

Shewanella sp. Ke75 Esterase with Specificity for *p*-nitrophenyl Butyrate: Gene Cloning and Characterization

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Abstract A bacterial strain that produces a cold-adapted esterase was isolated from tidal flats and identified as *Shewanella* sp. Ke75. In the present study, the corresponding gene was cloned using the shotgun method. The amino acid sequence deduced from the nucleotide sequence (957 bp) corresponded to a protein of 318 amino acid residues with a calculated molecular weight of 34875 Da. The esterase showed 68 and 57% identities with the putative esterases of *Shewanella amazonensis* SB2B and *Colwellia psychrerythraea* 34H, respectively. The esterase contained a putative leader sequence, as well as the conserved catalytic triad (Ser, His, Asp), consensus pentapeptide GX SXG, and oxyanion hole sequence (HG). The protein Ke75 was produced in both soluble and insoluble forms when *Escherichia coli* cells harboring the gene were cultured at 30°C. The enzyme showed specificity for C4 (butyrate) as a substrate, with little activity toward the other *p*-nitrophenyl esters tested. The optimum pH and temperature for enzyme activity were pH 9.0 and 30°C, respectively. Relative activity remained up to 60% even at 5°C with an activation energy of 6.29 kcal/mol, which indicated that it was a cold-adapted enzyme. Enzyme activity was enhanced in the presence of Mn²⁺ ions, but inhibited by Cd²⁺, Cu²⁺, Hg²⁺, and Zn²⁺ ions.

Keywords cold-adapted esterase · gene expression · *Shewanella* sp. · substrate specificity

Introduction

Lipases/esterases (EC 3.1.1.x) represent a diverse group of hydrolases that catalyze the cleavage and formation of ester bonds. They are ubiquitous in animals, plants, and microorganisms, including fungi and bacteria (Lee et al., 2006). The enzymes include esterase (EC 3.1.1.1) and lipase (EC 3.1.1.3), which catalyze the hydrolysis and synthesis of short-chain (<10) and long-chain acylglycerols (Arpigny and Jaeger, 1999), respectively. Certain enzymes exhibit narrow substrate specificity, position selectivity, and stereoselectivity, and therefore serve as useful biocatalysts in the pharmaceutical and fine chemical industries (Bornscheuer and Kazlauskas, 1999; Jaeger and Eggert, 2002; Gupta et al., 2004). 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. Psychrophilic lipases/esterases have attracted a great deal of attention because of their increasing use in organic synthesis of chiral intermediates due to their low optimum temperature and high activity at low temperatures, which are favorable properties for the production of relatively fragile compounds. In addition, cold-active enzymes cover a broad spectrum of biotechnological applications, including additives in detergents (cold washing), additives in food industries (fermentation, bakery, meat tenderizing), and environmental applications (digesters, bioremediation, bioaugmentation) (Joseph et al., 2008). Lipases of microbial origin represent the most extensively used class of these enzymes and are attracting

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increasing attention due to their relative ease of production and potential applications in biotechnology (Hasan et al., 2006). Cold-adapted microorganisms have been isolated from cold regions and studied as potential sources of cold-active lipases/esterases. Compared to other lipases, fewer cold-active bacterial lipases/esterases have been studied in detail. 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 lipolyticum* sp. nov. (Ryu et al., 2006), and *Salinisphaera* sp. P7-4 (Kim et al., 2011).

Recently, we isolated the psychrophilic bacterium, *Shewanella* sp. Ke75 from the intertidal flats of the KeWhaDo, Korea, which was shown to produce a cold-active esterase. Here, we describe the cloning and structural analysis of the unique *Shewanella* sp. Ke75 esterase gene and its overexpression in *Escherichia coli* along with characterization of its biochemical properties.

Materials and Methods

Bacterial strains and plasmids. Sludge samples taken near the intertidal flats of the KeWhaDo, Korea were collected, and colonies were screened for esterase activity by observing the clear zone on tributyrin (TBN)-marine agar plates (Ryu et al., 2006). Based on the level of esterase production, one strain, designated Ke75, was selected and identified based on 16S rDNA gene sequence analysis. *E. coli* XL1-blue was used as the cloning and expression host, and the pGEM-T easy vector (Promega, USA), and pGEX4T-1 (GE Healthcare Life Sciences, USA) were used as the cloning and expression vectors, respectively.

DNA manipulation and analysis of 16S rDNA. The genomic DNA from *Shewanella* sp. Ke75 was prepared for 16S rDNA analysis according to the method described by Saito and Miura (1963). Polymerase chain reaction (PCR) was performed to amplify the 16S rDNA coding region using the primers 5'-AGAG TTTGATCCTGGCTCAG-3' and 5'-ACGGTTACCTTGTTACG ACTT-3'. The reaction mixture was subjected to initial denaturation at 95°C for 10 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, with a final extension step at 72°C for 10 min. The PCR product was subcloned into the pGEM-T Easy vector (Promega, USA), and transformed into *E. coli* DH5a. DNA sequencing was performed using an Applied Biosystems Automated DNA Sequencer model 3130 with a dye-labeled terminator sequencing kit (Applied Biosystems, USA).

Cloning and sequence analysis of the esterase gene. Chromosomal DNA from *Shewanella* sp. Ke75 was partially digested with *Sau3AI*, ligated into the pUC118-HincII vector (TaKaRa, Japan) and used to transform *E. coli* XL1-Blue. A colony that formed a clear halo on a tributyrin (TBN)-Luria-Bertani (LB) plate containing ampicillin (100 µg/mL) was

selected. The recombinant plasmid (pUCKE75) was then purified from the transformant, and the insert DNA sequence was determined. 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.

Expression and purification of the esterase. The *E. coli* expression vector pGEX-4T-1 was used to express the recombinant Ke75 esterase. First, two oligomeric primers and the pUCKE75 plasmid from Ke75 shotgun library DNA were used to generate a PCR product carrying a *Bam*HI restriction site at its 5' end and *Xho*I site at its 3' end. The nucleotide sequences of the two primers were as follows: forward primer 5'-CCCGGATCCATGC GAGGAATCGTTTC-3' and reverse primer 5'-CCCCTCGAGGG CCTTGCCATAAACGC-3'. After digestion with *Bam*HI and *Xho*I, the PCR product was ligated into the pGEX-4T-1 expression vector. The resulting recombinant plasmid, pGEXKe75, was used for expression of the esterase gene in *E. coli* strain XL1-Blue. The transformed cells were grown at 30°C in 100 mL of LB medium containing 100 µg/mL ampicillin. When the cultures reached an optical density at 600 nm (OD₆₀₀) of 0.5, they were induced overnight with 0.5 mM isopropylthiogalactoside (IPTG). Cells were harvested by centrifugation (10000 × g, 20 min) at 4°C and stored at -70°C until lysis. The cell pellet was resuspended in 5 mL of ice-cold binding buffer (Phosphate buffered saline, PBS; 10 mM Na₂HPO₄, 140 mM NaCl, 2.7 mM KCl, 1.8 mM KH₂PO₄, pH 7.4) and lysed by sonication. The lysate was centrifuged at 15000 × g for 15 min at 4°C to remove cell debris and denatured protein. The soluble proteins were recovered from the cell extract by centrifugation and loaded onto a Glutathione *S*-Transferase (GST) SpinTrap purification module (GE Healthcare Life Sciences, USA). After washing in 1× PBS, the bound fusion protein was then eluted using glutathione elution buffer (50 mM Tris-HCl, pH 8.0, and 20 mM reduced glutathione). Proteins were then resolved by 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined by the bicinchoninic acid (BCA) method using a protein assay kit (Sigma-Aldrich, USA).

Esterase activity assays. Esterase activity was measured using *p*-nitrophenyl (*p*-NP) esters with fatty-acid chain lengths of C₂-C₁₈ (Ryu et al., 2006). The standard assay mixture (1.0 mL) contained 10 mM *p*-nitrophenyl butyrate (PNPB) in ethanol, 50 mM Tris-HCl (pH 8.0), and an appropriate amount (10 µL) of the enzyme. Blank reactions were performed with compositions identical to the assay mixture but without the enzyme. The mixture was incubated at 30°C for 5 min, and the absorbance of *p*-NP liberated was then measured at 405 nm. For long-chain *p*-nitrophenyl esters (C₁₂-C₁₈), 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 37°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 μ L 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 μ mol of *p*-nitrophenol per min from *p*-nitrophenyl ester.

Biochemical properties of the esterase. The optimum temperature of the Ke75 esterase was measured by assaying its hydrolytic activities toward *p*-nitrophenyl butyrate at temperatures of 10–70°C. To evaluate its thermostability, the enzyme was incubated at 10–70°C for 30 min in 50 mM Tris-HCl buffer (pH 8.0), then the residual activity was measured under standard assay conditions. The optimum pH for the Ke75 esterase was measured by assaying at 30°C with various pH buffers; *i.e.*, sodium acetate/acetic acid (pH 4–6), Tris/acetate (pH 6–7), Tris/HCl (pH 7–9), and sodium tetraborate/NaOH (pH 9–11) buffers. To evaluate its pH stability, the enzyme was incubated at various pH values (4–11) at 30°C for 30 min, and then residual activity was measured under standard assay conditions. The effects of various metal ions, chemical reagents, detergents, and organic solvents 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 to subtract the appropriate values for nonenzymatic hydrolysis of substrates from the results. **Nucleotide sequence accession numbers.** The nucleotide sequences of the *Shewanella* sp. Ke75 16S rDNA and esterase gene were deposited in the GenBank database under accession numbers JQ765408 and JQ765409, respectively.

Results and Discussion

Identification of Ke75 strain and cloning of the esterase gene. Intertidal flat sediments contain remarkable and unique bacterial diversity due to the dynamic physicochemical conditions (Kim et al., 2004). Of the many microbial strains isolated from the tidal flats of KeWhaDo, Korea, one showed a clear zone around its colonies after growing for 1–2 days on marine agar plates containing 1% tributyrin. Full-length sequencing of its 16S rDNA (GenBank accession no. JQ765408) indicated that it was most closely related to the genus *Shewanella*, with the highest similarity of 100% to *Shewanella vesiculosa*, 99.9% to *Shewanella livingstonensis*, 98.9% to *Shewanella frigidimarina*, and 98.5% to *Shewanella arctica*. Therefore, this strain was identified as *Shewanella* sp. Ke75. *Shewanella* strains are comprised of Gram-negative, rod-shaped, and facultatively anaerobic cells. To the best of our knowledge, no previous reports of esterases/lipases from *Shewanella* strains have been made; thus, we attempted to clone the corresponding gene and to characterize the new esterase.

A DNA library of *Shewanella* sp. Ke75 was constructed with the *Sau3A1* enzyme and used to transform *E. coli* XL1-blue. After 48 h of incubation, an *E. coli* transformant that showed a clear zone on TBN-LB plates was selected. The recombinant plasmid isolated from the cells was fully sequenced, and found to contain

a 3.1-kb insert DNA, with three major open reading frames (ORFs) corresponding to major facilitator transporter, putative esterase/lipase, and short-chain dehydrogenase/reductase SDR, based on the results of a homology search.

Sequence analysis of the esterase gene. The nucleotide and deduced amino acid sequences of the Ke75 esterase are shown in Fig. 1. Sequence analysis identified a 957-bp esterase ORF that encoded a polypeptide of 318 amino acids with a calculated molecular mass of 34875 Da. A putative ribosomal-binding site (RBS) was identified 7-bp upstream of the start codon (AGGAGG). No promoter-like motifs were identified in the sequence preceding the esterase coding region. The predicted esterase pre-protein contains a putative 23-aa leader peptide, which is cleaved to form a mature protein of 295 aa with a pI of 5.17. Support for identification of the esterase was provided by the presence of the semi-conserved active site pentapeptide, Gly-His-Ser-His-Gly, in the protein.

The predicted amino acid sequence of the cloned Ke75 esterase was compared with other protein sequences in GenBank using the basic local alignment search tool (BLAST) program (Fig. 2A). These putative esterases were identified by whole-genome sequencing, but none of them has been biochemically characterized. The Ke75 esterase exhibited the highest level of identity (68%) to the putative esterase of *Shewanella amazonensis* SB2B (YP_926405.1), followed by 57% identity to the putative esterase of *Colwellia psychrerythraea* 34H (YP_267690) and 45, 43, and 37% identities to the putative carboxylesterases of *Chromobacterium violaceum* ATCC 12472 (NP_900028), *Neisseria shayegani* 871 (ZP_08888002), and *Legionella longbeachae* D-4968 (ZP_06185889), respectively. In addition, signature sequences conserved in esterases/lipases were found in the ORF. The serine residue in the conserved pentapeptide GDSAG (from 158 to 162) appears to be one of the active sites. Asp 216 and His 291 represent the other two residues of the catalytic triad in the active sites. The N-terminal HG (from 87 to 88) may comprise an oxyanion hole to stabilize tetrahedral intermediates (Grochulski et al., 1993; Martinez et al., 1994). Many conserved regions were found within the entire sequences of all six protein sequences. Therefore, the Ke75 esterase seems to be a functional esterase/lipase 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 *Shewanella* sp. Ke75 esterase was more closely related to a putative esterases from *Shewanella amazonensis* SB2B and *Colwellia psychrerythraea* 34H than to other lipases and esterases. **Expression and purification of the esterase.** The esterase gene was inserted into the pGEX-4T-1 expression vector utilizing *Bam*HI and *Xho*I restriction enzymes, and the recombinant plasmid, pGEXKe75, was constructed. *E. coli* XL1-Blue cells were then transformed with the plasmid and induced to express the recombinant protein with IPTG. After induction of heterologous gene expression with 0.5 mM IPTG at 30°C, the cells were harvested and lysed by sonication. Soluble and insoluble fractions

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1  ATGCGAGGAATCGTTTCCCCTAAGCTGACTGATTTTCTAGCGTTAGCTAACAGCAATATTGCCTTGGCTAAACAACATAATGTCCAATTT  91
  M R G I V S P K L T D F L A L A N S N I A L A ↑ K Q H N V Q F  30
91  ACCCCAGCGATATTACGCGAAAATCTCAATAAACTTGGCGCACTAATGAGTGACTCTCCCTCAGTGAGCTATATTGCCGACAAGGCGTTT  181
  T P A I L R E N L N K L G A L M S D S P S V S Y I A D K A F  60
181  ACGTTAACCGACCGAGCTATTCCAGTCAGAGTGATAGCCCTGCACCAGAGCAGCCTTTGCCTGTGTTGGTGCACCTTCATGGTGGCGGT  271
  T L T D R A I P V R V Y S P A P E Q P L P V L V H F H G G G  90
271  CATATGTGTGGCAGTGTGGCGCTGTACGATCCGATTTGCCGTCATCTAGCCAGTATTGTGCAGTGGTGGTATTTCGGTTGAATACCGA  361
  H M C G S V A L Y D P I C R H L A S I V Q C V V I S V E Y R  120
361  CTCGCGCCTGAATTCCCATAACCCTGCAGGGCTTGATGACTGTGAATATGCACTGATCCATTATGCCGAGTTACTCGATGATGTTAACCAT  451
  L A P E F P Y P A G L D D C E Y A L I H Y A E L L D D V N H  150
451  CAAGACTCAGTCACCATTATTGGTGACAGTGCTGGTGGTGCTATTTGCACTAGCTTAAGCATGAAAAGTCTAACCGATAAACAGATTAAG  541
  Q D S V T I I G D S A G G A I C T S L S M K S L T D K Q I K  180
541  ATAGACCAACAAATTTTGTCTATCCAGTGTGCATTACACATTAAGCAGCCCATCACTTGATAGTAATGGCACTGGCTTCTTACTCGAA  631
  I D Q Q I L I Y P S V D Y T L S S P S L D S N G T G F L L E  210
631  ACACCTCGTATAAAGTGGTATTTTGGAGCAGTATTTCAACTGTCCGAATCATTGTCAAAGCAACAGATAGAGCAAGTTAAACAGGCTTCA  721
  T P R I K W Y F E Q Y F Q L S E S L S K Q Q I E Q V K Q A S  240
721  CCTTTGCTTGGTCCCATTAACGCTCACTTACCGAAAACACTGATTTTACCAGGTTGCGATCCGCTTAGGGATGAGGGCATAGCATAT  811
  P L L G P I N A H L P K T L I F T A G C D P L R D E G I A Y  270
811  GCTAATGCGTTAACTGCTGCGGGTGTAGAGGTTGAGCACCATGCGTTCGACGGCATGATCCATGCCTATATGTTGCTGCACGACTTAGTC  901
  A N A L T A A G V E V E H H A F D G M I H A Y M L L H D L V  300
901  AAAGAGGAATGATGGCAACCTCAACACATAGCTGCGTTTATGGCCAAGGCCTAA  957
  K E E C M A T Y Q H I A A F M A K A *  318

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Fig. 1 Nucleotide sequence of the Ke75 esterase gene and its deduced amino acid sequence. The vertical arrow indicates the putative signal peptide cleavage site. The conserved Ser, Asp, and His residues that comprise a putative catalytic triad are boxed. Both the 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 the accession number JQ765409.

were subjected to SDS-PAGE (Fig. 3). The results indicated that most of the recombinant esterases were produced as the soluble form in *E. coli* cells. The recombinant esterase fusion protein expressed from the vector pGEX-4T-1 containing a GST tag region at its amino-terminus was isolated by affinity chromatography in a one-step purification scheme using glutathione-sepharose. The purified esterase gave a single protein band of ~60 kDa on SDS-PAGE, in accordance with its predicted molecular mass of 35 kDa and GST protein with a molecular mass of 26 kDa, and was used to characterize the biochemical properties of the purified enzyme. This purified enzyme had a specific activity of 22.75 U/mg toward PNPB. Its activity was 4.4- and 3.3-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).

Biochemical characterization of KE75 esterase. The substrate specificity of the Ke75 esterase was determined using *p*-nitrophenyl esters with acyl chains of various lengths, C₂-C₁₈ (Fig. 4). The Ke75 esterase exhibited unique substrate specificity. That is, the purified enzyme had a strong preference for *p*-nitrophenyl butyrate (C₄), with little activity against short-chain esters, C₂ and C₈, and did not show any detectable activity against long-chain substrates (>C₁₀). The K_m and k_{cat} values of Ke75 esterase on *p*NP butyrate was respectively 0.222 mM and 793 s⁻¹. The value of k_{cat}/K_m was 3,572 s⁻¹ mM⁻¹. Determination of the K_m , k_{cat} and V_{max} values was not possible when other substrates were used. The EM2L8 esterase, which was isolated from the metagenome of a deep-sea sediment sample, most rapidly hydrolyzed PNPB (C₄), and hydrolytic activity decreased markedly with increasing chain length (Park et al., 2007). Carboxylesterases can be distinguished from lipases by their substrate spectra, using *p*-nitrophenyl

palmitate (cleaved by lipases) vs. *p*-nitrophenyl butyrate (cleaved by esterases and sometimes by lipases) (Bornscheuer, 2002). Based on the substrate preference profile, Ke75 was classified as a “true” carboxylesterase. The overall substrate specificity among carboxylesterases has been attributed to a number of features, including differences in the size, overall hydrophobicity or hydrophilicity of the substrate-binding pocket (Jaeger et al., 1999). The effect of temperature on Ke75 esterase activity was assessed using *p*-nitrophenyl butyrate as the substrate at temperatures of 5–70°C. Enzyme activity peaked at 30°C (Fig. 5A). The activation energy for lipase-catalyzed hydrolysis of *p*-NP butyrate was calculated from Arrhenius plots to be 6.29 kcal/mol at temperatures of 5–30°C (Fig. 5B), 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 hwoffii* 16C-1 (Kim and Kim, 2002). However, it is higher than those of *Salinisphaera* sp. P7-4 esterase (1.07 kcal/mol) (Kim et al., 2011) and *Psychrobacter* sp. Ant300 (4.7 kcal/mol) (Kulakova et al., 2004). These observations suggest that its catalytic efficiency is high in this temperature range. In fact, this esterase showed as much as 60% of the maximum activity at 5°C. These results indicated that the Ke75 esterase is a typical cold-adapted enzyme. The enzyme was stable with a residual activity greater than 50% after 30 min of incubation at 40°C. However, it was thermally unstable and lost its activity at temperatures above 50°C (Fig. 5C).

The activity of the recombinant Ke75 esterase under buffered conditions at pH 4–11 was determined (Fig. 5D and 5E). The enzyme exhibited at least 60% of its maximal activity at pH 8.0–

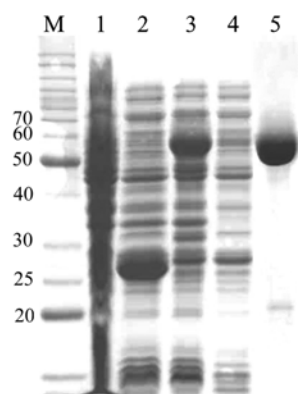


Fig. 3 Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of Ke75 esterase. Lane M, standard protein molecular weight markers; lane 1, lysate of a non-induced *E. coli* XL1-blue transformant; lane 2, supernatant of the lysate of an induced transformant harboring the pGEX4T-1 vector alone; lanes 3 and 4, supernatant and pellet of the lysate of an induced transformant harboring pGEXKe75; lane 5, Ke75 esterase purified by GST affinity chromatography.

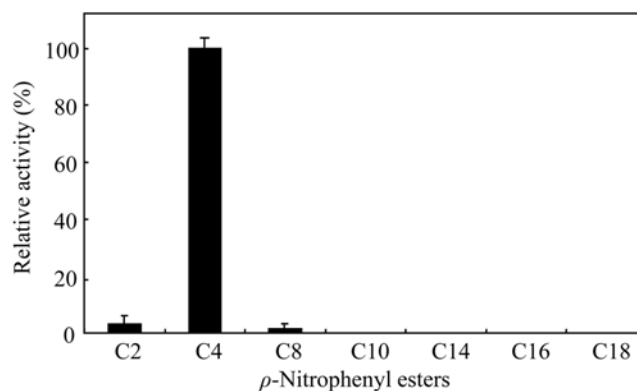


Fig. 4 Substrate specificity of the Ke75 esterase. Specific activity of hydrolysis of various *p*-nitrophenyl esters. Relative activities are shown as percentages of the activity toward *p*-nitrophenyl butyrate. All measurements were performed in triplicates.

10.0, with the highest activity at pH 9.0, indicating it to be an alkaline enzyme.

The effects of various metal ions and inhibitors on enzyme activity were determined (Fig. 6). The esterase showed a 1.7-fold increase in activity in the presence of 5 mM Mn^{2+} , whereas decreased by 50% in the presence of Co^{2+} and Ni^{2+} . Moreover,

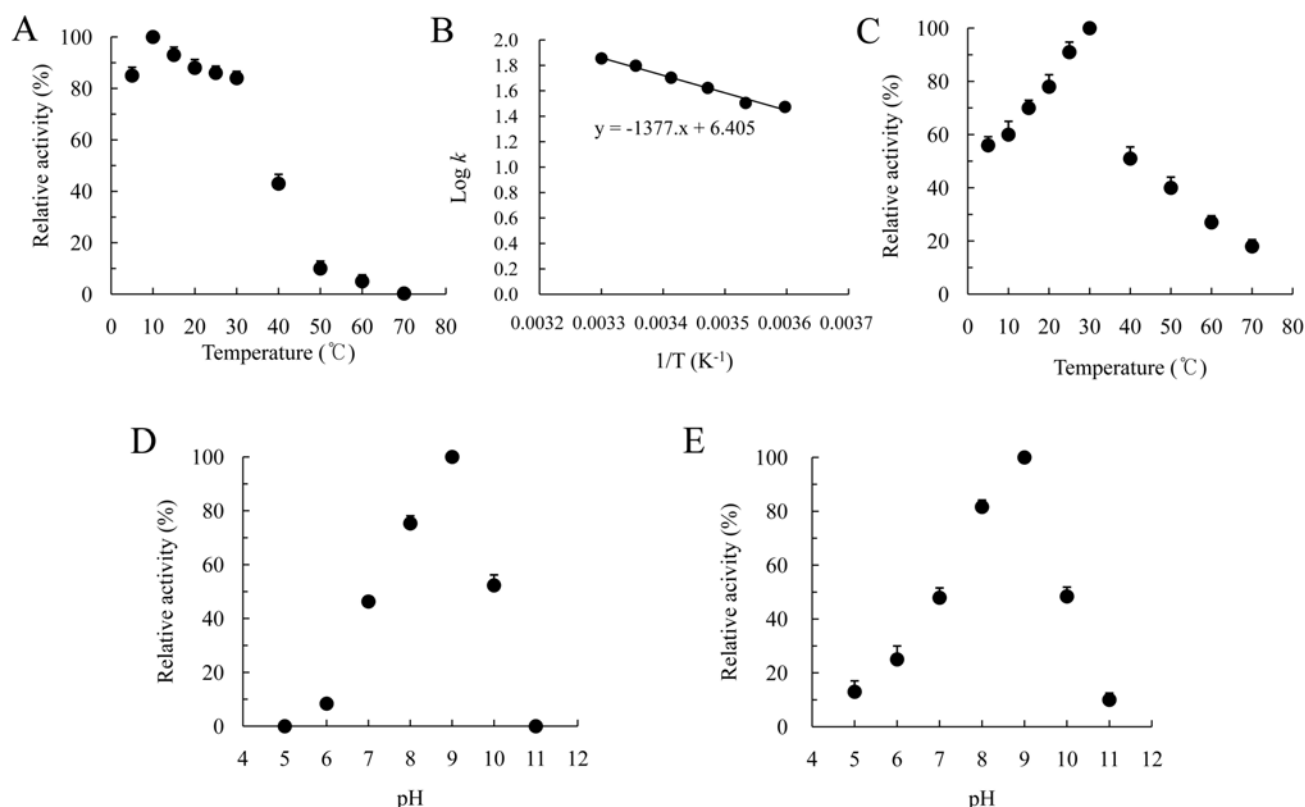


Fig. 5 Effects of temperature and pH on the Ke75 esterase. (A) Hydrolytic activity was measured at various temperatures and pH 8.0. (B) The logarithm of the enzyme turnover rate (k) (s^{-1}) was plotted against the reciprocal of absolute temperature (T). (C) Residual hydrolytic activity was measured after exposure to various temperatures for 30 min. (D) Hydrolytic activity at various pH values was measured at 30°C. (E) Residual hydrolytic activity was measured after exposure to various pH values for 30 min. All measurements were performed in triplicate.

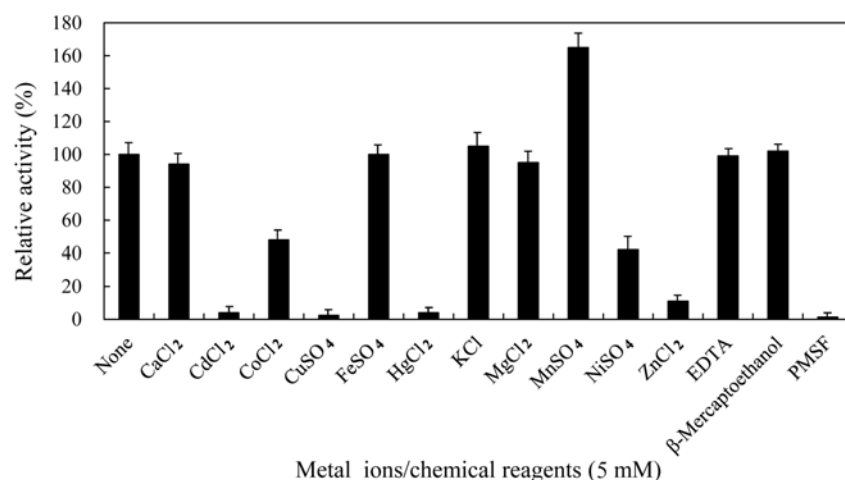


Fig. 6 Effects of metal ions and inhibitors on Ke75 esterase stability. The esterase activity in the presence of various metal ions and enzyme inhibitors was compared with the control (taken as 100%). All measurements were performed in triplicate.

compared with the control, the esterase activity was inhibited by Cd^{2+} , Cu^{2+} , Hg^{2+} , and Zn^{2+} . To confirm its identity as a serine hydrolase, the activity of the Ke75 esterase was determined in the presence of 5 mM phenylmethylsulfonyl fluoride (PMSF), a catalytic serine enzyme inhibitor. Similar concentrations of the metal-chelating agent, ethylenediaminetetraacetic acid (EDTA) and a reducing agent, β -mercaptoethanol, were also investigated to eliminate the possibility of involvement of metal cations or cysteine in the mechanism of action of the enzyme. The Ke75 esterase was significantly inhibited (90%) by PMSF, whereas EDTA and β -mercaptoethanol had no effect. The inhibitory effect of PMSF indicated the presence of serine-mediated catalytic activity. Thereafter, the effects of various organic solvents and detergents were determined (Table 1). The Ke75 esterase was exposed to various organic solvents for 30 min; activity was not

affected by all organic solvents tested. Various non-ionic detergents, such as Triton X-100, Tween 20 and 80, had no effect on activity, whereas the ionic detergent SDS reduced the activity by about 11.2%. Based on these results, we concluded that Ke75 esterase shows a high degree of stability in the presence of organic solvents and detergents, which is an important feature for industrial applications.

In this study, a novel esterase produced by *Shewanella* sp. Ke75, which showed the highest level of activity toward PNPB, exhibited high activity at low temperatures. In addition, it retained its activity in the presence of various organic solvents and detergents. These results suggest that this enzyme has potential for use as a biocatalyst and detergent additive for use at low temperatures.

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Table 1 Effects of organic solvents and detergents on Ke75 esterase activity¹⁾

Compounds	Concentration	Relative activity (%)
Organic solvents	15%	30%
Methanol	101±0.8	101±1.1
Ethanol	102±3.3	102±0.5
Isopropanol	103±2.9	100±3.8
Aceton	102±1.5	101±4.2
Acetonitrile	102±0.4	99±0.3
PEG-8000	101±1.6	102±3.1
DMSO	101±2.1	100±0.9
Detergents	0.05%	0.1%
Tween-20	102±0.7	102±2.9
Tween-80	101±1.8	104±7.1
SDS	15±4.3	11±5.4
Triton X-100	104±0.9	104±1.6

¹⁾Data are presented ± standard deviation

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