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BIOCATALYSIS

Identification and characterization of a novel cold-adapted esterase from a metagenomic library of mountain soil

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Abstract A novel lipolytic enzyme was isolated from a metagenomic library after demonstration of lipolytic activity on an LB agar plate containing 1% (w/v) tributyrin. A novel esterase gene (*estIM1*), encoding a lipolytic enzyme (EstIM1), was cloned using a shotgun method from a pFos-EstIM1 clone of the metagenomic library, and the enzyme was characterized. The *estIM1* gene had an open reading frame (ORF) of 936 base pairs and encoded a protein of 311 amino acids with a molecular mass 34 kDa and a p*I* value of 4.32. The deduced amino acid sequence was 62% identical to that of an esterase from an uncultured bacterium (ABQ11271). The amino acid sequence indicated that EstIM1 was a member of the family IV of lipolytic enzymes, all of which contain a GDSAG motif shared with similar enzymes of lactic acid microorganisms. EstIM1 was active over a temperature range of 1–50°C, at alkaline pH. The activation energy for hydrolysis of *p*-nitrophenyl propionate was 1.04 kcal/mol, within a temperature range of 1–40°C. The activity of EstIM1 was about 60% of maximal even at 1°C, suggesting that EstIM1 is efficiently cold-adapted. Further characterization of this cold-adapted enzyme indicated that the esterase may be very valuable in industrial applications.

Keywords Screening · Cold-adapted esterase · Metagenomic library · Cloning · Expression

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Introduction

The many complex microorganisms that live in soil [15] are valuable sources of antibiotics, pharmaceuticals, and biocatalysts [27]. Rossello-Mora and Amann [42] reported that about 10 billion microorganisms were to be found in just 1 g of soil, but most of these microbes have not been studied because 99–99.9% of such organisms grow only under fastidious laboratory conditions [10]. To access the beneficial anabolic pathways of uncultured microorganisms, genomic DNA isolated from soil microbes, termed a metagenome, has been studied without culture of the microbes [41]. Advances in modern metagenomic and gene cloning strategies have recently been reviewed in detail [10, 17, 45]. Metagenomics does not require cultivation of single microorganisms, or isolation thereof. Rather, metagenomics searches for novel enzymes, bioactive compounds, and ecologically and biotechnologically interesting phenotypes, synthesized/exhibited by soil microbes, in the absence of pure culture of such organisms [44].

Lipases (EC 3.1.1.3) hydrolyze and synthesize long-chain acylglycerols, and esterases (EC 3.1.1.1) can catalyze the hydrolysis of short-chain carboxylic acids. Many lipolytic enzymes, including esterases and lipases, have been identified in fungi, yeast, bacteria, plants, animals, and humans [21, 22, 47]. Lipolytic enzymes are important industrial biocatalysts in industries such as food manufacturing and the chemical, pharmaceutical, and cosmetics

industries [23, 39]. Useful features of such enzymes include an absence of cofactor requirement; stability under the sometimes aggressive conditions required to complete the reactions of organic chemistry; a broad substrate specificity range; and exquisite chemoselectivity, regioselectivity, and stereoselectivity [21, 22].

Cold-adapted enzymes have generated considerable interest, since they have potential to improve the efficiency of industrial processes and offer possible economic benefits through energy saving [6, 12, 16, 17, 30]. The cold-active esterase can be the key to the success in some of their applications. The esterases have been used in versatile industrial applications, e.g., as additives in laundry detergents for cold washing and catalysts for organic syntheses of unstable compounds at low temperatures [4, 36]. The demand for biocatalysts that are active under extreme conditions (temperatures, pH condition, and salt contents) has increased in the bioindustry.

Many useful enzymes, including lipases, proteases, and cellulases, have been characterized from unculturable microorganisms [9–11, 20, 28, 29, 31, 37]. Such enzymes differ in various characteristics compared with enzymes of culturable microorganisms and can be active over a wide temperature range, including low temperatures. In this manuscript, we identify a novel cold-adapted esterase expressed by cloned DNA from a metagenomic library constructed from soil. An esterase-encoding gene was initially identified, and the encoded protein was overexpressed, purified, and characterized in terms of temperature, pH, and substrate specificity.

Materials and methods

Chemicals

p-Nitrophenyl esters (*p*-NPEs) containing acetate, propionate, butyrate, valerate, hexanoate, octanoate, decanoate, laurate, myristate, palmitate, and stearate groups were purchased from Sigma-Aldrich, Inc. (St. Louis, MO) and Fluka (Buchs, Switzerland). All other chemicals were of the highest purity commercially available.

Construction of a metagenomic library

Genomic DNA was isolated from soil microorganisms using a modification of a sodium dodecyl sulfate (SDS)-based DNA extraction protocol [48]. Ibam mountain is situated in the Jeongeup, Jeonbuk, Republic of Korea. A soil sample (10 g) from Ibam mountain (35° 28′ 21.31″ N, 35° 50′ 04.57″ E) was suspended in 27 ml of extraction buffer (2% [w/v] CTAB, 20 mM EDTA, 1.4 M NaCl, 100 mM Tris–HCl [pH 8.0]) and 100 µl of proteinase K (10 mg/ml) was added. The mixture was shaken for 30 min at 200 rpm. Six milliliters of 10% (w/v) SDS was added and each sample was incubated at 65°C for 2 h, using gentle inversion. Each sample was centrifuged at 16,000×*g* for 20 min at 4°C. Each supernatant was admixed with 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v). The upper phase was recovered by centrifugation, mixed with 1 volume of isopropanol, and held at −20°C for 1 h. A pellet was obtained by centrifugation at maximum speed (20,000×*g*) for 20 min at 4°C, washed with 70% (v/v) ethanol, and resuspended in 200 µl of TE buffer. To remove humic substances, pulse-field gel electrophoresis (PFGE) using 1% (w/v) low melting point agarose with addition of 2% (w/v) polyvinyl polypyrrolidone (PVPP) was employed. PFGE was performed at 6 V/cm for 16 h using the CHEF-DR II system (Bio-Rad Laboratories, Hercules, CA) and DNA fragments approximately 30–40 kb in size were isolated using the GELase system (Epicentre Technologies, Madison, WI). A fosmid library of isolated DNA was constructed using the CopyControl fosmid library production kit (Epicentre Technologies). After PFGE, size-resolved DNA (30–40 kb) was end-repaired using an appropriate enzyme mix and ligated employing the Fast-Link DNA ligase into the pCC1FOS fosmid vector. Lambda packaging extracts were added to ligation mixtures and employed to infect phage EPI300-TI^{R}. Packaged fosmid clones were stored at 4°C.

Screening for lipolytic hydrolases from metagenomic library

To screen for lipolytic hydrolases, the fosmid library was spread onto LB agar plates containing 1% (w/v) tributyrin and 12.5 µg/µl chloramphenicol. After incubation at 37°C for 18 h, colonies showing haloes were selected for further study.

Shotgun cloning of *estIM1* and sequencing analysis

Fosmids from clones showing haloes upon plating with 1% (w/v) tributyrin were purified using an alkaline lysis method [3]. One gene (*estIM1*), encoding an esterase, isolated from the metagenomic library, was cloned by a shotgun method [14]. Isolated chromosomal DNA was partially digested with *Bfu*CI. The reaction was terminated by incubation at 65°C for 20 min. Fragments ca. 4–5 kb in size were then excised from a gel and purified using a gel extraction kit (QIAGEN Inc., Valencia, CA). Purified fragments were ligated with dephosphorylated pHSG298 (Takara, Shiga, Japan) digested with *Bam*HI. The plasmid library was transformed into *Escherichia coli* XL1-Blue cells and transformants were grown on Luria–Bertani (LB) agar containing 20 µg/ml kanamycin and 1% (w/v) tribu-

tyrin. Plasmids in clones of interest were purified using a QIAGEN plasmid midi kit and the nucleotide sequences of recombinant fragments were obtained by SolGent Co (Daejeon, Korea). BLAST analyses were performed using the NCBI server (http://www.ncbi.nlm.nih.gov/blast/). The sequences obtained were aligned using the multialignment program of BioEdit version 7.0.9.0. The BLAST search program was also used to search for protein sequences of family IV lipolytic enzymes from prokaryotes. Protein sequences thus obtained were aligned by employing ClustalV, with particular reference to MEGALIGN (DNAstar), and a neighbor-joining tree was generated using the same software. Units shown at the bottom of the tree indicate the number of substitution events.

Construction of a recombinant plasmid for the expression of EstIM1 in *E. coli*

PCR amplification was performed using a PCR thermal cycler (Tprofessional; Biometra, Goettingen, Germany), with the plasmid of a positive shotgun clone employed as template. The *estIM1* gene, encoding the EstIM1 enzyme, was amplified by PCR. Oligonucleotide primers were designed to amplify sequences encoding the EstIM1 enzyme. Such oligonucleotide primers were *estIM1*-F (5′-ATC ATA **TGC** CGC TCG ACC CAC AA-3′) and *estIM1*-R (5′-TAT CTC GAG TCG AGT AAA GGC GGC G-3′) (underlining indicates *Nde*I or *Xho*I sites, and bold text shows the *estIM1* start codon). Use of such primers resulted in amplification of a 0.95-kb fragment (the size is approximate) from a positive clone of the metagenomic library, by PCR employing *Taq* polymerase. Amplified DNA fragments were digested with *Nde*I and *Xho*I and ligated into pET-22b(+) (Novagen, Madison, WI). The resultant plasmid, pET-EstIM1, was introduced into *E. coli* BL21(DE3).

Nucleotide sequence accession number

The DNA sequence of the lipolytic enzyme-encoding gene (*estIM1*) has been submitted to EMBL under accession number HM802891.

Expression and purification of recombinant EstIM1 enzyme

The *E. coli* BL21(DE3) harboring pET-EstIM1 was grown in LB with 50 μg/ml ampicillin, at 37°C. Transcription of the recombinant gene was induced by addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After incubation for 4 h at 28°C, cells were harvested by centrifugation at 7,000×*g* for 10 min, and washed with ice-cold PBS (200 mM NaCl, 3 mM KCl, 2 mM KH_2PO_4, and 1 mM Na_2HPO_4; pH 7.5). Cells were again harvested by centrifugation and disrupted using a VCX750 sonicator (Sonics Materials Inc., Newtown, CT) in ice-cold PBS. The crude recombinant fusion protein bearing a His-tag at the C-terminus was purified using a HiTrap-chelating HP column (GE Healthcare, Piscataway, NJ) employing a gradient of 0–500 mM imidazole in PBS containing 500 mM NaCl. The purified protein was next desalted on a HiPrep 26/10 desalting column (GE Healthcare). All purification steps were performed on an FPLC system (ÄKTA Explorer; GE Healthcare). Protein concentration was measured by the method of Bradford, as employed in the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as standard [5].

Electrophoresis and protein analysis

Electrophoresis was performed on an Experion automated electrophoresis station (Bio-Rad Laboratories), and protein sizes were estimated according to the manufacturer's instructions. The Pro260 ladder, a modified version of the Precision-Plus Protein standard (Bio-Rad Laboratories), was used to quantify protein bands.

Characterization of the recombinant EstIM1 enzyme

The esterase activity of the purified enzyme was quantitatively measured using a spectrophotometric method employing *p*-NP-propionate (C_3) as substrate. Activity was assessed by incubating the enzyme with 1 mM substrate at 40°C in 100 mM sodium phosphate buffer at pH 8.0 [8]. Release of *p*-NP from *p*-NPE was continuously monitored, spectrophotometrically, at 405 nm using a microplate reader (Model 680, Bio-Rad Laboratories). One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of *p*-NP per min from *p*-NPE. The Michaelis–Menten constants (K_m values) of purified EstIM1 were determined from Lineweaver–Burk plots. The kinetic constants K_m, k_{cat}, and k_{cat}/K_m were estimated using standard formulae [33].

Purified protein was used to study all of enzyme kinetics, substrate specificity, optimum pH, optimum temperature, pH stability, and thermostability. All experiments were performed in triplicate. Substrate specificity was examined using *p*-NPEs of all of acetate (C_2), propionate (C_3), butyrate (C_4), valerate (C_5), hexanoate (C_6), octanoate (C_8), decanoate (C_{10}), laurate (C_{12}), myristate (C_{14}), palmitate (C_{16}), and stearate (C_{18}), dissolved in acetonitrile, over 5 min at 40°C. Initial reaction velocities were fitted to a Lineweaver–Burk equation reflecting substrate concentrations. An optimum temperature for enzyme activity was determined using 1 mM *p*-NP-propionate in 100 mM sodium phosphate buffer (pH 8.0) as substrate, at tempera-

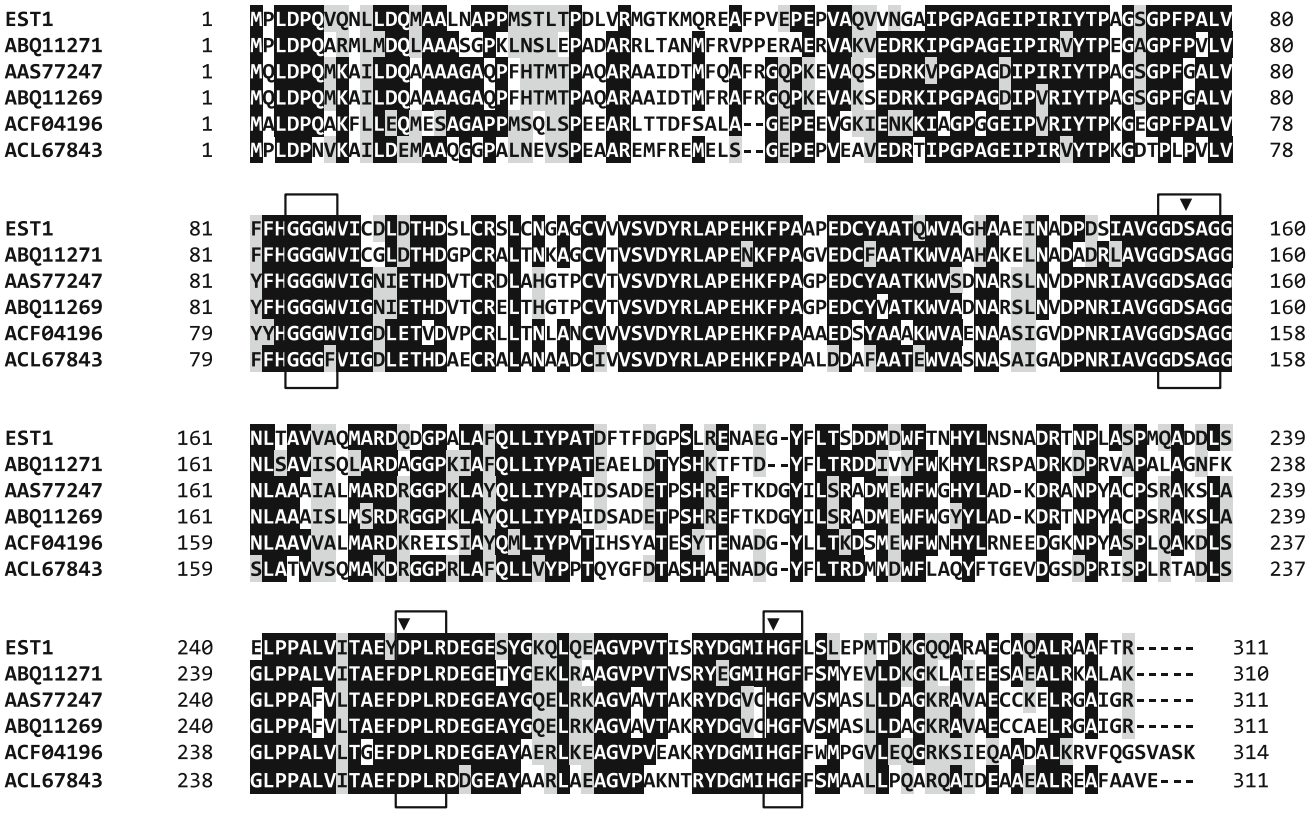

Fig. 1 Comparison of the amino acid sequence of the EstIM1 lipolytic enzyme cloned from a metagenomic library with those of various lipases/esterases of uncultured bacteria. Regions of identity or high similarity among sequences are shown as *black* or *gray columns*, respectively. The conserved motifs (GGGX, GXSXG, DPLR, and HGF) of family IV carboxylesterases and the putative catalytic triad residues of the hormone-sensitive lipase (HSL) family, composed of the Ser-157, Asp-252, and His-282 residues of lipases/esterases, are indicated in the *boxes* and by *inverted closed triangles*, respectively

tures of 1–70°C. The optimum pH was determined at 40°C, over 5 min, using 1 mM *p*-NP-propionate as substrate, dissolved in the following buffers: 100 mM sodium acetate buffer (pH 3.0–6.0); 100 mM sodium phosphate buffer (pH 6.0–8.0); 100 mM Tris–HCl buffer (pH 8.0–9.0); 100 mM borate buffer (pH 9.0–10.0); or 100 mM glycine-NaOH buffer (pH 10.0–12.0). Thermostability was evaluated by incubating the enzyme in 100 mM sodium phosphate buffer (pH 8.0) for 0–120 min at temperatures of 1–60°C. The effects of metal ions, detergents, and organic solvents were determined at final concentrations of 1 mM, 1% (w/v or v/v), and 15 or 30% (v/v), respectively. All assays were performed at the optima of pH and temperature.

Results

Construction of a metagenomic library and screening for lipolytically active clones

Novel esterases have previously been isolated from soil samples. Metagenomic libraries have been employed to screen for novel lipolytic genes. We constructed a metagenomic library from microbes in mountain soil using a fosmid library kit (as described in "Materials and methods"). This metagenomic library, consisting of 14,000 fosmid clones, was analyzed by restriction analysis of a randomly chosen sample of 20 clones, to verify both bank quality and insert size (data not shown). Insert DNA size was 30–40 kb, and the library thus covered approximately 500 Mb of total metagenomic DNA. To screen for lipolytic activity, fosmid clones were spread on an LB-agar plate containing 1% (w/v) tributyrin. A positive clone (pFosEstIM1) was identified by appearance of a clear zone around the colony.

Cloning and sequencing of the *estIM1* gene

To characterize the lipolytic activity of the pFosEstIM1 clone, the insert DNA of pFosEstIM1 was sequenced by shotgun cloning. A preliminary search for recombinants expressing lipolytic activity on 1% (w/v) tributyrin LB-agar plates identified a positive clone. On initial screening, we found only a single clone (pFosEstIM1) expressing

lipolytic activity. To identify other genes responsible for lipolytic activity, further subcloning and activity-based esterase screening on LB agar plates containing 1% (w/v) tributyrin were performed. We selected and sequenced recombinant DNA from one positive clone containing plasmid pHSGEstIM1, which contained a recombinant DNA fragment about 4 kb in size. An ORF (*estIM1*) of 936 bp, encoding a 311 amino acid protein of predicted molecular weight 34 kDa and with a p*I* value of 4.32, was identified. This sequence is registered in GenBank under accession number HM802891. A comparison with deduced amino acid sequences of other proteins, using the BLAST program, revealed that mature EstIM1 showed 62%, 61%, 59%, 61%, and 58% identity with lipase/esterase or lipolytic enzymes from uncultured bacteria (GenBank accession nos. ABQ11271, ACF04196, AAS77247, ACL67843, and ABQ11269; Fig. 1). Our putative protein was related in sequence to the hormone-sensitive lipase (HSL) of the family IV carboxylesterases. The *estIM1* gene contained the pentapeptide GDSAG motif (position 155–159), catalytic triad residues (Ser-157, Asp-252, and His-282), and a GGGX sequence (position 84–87), corresponding to the oxyanion hole conserved in many esterases. Further, phylogenetic analysis of EstIM1 and other family IV enzymes, based on amino acid sequences, revealed that EstIM1 was most closely related to an esterase/lipase from *Pseudomonas* sp. B11-1 (GenBank accession no. AAC38151; data not shown).

Expression and purification of recombinant EstIM1 enzyme

EstIM1 was most prominently expressed in the soluble fraction of recombinant cells when the pET-22b(+) vector was transformed into *E. coli* BL21(DE3), as described in "Materials and methods". The EstIM1 protein was purified by Ni–NTA affinity chromatography. The enzyme was obtained with a yield of 51% and was purified 17-fold to electrophoretic homogeneity (data not shown). The estimated molecular mass of purified EstIM1 was approximately 34 kDa, and protein purity was over 99% by Experion automated electrophoresis analysis (Fig. 2).

Biochemical characteristics of the purified recombinant EstIM1 enzyme

EstIM1 was active against all substrates from *p*-NP-acetate (C_2) through to *p*-NP-decanoate (C_{10}), and the greatest activity was apparent when *p*-NP-propionate (C_3) was employed as substrate (Fig. 3). EstIM1 substrate specificity was determined at 40°C in sodium phosphate buffer (pH 7.2) using various *p*-NPE compounds, ranging from *p*-NP-acetate (C_2) to *p*-NP-stearate (C_{18}). The hydrolytic activity of EstIM1 was

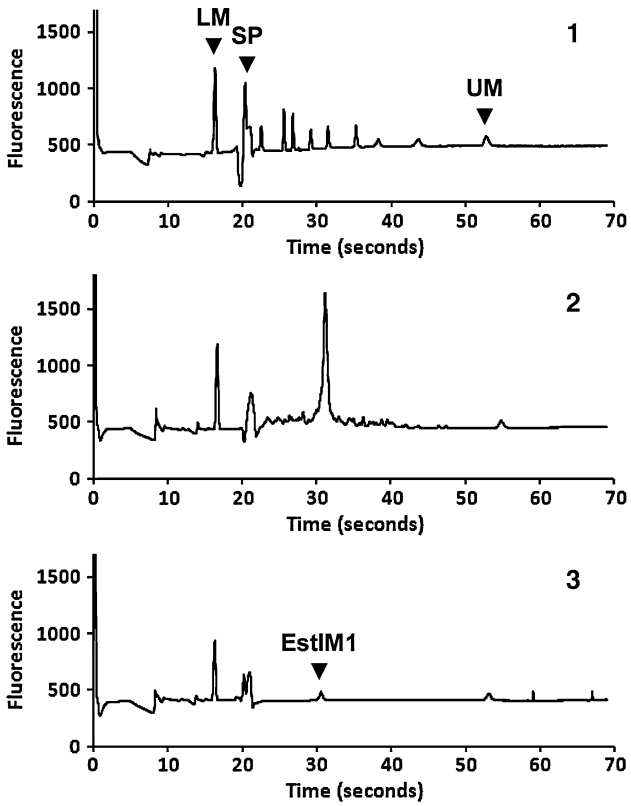

Fig. 2 Quantification of purified EstIM1 esterase using Experion Pro260 microfluidic technology. Electropherograms of molecular size markers (*1*), soluble protein (*2*), and purified recombinant EstIM1 (*3*), run under reducing conditions, are shown. Samples were separated by automated capillary electrophoresis, labeled with a fluorescent dye, and detected by laser-induced fluorescence. The first and last peaks detected correspond to the lower (*LM*) and upper markers (*UM*), which are incorporated into the sample buffer and permit alignment with the Pro260 ladder

optimal when *p*-NP-acetate was used a substrate, and, in general, higher activities were apparent when shorter-chain (C_2, C_3, C_4, C_5, C_6, C_8, and C_{10}) rather than longer-chain (C_{12}, C_{14}, C_{16}, and C_{18}) substrates were employed. Hydrolytic activity thus decreased as chain length increased, and activities toward substrates ranging in size from *p*-NP-laurate (C_{12}) to *p*-NP-stearate (C_{18}) were weak. The specific activity of EstIM1 was 2.51×10^7 U mg^{-1} when *p*-NP-propionate was used as substrate. EstIM1 activity was further investigated, both in terms of catalytic properties and by estimation of kinetic constants, using various *p*-NPEs as esterase substrates. The Michaelis–Menten constants (K_m values) of EstIMI were 290.3, 366.8, and 65.8 μM; the maximal reaction velocities (v_{max} values) 58.51×10^{-3}, 62.06×10^{-3}, and 18.24×10^{-3} μmol min^{-1}; a turnover number (k_{cat}) 1.008, 0.846, and 1.385 min^{-1}; and catalytic efficiency (k_{cat}/K_m) 3.473×10^{-3}, 2.307×10^{-3}, and 21.04×10^{-3} μM^{-1} min^{-1}, when *p*-NP-propionate, *p*-NP-butyrate, and *p*-NP-valerate, respectively, served as substrates (Table 1).

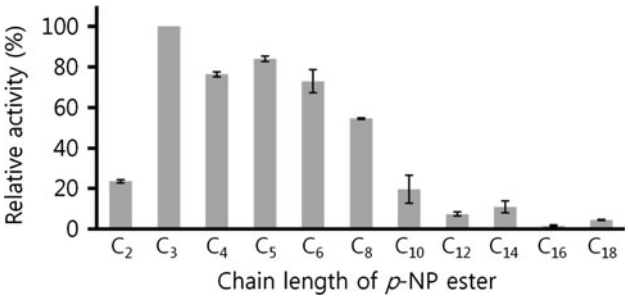

Fig. 3 Activities of EstIM1 toward *p*-NP esters of various chain length (C_2, acetate; C_3, propionate; C_4, butyrate; C_5, valerate; C_6, hexanoate; C_8, octanoate; C_{10}, decanoate; C_{12}, laurate; C_{14}, myristate; C_{16}, palmitate; and C_{18}, stearate) in reactions containing 100 mM sodium phosphate (pH 8.0), and 1 mM of the *p*-NP esters as substrates

Table 1 Kinetic parameters of EstIM1 determined using *p*-nitrophenyl esters as substrates

Substrate (*p*-nitrophenyl ester)	K_m (μM)	v_{max} (μmol min^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})
Propionate (C_3)	290.3	58.51×10^{-3}	1.008	3.47×10^{-3}
Butyrate (C_4)	366.8	62.06×10^{-3}	0.846	2.31×10^{-3}
Valerate (C_5)	65.8	18.24×10^{-3}	1.385	21.04×10^{-3}
Hexanoate (C_6)	166.1	29.57×10^{-3}	0.890	5.36×10^{-3}
Octanoate (C_8)	425.4	22.24×10^{-3}	0.261	0.61×10^{-3}

Figures 4 and 5a show the pH and temperature optima of the enzyme, and the effects of pH and temperature on enzyme stability. The activity of purified EstIM1 was measured over the temperature range 1–70°C and pH values of pH 3.0–11.0 using 1 mM *p*-NP-propionate as substrate. The optimal temperature and pH for EstIM1 activity were 40°C and pH 8.0, respectively (Figs. 4a, 5a). Evaluation of pH stability indicated that EstIM1 was stable over the pH range of 6.0–9.0, and more than 75% of activity was retained after incubation at 4°C for 24 h (Fig. 4b). The thermostability of purified EstIM1 was determined by analysis of residual activity after incubation for 1 h at various temperatures (1–60°C); the enzyme was stable at temperatures up to 30°C (Fig. 4c). However, EstIM1 was unstable when held above 35°C for 40 min; about 50% of activity was lost. Reactions catalyzed by enzymes of cold-adapted microorganisms generally require less activation energy than those catalyzed by analogous enzymes from the mesophilic counterparts of such bacteria [13]. The activation energy for hydrolysis of *p*-NP-propionate was 1.04 kcal/mol over the temperature range 1–40°C (Fig. 5b).

Esterase activity is frequently influenced by the presence of various molecules, including divalent metal ions and nonionic surfactants [7, 34]. The effects of metal ions and detergents on enzyme activity are shown in Table 2. The activity of the purified protein was inhibited by Hg^{2+} and Zn^{2+} (to 8 and 18% of the control value, respectively), whereas little or no inactivation by Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, K^+, Rb^+, Tween 20, or Tween 80 was evident. Various nonionic detergents, including Tween 20, Tween 80, and Triton X-100, weakly inhibited esterase activity, whereas an ionic detergent (SDS) almost completely eliminated activity. EstIM1 was incubated with various water-miscible organic solvents at concentrations of either 15 or 30% (both v/v) at 40°C for 5 min (Table 3). Activity was stimulated by methanol, but severely inhibited by 30% (v/v) acetonitrile. No activity was detected after incubation of enzyme with 30% (v/v) isopropanol. EstIM1 was rather stable in ethanol (15% v/v), acetonitrile (15% v/v), and dimethyl sulfoxide (DMSO; 15 and 30%, both v/v).

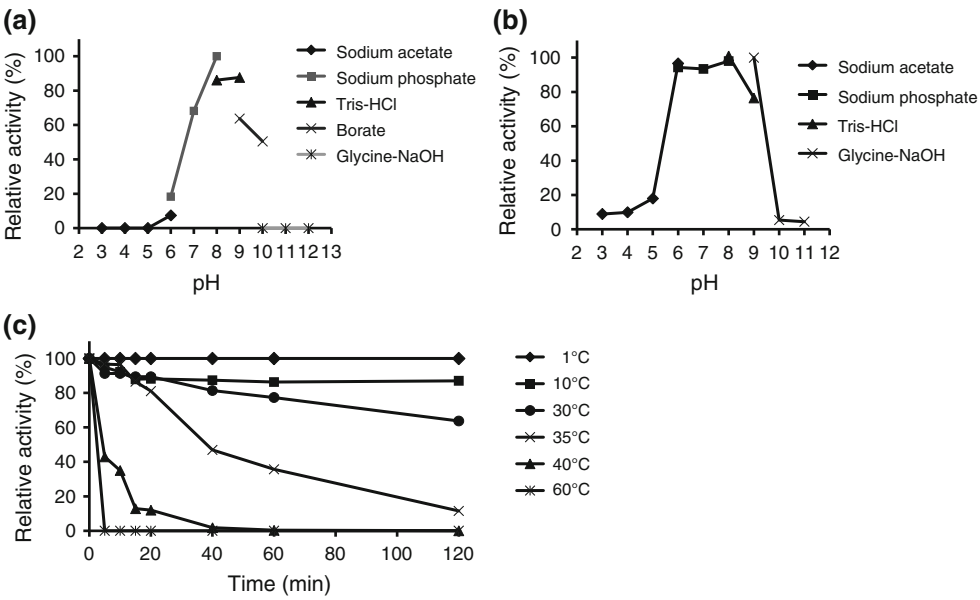

Fig. 4 Biochemical characterization of the EstIM1 enzyme. **a** Optimal pH for activity was determined by incubating the enzyme in buffers of various pH values at 40°C for 5 min. **b** pH stability of EstIM1 was assessed under optimal conditions (sodium phosphate buffer at pH 8.0; 40°C for 5 min) after the enzyme had been incubated in various buffers at 4°C for 24 h. **c** Thermotolerance of EstIM1. The thermal stability of EstIM1 was explored by incubation at various temperatures for 0–120 min. Enzyme activity was measured using *p*-nitrophenyl propionate as substrate

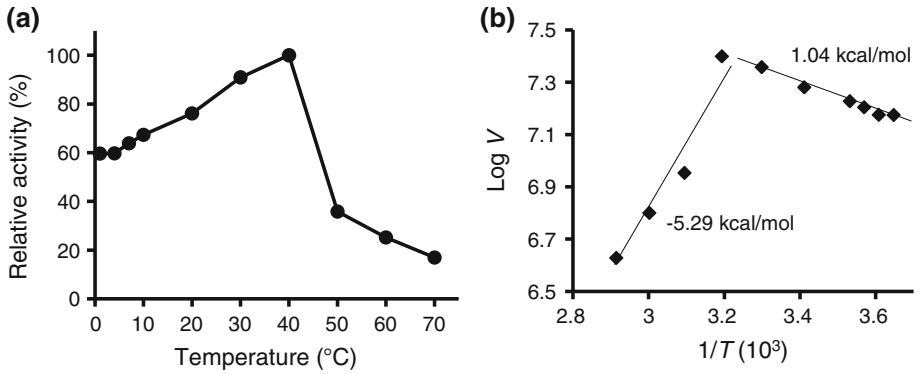

Fig. 5 Effect of temperature on the activity of the EstIM1 enzyme. **a** Enzyme activity was assayed in 100 mM sodium phosphate buffer (pH 8.0) using *p*-nitrophenyl propionate as substrate, at various temperatures, for 5 min. The value obtained at 40°C was taken as 100%. **b** The logarithms of specific activity values (*V*; in micromoles per milligram per minute) were plotted against the reciprocals of absolute temperature (*T*). The values shown are activation energies calculated from the linear region of the plot

Table 2 Effects of metal ions and detergents on enzyme activity

Ion or detergent	Relative activity (%)
None	100
Ca^{2+}	95
Co^{2+}	87
Cu^{2+}	76
Fe^{2+}	90
Hg^{2+}	8
Mg^{2+}	88
Mn^{2+}	90
Ni^{2+}	84
K^{+}	87
Rb^{+}	89
Zn^{2+}	18
SDS	0
Tween 20	76
Tween 80	81
Triton X-100	54

Metal ions and detergents were added to final concentrations of 1 mM and 1% (either w/v or v/v), respectively. All assays were performed at the optimum pH and temperature. The EstIM1 enzyme was treated with 1 mM EDTA before use, to remove metal ions

Table 3 Effects of organic solvents on EstIM1 activity

Organic solvent	Relative activity (%)	
	15% v/v	30% v/v
None	100	100
Methanol	166	111
Ethanol	87	21
Acetonitrile	71	13
Dimethyl sulfoxide	76	99
Dimethyl formamide	37	27
Isopropanol	58	0

Enzyme activities were determined using the substrate 1 mM *p*-nitrophenyl propionate in 100 mM sodium phosphate buffer (pH 8.0), at 40°C, and are expressed as percentages of control values (obtained in the absence of organic solvent)

Discussion

We isolated *estIM1*, encoding an esterase, from a metagenomic library constructed from soil samples collected on Mount Ibam in South Korea. The deduced amino acid sequence showed that EstIM1 had 62% identity with a lipase/esterase from an uncultured bacterium (GenBank accession no. ABQ11271). Biochemical characterization of EstM1 esterase identified it as a cold-adapted esterase.

Family IV carboxylesterases include hormone-sensitive lipases (HSLs). Enzymes in this family are similar to the mammalian HSL enzyme [1, 38], which is involved in lipid metabolism and energy homeostasis. HSLs control release of fatty acids from triacylglycerols stored in adipose tissue. Mammalian HSLs contain a catalytic domain and an associated unique regulatory module in the N-terminal domain [35]. Family IV carboxylesterases have four conserved sequence motifs: the oxyanion region GGGX, the pentapeptide –GDSAG– signature motif, and two C-terminal conserved motifs, –DPLR– and –HGF– [1, 38, 46]. The deduced amino acid sequence indicates that EstIM1 is similar to other esterases of family IV, as it also contains a GGGX (N-terminal oxyanion region) motif, the pentapeptide GXSXG (a nucleophilic elbow), a DPLR motif (at position 252–255), and an HGF motif (at position 282–284) (Fig. 1). The putative residues of the catalytic triad are marked by inverted closed triangles in Fig. 1. On the basis of alignment with other esterase sequences, His-282 most likely serves as the catalytic histidine, and the catalytic aspartic acid is tentatively identified as Asp-252. Phylogenetic analysis in comparison with genes encoding eight

other esterases/lipases in the same family showed that EstIM1 clusters closely with the family IV esterase/lipase of *Pseudomonas* sp. B11-1 (GenBank accession no. AAC38151). Carboxylesterases in family IV hydrolyze acyl chains less than 10 carbon atoms in length [32], and can be either thermolabile [18] or thermostable [38, 40].

We used a spectrophotometric assay to search for new genes encoding lipases/esterases in a metagenomic library, overexpressed one such gene, and characterized the gene product. The substrate specificity of EstIM1 using various forms of *p*-NPE, C_2–C_{18} in chain length, revealed an enzymic preference for short-chain substrates (Fig. 3). EstIM1 hydrolyzed a wide range of *p*-NPE substrates (C_2 to C_{18} in chain length), but hydrolysis rates were highest when the substrates *p*-NP-propionate (C_3) to *p*-NP-octanoate (C_8) were used, whereas lower activities were evident toward the longer *p*-NP-laurate (C_{12}) to *p*-NP-stearate (C_{18}) substrates. This suggests that EstIM1 is an esterase and not a true lipase.

EstIM1 had a Michaelis–Menten constant (K_m value) of 290.3 μM when *p*-NP-propionate was used as substrate, compared with the value of and 550.0 μM exhibited by the cold-adapted esterase CHA3 obtained from a metagenomic library [19], and the value of 14.44 μM of the esterase EstB28 of *Oenococcus oeni* [46]. EstIM1 was strongly inhibited by Hg^{2+}, Zn^{2+}, SDS, and Triton X-100, whereas little or no inactivation was observed upon incubation with the other ions and detergents tested (Table 2). The effects of Fe^{2+} and Mn^{2+} on EstIM1 activity differ from those on the EstAT1 and EstAT11 esterases obtained from the Arctic sediment metagenome [24, 25]. EstIM1 was stable in methanol (15 and 30%, both v/v), ethanol (15% v/v), and DMSO (30% v/v), retaining over 80% of activity (Table 3). Stability in the presence of organic solvents is important if an enzyme is to be used in biotechnological and industrial applications [43].

In comparison with the other metagenome-derived esterases, EstAT1 and EstAT11 [38], EstIM1 is much more active under both neutral and alkaline conditions (Fig. 4a), suggesting that EstIM1 is alkaliphilic. EstIM1 activity was higher at pH 8.0 than under acidic conditions, and no activity was evident below pH 5.0.

In addition, the activity of EstIM1 was optimal at 40°C, and about 60% of maximal activity was retained even at 1°C, suggesting that EstIM1 is well-adapted to cold (Fig. 5a). The activation energy of an enzymic reaction reflects the enzyme catalytic efficiency. The activation energies of reactions catalyzed by enzymes from cold-adapted organisms are usually lower than those catalyzed by the corresponding enzymes from mesophilic counterparts [13]. The activation energy of EstIM1 for hydrolysis of *p*-NP propionate was 1.04 kcal/mol over the temperature range 1–40°C, which is lower than the values exhibited by rEstAT1 (12.65 kcal/mol) and rEstAT11 (11.26 kcal/mol) cloned from an Arctic sediment metagenome, and LipP (11.2 kcal/mol) of *Pseudomonas* sp. B11-1 [8] (Fig. 5b). This indicates that the catalytic efficiency of EstIM1 is much greater than those of other similar enzymes, again because low activation energy is associated with high catalytic efficiency.

Our results thus indicate that EstIM1 is a novel family IV esterase, with unique characteristics, and that the enzyme will be valuable as a new biocatalyst. Lipolytic enzymes find biotechnological applications in the food, detergent, cosmetics, chemical, and pharmaceutical industries [2, 21–23, 26, 39]. The applications are varied and include use as detergent additives, for environmental bioremediation, in food processing, and in biomass and plant waste degradation for production of useful organocompounds [2, 26]. The application of a cold-adapted esterase offers appreciable potential to the biotechnology industry. The enzyme will be utilized for the production of fine chemicals in the detergent and food industries and in bioremediation processes. In the present study, we screened a metagenomic library from soil to identify a novel cold-adapted EstIM1 esterase, and we conclude that EstIM1 will be a useful industrial biocatalyst.

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