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Screening and Identification of a Novel Esterase EstPE from a Metagenomic DNA Library

So-Youn Park[1], Hyun-Jae Shin[2], and Geun-Joong Kim[1]*

[1]*Department of Biological Sciences, College of Natural Sciences, Chonnam National University, Gwangju 500-757, Republic of Korea*
[2]*Department of Chemical Engineering, Chosun University, Gwangju 501-759, Republic of Korea*

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Esterases represent a large family of hydrolases with broad substrate specificity and functional sequence space. Although many attempts to screen new esterases have been conducted, there have been few reports conducted to discriminate unique enzymes from typical ones based on novel structure and function. In this study, we discovered an esterase and a novel family through a successive assay of whole cells and crude lysates (oxidative open condition). The screened putative esterases from the metagenomic DNA of salted shrimp consisted of 753 bp encoding 27 kDa of polypeptide, namely PE esterase. Sequence analyses revealed that an identical gene was reported from whole genome sequencing of *Stenotrophomonas maltophilia* K279a. However, its biochemical and phylogenetic characteristics have not yet been evaluated. PE esterase was overexpressed only by the MBP fusion state in *E. coli* and was easily purified using an affinity column. This enzyme showed a typical spectrum of substrate specificity and possessed the consensus motifs, Ser-Asp-His and GXSXG, which are essential for most esterase/lipase superfamilies. Interestingly, the entire organization of the ORF and consensus sequence around the active site were distinct from the related enzymes, and its structure could be affected by a reducing agent, DTT.

***Keywords*:** esterase, oxidative modulation, novel family, metagenome

Metagenomics is an extended field of research into actual biodiversity that has been growing since it was recognized that the majority of all microbes have not yet been cultivated (Handelsman, 2005). During the last decade, metagenomics have primarily focused on redefining and expanding our knowledge of natural diversity and ecofunction, as well as screening novel enzymes and functional biomolecules. Thus, metagenomics has allowed evaluation of natural microbial ecology and entire metabolic networks, thereby providing a diverse biosynthetic or degradative pathway (Quinn *et al*., 2007; Nguyen *et al*., 2008; Fisch *et al*., 2009; Hertweck, 2009). Specifically, metagenome resources have a higher potential to show the sequence, scaffold and activity space than proteins deposited in databanks or that have evolved *in vitro*. The narrow activity and/or sequence space of the mined proteins might be closely linked to the limitations of conventional screening approaches from libraries and the expression of interests in heterologous hosts. Therefore, metagenomics can yield more meaningful insight and/or products to help enable a better understanding of natural diversity and structural space, especially when coupled with improved access strategies and newly developed mining methods.

Esterase is a generic term for a hydrolase that catalyzes the cleavage and formation of ester bonds. In a narrow sense, esterases are represented by carboxyl esterase, and then further classified into 20 subfamilies based on their substrate specificity or sequence alignments (Bornscheuer, 2002). Esterases are widely distributed in nature and play a major role in the degradation of natural compounds or industrial pollutants owing to their high stereoselectivity and broad range of substrate spectrum. Thus, esterases dominate the industrial market for the food, medicine, biodiesel, and agricultural industries (Bornscheuer, 2002; Toke *et al*., 2007). The attractive features of esterases, including lipase, are that they do not require cofactors and are usually stable, and can even be active in organic solvents. Therefore, many esterases have consistently been screened from various resources including metagenomes. However, screening of esterases with new functional sequences and/or structures is rare because of the many barriers that exist in typical screening procedures (Chu *et al*., 2008; Li *et al*., 2008; Tirawongsaroj *et al*., 2008).

In this study, the fermented food, salted shrimp, was selected as a resource for new enzymes with novel properties. It is well known that the transition of aerobic to anaerobic conditions occurs during this fermentation process. During the fermentation of salted shrimp, microorganisms produce or secrete some enzymes to break the cell wall or membrane of the shrimp. This means that hydrolases (esterease, lipase, and protese, etc.) are involved in this step, and their structure or activity can be influenced by environmental oxidation potential.

Here, we describe the screening and identification of a metagenome-derived gene encoding a novel esterase, EstPE. The enzyme exhibited a typical esterase activity and consensus sequence, GXSXG, but had a unique feature of a primary structure throughout the entire region and composition of the consensus sequence (Met was present in both X residues). Based on analyses of the relationship between the structure and function, the enzyme showed plausible evidence that structure

* For correspondence. E-mail: gjkim@chonnam.ac.kr; Tel: +82-62-530-3403; Fax: +82-62-530-3409

and/or function could be modulated when treated with a reducing agent. Thus, the results presented here provide a first clue into the functional modulation of the esterase by oxidation.

Materials and Methods

Plasmids, strains, and culture conditions

E. coli XL1-Blue (Stratagene) was used as the host for construction of a metagenomic DNA library and as the expression host. The plasmid pMAL-c2x (New England Biolabs) was used for high level expression and simple purification. *E. coli* cells were routinely cultured at 37°C in LB media supplemented with 50 μg/ml ampicillin. Competent cells were prepared by washing with Inoue transformation buffer after cultivation in SOB medium (Inoue *et al*., 1990).

Purification of metagenomic DNA

The total chromosomal DNA was isolated from a resource (salted shrimp) according to a typical procedure (Miller *et al*., 1999). The sample was mixed with 100 mM Tris-HCl (pH 8.0) and incubated at 37°C for 30 min with constant shaking (220 rpm). To remove the shrimp, the sample was centrifuged at 500×g for 1 min. The supernatant was then transferred to a new tube and centrifuged at 13,000×g for 10 min. Next, the recovered cells were resuspended in lysis buffer containing 100 mM EDTA (pH 8.0), 15 mg/ml lysozyme and 10% SDS, after which they were incubated for 1 h at 65°C. The crude extract was then centrifuged at 10,000×g for 30 min, and the resulting solution was transferred to a new tube containing 0.5 M potassium acetate. To precipitate the proteins and polysaccharides, the mixture was centrifuged at 10,000×g for 10 min. The recovered solution was then treated with an equal volume of isopropanol, after which the precipitate was harvested by centrifugation and washed with 70% (v/v) ice-cold ethanol. The resulting metagenome was dried and resuspended in pure water when needed.

Construction of a cloning vector

A scaffold of expression vector, pTrc99A, was employed for construction of a cloning vector that discriminated positive clones by insertional inactivation of a fluorescent reporter. To accomplish this, the GFPuv gene was amplified by PCR using a forward 5′-TGGATG AGTAAAGGAGAAGAACT-3′ and reverse primer 5′-TTTAAGCTT TTATTTGTAGAGCT-3′ from pGFPuv (Clonetech). The amplified DNA fragment obtained using *Pfu* polymerase was treated with *Hind*III. The resulting gene, which had a blunt end-*Hind*III terminus, was introduced into pTrc99A at the same sites. The blunt end terminus of pTrc99A was generated using the Klenow fragment (Promega) after it was digested with *Nco*I. After being confirmed by DNA sequencing, this construct (pD3-P2) was further used to prepare a library by insertion of metagenomic DNA into the innate *Bam*HI site of the ORF encoding GFPuv.

Library construction and screening

The metagenomic DNA was randomly digested with *Bam*HI. The resulting DNA fragments (2-6 kb) were fractionated on agarose gel and purified using a DNA clean up system (Promega). The eluted fragments were cloned into the *Bam*HI site of pD3-P2 and transformed into *E. coli* XL1-Blue. The transformed cells were then cultured at 37°C in LB agar medium for 20 h. To screen for clones with esterase activity, activity staining was conducted using an overlaid soft agar (0.8%) containing α-naphthyl acetate (45 μg/ml) and fast blue RR (45 μg/ml) (Kim *et al*., 2004). These clones were further assayed for final selection under oxidation-sensitive open conditions with cell lysate.

The insert of the finally screened clone was sequenced using universal primers specific for pTrc99A. The open reading frames and their probable functions were predicted using ORF finder and the BLAST programs of the NCBI.

Cloning and expression of an esterase gene

To amplify a predicted esterase gene, the synthetic primers H-HCPB (5′-TATGGATCCATGATGAGCGGTTG-3′) and H-CHP (5′-TATAA GCTTTCAGCGCTTCTGCG-3′) were used for PCR. The resulting gene was purified and digested with *Bam*HI and *Hin*dIII, after which it was subcloned into an expression vector, pTrc99A, to produce pTrc-PE11. To analyze the expression pattern and solubility, this gene was also subcloned into the same sites of the expression vectors pMAL-c2x and pQE30, yielding two constructs, pMAL-PE13 and pQE-PE11, respectively.

For protein expression, a single colony of transformants was seeded in 5 ml of LB medium and then incubated overnight. After 1% of the culture broth was reseeded in 15 ml of the same medium, the recombinants cells were further grown at 37°C to an OD_{600} of 0.4-0.5 and then induced with 1 mM IPTG for 90 min. Following centrifugation, an aliquot (1 mg of wet weight) of harvested cells was loaded onto a 10% SDS-PAGE gel. Transformants harboring empty vector or a previously reported pQE-1767 containing a typical esterase were used as controls (Kim *et al*., 2003).

Protein purification

The MBP-fused protein was single-step purified using a MBP-trap affinity column (GE Healthcare). To accomplish this, recombinant cells were seeded in LB liquid medium and then incubated at 37°C overnight. After 1% of the culture medium was reseeded in 1 L of the same medium, the recombinant cells were further grown at 37°C to an OD_{600} of 0.45-0.5 and then induced with 0.3 mM IPTG for 90 min. The cells were recovered using a centrifuge and washed twice with DDW, after which they were suspended in 50 ml of lysis buffer (20 mM Tris-HCl, 1 mM EDTA, and pH 7.0) and disrupted by ultrasonification. After centrifugation at 15,000×g for 30 min, the supernatant was treated with 1 g of CDR (cell debris remover) and then again centrifuged at 15,000×g for 30 min. The protein solution was subsequently diluted 1:10 using a column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, and pH 7.4) and loaded onto an affinity column using the FPLC system at a flow rate of 1.5 ml/min. The column was completely washed with the same buffer and then eluted with a buffer containing 10 mM maltose. The fusion protein was dialyzed against 50 mM Tris-HCl (pH 7.4) buffer and treated with Factor Xa for 2 days at 4°C. The resulting mixture was loaded onto the same affinity column to remove the MBP. The active fraction having an esterase was quantitatively analyzed using a protein assay kit (Pierce).

Gel electrophoresis and activity staining

The apparent homogeneity and molecular mass of the purified PE esterase was determined by 10% SDS-PAGE according to a general procedure. For the zymogram assay, the purified enzyme was mixed with a native sample buffer containing 50% glycerol and resolved on 8% native PAGE. After electrophoresis, the separating gel was washed completely with a 50 mM Tris-HCl (pH 7.3) buffer, after which it was soaked in the same buffer containing α-naphthyl acetate (45 μg/ml) and Fast Blue RR (45 μg/ml). The active band position

was rapidly (<10 min) visualized as a deep brown color (Kim *et al*., 2004).

DTT effect on structure

The conformational change in PE esterase was analyzed using a size exclusion chromatography column (Superose12, GE Healthcare) and a fluorescence spectrophotometer (RF-5301PC, Shimadzu) after pre-incubation with DTT (dithiothreitol, 1-5 mM) at 37°C for 10 min. When size exclusion chromatography was conducted, the flow rate of the mobile phase containing 50 mM Tris-HCl (pH 7.3) was fixed at 0.5 ml/min and the elution profiles were monitored at 280 nm. The fluorescence of the PE esterase was analyzed according to a general procedure, with excitation at 280 nm and emission at 300-450 nm (Leal *et al*., 2005).

Enzyme assay and analysis

The specific activity and substrate specificity were determined by releasing the amount of PNP (*p*-nitrophenol) from *p*-nitrophenyl butyrate (C4), *p*-nitrophenyl decanoate (C10), *p*-nitrophenyl myristate (C14) and *p*-nitrophenyl palmitate (C16). The purified enzymes (1 μg) were incubated with an ester derivative (1 mM) in a Tris-HCl buffer (pH 7.3) at 37°C for 10-20 min (Gupta *et al*., 2002; Kim *et al*., 2004). After incubation, a reaction solution was mixed with methanol to stop the reaction. The concentration of *p*-nitrophenol was then determined by HPLC. The column and mobile phase were a C18 column (HPLC, Shimadzu) and 50% acetonitrile, respectively. PNP was monitored at 405 nm at a constant flow rate (0.5 ml/min). One unit of enzyme activity was defined as the amount of enzyme producing 1 μmole of *p*-nitrophenol per minute at 37°C.

Nucleotide sequence accession number

The insert sequences of pTH5-2 have been deposited in the GenBank database under accession number GU564231.

Results and Discussion

Construction of a metagenomic DNA library

For library construction, the *Bam*HI site in the GFPuv gene was used as the cloning site for the metagenomic DNA fragment. In this case, cloning was simply identified by fluorescence using a UV hand lamp (365 nm) because the reporter was inactivated by insertion of foreign DNA. As a preliminary experiment, a pD3-P2 vector harboring a fluorescence protein was constructed and its fidelity was evaluated by counting the insert-harboring frequency of the no-fluorescence clones. Almost all clones (>98%) with no-fluorescence had an insert. Thus, the pD3-P2 vector was found to be an efficient system for cloning metagenomic DNA due to simple detection and high fidelity. For this reason, we constructed a metagenomic library using pD3-P2 vector by insertion of *Bam*HI-digested fragments (2-6 kb) of metagenomic DNA. The pool of recombinant genes was transformed into *E. coli* XL1-Blue, and no-fluorescence clones were selected and transferred onto freshly made solid media, resulting in >4,000 independent clones.

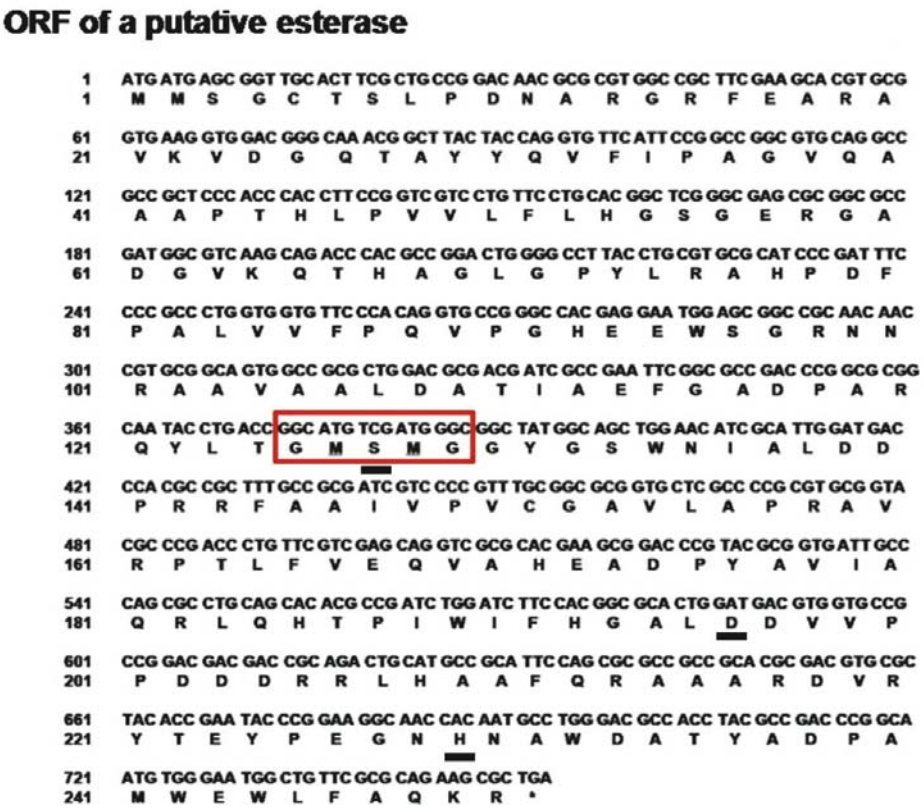

Fig. 1. Nucleotides and deduced amino acid sequence of a putative esterase from the screened clone. The putative active site and catalytic triad were indicated by the box and underlines, respectively.

Screening and analyses of a gene encoding an esterase

We identified four active clones from the metagenome library by typical activity staining of whole cells in solid plates. Subsequent assays in solution also revealed a distinct activity when compared with those of solid plates. Two of the four clones were determined to have decreased activity of less than 30-60% of the whole cell enzymes in cell lysate (oxidative open condition), although some fluctuation was observed. Generally, cell lysates have more activity than whole cell enzymes because the barrier (cell wall and membrane) for substrate transport has been removed. Thus, these clones were chosen as candidates for further analyses and found to be the same gene upon restriction enzyme mapping (data not shown). Serial deletion and/or subcloning of various fragments further confirmed that subclones harboring a plasmid (pTH5-2) with a *Bam*HI and *Nco*I digested fragment retained their original activity on the substrate.

The complete nucleotide sequence of the 4 kb insert from pTH5-2 was analyzed using an ORF finder and the BLAST program, which predicted a hypothetical protein as a putative ester-hydrolase composed of 250 amino acids (Fig. 1). Surprisingly, a homology search of this ORF revealed that it was 99% homologous with a predicted ORF in the whole genome sequence of *Stenotrophomonas maltophilia* K279a (YP_001970974.1). The origin of the insert has not yet been defined exactly because there was no sequence of 16S rRNA or comparable signature in the sequence of the insert. However, a much higher sequence identity and codon usage provided a possibility of close relationships between the DNA fragment of the insert and that of *S. maltophilia* K279a, although minor deletion and insertion of nucleotides were observed around the putative ORF (data not shown). To date, the biochemical and phylogenetic data of the putative ORF from *S. maltophilia* K279a have not been reported.

Further attempts to analyze the sequence of putative esterase, designated PE esterase (EstPE), revealed a typical catalytic triad Ser^{127}-Asp^{196}-His^{229} that was commonly observed in most esterase/lipase family enzymes and contained a conserved GXSXG motif (Fig. 2A). Interestingly, two X residues were occupied by oxidation sensitive methionine. Querying results for homologous enzymes using this sequence revealed some sequences that were predicted to have a considerable homology (>35-40%) and thus presumed to have a common ancestor (Fig. 2B). Among them, two enzymes, a thermostable esterase from *Thermotoga maritina* (Levisson *et al*., 2009) and a peptidase from *Nostoc commune* UTEX 584 (Wright *et al*., 2005),

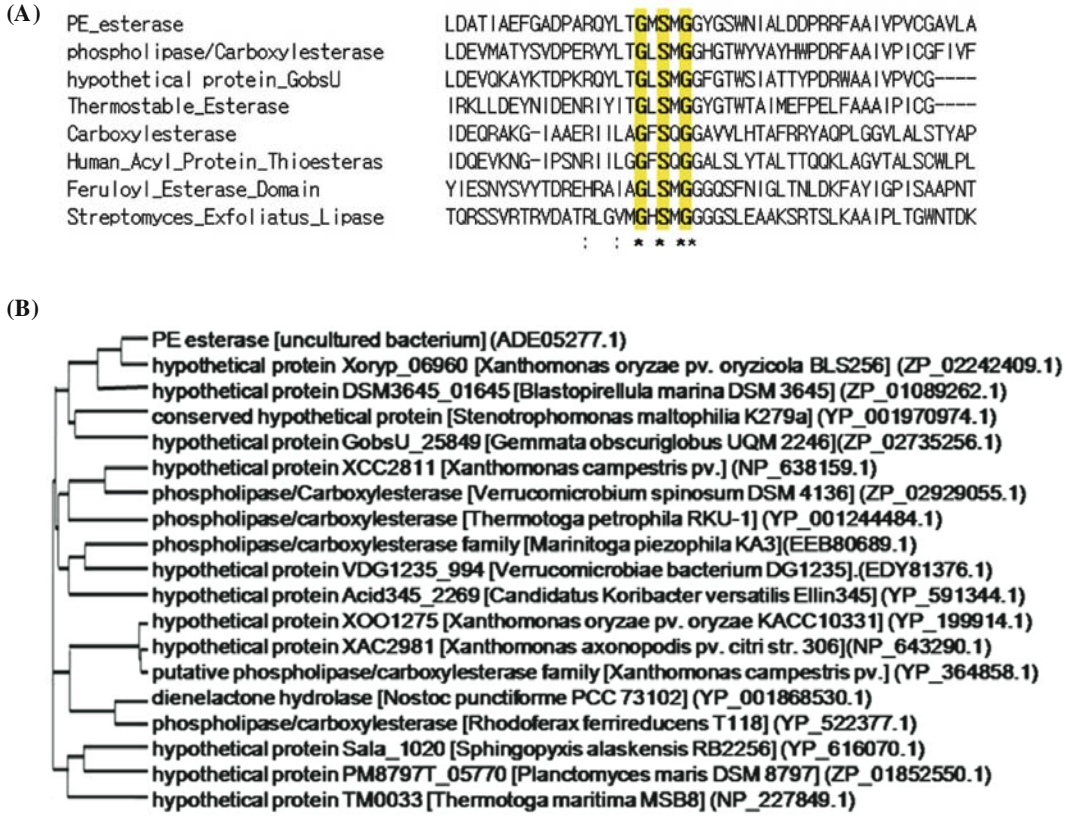

Fig. 2. Multiple sequence alignment and evolutionary relationships of related esterases. (A) The amino acid sequence spanning the putative active region of EstPE was aligned with homologous proteins. The sequence alignment of the entire ORF was intentionally excluded from this figure because the overall homology with those of the known family of enzymes was extremely low. The aligned sequences included a typical sequence of each family: phospholipase/carboxylesterase from *Rhodothermus marinus* DSM 4252 (YP_003289701.1), hypothetical protein GobsU from *Gemmata obscuriglobus* UQM 2246 (ZP_02733028.1) and other well known proteins. The GXSXG motif was marked by bold letters. (B) Phylogenetic tree analysis of a novel family including EstPE. All aligned sequences shared considerable homology (>35-40%).

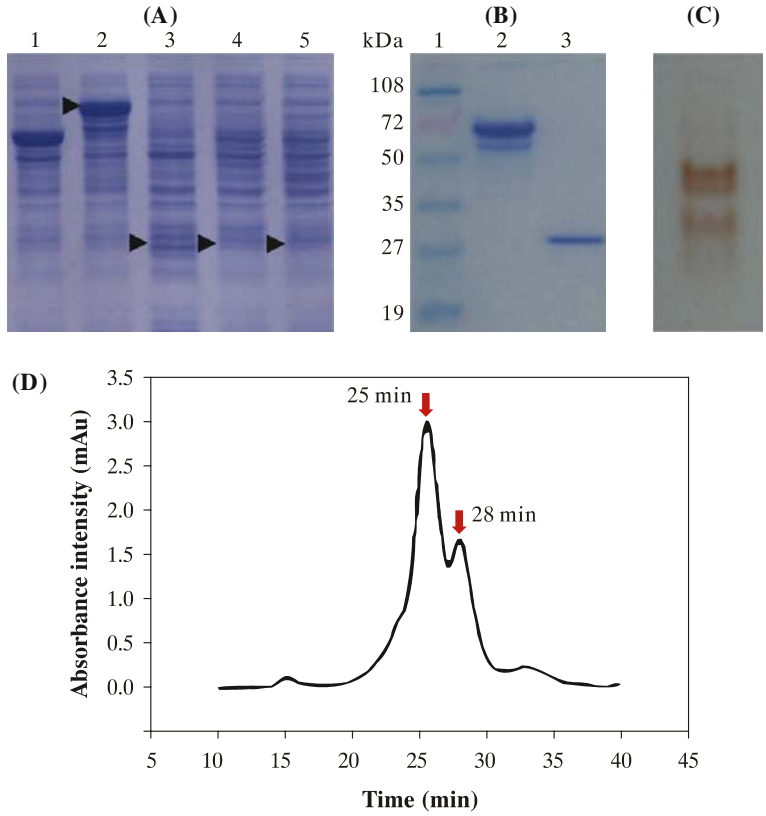

Fig. 3. Analysis of protein expression and oligomeric structure. (A) Transformed cells were induced with 1 mM IPTG, and the expression level of EstPE in the induced cells was analyzed by 10% SDS PAGE. Lanes: 1, pMAL-c2x; 2, pMAL-EstPE; 3, pQE-EstPE; 4, pTrc-EstPE; 5, pTrc-1767. (B) Apparent homogeneity of the purified EstPE. Lanes: 1, protein size marker; 2, purified MBP-EstPE; 3, purified EstPE. (C) Activity staining of the purified EstPE on a 8% native PAGE using α-naphthyl acetate (45 μg/ml) and Fast Blue RR (45 μg/ml). (D) Elution profile of EstPE from a Superose 12 gel filtration column.

had previously been identified. However, the structural properties and physiological roles of closely related enzymes, including PE esterase, have not previously been reported. Comparison of the sequences revealed a very low sequence similarity (<5-11%) to those of established esterase family enzymes, which are related to typical esterase/lipase superfamily members that have been reported to date. The phylogenetic tree analyses generated using the neighbor-joining and maximum-likelihood methods also showed that the putative esterase of pTH5-2 was uniquely positioned to provide a possibility of new enzymes with a novel sequence space. Based on these observations, we propose that these esterases comprise a novel family of enzymes with no reported function. We also analyzed the entire sequence of the insert in detail. However, we could not find a complete signal of a putative promoter in the upsteam region of the gene encoding EstPE. Thus, it was presumed that the expression of the enzyme in the screening step was due to a promoter like signal located within the ligation site or a strong promoter of the reporter enzyme GFPuv.

Functional expression of PE esterase

To identify the putative esterase, the corresponding gene was expressed using several expression vectors (pTrc99A, pQE-30, and pMAL-c2X). As shown in Fig. 3A, PE esterase was determined to be overexpressed only by MBP fusion, although a moderate increase in the level of activities (4-7 fold higher than the controls) was observed in other cases (pTrc99A and pQE-30). Furthermore, the enzyme activities of the crude extracts were well correlated with those of the whole cell enzymes. However, these expression patterns were not improved when subjected to various culture or induction conditions. Most of the cases, especially in pMAL-PE13, did not influence cell growth. Therefore, there was no growth retardation when compared with that of empty vectors. These results indicate that the putative esterase had an innate low stability or was susceptible to proteolysis. Additionally, there is the possibility that expression factors such as chaperones and/or reduction potential were needed for functional folding.

Purification and characterization of PE esterase

As shown in Fig. 3B, the putative esterase was readily purified and seen as a single band under denaturing SDS-PAGE. However, an interesting result was observed upon native gel analysis by activity staining (Fig. 3C). Specifically, separate bands (two major and one minor) were developed and showed distinct activity. This trait was consistently observed when the developing conditions were changed by alteration of the con-

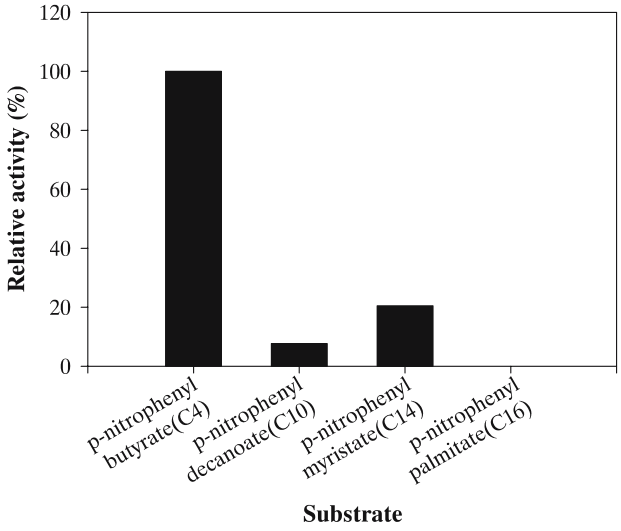

Fig. 4. Substrate specificity of PE esterase. The substrate specificity was determined according to the procedure described in the methods section. Each value represent the average of three independent assays.

centration of proteins. We also conducted size exclusion chromatography to more correctly determine the oligomeric structure of PE esterase. Consistent with the native gel analyses, the elution profile of the size exclusion chromatography primarily generated two protein peaks at 25 and 28 min (Fig. 3D). When these retention times were compared with those of the size-marker proteins, each peak corresponded to a monomer and a dimer, respectively. There were no more molecular aggregates or oligomeric structures observed under other conditions. These results indicated that the PE esterase primarily coexisted in the monomer and dimer state. It is generally recognized that the original source (strain) cannot be easily identified from metagenome resources due to their complex nature consisting of various types of microorganisms. Therefore, a comparative study of the oligomeric structure of the parent and recombinant enzymes could not be conducted. Nevertheless, it is likely that the structure and/or function of the enzyme were closely linked with oxidation or reduction, as observed during screening (also see the last section of the results).

Figure 4 shows the relative activity of purified PE esterase toward some typical substrates. PE esterase showed higher activity toward the short carbon chain substrate, *p*-nitrophenol butyrate (1.344 μmole/min/mg protein). However, this enzyme did not show any measurable activity toward *p*-nitrophenol palmitate. Considering the substrate spectrum, it is likely that the enzyme belonged to a family of esterases. The unknown functional roles and actual substrates can primarily be attributed to the relatively lower activity toward typical substrates.

Effect of DTT on oligomeric structure

It is well known that cysteine and methionine residues in protein are readily oxidized and can influence protein structure or function according to environmental conditions. As shown in Fig. 2A, two residues of the active site consensus sequence in the PE esterase were determined to be methionines.

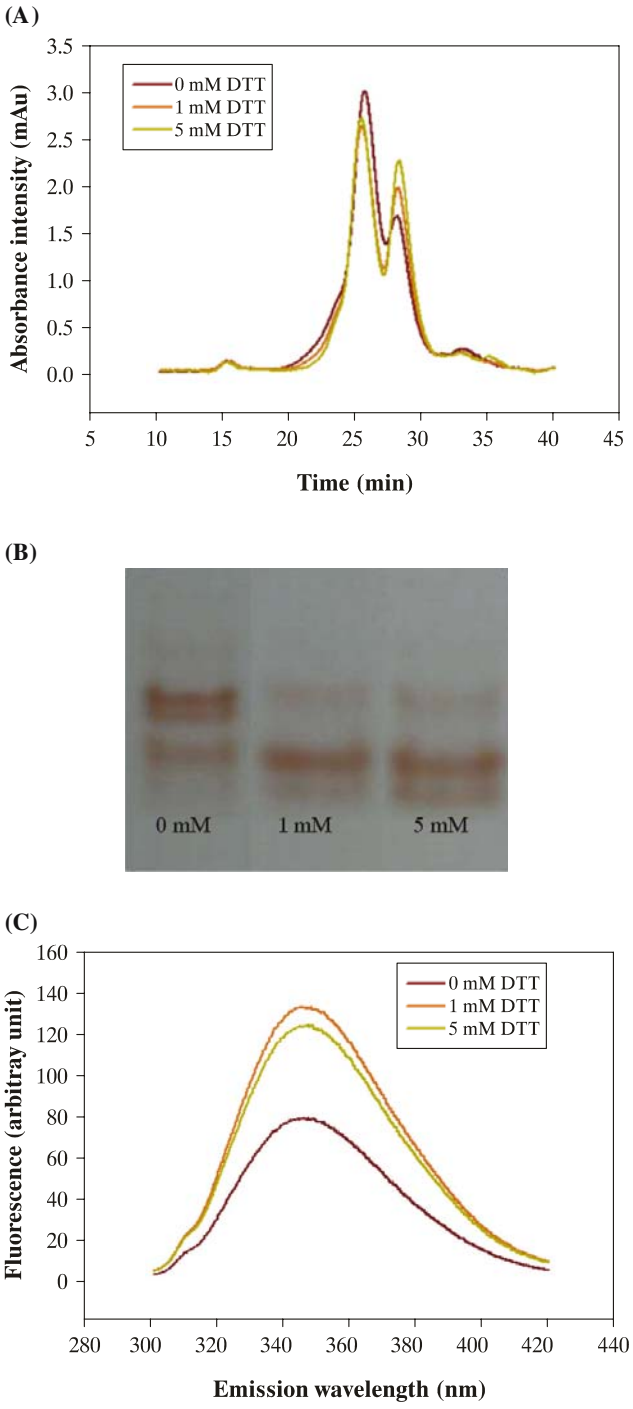

Fig. 5. Effect of DTT on the conformational change of PE esterase. After treatment of EstPE with DTT (1-5 mM) at 37°C for 10 min, the conformational changes were monitored by gel filtration column chromatography (A), activity staining on a native gel (B) and fluorescence spectrophotometer (C), according to the procedure described in the methods section. All experiments were conducted in duplicate and their average values are shown.

Therefore, there is a possibility of oxidative modulation of structural or functional properties as observed in other proteins (Gan *et al*., 1995; Sacksteder *et al*., 2006). To monitor this possibility, the retention time of PE esterase following treatment with DTT was analyzed by size exclusion chromatography. As shown in Fig. 5A, the elution peak ratio of the enzyme was modulated according to the concentration of DTT, resulting in an increase of peak 1 corresponding to the monomer. Treatment with DTT also revealed a change in migration profiles upon activity staining of the native gel (Fig. 5B), as well as a distinct decrease in the dimer fraction. These observations strongly suggested that the structural change of PE esterase occurred either in the inter- or intra-subunit. Subsequently, the fluorescence spectra of the PE esterase were measured according to the increasing concentration of DTT. As shown in Fig. 5C, the protein fluorescence was also sensitive to reduction. Upon reduction after treatment of DTT in normal buffer solutions, the protein fluorescence increased with the concentration of DTT and was shifted to slightly longer wavelengths. Based on these observations, further attempts to determine the activity of the enzyme were made in the presence of DTT. Unfortunately, since the oligomeric structure was heterogeneous and dependent on the reduction state, direct comparisons of the kinetic parameters between the oxidized and reduced enzymes were difficult, although a preliminary experiment showed a decrease of one order of magnitude in Km when compared to that of a typical esterase (Kim *et al*., 2003). *In-vitro* cross linking and subsequent size fractionation provided a method to resolve this problem, although these states do not represent the native structure.

As shown above, there was a conformational change induced by reduction of PE esterase. Although the exact mechanism by which this occurred is currently unclear, it is likely that reduction by DTT was closely linked to the structural change between the oligomers (mainly dimers) and monomers. A plausible mechanism of oligomerization between the inter-subunit is that it is occasionally mediated by oxidation of cysteine. In PE esterase, this possibility is negligible because of the low content of cysteine and the absence of the effects of chemical modification by DTNB. Generally, methionine present near the active site or surface of the quaternary structure can function to regulate the biological activity or structure of proteins. This is because oxidation of the sulfur-containing side chain to sulfoxide affects the conformation through changes in the hydrophobicity and steric hindrance. This effect is known to be protected by reducing agents such as DTT (Kim *et al*., 2001). Methionine can also play a crucial role in cellular functions as an antioxidant and is involved in regulation, signaling, protein degradation, and oxidation-sensitive switches (Levine *et al.*, 2000). More detail regarding the mechanisms involved in the oxidative modulation of PE esterase will be elucidated by resolving the 3D structure and site-directed mutagenesis of methionine. These studies are currently in progress.

Metagenome-derived protein is expected to have the potential to enable discovery of new proteins with novel sequences that differ from already known proteins from cultivable resources. However, conventional screening processes commonly exclude this possibility due to limitations in demand to acquire highly active proteins based on activity or sequence-based screening approaches. In particular, oxidation-dependent proteins are intentionally excluded for practical applications. This is a barrier that prevents access to proteins with novel sequence spaces. For example, PE esterase screened by the simple screening strategy employed in this study shows novel organization and properties that have not yet been reported and provides a chance to improve our understanding of the structural space of protein and cell physiology.

Acknowledgements

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