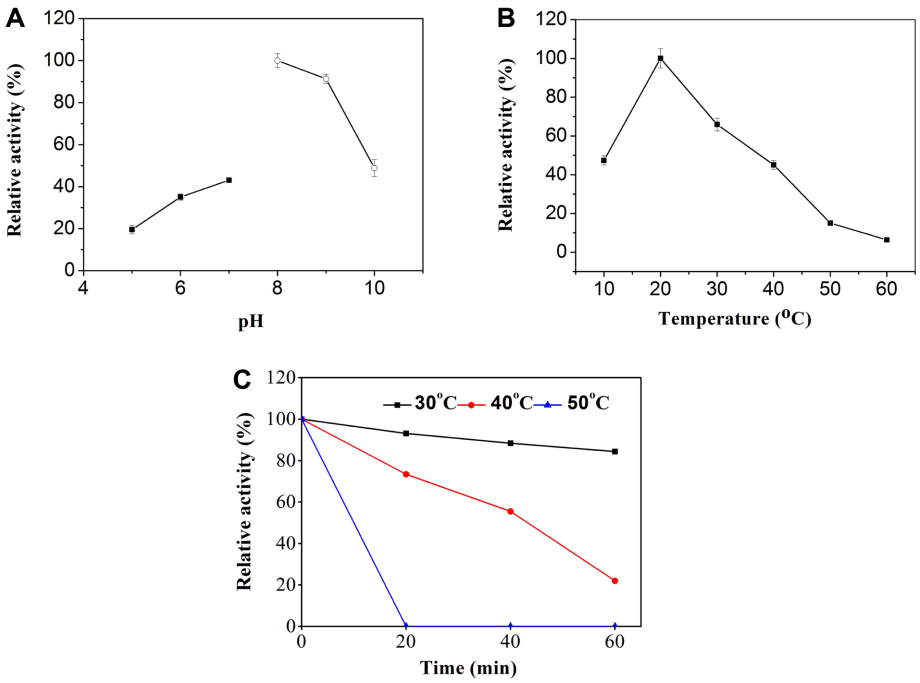

Fig. 5. Effects of temperature and pH on the enzyme activity of Est01, and the thermostability of Est01.
(**A**) Optimum pH; (**B**) optimum temperature; and (**C**) thermostability of Est01.

Substrate Specificity and Lactamase Activity of Est01

The substrate specificity of purified Est01 was studied using *p*-nitrophenyl esters as substrates. The results indicated that Est01 had high hydrolytic activity against short-chain fatty acids esters, and the relative activities toward C4, C8, and C2 were 100%, 47.6%, and 35.9%, respectively. Unlike esterases isolated from other environments, Est01 had no esterase activity toward long-chain fatty acids (≥ C10) (Table 1). This strong short-chain fatty acids preference of substrate utilization means that Est01 is a typical carboxylesterase rather than a lipase. The β-lactamase activity of Est01 against ampicillin was also measured. Surprisingly, the results showed that Est01 had no β-lactamase activity toward ampicillin, although it shares the highest identity to a β-lactamase. This is consistent with the case of EstA3, which also showed the highest identity to a β-lactamase but with no β-lactamase activity [6].

Effects of pH and Temperature on Est01 Activity

Est01 was active over a pH range of 5.0 to 10.0 and displayed maximal activity at pH 8.0 and with nearly 90% remaining activity at pH 9.0 (Fig. 5A), which means Est01 is an alkaliphilic esterase like these members reported previously [13, 18]. Est01 was active within a broad temperature range from 10°C to 60°C, with the highest hydrolytic activity found at 20°C. Above 20°C, as the temperature raised, the activity of Est01 decreased gradually (Fig. 5B). Est01 showed poor thermostability, since it lost 80% activity with only an incubation at 40°C for 1 h, and no activity could be measured only after 20 min incubation at 50°C (Fig. 5C), which is comparable to the case of Est2k [13]. Promisingly, it retained 43% residual activity when the temperature was as low as 10°C.

Moreover, the optimal temperature of Est01 was even lower than the esterase CHA3 [10] that was isolated from Antarctic desert soil, where the temperature is much lower. Taken together, these results suggest that Est01 is a low-temperature-adapted esterase. Considering the origin of the sample (collected from a well-operated biogas digester in the winter with average temperature nearly 15°C [4]), in which the Est01 producer has adapted well to low-temperature environment conditions, we conclude that Est01 displays some habitat-related characteristics.

In this study, we have confirmed that the cold-adapted esterase Est01 is a novel member of family VIII esterases. This promising enzyme could have a potential broad application in low-temperature hydrolysis systems related to various industrial processes for cost and energy savings. In addition, it also could be potentially re-introduced into rural household biogas digester systems to improve the

biogas yield in the winter, which may encourage approaches targeting the exploration of microbial enzymes or transgenic microorganisms in the process of biogas fermentation in the future. Lastly, this study also demonstrated that the metagenomic approach is very useful for obtaining special habitat-adapted enzymes using special-environment samples directly.

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