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Functional expression and refolding of new alkaline esterase, EM2L8 from deep-sea sediment metagenome

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Abstract

A metagenomics approach is an efficient method of isolating novel and useful genes from uncultured microorganisms in diverse environments. In this research, a gene encoding a new esterase (EM2L8) was cloned and characterized from the metagenomic DNA library of a deep-sea sediment. The gene consisted of 804 bp encoding a polypeptide of 267 amino acids with a molecular mass of 28,952. The deduced amino acid sequence showed similarities with the BioH of *Kurthia*, the 3-oxoadipate enol-lactonase of *Haloarcula* and the acyltransferase of *Thermoanaerobacter*, which feature identities of 38%, 32%, and 33%, respectively. Residues essential for esterase activity, such as pentapeptide (GXSXG) and catalytic triad sequences, were uncovered. While the protein was overproduced mainly as inclusion body at 37 °C, it was mainly produced as a soluble active enzyme at 18 °C. A zymogram analysis revealed that purified EM2L8 taken from the soluble fraction could hydrolyze tributyrin substrate. Furthermore, the protein from the inclusion body fraction also showed strong activity on gel, thus indicating that the protein was refolded during SDS-gel electrophoresis and the ensuing incubation period. When the inclusion body was mixed with some anionic detergent solutions and diluted with a non-detergent buffer, the insoluble EM2L8 refolded rapidly and recovered its full esterase activity. Although EM2L8 had an optimum temperature of 50–55 °C, its activation energy in the range of 10–40 °C was 8.34 kcal/mol, indicating that it is a cold-adapted enzyme. Moreover, it was found to have an optimum pH of 10–11, thus revealing that it is an alkaline enzyme. In this paper, the new esterase EM2L8 buried in a deep-sea sediment became known on the surface and was characterized biochemically.

Keywords: Esterase; Metagenome; Refolding; Alkaline

Lipases/esterases are known to catalyze both the hydrolysis and synthesis of ester compounds. Certain enzymes exhibit a narrow substrate specificity, position selectivity, and stereoselectivity, and therefore serve as useful biocatalysts within the pharmaceutical and fine chemical industries [1,2]. Recently, an increasing number of drugs and organic materials have been produced through enzyme-catalyzed reactions. In this regard, the use of target-specific enzymes for the efficient production of such target materials is essential.

Many enzymes, some of which are available commercially, have been screened from animals, plants, and microorganisms. However, despite this denouement, it remains difficult to find a suitable one. Accordingly, the construction of a lipases/esterase toolbox through the addition of more enzymes remains very important with regards to the efficient synthesis of various ester compounds [3].

Some useful enzymes have been reported from microorganisms living in extreme environments such as hot springs, the deep-sea, and the Antarctic region [4]. Because the number of microorganisms cultured to date remains only a small fraction of all the microorganisms on earth, the number of novel enzymes is expected to increase continuously.

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Some enzymes taken from uncultured microorganisms are expected to become novel enzymes that exhibit quite different properties. Metagenomic screening represents an efficient method of isolating novel and useful genes from environmental DNA libraries. The advantage of this technique is that significantly diverse genes can be isolated directly from the environmental sources. Some novel lipase/esterase genes have already been isolated successfully through this method [5–7].

One of the drawbacks to metagenomic screening is the difficulty associated with enzyme production using heterologous host systems [8]. On certain occasions, no protein was produced at all because of the difference in codon usages and/or the formation of a stable secondary structure of mRNA [9–11]. In addition, as a result of their slow folding rate in the host cell, inclusion bodies have frequently been made [12,13]. Some lipases are known to require a chaperone protein such as lipase-specific foldase (Lif)[1] for their correct folding [14,15]. Thus, an efficient method of protein production should be developed in order to ensure successful applications of the diverse metagenome.

In this research, a gene encoding a new esterase was isolated from the metagenome of a deep-sea sediment sample of a clam bed community, and the soluble enzyme was overexpressed in *Escherichia coli* at 18 °C. In addition, most of the inclusion body protein produced was refolded through detergent treatment and a subsequent dilution process. The active enzyme was then purified, and its biochemical properties were characterized.

Materials and methods

Bacterial strains and plasmids

Escherichia coli DH5α (Stratagene, LaJolla, CA), *E. coli* BL21 (DE3) (Stratagene) and *E. coli* EPI300 (Epicentre, Madison, WI) were used in this study. Moreover, the pBluescript SK (Stratagene) was used for the construction of the mini library for the subcloning of the recombinant fosmid clone, and a plasmid pET-24a (Novagen, Madison, WI) was used for gene expression.

The construction of a metagenomic library and the screening of a lipase/esterase gene

Deep-sea sediment samples taken from a clam bed community from Edison Seamount, south of Lihir Island in the New Ireland Fore-arc near Papua New Guinea were received from the Korea Ocean Research and Development Institute [16,17]. The DNA extraction method developed by Hurt et al. [18] was used with minor modifications, and further purification of the DNA was performed through direct extraction from the agarose gels. The metagenomic library was prepared in the following manner using a fosmid vector, pCC1FOS (Epicentre). The purified DNA was sheared randomly and end-repaired. About 40 kb-sized DNA was then isolated and ligated using the pCC1FOS vector. Lambda phage packaging extracts were added to the ligation mixtures and the infection of *E. coli* EPI300 was performed. For the direct screening of lipase/esterase activity, the transformants were plated on an LB agar medium supplemented with 1% tributyrin.

Subcloning of a lipase/esterase-positive clone

A transformant containing a recombinant fosmid, pES02L8 with tributyrin-lytic activity was cultured overnight in 20 mL of LB medium (12.5 μg/mL chloramphenicol) using a shaking incubator at 37 °C. For the amplification of the plasmid DNA, 180 mL of fresh LB media (12.5 μg/mL chloramphenicol) containing 200 μL of induction solution (Epicentre) was added to the culture, which was then cultivated for an additional 5 h. The plasmid DNA was purified using the alkaline lysis method [19] and the Nebulizer (Invitrogen, Carlsbad, CA) was then used to fragment it into 2–4 kb in size. The fragmented DNA was ligated into pBluescript SK(-) plasmid digested with *Eco*RV, and the recombinant plasmids were transformed into *E. coli* DH5α. The lipase/esterase-positive clone, designated as pBES02L8, was obtained. The full sequence of the insert DNA of pBES02L8 was determined utilizing a primer walking method involving an automated sequencer (ABI3100) which made use of a BigDye Terminator kit (Applied Biosystems, Foster City, CA). The DNA sequence was assembled using the Vector NTI Advance program (InforMax Inc., Frederick, MD). Open reading frame (ORF) analysis was then performed using the NCBI's ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Thereafter, the nucleotide sequence was submitted to GenBank under accession number DQ846908.

Expression and purification of esterase EM2L8

The full length of the putative esterase gene flanked by the *Nde*I and *Xho*I sites was amplified by PCR with pES02L8 fosmid DNA and two primers (5′-GGC GGT AGC ATA TGC CAA TTG TAA GTG CGA ATG GGA TAG-3′ and 5′-CTC ACG GCCC TCGA GGCT GCG CAA GAA GAA TTC ACG-3′). The thermal cycles of denaturation (95°C, 1 min), annealing (50 °C, 1 min), and polymerization (72 °C, 1 min) were performed 25 times. The amplified DNA fragment was digested with *Nde*I and *Xho*I. The fragment was then ligated with *Nde*I/*Xho*I-digested plasmid pET-24a, and the resultant recombinant plasmid was used to transform *E. coli* DH5α. Once the sequence had been confirmed, the expression was then done by introducing the recombinant plasmid into the *E. coli* BL21(DE3). The recombinant *E. coli* was cultured in an LB medium containing kanamycin (50 μg/mL) overnight at 18 °C. It was used as a seed culture (1%) and then the main

[1] *Abbreviations used:* Lif, lipase-specific foldase; ORF, open reading frame; IPTG, isopropyl-β-D-thiogalactopyranoside; NTA, nitrotriacetic acid; pNPB, *p*-nitrophenyl butyrate; pNPEs, *p*-nitrophenyl esters.

culture was successively performed at 18 °C. The target enzyme expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) during the mid-exponential growth phase (OD_{600}, 0.5) and then incubating it for 12 h at 18 °C. The cells were harvested by centrifugation (6,000*g* for 20 min at 4 °C) and resuspended in 50 mM Tris–HCl buffer (pH 7.5). It was then lysed with a Vibra Cell™ (Sonics & Materials Inc., Danbury, CT) for 20 min and centrifuged (10,000*g* for 20 min at 4 °C). The resulting supernatant was applied to a nickel–nitrilotriacetic acid (Ni–NTA) column (QIAGEN GmbH, Hilden, Germany). After washing with 50 mM imidazole, 300 mM NaCl, and 50 mM NaH_2PO_4 buffer (pH 7.5), the bound esterase was eluted using 350 mM imidazole in the same buffer. The pooled fractions were dialyzed against a 50 mM Tris–HCl (pH 8.0) buffer.

Esterase activity assay

Activity was measured using a *p*-nitrophenyl butyrate (pNPB) or other *p*-nitrophenyl esters (pNPEs) as substrates [20]. The reaction mixture consisted of 0.01 mL of 10 mM substrate in acetonitrile, 0.04 mL of ethanol, and 0.95 mL of 50 mM Tris–HCl buffer (pH 8.0) containing an appropriate amount of the enzyme. Unless otherwise specified, the enzyme reaction was performed for 3 min at 35 °C. The amount of *p*-nitrophenol liberated during the reaction was measured by its absorbance at 405 nm. 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.

Electrophoresis and zymographic analysis

SDS–PAGE was conducted on 12% running gels using the Laemmli method. A low range protein standard (Bio-Rad, Hercules, CA) was used as a molecular weight marker. The protein concentration was determined through the Bradford method using bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as the standard.

The lipolytic activity of the protein on the SDS–PAGE gel was detected through a zymographic analysis using tributyrin agar plates [21]. After running, the gel was washed sequentially for 10 min with a Tris–HCl buffer (50 mM, pH 8.0) containing 1% Triton X-100 and twice with a detergent-free Tris–HCl buffer. The gel was overlaid on a TBN agar plate prepared with agar (1.5%) and tributyrin emulsion (1% TBN, 20 mM NaCl, 1 mM $CaCl_2$, and 0.5% gum arabic) and incubated at 37 °C until clear bands appeared.

Effects of temperature, pH, and chemicals on EM2L8 esterase

The optimum temperature for enzyme activity was determined by assaying esterase activities toward pNPB (C_4) in a Tris–HCl buffer (pH 8.0) at various temperatures.

To determine the optimum pH of the enzyme, esterase activity toward *p*-nitrophenyl caprate (pNPC, C_{10}) was measured at 37 °C with various pH buffers; namely, 0.1 M sodium acetate (pH 4–6), potassium phosphate (pH 6–7.5), Tris–HCl (pH 7.5–9), glycine–KCl–KOH (pH 9–11), and K_2HPO_4– K_3PO_4 (pH 11–12.5) buffers. In this experiment, pNPC (C_{10}) was used for its high stability at alkaline pHs ($\geqslant$pH 10), at which pHs pNPB (C_4) was auto-hydrolyzed rapidly.

To test enzyme stability for various metal ions and detergents, enzyme solutions were, after pre-incubation for 30 min with each reagent, assayed at pH 8.0 and 37 °C.

Refolding of inclusion body

Cell culture (10 mL) was centrifuged, and the harvested cell was resuspended with 0.5 mL of a 50 mM Tris–HCl (pH 8.0) buffer. It was then sonicated and the precipitate was recovered through centrifugation. After washing with the same buffer, the insoluble fraction was resuspended with a 0.09 mL buffer. Ten microliters of detergent solutions (10× concentration) were added and thoroughly mixed. The cleared solution was diluted with a 900 μL buffer, and the recovered esterase activity was measured.

Results

Screening of novel esterase gene

A DNA library using a sediment sample collected from a deep-sea clam bed community was constructed in a fosmid vector, pCC1FOS, and followed through the screening of the lipolytic activity of the clones on a tributyrin agar plate. As a result, a lipase/esterase-positive clone, pES02L8, was selected. Through subsequent subcloning experiments with a pBluscript plasmid, a recombinant plasmid (pBES02L8) with a short insert of 1170 bp was constructed. The sequence analysis of the insert DNA showed the presence of one open reading frame (ORF) of 804 bp, encoding a polypeptide of 267 amino acids with a deduced molecular mass (Mr) of 28,952.

The BLAST search against the NCBI nonredundant protein database (http://www.ncbi.nlm.nih.gov/BLAST/) revealed that the deduced amino acid sequence of the ORF was similar to the BioH of *Kurthia* sp. (BAB39459) [22], the 3-oxoadipate enol-lactonase of *Haloarcula marismortui* (AAV45586) [23], the acyltransferase of *Thermoanaerobacter tengcongensis* (NP_622224) [24] and the 3-oxoadipate enol-lactonase of *Chromobacterium violaceum* (AAQ59006) [25], which feature identities of 38%, 32%, 33%, and 34%, respectively (Fig. 1). In addition, the signature sequences conserved in lipase/esterase could also be found in the ORF (Fig. 2). That is, serine residue in the conserved pentapeptide GVSMG (from 93 to 97) appears to be one of the active sites [8]. Asp 218 and His 246 comprise the other two residues of the catalytic triad in the active sites. N-terminal RG (from 56 to 57) might compose an oxyanion hole. Amongst all five protein sequences, many conserved

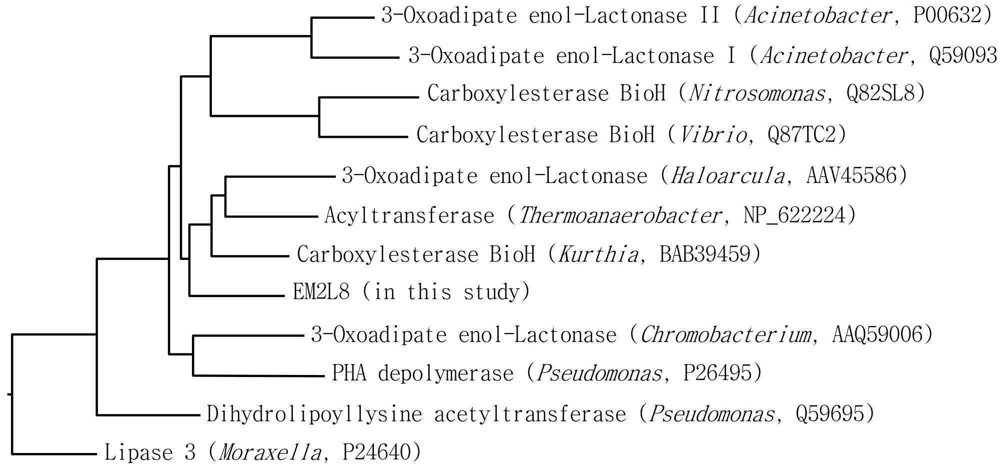

Fig. 1. Phylogenetic tree of esterase EM2L8 and similar enzymes. The protein sequences of esterase EM2L8 and 11 enzymes were compared and the phylogenetic tree was drawn using the DNAStar program's ClustalW method.

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                                 **         ***  *     ** *  *       *      *   * **  * ** * *
EM2L8        1  -----------------MPIVSANGIEIYYETAGSPDAPPLLLIQGLSGYTEGWLMQVPALKEDFYVIYFDNRGAGKSTQ 63
BAB39459     1  -----------------MPFVNHDNESLYYEVHG--QGDPLLLIMGLGYNSLSWHRTVPTLAKRFKVIVFDNRGVGKSSK 61
AAQ59006     1  -----------------MPHAQLAGYQVYYEESG-HGSQPLLLLNGITMSTVGWTLMMPLLEPHFRVIRMDFLGQGQSEH 62
NP_622224    1  -----------------MPKITVNNVSLYYEIHG—NGSPLVLIEGLGCSKWMWFKQIDELKKHFKVIVFDLRGVGDSEK 61
AAV45586     1  MNAEPNYLKNIQPEPVSMHYATNDGVSLAYEREGPPDAETVVFVEGIGYGRWMWLWQQEALVEEYQTIVWDNRGTGDSDE 80

                                              ●●●●●
                     *     * *  * **      * * * ******** **   *     * *  *  **
EM2L8       64  S-EPGYMMVDMADDTAALLDTLEIPQAYVLGVSMGGMIALNLAMQHPQKVNKLALGCTTAGGASAVWADEKVSAALITPS 142
BAB39459    62  P-EQPYSIEMMAEDARAVLDAVSVDSAHVYGISMGGMIAQRLAITYPERVRSLVLGCTTAGGTTHIQPSPEISTLMVSRA 140
AAQ59006    63  VPGELYALADQADLAAALLDWLQLPQAYVAGLSYGGMVAQHLAHRHPGRLSRLLLAGTLAWADTVN---HHIGDSWIAAN 139
NP_622224   62  P-DMEYSIKLLADDTAALVAELGFKKVHVLGVSMGGYIAQELALEYPDLVDRLILCSTHYGGPNIVPIPLSTLNIILNGA 140
AAV45586    81  P-GGPYTMSQMASDLEAVLDDAGIEQAHVVGASMGGMIAQQYALEY-DRARSLTLMCTSPGGPEAVPVPEETQQRMFAVP 158

                                                                                           ●
                                  *                             *  *    *    *     * *  *  *
EM2L8      143  --SGDLRQDFYDSAWFLLAPDTIENNSRLVEQLAENAGNNPQTPTGFMGQLQAISTHDVAGALPELSMPTLVMHGDLDLL 220
BAB39459   141  SLTGSPRDNAWLAAPIVYSQAFIEKHPELIQEDIQKRIEIITPPSAYLSQLQACLTHDTSNELDKINIPTLIIHGDADNL 220
AAQ59006   140  N-QGGLDLRYQVSVPWLFSSRFLAGNAAMLDDLKLLAG--MVDWDAVIRLINGVRQHDARTWLPQLQLPVHILVGDEDRL 216
NP_622224  141  GLRNA-LENLRIAMSLNFSDEYLSTHKDEFEQIVKWKFEKPQPFYAYRRQLYAALTFDEEARIHLIKHPTLIMAGKDDKV 219
AAV45586   159  DDLDE-RELRRYKMQPALSDSFMEAHEDLIERIIDWRIDSDASDQALDWQGAAVQAFNASDRLGEITVPALVIHGTADEV 237

                                         ●
                 *  *   *   *            *    *     *    *
EM2L8      221  IPLQNGRFLAEQIPHAEFKNYPNTGHLFFVEQAVPVNDDLREFFLRS                               267
BAB39459   221  VPYENGKMLAERIQGSQFHTVSCAGHIYLTEAAKEANDKVIQFLAHL                               267
AAQ59006   217  TPLYQARLLQELIPGAELEVLPGAAHVLHIEAVEAFARAIVRFRQD                                262
NP_622224  220  VPYENALLLHSKIENSEIELFSNAGHMFFIEKAEEVNRKIIEFLTKPIGGDKKWEEKMLM                  279
AAV45586   238  VPYENGKLLARGLPNADFITVHGGPHLLFIEEYERVNTQIQEFIDDV                               284
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Fig. 2. Protein sequence alignment of esterase EM2L8 and four similar enzymes. Asterisks (*) indicate amino acids conserved in at least four enzymes. Filled circles (●) indicate putative oxyanion hole and active site serine, aspartic acid, and histidine.

regions were found within the entire sequence. When taken together, the protein seems to be an esterase enzyme with a considerably novel sequence which has been denoted as EM2L8 esterase.

Expression and purification of the esterase EM2L8

When the EM2L8 esterase gene was expressed in *E. coli* BL21 (DE3) through IPTG induction at 37 °C, a His-tagged EM2L8 recombinant protein was over-produced (Fig. 3A). Although the total expression level of the EM2L8 protein was very high, more than half of the protein was uncovered in the inclusion body (Fig. 3A, lanes 3 and 4). That is, although the amount of the EM2L8 protein in the insoluble pellet was much more than that in the soluble fraction, its activity (0.36 U/mL) was too low to be compared with the soluble fraction's activity (10 U/mL) (Table 1). Various factors (culture temperatures, induction temperatures, and IPTG concentrations) related to the expression were tested in order to improve the solubility of the EM2L8. The induction at 18 °C was effective in producing the soluble enzyme shown in Fig. 3A. The specific activity of the soluble fraction was 5.1 U/mg toward pNPB (Table 1).

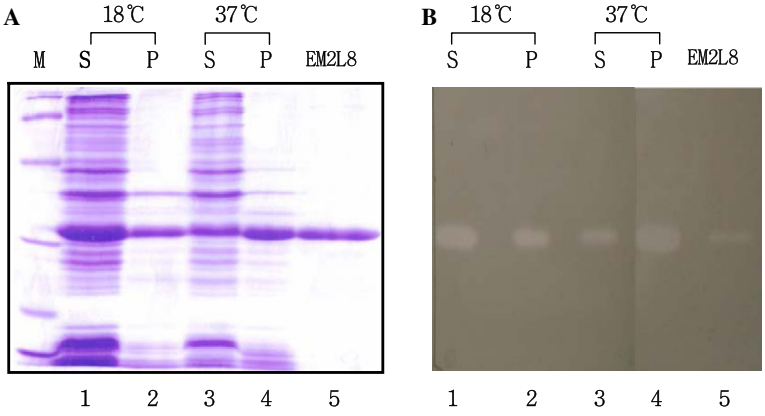

Fig. 3. SDS–PAGE of esterase EM2L8. (A) Coomassie brilliant blue staining was done after the SDS–PAGE. Lanes 1 and 3 indicate the soluble fractions and lanes 2 and 4 the insoluble fractions after cell lysis. Lane 1 and 2 were obtained from cells cultured at 18°C and lanes 3 and 4 from cells cultured at 37 °C. Lane 5 indicates purified esterase EM2L8. (B) TBN-zymogram was done after the SDS–PAGE. Lanes 1–5 were the same as in panel A. There were blank lanes between the sample lanes.

Table 1
Esterase activities in soluble and insoluble fractions

	18 °C culture		37 °C culture		Purified EM2L8
	Sup[a]	Pellet[b]	Sup[a]	Pellet[b]	
Activity (U/mL)	31	2.4	10	0.36	39
Protein (mg/mL)	6.1	0.35	3.8	0.46	0.25

[a] Cell (10 mL culture) was harvested and resuspended in 0.5 mL buffer. After cell lysis and centrifugation, supernatant was obtained.
[b] Insoluble pellet was resuspended in 0.5 mL buffer.

The His-tag fusion protein in the soluble fraction was purified using an Ni–NTA agarose column and elution buffer containing imidazole (350 mM). A SDS–PAGE analysis showed that EM2L8 protein was purified to apparent homogeneity (Fig. 3A, lane 5). This purified enzyme had a specific activity of 156 U/mg toward pNPB (Table 1).

The purified protein from the soluble fraction showed a clear band (Fig. 3B, lanes 1 and 3) when a zymographic analysis was performed as described in the Methods, thus indicating protein-retained esterase activity. Surprisingly, despite the low esterase activity of the inclusion body fraction (Table 1), the 32 kDa-protein from the insoluble fraction also formed a clear band when the zymographic analysis (Fig. 3B, lanes 2 and 4) was conducted, indicating that the inclusion body protein was properly refolded during SDS-gel electrophoresis and the successive incubation step.

To prove the possibility that some detergents may be effective in refolding the protein, the inclusion body was thoroughly mixed with various detergent solutions, and then diluted subsequently with a non-detergent buffer. When the inclusion body was mixed with SDS (1%, denaturing concentration) or Sarkosyl (1%, denaturing concentration), it was completely denatured and the subsequently diluted solutions exhibited high relative activity which marked a 7-fold increase when compared with the original insoluble fraction (Fig. 4). In fact, the specific activity of the refolded solution was measured as 142 U/mg, whose value

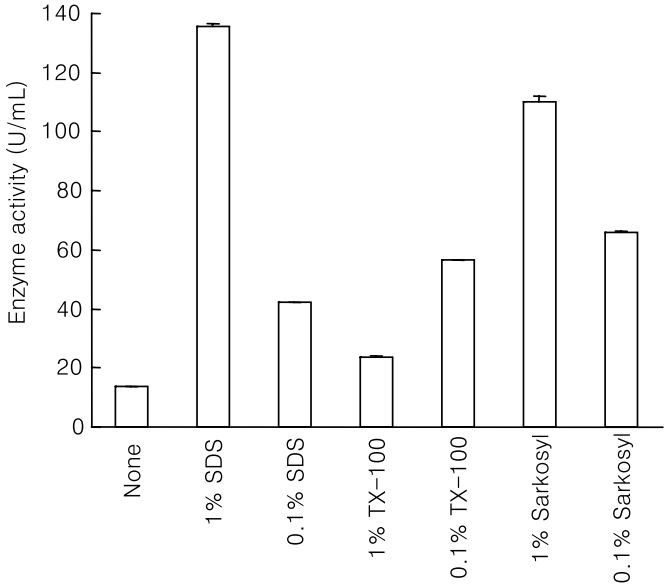

Fig. 4. Esterase activities after refolding. Recombinant *E. coli* cells (10 mL culture) were lysed and precipitate was resuspended in a 0.09 mL buffer. After the inclusion body was mixed with various detergents (0.01 mL, 10×), the final volume was adjusted to 1 mL. The experiments were conducted triplicate.

was as much as 90% of the specific activity (156 U/mg) of the Ni column-purified soluble enzyme.

Biochemical characterization of esterase EM2L8

The purified enzyme hydrolyzed various *p*-nitrophenyl esters from pNPA (C_2) to pNPL (C_{12}). Among them, pNPB (C_4) was most rapidly hydrolyzed, and as the chain length increased, hydrolytic activity decreased dramatically, therefore indicating that the enzyme was an esterase, not a lipase (Fig. 5).

The effect of temperature on enzyme activity was tested using pNPB as a substrate. The enzyme boasted maximum hydrolytic activity at 50–55 °C and an activation energy of

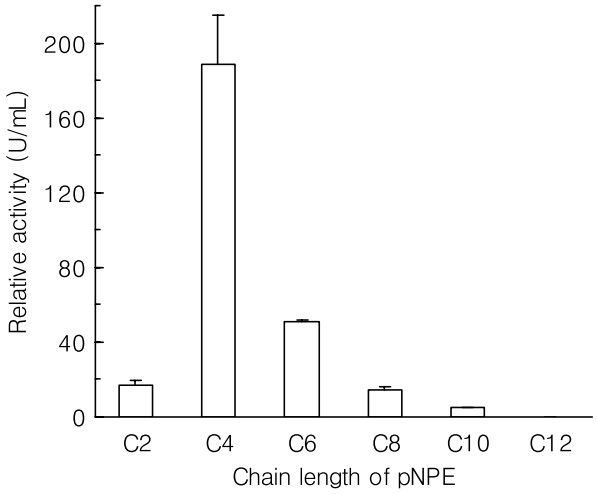

Fig. 5. Chain length specificity of the esterase EM2L8. The esterase activity of the purified EM2Ls enzyme was assayed toward various pNPEs at 35 °C and pH 8.0. The experiments were conducted triplicate.

8.34 kcal/mol in the range of 10–40 °C (Fig. 6 A and B). The inactivation energy, which reflected protein denaturation, was calculated to be −15.9 kcal/mol in the range of 55–65 °C. The optimum activity occurred at an alkaline pH of 10–11, which was five times higher than that at pH 8, thus indicating an alkaline esterase (Fig. 6C). Thereafter, the effect of various metal ions and detergents were tested (Table 2). Most metal ions were found to have little effects on the enzyme except cadmium and zinc ions, which displayed inhibitory effects. Anionic detergents (SDS and Sarkosyl) showed a very weak inhibitory effect, and most neutral detergent had little activating effect.

Table 2
Effect of detergents and metal ions on enzyme stability

Detergent	Relative activity (%)	Metal ion (1 mM)	Relative activity (%)
None	100	$CaCl_2$	98
Sodium dodecylsulfate (1%)	97	$Cd(NO_3)_2$	22
Sodium dodecylsulfate (0.1%)	93	$CoSO_4$	103
Sarkosyl (1%)	108	$CuSO_4$	110
Sarkosyl (0.1%)	93	$FeSO_4$	106
Triton X-100 (1%)	130	$MgCl_2$	97
Tween 20 (1%)	124	$MgSO_4$	81
Tween 40 (1%)	115	$MnCl_2$	94
Tween 60 (1%)	120	$NiSO_4$	77
Tween 80 (1%)	123	$ZnSO_4$	34

Discussion

Through its substrate specificity (Fig. 5) and the presence of esterase signature sequences (Fig. 2), this protein, EM2L8, was determined to be a typical esterase enzyme. As the most similar enzyme had an identity less than 38%, it was also determined to have a considerably novel amino acid sequence.

Like most enzymes screened from metagenome libraries, no information about a source microorganism could be obtained. In fact, the microorganisms producing the four most similar enzymes are quite remote from one another in

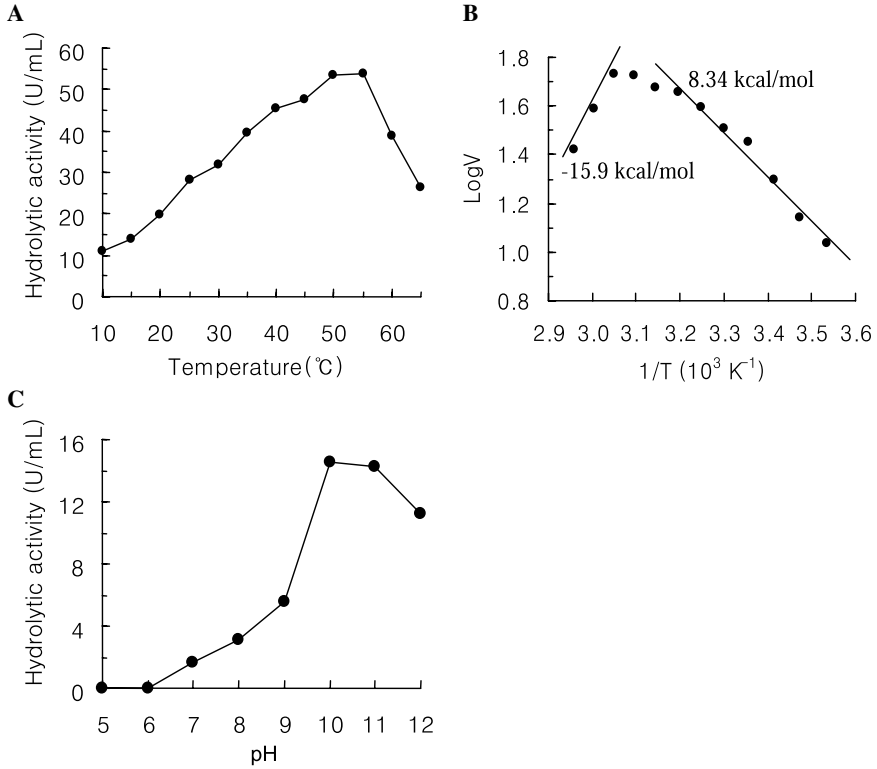

Fig. 6. Effects of temperature and pH on esterase EM2L8. (A) Esterase activity was assayed toward pNPB (C_4) at pH 8.0. (B) Activation and inactivation energy was calculated from panel A. (C) Esterase activity was assayed toward pNPC (C_{10}) at 35 °C. The experiments were conducted triplicate.

the phylogenetic tree. In addition, the physiological function of EM2L8 esterase could not be addressed for their different roles in each microorganism (Fig. 1).

Many novel genes have been found directly from genome information as well as metagenome library. However, as mentioned above, many genes are frequently not expressed as active proteins in heterologous hosts such as *E. coli* cells. Such difficulties in protein expression have usually been caused by the difference in codon usage between the expressed gene and the expression host [9–11]. It was also known that the stability of mRNA secondary structure affected expression level in *E. coli* [10]. Although these two problems have now been resolved, another problem remains; the fact that inactive inclusion body may frequently be produced [12,13]. Based on the amount of the total expressed esterase (Fig. 3A, lanes 3 and 4), codon usage and stability of mRNA structure do not appear to be limiting factors for enzyme production in the case of esterase EM2L8. It was believed that the problem emanating from the fact that a plentiful inclusion body was created at 37 °C could potentially be resolved by lowering the temperature to 18 °C; although a small amount of inclusion body was still produced, a large amount of EM2L8 protein in soluble active form was made (Fig. 3A, lanes 1 and 2).

The zymographic analysis revealed that the SDS-denatured EM2L8 proteins from both the soluble and insoluble fractions refolded rapidly and showed esterase activities on gel (Fig. 3B). These unexpected results urged us to perform the next experiment. When the washed inclusion body solution was thoroughly mixed with anionic detergent solutions and the mixtures were then diluted, the insoluble inclusion body was refolded. That is, the inclusion body was completely denatured by the anionic detergent and refolded into its native state as the detergent was removed through dilution (Fig. 4). Similar result was already reported with *E. coli* RNA polymerase σ factor and hygromycin B phosphotransferase (HPT) [13,26]. These inclusion bodies produced in *E. coli* were solubilized by 0.3% Sarkosyl, diluted, dialyzed, and then purified by anion-exchange chromatography. Recoveries of refolded proteins were about 50 and 37%, respectively.

In fact, inclusion bodies sometimes possess innate advantages in that, the yield of inclusion bodies is often very high, they can be protected from proteolytic degradation, and can easily be separated from soluble proteins. But to utilize such an inclusion body, the inactive and insoluble inclusion body has to be converted into an active and soluble protein. Therefore, the detergent treatment and dilution methods employed in this experiment and by other researchers can be utilized as a method of using inclusion bodies.

Taken together, the high production yield of the active EM2L8 enzyme was achieved through temperature-shift induction as well as the refolding of the inclusion body.

The purified protein EM2L8 showed little hydrolytic activity toward olive oil (data not shown), which meant that it had no hydrolytic activity toward long chain triglycerides. As for *p*-nitrophenyl esters, it showed a narrow chain length specificity toward C_4 among C_2–C_{10} (Fig. 5).

As the gene source was a deep-sea sediment sample, the esterase EM2L8 was at first expected to be a psychrophilic enzyme. However, it had somewhat of a high optimum temperature of 50–55 °C (Fig. 6A). Nevertheless, the activation energy in the range of 10–40 °C was calculated to be 8.34 kcal/mol (Fig. 6B), thus indicating that it is a cold-adapted enzyme [27].

To summarize, a new alkaline esterase was found from a deep-sea sediment metagenome, and a large amount of soluble active enzyme was obtained. Although we do not know the true substrate and physiological function of this enzyme, it could potentially be used as a biocatalyst in hydrolysis and synthesis reactions performed within the pharmaceutical and fine chemical industries.

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