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Screening and Characterization of an Esterase from a Metagenomic Library

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Abstract A metagenomic library was constructed using a fosmid vector, and total genomic DNA was extracted directly from soil at Cisolok (hot spring area, Indonesia). This library was composed of 10,214 clones and screened for lipolytic enzyme on tributyrin agar plates. An esterase gene (*estMa*) was subcloned and sequenced from a positive lipolytic active clone. Esterase EstMa was encoded by a 954-bp open reading frame and showed low (11–33%) amino acid similarity to known esterases. The amino acid sequence analysis demonstrated that the enzyme is a new member of lipolytic enzyme family VI. The *estMa* gene encodes a preprotein of 317 amino acids with a predicted molecular mass of 34,799 Da. The purified enzyme exhibited optimal activity at 50°C and pH 6.5. The K_m and V_{max} values of EstMa for the hydrolysis of ρ-nitrophenyl valerate were 45.3 μM and 4.45 U/mg, respectively.

Key words: Esterase, library, metagenome, soil

The structure of microbial communities in many environmental samples is highly complex and diverse. A recent study estimated that 1 g of soil may contain up to 4,000 different species [22]. The complexity of these communities is not only an intriguing subject, but also a big challenge to biotechnology. Current estimates indicate that less than 1% of the microorganisms existing in many environments are readily culturable [1].

Metagenomics is a new and rapidly developing field that tries to analyze the complex genomes of microbial niches. Although the term "metagenome" has been introduced only recently for describing the genomes of noncultivated microbes existing within a soil microbial community [5], the new technology has been used to overcome the difficulties of culturing microorganisms from natural environments and cloning and expressing their DNA in surrogate expression hosts [12, 16, 17]. Metagenomic DNA libraries that recover functional genes from uncultivated bacteria provide a promising discovery tool, and recent studies have begun to confirm the enormous potential of this technology for discovering new enzymes and small molecules [12, 17, 19, 20, 23].

Esterases (E.C. 3.1.1.1) display a broad spectrum of substrate specificities and mediate reactions to synthesize or cleave the ester bond of molecules. These enzymatic reactions have versatile applications in the food, pharmaceutical, and fine chemical industries [7, 14, 15].

Accordingly, we attempted to study esterases from metagenomic libraries. For this purpose, we constructed a metagenomic library by direct cloning of environmental DNA that contains large DNA inserts, and then screened it for the presence of genes conferring lipolytic activity. The novel gene encoding lipolytic activity was recovered, sequenced, and then the corresponding gene product was expressed in *E. coli* and partially characterized.

Materials and Methods

Bacterial Strains, Vectors, Media, and Environmental Sample

The vectors and *E. coli* strains used in this experiment are described in Table 1. The recombinant *E. coli* was grown at 37°C on LB medium supplemented with appropriate antibiotics. A soil sample from the hot spring area (70°C, pH 7.0–7.5) was obtained at Cisolok in Indonesia, August 2003. The sample was taken from the top 10 cm of soil after removing leaf litter.

DNA Extraction from Soil

Soil (100 g) was suspended in 150 ml of soil DNA extraction buffer and incubated at 65°C for 2 h with occasional gentle shaking. The suspension was extracted with an equal volume of chloroform, and DNA in the

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Table 1. Microbiological materials used in the experiment.

Material	Relevant trait (s)	Source
E. coli strains		
Top10	F⁻, *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ (*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
EPI300	[F⁻ mcrA Δ(*mrr-hsd*RMS-*mcr*BC) φ80d *lac*Z ΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ (*ara, leu*)7697 *gal*U *gal*K λ⁻ *rps*L *nup*G *trf*A]	Epicentre
M15 (pREP4)	Expression host for pQE30 vectors	Qiagen
Plasmids and fosmid		
pBKS	pBluescript II KS multicopy cloning vector, Amp^r	Stratagene
pQE30	Expression vector	Qiagen
pCC1Fos	Fosmid cloning vector for the construction of DNA libraries, Cm^r	Epicentre

supernatant was precipitated with isopropanol [25]. Precipitated DNA was dissolved in 500 μl of water.

PFGE (Pulsed Field Gel Electrophoresis)

Genomic DNA plugs, which were mixed with low-melting-point agarose (Bio-Rad Laboratories, Richmond, CA, U.S.A.), were loaded in a 1.0% PFGE agarose gel in 0.5×TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA) and subjected to electrophoresis on a CHEF DR II apparatus (Bio-Rad) (initial switching time, 10 sec; final switching time, 100 sec; run time, 16 h; temperature, 14°C; angle, 120°; voltage gradient, 4.5 V/cm). After electrophoresis, DNA-containing regions (>30 kb) was cut from the gel and electroeluted to improve cloning efficiency.

Construction and Screening of Metagenomic Library

The electroeluted DNA was enzymatically treated to prepare 5' phosphorylated blunt end and ligated into fosmid vector pCC1FOS (Epicentre, U.S.A.) for 2 h at room temperature. After heat inactivation, the ligated DNA was packaged using λ packaging extract at 30°C. The fosmid particles were introduced into EPI300 host cell according to the manufacturer's protocol, and the cells were spread onto LB agar plates containing 12.5 μg/ml chloramphenicol (Cm). To detect lipolytic activity, the library was spread to plates containing LB agar plus 1% tributyrin (ACROS, U.S.A.) and detected for the presence of a clear halo after 3 days of culture [11].

Shotgun Cloning of Selected Fosmid CL2 Plasmid

A shotgun cloning library of fosmid clone CL2 was prepared by partially digesting with *Sau*3AI. DNA fragments of 2–3 kb and 3–5 kb were ligated into the dephosphorylated *Bam*HI site of pBluescript II KS. The ligation products were transformed into *E. coli* Top10. The transformants were examined and confirmed again for lipolytic activity and named pCL2. The DNA sequencing was performed using a Basestation sequencer (MJ research, Inc., U.S.A.), and open reading frames were identified by the vector NTI Suite program and translated using the NCBI databases.

Subcloning and Expression of Esterase Gene (*estMa*) in *E. coli*

PCR was performed with two primers, CL2 FW (5'-GGA TCC ATG AAG CCA AAA CAT GAA-3') and CL2 RV (5'-CTG CAG CTA GTG GCG TGC GGG TTT-3'), which included restriction enzyme sites (underlined) for *Bam*HI and *Pst*I, respectively, with pCL2 plasmid DNA as a template. The PCR product was cloned into the pGEM-T Easy vector and transformed into *E. coli* Top10. The selected transformant was on LB medium-ampicillin (100 μg ml^{-1}) plates containing 0.01% X-Gal digested with *Bam*HI and *Pst*I and ligated into pQE30 cut with *Bam*HI and *Pst*I, yielding pQECL2. For expression of the recombinant enzyme, *E. coli* M15 cells transformed with pQECL2 were aerobically grown at 37°C in 1 l LB broth containing 100 μg of ampicillin and 50 μg of kanamycin per ml, and induced with 1 mM IPTG. After 5 h, cells were harvested, disrupted, and pQECL2 was purified using the His-Bind resin (Novagen) column following the manufacturer's instructions.

Molecular Mass Measurement

The relative molecular mass of the purified esterase was estimated by SDS-PAGE, using 12% (w/v) acrylamide gel according to the method of Laemmli [10]. Proteins were stained with Coomassie brilliant blue R-250 (Sigma).

Enzyme Assay

Activity was measured spectrophotometrically with ρ-nitrophenyl butyrate (pNPB) as a substrate, which was dissolved in acetonitrile at 10 mM concentration. Subsequently, ethanol and 50 mM potassium phosphate buffer (pH 7.5) were added to a final composition of acetonitrile/ethanol/buffer at 1:4:95 (v/v/v) [4, 9]. An appropriate amount of enzyme (0.3 ml) was added to the substrate solution (0.9 ml), and the mixture was then incubated at 50°C. After 10 min, the enzyme activity was measured by monitoring the change in absorbance at 405 nm that represents the amount of released ρ-nitrophenol (PNP). One unit of lipase activity is defined as the amount of enzyme to release 1 μmol of PNP per min under the assay conditions [24].

Characterization of Enzyme

Optimum temperature for the enzyme was evaluated using the above enzyme activity assay with pNPB at different temperatures. The assay was performed at pH 7.5.

The pH optimum of the enzyme was measured at 50°C and 348 nm using the buffer solutions of different pH values. The following buffers (50 mM) were used: sodium acetate (pH 5.0–6.0), potassium phosphate (pH 6.0–8.0), and Tris-HCl (pH 8.0–9.0).

Substrate specificity towards different p-nitrophenyl esters (pNPE)s was spectrophotometrically analyzed as described above. The pNPE substrates (C4-C18) were dissolved in acetonitrile at 10 mM concentration [8].

Kinetic Parameters

The Michaelis-Menten kinetic parameters V_{max} and K_m for the enzyme were calculated using pNPE (C4-C6) as a substrate. Lineweaver-Burk plots were used to determine the parameters, assuming that simple Michaelis-Menten kinetics was followed.

Nucleotide Sequence Accession Number

The nucleotide sequence data reported here are available in the GenBank databases under accession no. AY833094.

Results and Discussion

Construction of Metagenomic Fosmid Library

To obtain genomic information on the soil microbes that are not readily culturable, we extracted and cloned large DNA fragments directly from hot spring soil. We then constructed the Cisolok metagenomic library in pCC1FOS. This library was composed of 10,214 clones and contained insert DNA with an average insert size of 40 kb. We estimated that there is approximately 409 Mbp DNA contained.

Screening Library for Lipolytic Activity

To investigate the functional diversity and identify clones expressing metagenomic DNA, we initially screened on the plate assay and found 10 clones expressing lipolytic activities. Among the 10 positive clones, the *E. coli* clone that showed the highest activity by clear zone was designated as CL2. To confirm the lipolytic activity, two types of indicator plates were employed: Esterase activity was detected on tributyrin agar plates, and lipase activity was detected on LB agar plates containing olive oil and rhodamine B. A clear halo was detected only on the tributyrin plates, and therefore, we concluded that CL2 was esterase.

Cloning and Sequence Analysis of EstMa

In order to isolate the gene that was responsible for the esterase activity, DNA from fosmid clone CL2 was digested with *Sau*3AI and inserted into pBluescript II KS. The ligation mixture was transformed into *E. coli* Top10. The recombinant *E. coli* strains were screened again on LB tributyrin agar plates. Among the transformants, the clone pCL2 that formed a clear halo was sequenced, and its sequence was compared with the sequences available in the National Center for Biotechnology Information (NCBI) databases. The restriction map and the localization of the identified gene are given in Fig. 1.

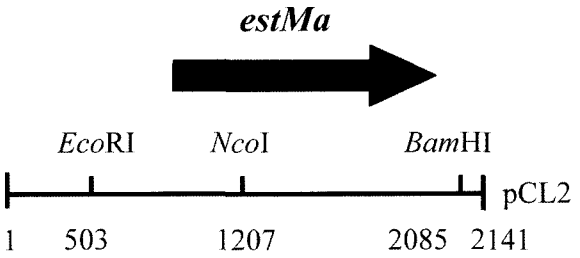

Fig. 1. Genetic organization and the length of the insert of pCL2.
The arrow and arrowhead represent the length, location, and orientation of the potential gene within the insert. The arrow of potential gene, which was identified as being responsible for the esterase of the *E. coli* clones, is shaded gray.

The plasmid pCL2 (2,141 bp) contained an open reading frame of 954 bp, designated *estMa*. The deduced amino acid sequence, consisting of 317 residues, showed 32% and 30% identity to the hypothetical protein from *Thermotoga maritima* (accession no. F72424) [13] and the predicted peptidase from *Anabaena variabilis* (accession no. ZP_00160764), respectively. Among the lipolytic enzymes sequences, esterase EstMa is the closest (33%) to the LipC produced by *Mycobacterium avium* (accession no.

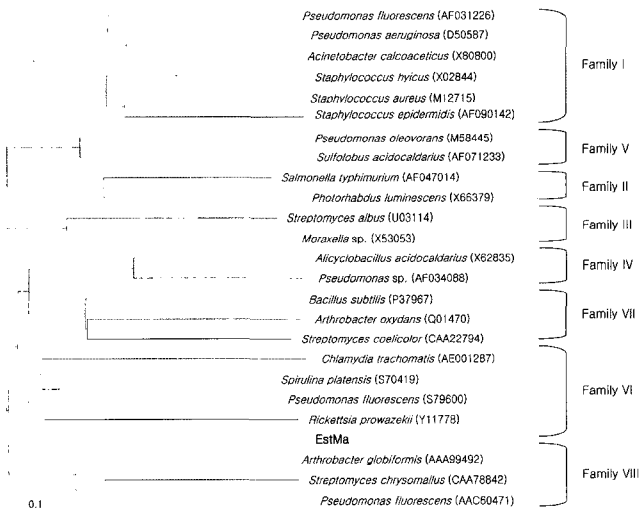

Fig. 2. Phylogenetic relationship between EstMa and related microorganisms.
The branching pattern was generated by the neighbor-joining method. The accession numbers of the aligned sequences are shown in parentheses. The scale bar indicates 0.1 nucleotides substitutions per nucleotide position.

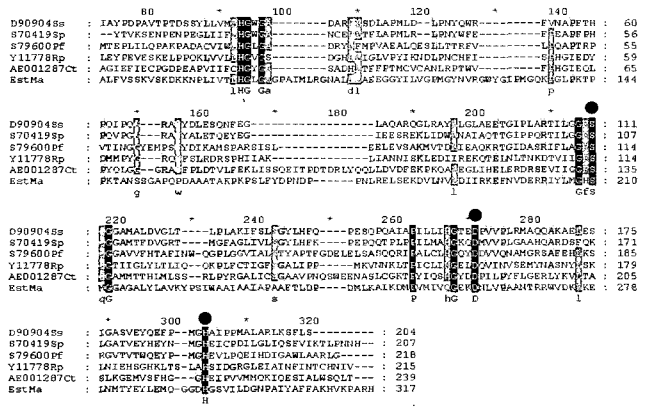

Fig. 3. Alignment of amino acid sequences in the family VI from various microorganisms.
D90904Ss, esterase from *Synechocystis* sp.; S70419Sp; serine esterase from *Spirulina platensis*; S79600Pf, esterase II from *Pseudomonas fluorescens*; Y11778Rp, esterase from *Rickettsia prowazekii*; AE001287Ct, predicted lysophospholipase esterase from *Chlamydia trachomatis*; EstMa, purified esterase. Symbol: ●, amino acid residues belonging to the catalytic triad. Compare the motifs surrounding the active-site serine residue.

AAS06206) and more distantly (27%) to a putative esterase from *Photobacterium profundum* (accession no. CAG19196). Lipolytic enzymes from prokaryotes are classified into eight families (I to VIII) according to their amino acid sequences [2]. Based on this classification, phylogenetic analysis of EstMa showed that this enzyme is close to the family VI (Fig. 2). In comparison with enzymes belonging to the family VI, EstMa showed very low similarities to esterase II from *Pseudomonas fluorescens* (15.1% identity) [6], esterase from *Synechocystis* sp. (11.1% identity), serine esterase from *Spirulina platensis* (17.5% identity) [18], esterase from *Rickettsia prowazekii* (16.1% identity), and predicted lysophospholipase esterase from *Chlamydia trachomatis* (13.7% identity) [21], indicating that EstMa is a new member of the family VI lipolytic enzymes. These members commonly harbor the GXSXG motif. The pentapeptide GHSMG, including catalytic serine, was conserved in the enzyme at positions 207 to 211, suggesting that Ser210 is the catalytic residue in the enzyme [3]. A sequence alignment among the family VI enzymes indicated that the other catalytic components (Asp261 and His293) are conserved (Fig. 3).

Overexpression and Purification of Recombinant EstMa

Plasmid pQECL2 harboring the *estMa* gene was constructed in *E. coli* M15. From the crude extract, the enzyme was easily purified by NI–NTA affinity chromatography to give a single band on SDS-PAGE (Fig. 4). The apparent molecular weight of EstMa was 34 kDa when analyzed on SDS-PAGE, which was in good agreement with the deduced amino acid sequence (34,799 Da). Sequence analysis of the SignalIP server (http://www.cbs.dtu. dk) revealed a putative

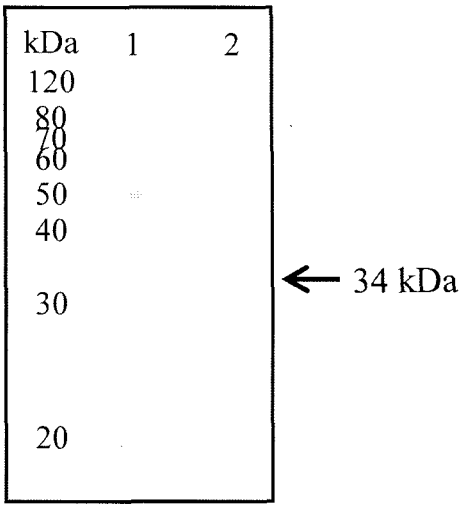

Fig. 4. SDS-PAGE of the purified EstMa.
Lanes: 1, molecular weight markers; 2, purified EstMa after NI-NTA affinity chromatography.

signal sequence of 29 amino acids and 288 amino acids processed enzyme, and a molecular mass of 31,615 Da was predicted.

Characterization and Kinetic Properties of Recombinant EstMa

The effect of pH on EstMa activity was investigated using p-nitrophenyl butyrate as a substrate. Absorption of p-

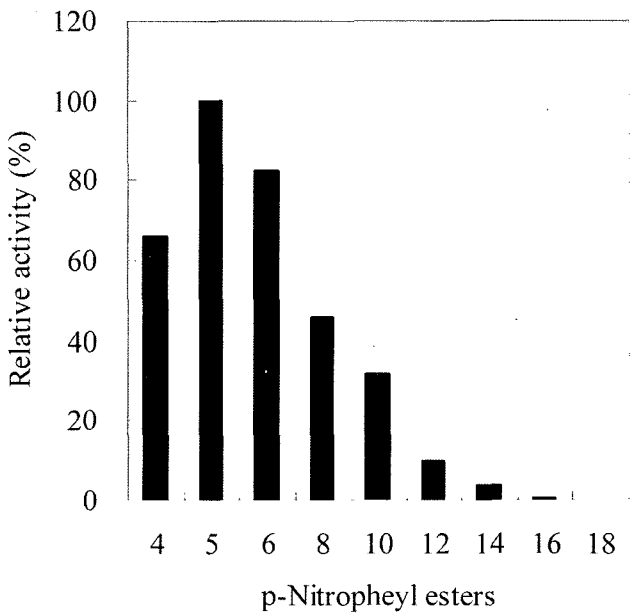

Fig. 5. Effect of various p-nitrophenyl esters (pNPE)s on the activity of the purified EstMa.
The enzyme activity on various substates was calculated relative to that on C5. C4, pNP butyrate; C5, pNP valerate; C6, pNP caproate; C8, pNP caprylate; C10, pNP caprate; C12, pNP laurate; C14, pNP myristate; C16, pNP palmitate; C18, pNP stearate.

Table 2. Kinetic parameters for recombinant EstMa.

Substrates*	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)
pNP-C4	186.7	195.5	1,047.1
pNP-C5	45.3	155.8	3,439.3
pNP-C6	172.0	367.3	2,135.5

* pNP-C4: ρ-nitrophenyl butyrate; pNP-C5, ρ-nitrophenyl valerate; pNP-C6, ρ-nitrophenyl caproate. Data are reported as the mean of two independent experiments.

nitrophenol varies, when pH is altered. Therefore, the release of p-nitrophenol was monitored at 348 nm, the isosbestic point of p-nitrophenol and p-nitrophenoxide. The activity was measured in the pH 5.0 to 9.0 range. EstMa showed strong activity at acidic pH with an optimal pH of 6.5.

The effect of temperature on esterase activity was investigated using p-nitrophenyl butyrate as a substrate. The activity was measured in the range of 30°C to 80°C, and the enzyme showed an optimal temperature of 50°C. The *Pseudomonas fluorescens* esterase II [6] belonging to this family has its optimum pH from 6.0 to 9.0 and maximum activity at 45°C.

We examined the substrate specificity of the enzyme using pNPEs with varying acyl chain lengths (C4-C18) as substrates (Fig. 5). EstMa showed higher hydrolytic activity towards esters with short to medium chain lengths, with pNP-C5 the best substrate, whereas it showed little hydrolytic activity towards those with more than 12 acyl chain. Therefore, this result indicates that EstMa harbored the esterase gene.

Kinetic experiments were performed by the standard activity assay with pNP-C4, pNP-C5, and pNP-C6 as substrates. As summarized in Table 2, the K_m and V_{max} values for this enzyme were determined to be 45.3 μM and 4.45 U/mg, respectively, with pNP-C5 as a substrate. It was found that the EstMa had higher affinity but lower turnover number for pNP-C5. The specificity constant k_{cat}/K_m showed a maximum value of 3,439.3 ($s^{-1}mM^{-1}$) for pNP-C5, and this value was 3.3-fold and 1.6-fold higher than those of pNP-C4 and C6, respectively.

To date, only two bacterial esterases belonging to this family of lipolytic enzymes have been characterized: an esterase II from *Pseudomonas fluorescens* [6] and an esterase from *Spirulina platensis* [18]. Optimum pH and temperature were similar to esterase II from *Pseudomonas fluorescens*. However, as described in Table 3, some of the properties of this enzyme, such as molecular weight and substrate specificity, were different from the reported two esterases. Enzymes belonging to the family VI are known to be the smallest esterases with molecular mass in the range of 23–26 kDa [2]. However, the molecular weight of EstMa is larger than already known family VI enzymes. Compared with EstMa in this study, the esterase II from *Pseudomonas fluorescens* showed higher substrate specificity toward pNP-C2 than pNP-C5, and the highest activity was observed with pNP-C2 (relative activity, 131%), but hydrolysis of pNP-C5 resulted in significantly lower activities (relative activity, 15%). Based on amino acid identity and preference for pNP-C5 as a substrate in this study, EstMa appears to have unique features that are different from previously known esterases.

In conclusion, a novel EstMa was uncovered during the screening of a metagenomic library. The deduced amino acid sequences revealed only little identity (<33%) with any other sequence available in the databases. Our results indicate that the constructed DNA libraries harbor genes from microorganisms of which many have not been reported or even cultivated, and demonstrate that the gene expression of foreign DNA cloned into fosmid vector was maintained in *E. coli*. The fact that the presently identified esterase gene has a novel sequence suggests that the metagenomic library provides a rapid and effective method to find novel and functional genes.

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Table 3. Comparison of properties of various family VI enzyme-producing strains.

Enzymes	Opt. temp. (°C)	Opt. pH	Substrate* specificity	Molecular weight (kDa)	Kinetic data
EstMa	50	6.5	pNP-C5	34	Table 2
Esterase II from *Pseudomonas fluorescens*	45	6.0–9.0	pNP-C2	23	-
Esterase from *Spirulina platensis*	-	-	-	23	-

* pNP-C2: ρ-nitrophenyl acetate; pNP-C5, ρ-nitrophenyl valerate.
-: No result.

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