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Molecular Cloning, Purification, and Characterization of a Cold-Adapted Esterase from *Photobacterium* sp. MA1-3

Young-Ok Kim*, Yu Li Heo, Bo-Hye Nam, Dong-Gyun Kim, Young-Ju Jee, Sang-Jun Lee and Cheul-Min An

Biotechnology Research Division, National Fisheries Research and Development Institute, Busan 619-902, Korea

Abstract

The gene encoding an esterase from *Photobacterium* sp. MA1-3 was cloned in *Escherichia coli* using the shotgun method. The amino acid sequence deduced from the nucleotide sequence (948 bp) corresponded to a protein of 315 amino acid residues with a molecular weight of 35 kDa and a pI of 6.06. The deduced protein showed 74% and 68% amino acid sequence identities with the putative esterases from *Photobacterium profundum* SS9 and *Photobacterium damselae*, respectively. Absence of a signal peptide indicated that it was a cell-bound protein. Sequence analysis showed that the protein contained the signature G-X-S-X-G included in most serine-esterases and lipases. The MA1-3 esterase was produced in both soluble and insoluble forms when *E. coli* cells harboring the gene were cultured at 18°C. The enzyme was a serine-esterase and was active against C_2, C_4, C_8 and C_{10} *p*-nitrophenyl esters. The optimum pH and temperature for enzyme activity were pH 8.0 and 30°C, respectively. Relative activity remained up to 45% even at 5°C with an activation energy of 7.69 kcal/mol, which indicated that it was a cold-adapted enzyme. Enzyme activity was inhibited by Cd^{2+}, Cu^{2+}, Zn^{2+}, and Hg^{2+} ions.

Keywords: *Photobacterium* sp., Cold-adapted esterase, Gene expression, Substrate specificity

Introduction

Lipases and esterases (glycerol ester hydrolases, E.C. 3.1.1.) are hydrolases that act on the carboxyl ester bonds present in acylglycerols to liberate organic acids and glycerol. Esterases (E.C. 3.1.1.1) differ from lipases (E.C. 3.1.1.3) mainly based on their substrate specificity and interfacial activation (Long, 1971). Lipases, which have a hydrophobic domain covering the active site, show a preference for triglycerides of long chain fatty acids, and thus have different properties to esterases, which have an acyl binding pocket (Pleiss et al., 1998). Lipases and esterases have been recognized as useful biocatalysts because of their versatility in a wide range of industrial applications, including their use in detergents or as additives in the food industry (Harwood 1989; Jaeger and Reetz 1998). Due to their wide diversity enzymatic properties, large numbers of lipases/esterases isolated from bacteria, fungi, plants, and higher animals have been reported (Jaeger et al., 1999; Schmidt and Verger, 1998; Villeneuve et al., 2000). In particular, lipases/esterases of microbial origin represent the most extensively used class of these enzymes and are attracting increasing attention due to their relative ease of production and potential applications in biotechnology (Hasan et al., 2006). Microorganisms that thrive at low temperatures produce cold-adapted enzymes, which have high catalytic efficiency, generally associated with low thermal stability (Feller et al., 1996). Among these enzymes, cold-adapted lipases/esterases are useful in industrial applications as additives in laundry detergents to allow washing in cold water, the food industry, bioremediation processes, and biodiesel applications, based on their high catalytic activity at low temperature and low thermostability as well as unusual specificities (Knothe, 2005; Hasan

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***Corresponding Author**
E-mail: yobest12@korea.kr

et al., 2006; Margesin, 2007). In addition, these enzymes can potentially be used as catalysts in organic synthesis of chiral intermediates, allowing relatively unstable compounds to be produced at low temperatures (Ryu et al., 2006). Compared to other lipases, few cold-adapted lipase/esterase have been studied. These include the enzymes from *Moraxella* sp. strain TA144 (Feller et al., 1991), *Aeromonas* sp. LPB4 (Lee et al., 2003), *Pseudomonas* sp. strain B11-1 (Choo et al., 1998), *Acinetobacter* sp. No. 6 (Suzuki *et al*., 2001, 2002a, 2002b), *Psychrobacter* sp. Ant300 (Kulakova et al., 2004), *Photobacterium* sp. (Ryu et al., 2006), *Salinisphaera* sp. P7-4 (Kim et al., 2011), and *Shewanella* sp. Ke75 (Kim et al., 2013).

The strain *Photobacterium* sp. MA1-3, previously isolated from the intestine of a blood clam, possesses a cold-adapted lipase (Kim et al., 2012). Recently, we isolated another recombinant clone bearing lipolytic activity from a gene library from this strain, and the gene was heterologously expressed in *Escherichia coli* cells. Here, we report the cloning, sequencing, and biochemical properties of the cloned enzyme.

Materials and Methods

Materials

Tributyrin, *p*-nitrophenyl (*p*-NP) acetate (C_2), butyrate (C_4), caprylate (C_8), caprate (C_{10}), palmitate (C_{16}), and stearate (C_{18}) were purchased from Sigma (St. Louis, MO, USA), and *p*-NP myristate (C_{14}) was from Fluka (Milwaukee, WI, USA). All other chemicals and solvents were analytical grade and are commercially available.

Gene cloning and sequence analysis

Chromosomal DNA from *Photobacterium* sp. MA1-3 was partially digested with *Sau*3AI, ligated into a pUC118-HincII vector (Takara, Kyoto, Japan), and used to transform *E. coli* XL1-Blue. A colony, forming a clear halo on a Luria Bertani (LB) plate containing tributyrin and ampicillin (100 μg/mL), was selected. The recombinant plasmid (pUCMA1-3) was then purified from the transformant, and the insert DNA sequence was determined. DNA sequencing was performed with an Applied Biosystems Automated DNA Sequencer model 3130 with a dye-labeled terminator sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequence analysis and database similarity searches were performed using the server at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignments were performed using ClustalW (Thompson et al., 1994), and the signal peptide sequence was deduced by SignalP version 3.0.

Construction of the expression vector and overexpression

The DNA corresponding to the coding region was amplified by PCR. The putative MA1-3 esterase gene was amplified from the pUCMA1-3 plasmid using the primers: 5′-TTCATATGGATAGTTGGCGCAATAGG -3′ (*Nde*I adaptor restriction enzyme site is underlined) and 5′-TTCTCGAGGCTAGCTTCTTTATGTTC-3′ (*Xho*I). After digestion with *Nde*I and *Xho*I, the PCR product was ligated into the pET22b(+) vector (Novagen, Madison, WI, USA). The resulting recombinant plasmid, pETMA1-3, was transformed into *E. coli* BL21 (DE3) cells.

Purification of the recombinant protein

E. coli BL21 (DE3) cells transformed with pETMA1-3 were cultivated in LB medium containing ampicillin (100 μg/mL) at 30°C. When the optical density at 600 nm reached 0.6, 1 mM isopropylthiogalactoside (IPTG) was added, and the cultures were further incubated overnight at 18°C. *E. coli* cells were then harvested and ruptured by ultrasonic cell lysis. The soluble proteins were recovered from the cell extract by centrifugation (10,000 *g*, 20 min) and loaded onto a nickel-nitrilotriacetic (Ni-NTA) column. After washing with 60 mM imidazole, 500 mM NaCl, and 50 mM Tris-HCl buffer (pH 7.9), the bound esterase was then eluted using 1000 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl buffer (pH 7.9) and dialyzed against a 50 mM Tris-HCl buffer (pH 8.0) to characterize its biochemical properties. The protein concentration was determined by the BCA method with bovine serum albumin as the standard (Sigma).

Esterase assay

Esterase activity was measured using *p*-nitrophenyl (*p*-NP) esters with fatty-acid chain lengths of C_2-C_{18} (Ryu et al., 2006). The standard assay mixture (1.0 ml) contained 10 mM *p*-NP butyrate (C_4) in ethanol, 50 mM Tris-HCl buffer, pH 8.0, and 10 μl of purified enzyme. Blank reactions were also run with a composition identical to the assay mixture without the enzyme. The mixture was incubated at 30°C for 5 min and the absorbance of *p*-NP librated was then measured at 405 nm. For long-chain *p*-nitrophenyl esters (C_{12}-C_{18}), 20 μl of esterase solution was added to 880 μL of reaction buffer containing 50 mM Tris-HCl (pH 8.0), 0.1% gum Arabic, and 0.2% deoxycholate. After 5-min incubation at 30°C, the reaction was initiated by adding 100 μl of 8 mM substrate in isopropanol. The reaction was stopped by addition of 0.5 ml of 3 M HCl. After centrifugation, 333 μl of supernatant was mixed with 1 ml of 2 M NaOH, and the absorbance at 405 nm was measured. One unit of enzyme activity was defined as the release of 1 μmol of *p*-nitrophenol per min from *p*-NP ester.

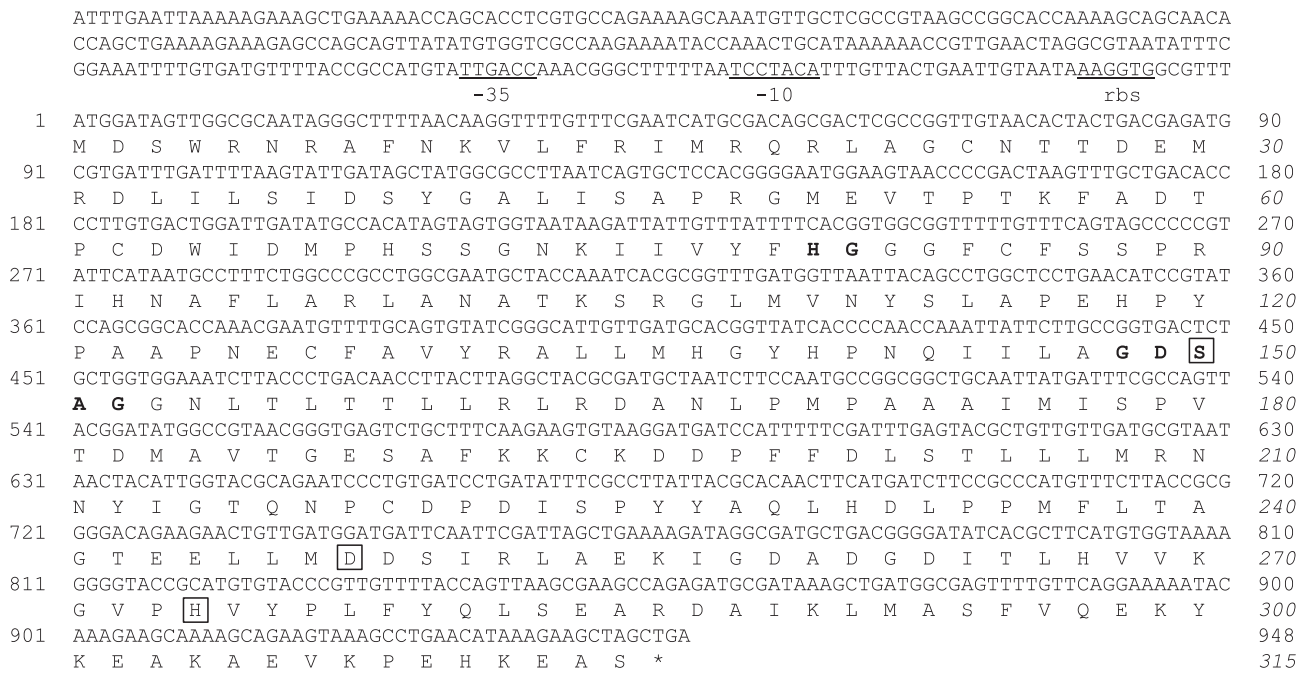

Fig. 1. Nucleotide sequence of the MA1-3 esterase gene and its deduced amino acid sequence. Potential promoter regions (-10, -35) and ribosomal binding-site (RBS) are labeled and underlined. The conserved Ser, Asp, and His residues that comprise a putative catalytic triad are boxed. Both conserved pentapeptide sequence (Gly-X-Ser-X-Gly) and HG sequence (oxyanion hole) are indicated in bold. The sequence has been submitted to GenBank under accession number KF431955.

Biochemical properties of recombinant esterase

The optimum temperature was assayed at various temperatures in the range of 5-80°C at 50 mM Tris-HCl (pH 8.0). For thermostability, the enzyme was preincubated at various temperatures for 30 min in 50 mM Tris-HCl buffer (pH 8.0). After rapid centrifugation, they were removed and the residual enzyme activity of the supernatant was measured as standard assay. Various buffers were used to study the effects of pH: sodium acetate/acetic acid (pH 4-6), Tris/acetate (pH 6-7), Tris/HCl (pH 7-9), and sodium tetraborate/NaOH (pH 9-11). To determine pH stability, the enzyme was preincubated at 25°C in buffers of various pH for 1 h and the remaining activity was measured by standard assay. The effects of various metal ions and inhibitors on enzyme activity were assessed after preincubation in 50 mM Tris-HCl buffer (pH 8.0) at 25°C for 30 min. Blank reactions were performed with each measurement under different conditions and the values for nonenzymatic hydrolysis of substrates were subtracted from the results.

GenBank accession number

The nucleotide sequence of the *Photobacterium* sp. MA1-3 esterase gene has been deposited in the GenBank database under accession number KF431955.

Results and Discussion

Gene cloning and sequence analysis

A DNA library of *Photobacterium* sp. MA1-3 was constructed with the *Sau*3A1 enzyme and used to transform *E. coli* XL1-Blue. *E. coli* transformant that showed a clear zone on TBN-LB plates after 48 h of incubation were selected. The recombinant plasmid from a clone was shown to contain a DNA insert of 3.2 kb, with one complete open reading frame (ORF) corresponding to a putative esterase/lipase gene based on the results of a homology search. The complete DNA sequence of the MA1-3 esterase was determined (Fig. 1). It contained a ORF of 948 bp, encoding a polypeptide chain of 315 amino acids with a predicted molecular weight of 35,020 and a pI value of 6.06. No signal sequence was found, as determined by SignalP version 3.0, suggesting that the MA1-3 esterase is a cell-bound protein. A putative ribosomal binding-site (RBS) was located 5 bp upstream of the MA1-3 esterase. In addition, a putative transcriptional initiation site with a -10 region was located 32 bp upstream of the MA1-3 esterase start codon and separated from the –35 region by 15 bp. The predicted amino acid sequence of the cloned MA1-3 esterase was compared with other protein sequences deposited in GenBank using the basic local alignment search tool (BLAST) program (Fig. 2A). The esterase showed 74%, 68%, 52%, and 36% identities with

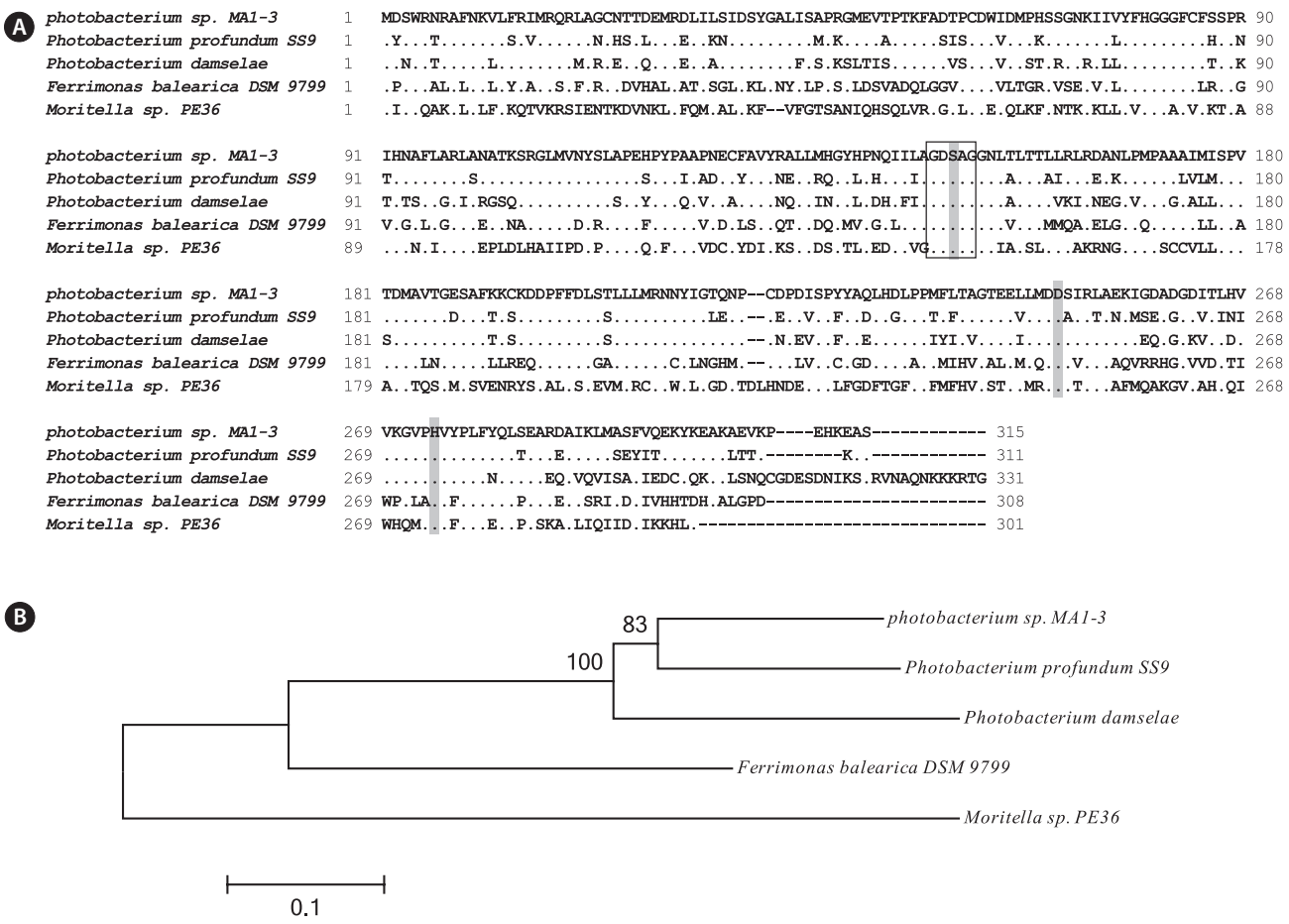

Fig. 2. Protein sequence alignment (A) and phylogenetic tree (B) of MA1-3 esterase and five similar enzymes. (A) Identity sequences are displayed by dots (...), and deletions of amino acid residues are indicated by dashes (---). Residues involved in the catalytic triad are shaded. Conserved pentapeptide containing the catalytic serine is shown in box. The accession numbers for each sequence are as follows: *Photobacterium* sp. MA1-3 (KF431955), *Photobacterium profundum* SS9 (YP_130084), *Photobacterium damselae* (WP_005299527), *Ferrimonas balearica* (YP_003912034), *Moritella* sp. PE36 (WP_006032431). (B) A phylogenetic tree of the aligned sequences was constructed using the Neighbor-Joining algorithm in MEGA (version 4.0). The degree of confidence for each branch point was determined by bootstrap analysis (1,000 repetitions).

the putative esterase/lipases from *Photobacterium profundum* SS9, *Photobacterium damselae*, *Ferrimonas balearica*, and *Moritella* sp. PE36, respectively. The MA1-3 esterase was only 44% identical to the lipases of the same strain *Photobacterium* sp. MA1-3 (Kim et al., 2012).

The MA1-3 esterase primary structure contained a -G-D-S-A-G- sequence (positions 148-152), which corresponds well with the pentapeptide -G-x-S-x-G- signature motif that is generally conserved in many esterase enzymes. Based on sequence comparisons with other esterases, it was concluded that Ser 150 (in the motif GDSAG), Asp 248, and His 274 comprise the catalytic triad. Finally, an HG sequence (His80, Gly81), which constitutes an oxyanion hole in the three-dimensional protein structure, was found in the esterase (Grochulski et al., 1993; Martinez et al., 1994). Sequence analysis suggested that MA1-3 esterase may be a functional esterase with a novel amino acid sequence.

Fig. 2B shows the phylogenetic tree, indicating the evolutionary relationship with other bacterial esterases based on the amino acid sequence. The phylogram generated using Phylip showed that *Photobacterium* sp. MA1-3 esterase was more closely related to a putative esterase from *Photobacterium profundum* SS9 than to other lipases and esterases identified to date.

Expression and identification of the recombinant esterase

E. coli BL21 (DE3) was transformed with the plasmid, pETMA1-3, and was induced to express the recombinant protein using 1 mM IPTG. When cultivated and incubated at 37°C, the resulting protein was insoluble; however, at the lower culture temperature of 18°C, the resulting protein was both soluble and insoluble (Fig. 3). Approximately 20% of the

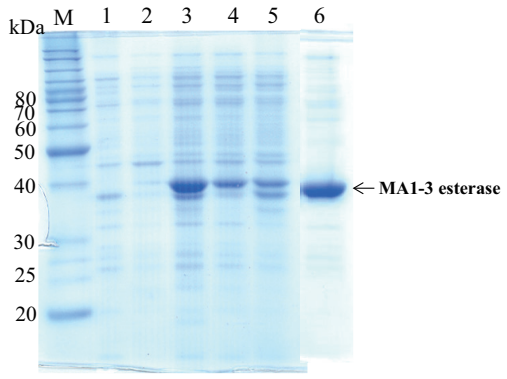

Fig. 3. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of MA1-3 esterase. Lane M, Standard protein molecular weight markers; lane 1, lysate supernatants of uninduced transformant; lane 2 and 3, soluble and insoluble protein induced by isopropylthiogalactoside (IPTG) at 37°C ; lane 4 and 5, soluble and insoluble protein induced by IPTG at 18°C lane 6, purified esterase by nickel-nitrilotriacetic (Ni-NTA) affinity column chromatography. Arrow indicates MA1-3 esterase.

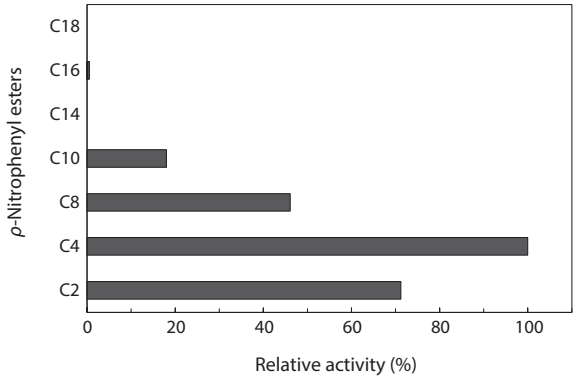

Fig. 4. Substrate specificity of MA1-3 esterase. Hydrolysis activity was measured toward various *p*-nitrophenyl esters.

recombinant esterase protein was produced in soluble form in *E. coli* cells. The recombinant enzyme was then purified to homogeneity by His-Bind resin affinity chromatography with a six-histidine tag at the C-terminus. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the eluted fraction showed a distinctly expressed protein band of 39 kDa (Fig. 3). This mass is in agreement with the mass of the esterase (35 kDa) with an additional 36 amino acid residues corresponding to the N-terminus 6× histidine-tag and the linker region. The specific activity of the purified enzyme was 20.2 U/mg toward *p*-NP butyrate at 30°C. Its activity was 3.8- and 2.9-fold higher than those of PWTSB and PWTSC, which had specific activities of 5.3 and 6.9 U/mg toward PNPB, respectively (Wei et al., 2009). In contrast, Ke75 esterase showed a lightly higher specific activity of 22.75 U/mg (Kim et al., 2013).

Substrate specificity

The substrate specificity of MA1-3 esterase was examined with various *p*-NP esters with aliphatic acyl-chains of different lengths from 2 to 18 carbon atoms (Fig. 4). Analysis of the enzyme hydrolytic activity against these *p*-NP esters showed a strong preference toward short acyl chains of *p*-NP-2, *p*-NP-4 and *p*-NP-8 esters, with *p*-NP-C_4 being the most easily hydrolyzed substrate. The reactivities of these substrates to the enzyme were dependent on their acyl-chain length, and the substrates with C_{14}, C_{16}, and C_{18} acyl groups were virtually inert as substrates for the enzyme. Based on the substrate preference profile, MA1-3 esterase was classified as a true carboxylesterase (Jaeger et al., 1993, 1999; Bornscheuer, 2002).

Effects of pH and temperature on enzyme activity and stability

The temperature activity profile of MA1-3 esterase was examined over the temperature range of 5–80°C under assay conditions with *p*-NP butyrate as the substrate (Fig. 5A). Enzyme activity peaked at 30°C. The activation energy of the enzymes derived from cold-active organisms is usually lower than those from their mesophilic counterparts (Feller et al., 1996). As expected, the activation energy of MA1-3 esterase was 7.69 kcal/mol in the range of 5°C to 30°C, which is lower than those of other cold-adapted esterases: 11.2 kcal/mol for the esterase of *Pseudomonas* sp.B11-1 (Suzuki et al., 2003); 9.0 kcal/mol for the esterase of *Acinetobacter* sp. No. 6 (Suzuki et al., 2002a); and 11.25 kcal/mol for the esterase of *Acinetobacter lwoffii* 16C-1 (Kim and Park, 2002). These observations suggest that the catalytic efficiency of this esterase is high over this temperature range. In fact, this esterase showed as much as 45% of the maximum activity at 5°C. The enzyme was stable within the temperature range 5~40°C. However, it was thermally unstable and lost its activity at temperatures above 50°C (Fig. 5C). Taken together, these observations indicated that MA1-3 esterase is a typical cold-adapted enzyme

The optimal pH of MA1-3 esterase was determined to be 8.0, and it retained at least 80% of its maximum activity between pH 8.0 and 9.0, indicating that it is an alkaline enzyme (Fig. 5D). Although its esterase activity was somewhat different depending on the various incubation buffers used, the MA1-3 esterase was fairly stable after incubation in buffers ranging in pH from 7.0 to 10.0 (Fig. 5E). The maximum activity at alkaline pH is a useful characteristic for detergent applications.

Effects of metal ions and inhibitors on esterase activity

The effects of various metal ions and inhibitors on enzyme activity were determined (Table 1). Divalent salts, such as $MgCl_2$, simulated the esterase activity to 119% compared to

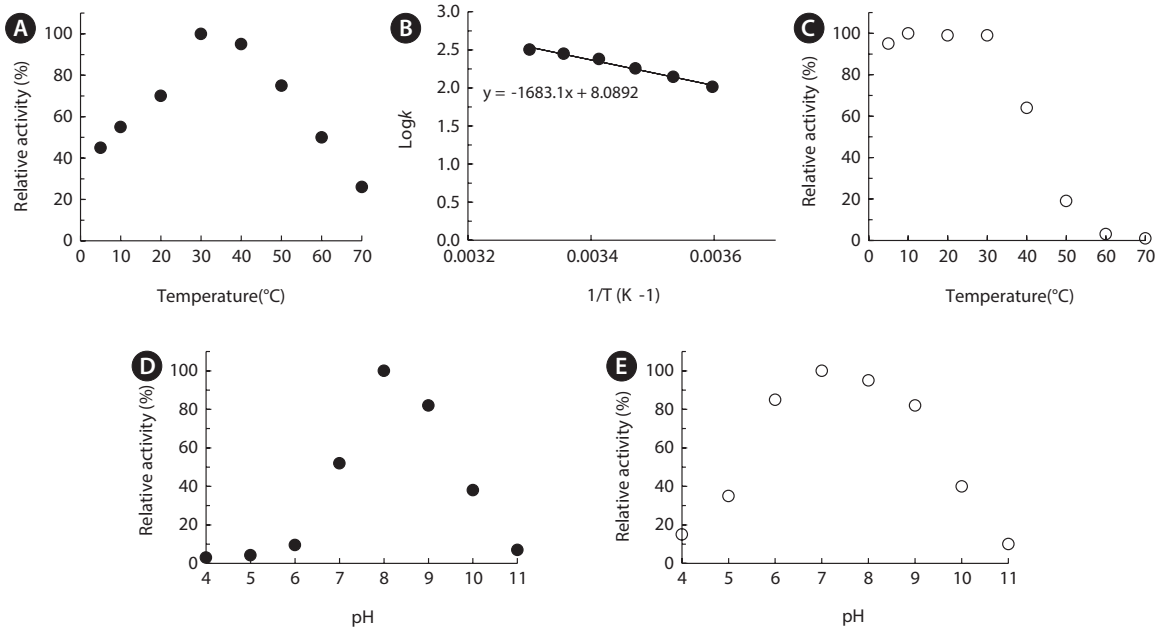

Fig. 5. Effects of temperature and pH on the activity and stability of MA1-3 esterase. (A) Hydrolytic activity was assayed at various temperatures (B) The logarithm of the enzyme turnover rate (k) (s^{-1}) was plotted against the reciprocal of absolute temperature (T). The values shown are activation energy calculated from the linear part of the Arrhenius plot. (C) Residual hydrolytic activity was measured after treatment for 30 min at a range temperatures (D) Hydrolytic activity was assayed at various pHs (E) Residual hydrolytic activity was measured after treatment for 30 min at a range pH values.

control, whereas the activity was decreased by 50~60% in the presence of $MnSO_4$ $NiSO_4$, and $CoCl_2$ Moreover, its activity was strongly inhibited by 5 mM metal ions such as $CdCl_2$, $CuCl_2$, $ZnCl_2$, and $HgCl_2$.

To confirm that the enzyme was a serine hydrolase, the activity of MA1-3 esterase was determined in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF), a catalytic serine enzyme inhibitor. Similar concentrations of a metal-chelating agent, ethylenediaminetetraacetic acid (EDTA), and a reducing agent, dithiothreitol (DTT), were also investigated to eliminate the possible involvement of metal cations or cysteine in the enzyme mechanism. MA1-3 esterase was significantly inhibited (98%) by PMSF, while the other two additives (DTT and EDTA) had little effect on its activity. The inhibitory effect of PMSF on MA1-3 esterase indicated the involvement of serine-mediated catalytic activity in this enzyme.

Table 1. Effects of various metal ions and inhibitors on MA1-3esterase activity

Metal ions or reagents (5 mM)	Relative activity (%)*
$CaCl_2$	63 ± 4.2
$CdCl_2$	1 ± 0.8
$CuSO_4$	6 ± 2.1
$CoCl_2$	39 ± 1.7
$FeSO_4$	95 ± 2.5
$HgCl_2$	2 ± 1.3
KCl	99 ± 4.2
$MgCl_2$	119 ± 0.4
$MnSO_4$	60 ± 1.5
$NiSO_4$	54 ± 3.7
$ZnCl_2$	4 ± 2.3
EDTA	105 ± 0.6
PMSF	3 ± 1.1
DTT	102 ± 1.6

*Data are presented ± standard deviation.

In this study, a novel esterase produced by *Photobacterium* sp. MA1-3 exhibited high activity at low temperatures and alkaline pH. Its low activation energy in the range of 5-30°C indicated that it is a cold-adapted enzyme. These results suggest that this enzyme may be useful as a biocatalyst and detergent additive for low temperature applications.

Acknowledgements

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