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A novel esterase from a psychrotrophic bacterium, *Acinetobacter* sp. strain no. 6, that belongs to the amidase signature family

Yun-lin Wei, Tatsuo Kurihara, Takeshi Suzuki, Nobuyoshi Esaki*

Laboratory of Microbial Biochemistry, Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan

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Dedicated to Professor Dr. Kenji Soda in honor of his 70th birthday

Abstract

A novel esterase that belongs to the amidase signature family was found in a psychrotrophic bacterium, *Acinetobacter* sp. strain no. 6, isolated from Siberian soil. The gene coding for the esterase, named EstA8, was cloned, and an open reading frame of 1488 bp corresponding to 496 amino acid residues was identified. EstA8 showed 30% sequence identity with 6-aminohexanoate-cyclic-dimer hydrolases from *Pseudomonas* sp. strain NK87 and *Flavobacterium* sp. strain K172, which degrade a by-product of the nylon-6 industry. EstA8 was overproduced in *Escherichia coli* JM109 under the control of the *lac* promoter of pUC118 and purified. Consistent with the fact that the source microorganism is cold-adapted, the enzyme was unstable at moderate temperatures. It lost 75% of its original activity by incubation at 40 °C for 30 min. Despite its structural similarity to 6-aminohexanoate-cyclic-dimer hydrolase, 6-aminohexanoate cyclic dimer did not serve as the substrate. EstA8 is a member of the amidase signature family, but its esterase activity toward *p*-nitrophenyl esters, such as *p*-nitrophenyl acetate, was much higher than its amidase activity toward *p*-nitroanilides, such as *p*-nitroacetanilide.

Keywords: Esterase; Amidase; Amidase signature family; Psychrotroph; *Acinetobacter* sp.

1. Introduction

Esterases and amidases, which catalyze the hydrolysis/formation of the ester bonds and the hydrolysis/formation of the amide bonds, respectively, are useful in organic synthesis. For example, peptide amidase can be used for synthesizing the C-terminal amide in peptide hormones [1]. It is also useful for deprotecting peptide amides during the peptide synthesis with amino acid amides as nucleophiles [2]. Some esterases can be used for the production of optically pure compounds by kinetic resolution of the corresponding racemic ester compounds [3]. In addition, transesterification of fatty acid esters with esterases is an important method for the synthesis of various esters [4].

In order to develop new biotechnological processes suitable for thermolabile compounds, we have been searching for enzymes with high activity at low temperatures [4–8]. Psychrophilic and psychrotrophic microorganisms, which inhabit cold environments, such as the deep sea and the Arctic, Antarctic, and Alpine regions, are good sources of cold-active enzymes [9–12]. We carried out extensive screening for the cold-adapted microorganisms producing useful esterases and isolated a psychrotrophic strain, *Acinetobacter* sp. strain no. 6, from Siberian tundra soil [7].

* Corresponding author. Tel.: +81-774-38-3240; fax: +81-774-38-3248.
E-mail address: esaki@scl.kyoto-u.ac.jp (N. Esaki).

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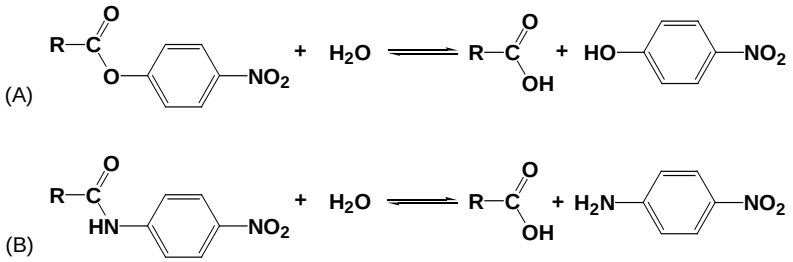

Fig. 1. The reactions catalyzed by EstA8. (A) Hydrolysis of *p*-nitrophenyl esters. (B) Hydrolysis of *p*-nitroanilides.

The bacterium efficiently hydrolyzes the ester bonds of triacylglycerols at low temperatures and was expected to be a good source of lipases and esterases. Indeed, we succeeded in obtaining two novel esterases from this strain [4,8].

In this report, we describe another novel esterase from *Acinetobacter* sp. strain no. 6. The enzyme, named EstA8, was found to belong to the amidase signature family, showing significant sequence similarity to 6-aminohexanoate-cyclic-dimer hydrolase, which degrades 6-aminohexanote cyclic dimer, a by-product of the nylon-6 industry [13]. The enzyme exhibited both esterase and amidase activities (Fig. 1). Although the enzyme was structurally more closely related to amidases, the activity toward the ester compounds was much higher than that toward the amide compounds. The primary structure and properties of this novel esterase are described.

2. Experimental

2.1. Bacterial strains and plasmid

Acinetobacter sp. strain no. 6, a psychrotrophic bacterium, was isolated from Siberian tundra soil as described previously [7]. *Escherichia coli* C600 was used for constructing the genomic DNA library of *Acinetobacter* sp. strain no. 6. *E. coli* JM109 was used as the host for the production of EstA8. pUC118 was used as the cloning vector.

2.2. Chemicals

6-Aminohexanoate cyclic dimer was kindly provided by Prof. Seiji Negoro (Himeji Institute of Technology, Himeji, Japan). Gigapite was obtained from the Seikagaku Corporation (Tokyo, Japan). *p*-Nitrophenyl esters and all other chemicals were purchased from Nacalai Tesque (Kyoto, Japan), Wako Pure Chemical Industries (Osaka, Japan), and Sigma–Aldrich, Inc. (St. Louis, MO, USA).

2.3. Cloning and sequencing of the EstA8 gene

The genomic DNA library of *Acinetobacter* sp. strain no. 6 was constructed with pUC118 as the cloning vector and *E. coli* C600 as the host cell as described previously [4,8]. The recombinant *E. coli* cells were grown on agar plates containing tributyrin at 20 °C and incubated at 4 °C. Several colonies formed a clear halo, indicating the production of lipolytic enzymes. These colonies were isolated, and one of them, named A8, was used in the present study. The lipolytic enzyme produced by A8 was named EstA8, and the plasmid coding for EstA8 was named pEstA8.

The nucleotide sequence of the EstA8 gene was determined with an Applied Biosystems DNA Sequencer 373A with a Dye Terminator sequencing kit. The potential promoter sequence was identified with the Neural Network Promoter Prediction program (http://www.fruitfly.org/seq_tools/promoter.html). Homology search was done with the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). The multiple sequence alignment was carried out with the MegAlign program (DNASTAR Inc., Madison, WI, USA) by the Clustal method.

2.4. Enzyme assay

p-Nitrophenyl butyrate was used as the substrate for routine assays. The enzymatic hydrolysis of

p-nitrophenyl butyrate was monitored at 25 °C in the solution containing a 50 mM potassium phosphate buffer (KPB) (pH 7.3), 10% acetonitrile, 1 mM *p*-nitrophenyl butyrate, and an appropriate amount of the enzyme. The reaction was started by the addition of the substrate to the prewarmed reaction mixture, and increase in the absorbance at 400 nm, indicating the formation of *p*-nitrophenol (ε_M = 14, 300 M^{-1} cm^{-1}), was recorded.

The enzyme activities toward various *p*-nitrophenyl esters were determined with the reaction mixture containing a 50 mM KPB (pH7.3), 15% acetonitrile, 0.3% Triton X-100, 0.2 mM *p*-nitrophenyl esters, and an appropriate amount of the enzyme. Increase in the absorbance at 400 nm was monitored at 25 °C.

The enzyme activities toward *p*-nitroanilides were determined as follows. The reaction mixture contained a 50 mM KPB (pH 7.3), 1 mM *p*-nitroanilides, and an appropriate amount of the enzyme. The reaction temperature was 25 °C. The reaction was started by the addition of the substrate to the prewarmed reaction mixture, and the formation of *p*-nitroaniline was monitored by measuring the absorbance at 412 nm (ε_M = 9400 M^{-1} cm^{-1}).

The enzyme activities toward various amide compounds were determined as follows. The reaction mixture contained a 50 mM KPB (pH 7.3), 1 mM amide compounds, and an appropriate amount of the enzyme. The reaction was performed at 25 °C. The ammonia produced was quantified with an Ammonia Test Kit (Wako Pure Chemical Industries). Primary amines produced were quantified with 2,4,6-trinitrobenzenesulfonic acid by the method described by Fields [14]. Hydrolysis of 6-aminohexanoate cyclic dimer was also monitored with an ionspray mass spectrometer, PE-Sciex API 3000 (Sciex, Thornhill, Ont., Canada).

One unit of the enzyme was defined as the amount of the enzyme catalyzing the hydrolysis of 1 μmol of the substrate in 1 min.

2.5. Protein assay

Protein concentration was measured with a Bio-Rad Protein Assay Kit (Hercules, CA, USA). Bovine serum albumin was used as the standard.

2.6. Purification of EstA8

pEstA8 was introduced into *E. coli* JM109. The recombinant cells were cultivated at 37 °C for 12 h in 10 ml of the LB medium supplemented with 100 μg/ml ampicillin, and the culture was transferred into 1 l of the LB medium containing 200 μg/ml ampicillin and 0.1 mM IPTG. After 12-h cultivation at 28 °C with reciprocal shaking, the cells were harvested. All the following operations were done at 0–4 °C, and a 10 mM KPB (pH 7.3) was used as the standard buffer. The cells were washed with the standard buffer, suspended with 25 ml of the same buffer, and disrupted by sonication for 5 min with a Biomic 7500 Ultrasonic Processor (Seiko, Tokyo, Japan). The cell debris was removed by centrifugation, and the supernatant was used as the cell-free extract.

Solid ammonium sulfate was added to the cell-free extract to give 20% saturation. After stirring for 1 h, the precipitate was removed by centrifugation, and ammonium sulfate was added to the supernatant to give 50% saturation. After stirring for 1 h, the precipitate was recovered by centrifugation, dissolved with 20 ml of the standard buffer, and dialyzed against the standard buffer.

The enzyme solution was applied to a Gigapite column (2.5 cm × 15 cm) equilibrated with the standard buffer. After the column was washed with the same buffer, EstA8 was eluted with a linear gradient of a 10–300 mM KPB (pH 7.3) with a total volume of 500 ml. The active fractions were pooled, concentrated, and used as the purified enzyme.

2.7. Determination of molecular weight

The molecular weight of the native enzyme was estimated by gel filtration with a Superose 12 HR 10/30 column (1 cm × 30 cm). The molecular weight markers used were as follows: cytochrome c (12,400), myokinase (32,000), enolase (67,000), lactate dehydrogenase (142,000), and glutamate dehydrogenase (290,000) (Oriental Yeast Co. Ltd., Osaka, Japan). The molecular weight of the subunit was determined by SDS–polyacrylamide gel electrophoresis with the following marker proteins: phosphorylase b (molecular weight: 94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin

inhibitor (20,100), and α-lactalbumin (14,400) (Amersham Pharmacia Biotech, Uppsala, Sweden).

2.8. Amino acid sequencing

The N-terminal amino acid sequence was determined with a fully automated protein sequencer Shimadzu PPSQ-10 (Kyoto, Japan).

2.9. Effects of temperature and pH on EstA8

The enzyme activities at different temperatures were measured with a reaction mixture containing a 50 mM KPB (pH 7.3), 10% acetonitrile, and 1 mM *p*-nitrophenyl butyrate. For the thermal stability studies, the enzyme was incubated in a 10 mM KPB (pH 7.3) at various temperatures ranging from 4 °C to 60 °C. After 30- and 60-min incubation, an appropriate amount of the enzyme was withdrawn and kept on ice for 1 h. The remaining activity was measured with *p*-nitrophenyl butyrate as the substrate.

The enzyme activity at various pHs was measured at 25 °C with a reaction mixture containing 1 mM *p*-nitrophenyl butyrate, 10% acetonitrile, and one of the following buffers: a 50 mM potassium acetate buffer, pH 5–5.4; or a 50 mM KPB, pH 5.8–8. For the pH stability studies, the enzyme was incubated at 4 °C for 24 h in one of the following buffers: a 50 mM potassium acetate buffer, pH 4.5–5.5; a 50 mM KPB, pH 6–8; a 50 mM Tris–HCl buffer, pH 8.5–9.5; or a 50 mM glycine–NaOH buffer, pH 10–10.5. The remaining activity was measured with *p*-nitrophenyl butyrate as the substrate.

2.10. Effects of organic solvents, salts, and various chemicals on EstA8

The stability of the enzyme in organic solvents was examined as follows. The enzyme was incubated in 10% and 30% water-miscible organic solvents for 30 min at 25 °C, and the remaining activity was determined.

To examine the effect of salts on the stability of the enzyme, the enzyme was incubated in a 10 mM KPB (pH 7.3) containing various salts for 48 h at 4 °C, and the remaining activity was determined.

The effect of various chemicals on the enzyme activity was examined as follows. The enzyme was incubated with various chemicals in a 50 mM KPB (pH 7.3) at 25 °C for 30 min, and the remaining activity was determined.

3. Results and discussion

3.1. Nucleotide sequence of the EstA8 gene and primary structure of EstA8

The recombinant *E. coli* cells producing the lipolytic enzymes of *Acinetobacter* sp. strain no. 6 were isolated as described in the Experimental section by screening of the genomic DNA library on the tributyrin-containing agar plates. Two of the lipolytic enzymes have already been characterized, and their primary structures and properties have been reported [4,8]. In the present study, we characterized another lipolytic enzyme named EstA8.

The plasmid coding for EstA8, named pEstA8, contained a 1.8-kbp insert with an open reading frame (ORF) consisting of 1488 bp, which encodes a protein of 496 amino acid residues (Fig. 2). The deduced molecular weight of EstA8 was 54,038. A potential promoter sequence and a probable Shine-Dalgarno sequence were found in the upstream region of the ORF.

EstA8 was found to belong to the amidase signature family, which includes 6-aminohexanoate-cyclic-dimer hydrolase [13], Glu-tRNA^{Gln} amidotransferase [15], fatty acid amide hydrolase [16], malonamidase [17], and peptide amidase [18]. These enzymes catalyze the hydrolysis of the amide bonds of their substrates. Among them, EstA8 showed the highest sequence similarity to 6-aminohexanoate-cyclic-dimer hydrolases from *Pseudomonas* sp. strain NK87 and *Flavobacterium* sp. strain KI72, which degrade a by-product of the nylon-6 industry (30% identity to each enzyme) [13] (Fig. 3). Although EstA8 catalyzed the hydrolysis of tributyrin and *p*-nitrophenyl esters (described below), no enzyme belonging to the lipase and esterase families showed homology with EstA8.

The following sequence is the consensus sequence of the proteins in the amidase signature family (the PROSITE database (Accession code: PS00571)): Gly-[Gly/Ala]-Ser-[Gly/Ser]-[Gly/Ser]-Gly-Xaa-[Gly/Ser/Ala]-[Gly/Ser/Ala/Val/Tyr]-Xaa-[Leu/Ile/Val/Met]-

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-133                                    TCAATTCCTGTTGCCGTGCCCAAAATCTCATTTATAAACTTAAACCTTAAGCAG  -80

 -79 CTCATCTCATCTACCTTGCTTCTTAAGGCAATATCGCTTAAAACAACAGCAGTTTATAATAATTTCAGTCAGGATGATG   -1

   1 ATG AAT TTT TTA GAA TAC TTA CAA TAT GAT GGC CTA GGA TTG GCC GAA TTA GTG GCA AAG   60
   1 Met Asn Phe Leu Glu Tyr Leu Gln Tyr Asp Gly Leu Gly Leu Ala Glu Leu Val Ala Lys  20

  61 CAA GAA GTT AGC GCA GCT GAG CTA TTA GAC TTG GCA TTA AAA CGT GCA GCT GAG GTC AAT  120
  21 Gln Glu Val Ser Ala Ala Glu Leu Leu Asp Leu Ala Leu Lys Arg Ala Ala Glu Val Asn  40

 121 CCC AGC TTA AAT GCC ATT ATT ATC CCC ATG CAT GAT TAT GCA CAT CAA CGT ACA CAA CAA  180
  41 Pro Ser Leu Asn Ala Ile Ile Ile Pro Met His Asp Tyr Ala His Gln Arg Thr Gln Gln  60

 181 ACA TTA ACT GGC GCT TTT GCC GGT GTG CCT TTT TTG GTC AAA GAT CTA TTT CAA GAG TAT  240
  61 Thr Leu Thr Gly Ala Phe Ala Gly Val Pro Phe Leu Val Lys Asp Leu Phe Gln Glu Tyr  80

 241 GCC GGC TAT CCA ACC TCT TAT GGC AGT AAA GCC TTA AAA CGT ATT GGC CAT CTT TCT GAG  300
  81 Ala Gly Tyr Pro Thr Ser Tyr Gly Ser Lys Ala Leu Lys Arg Ile Gly His Leu Ser Glu 100

 301 CAA AAT TCC GAG ATT GTG AAC CGC TGG GAA AAA GCC GGA ATT GTC ACG TTC GGG CGC ACC  360
 101 Gln Asn Ser Glu Ile Val Asn Arg Trp Glu Lys Ala Gly Ile Val Thr Phe Gly Arg Thr 120

 361 AAC ACG CCA GAA TTT GGT ATT AAA GGC GTT ACT GAG CCA GAA GCT TGG GGC GCT TGT CAT  420
 121 Asn Thr Pro Glu Phe Gly Ile Lys Gly Val Thr Glu Pro Glu Ala Trp Gly Ala Cys His 140

 421 AAT CCG TGG AAT ATC AGC CAC AAT AGT GGT GGA TCG TCT GGC GGT TCT GCA TCT GCG GTG  480
 141 Asn Pro Trp Asn Ile Ser His Asn Ser Gly Gly Ser Ser Gly Gly Ser Ala Ser Ala Val 160

 481 GCA GCA GGT ATT GTA CCT ATT GCA GGT GCA GGA GAT GGT GGT GGA TCA ATT CGT ATT CCG  540
 161 Ala Ala Gly Ile Val Pro Ile Ala Gly Ala Gly Asp Gly Gly Gly Ser Ile Arg Ile Pro 180

 541 GCC TCT TAT TGC GGA TTA TTT GGA CTC AAA CCC AGC CGT GGG CGC ACG CCT TGG GGA CCG  600
 181 Ala Ser Tyr Cys Gly Leu Phe Gly Leu Lys Pro Ser Arg Gly Arg Thr Pro Trp Gly Pro 200

 601 CAA CTC AGT GAA GCC ATG CAT GGT GCA GCC GTT CAG CAC GTC TTA AGC AAA ACA GTC CGT  660
 201 Gln Leu Ser Glu Ala Met His Gly Ala Ala Val Gln His Val Leu Ser Lys Thr Val Arg 220

 661 GAT AGC GCA GCC ATG TTA GAT GCC ACG CAA GGT GCA GAA CAT AGT TCA TTG TTT AAA ATT  720
 221 Asp Ser Ala Ala Met Leu Asp Ala Thr Gln Gly Ala Glu His Ser Ser Leu Phe Lys Ile 240

 721 GAA CCG CCA CAA GGT CGC TAT TTA GAC CTC ATT CAA AAG CCA CCT AAA AAG CTG AGC ATT  780
 241 Glu Pro Pro Gln Gly Arg Tyr Leu Asp Leu Ile Gln Lys Pro Pro Lys Lys Leu Ser Ile 260

 781 GCG TTT AGT ACA CGC TCA CCC ATT GGT ACA ACG GTA TCG GCA GAT GCG ATA GCT GCG GTT  840
 261 Ala Phe Ser Thr Arg Ser Pro Ile Gly Thr Thr Val Ser Ala Asp Ala Ile Ala Ala Val 280

 841 CAG CAT ACT GCA AAA CTA TTG GAG TCA TTG GGT CAT ACG GTG GTC GAA GAT ACG CCC GCG  900
 281 Gln His Thr Ala Lys Leu Leu Glu Ser Leu Gly His Thr Val Val Glu Asp Thr Pro Ala 300

 901 ATC GAT GGT ATG GCA TTG GCC AAG GAT TTT ATC ACC ACT TGG TTT AGC CAA TTT GCT TAT  960
 301 Ile Asp Gly Met Ala Leu Ala Lys Asp Phe Ile Thr Thr Trp Phe Ser Gln Phe Ala Tyr 320

 961 GTG CTT GAT CAA ACC AAG CGA CAA TAT TCA ATA AAA GCC AAT GAT TTT GAA CTT GAT TCA 1020
 321 Val Leu Asp Gln Thr Lys Arg Gln Tyr Ser Ile Lys Ala Asn Asp Phe Glu Leu Asp Ser 340

1021 TTG GCA CTG GCA GCC TTT GGT CAG CAA ACC ACA GCA ATA CAA TAT ATT CAC AAT TTA AAT 1080
 341 Leu Ala Leu Ala Ala Phe Gly Gln Gln Thr Thr Ala Ile Gln Tyr Ile His Asn Leu Asn 360

1081 AAT TGG GGT GTC TAC ACC ACA CAA ATG AAT CAT TTT TTT GAT CGT TAT GAT TTA TAT TTA 1140
 361 Asn Trp Gly Val Tyr Thr Thr Gln Met Asn His Phe Phe Asp Arg Tyr Asp Leu Tyr Leu 380

1141 ACA CCC GCC ACT GCT TCT GTA GCG CCT AAG AAT GGT GAA ATA AAA ATA CCA ATC TGG CAA 1200
 381 Thr Pro Ala Thr Ala Ser Val Ala Pro Lys Asn Gly Glu Ile Lys Ile Pro Ile Trp Gln 400

1201 AAA CCA ATT TTA AAA GGC TTA TTA AAA CTG GGT AAA GCA CAT CTC TTG GCA CAA GGT AAA 1260
 401 Lys Pro Ile Leu Lys Gly Leu Leu Lys Leu Gly Lys Ala His Leu Leu Ala Gln Gly Lys 420

1261 TTG GTC GAA AAA ATG GTT AAA GAG AAT ATG GCA CGC GTA CCG TTT ACA CAG TTG GCC AAT 1320
 421 Leu Val Glu Lys Met Val Lys Glu Asn Met Ala Arg Val Pro Phe Thr Gln Leu Ala Asn 440

1321 ATT ACT GGA CTG CCA GCG ATG TCA GTA CCG CTA TAT TGG AAT CAG GAT AAT TTA CCT TTA 1380
 441 Ile Thr Gly Leu Pro Ala Met Ser Val Pro Leu Tyr Trp Asn Gln Asp Asn Leu Pro Leu 460

1381 GGT TCA CAA TTT ATT GCG CCT TTC GCT CGT GAA GAT GTA CTG CTG CAA TTG GCA GCA CAG 1440
 461 Gly Ser Gln Phe Ile Ala Pro Phe Ala Arg Glu Asp Val Leu Leu Gln Leu Ala Ala Gln 480

1441 CTA GAA CAA GCA CAG CCG TGG TTT AAC CGT TAC AGT CAA ATT CAA GTT TAG CGC TTA TTA 1500
 481 Leu Glu Gln Ala Gln Pro Trp Phe Asn Arg Tyr Ser Gln Ile Gln Val ***              496

1501 TCTCGCTAATATTTATTTAACCTTTTATTTAACCTTTTATTGGGCCGGTTGAAGCTGTTTGGGCAACCAGCGCACAAAC 1579

1580 AACAAGCCCAATAAGGTTGAACATCCAGCAAAAACAAAGACTATTTCACCAGAAAACACATTCCAATAACGTCCCGCCA 1658

1658 AAATACTCCCGATCCTCT                                                              1676
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Fig. 2. Nucleotide sequence of the EstA8 gene and deduced amino acid sequence. The underlined and boldfaced nucleotide sequences are the potential promoter sequence and the probable Shine-Dalgarno sequence, respectively. The boldfaced amino acid residues (Ser176, Ser152, and Lys74) presumably constitute a catalytic triad.

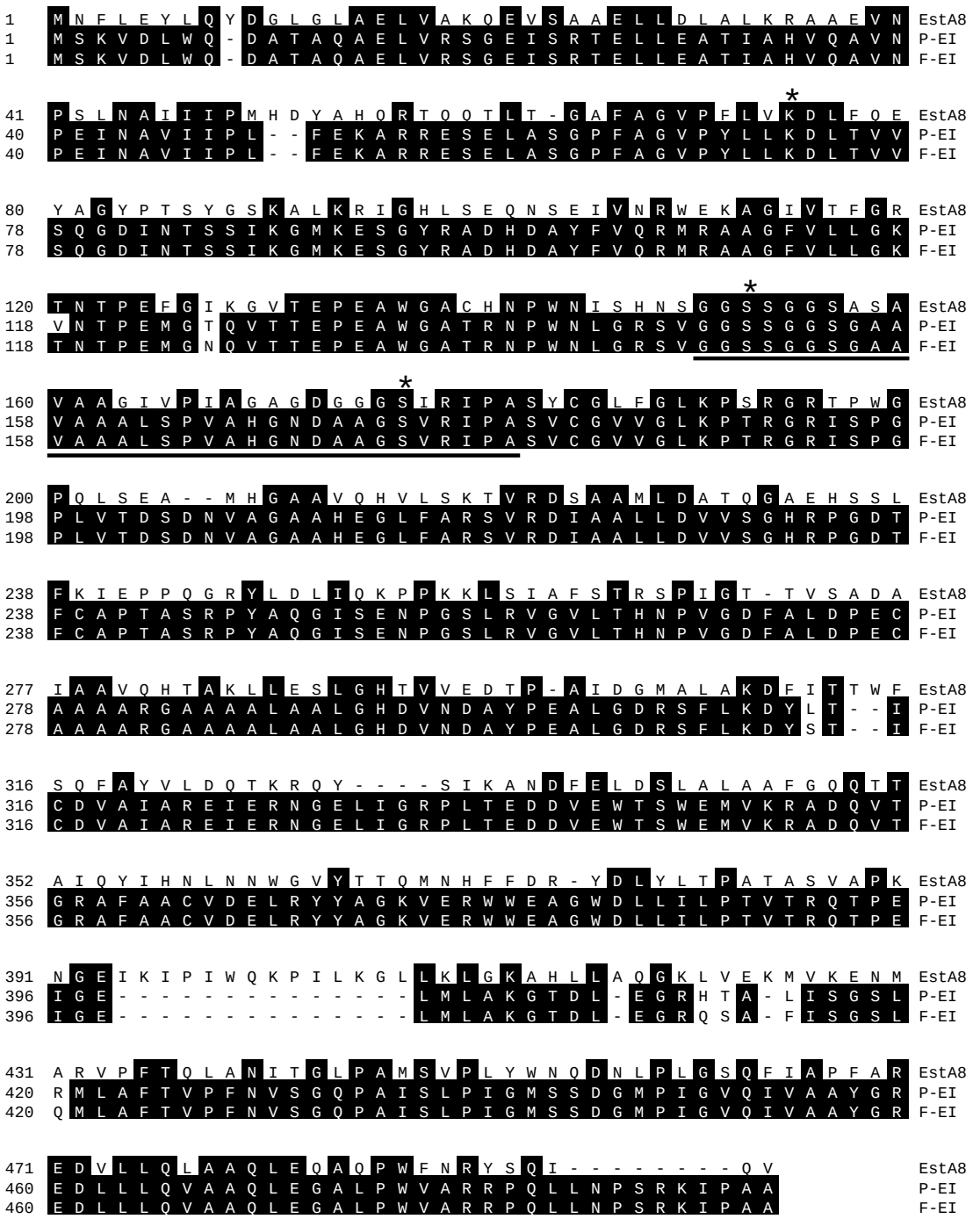

Fig. 3. Sequence alignment of EstA8 and 6-aminohexanoate-cyclic-dimer hydrolases from *Pseudomonas* sp. strain NK87 (P-EI) and *Flavobacterium* sp. strain KI72 (F-EI). The conserved residues are shown in the black boxes. The consensus sequence of the amidase signature family is underlined. The putative catalytic triad (Ser176–Ser152–Lys74) is indicated by asterisks.

[Gly/Ser/Ala]-$(Xaa)_6$-[Gly/Ser/Ala/Thr]-Xaa-[Gly/Ala]-Xaa-[Asp/Glu]-Xaa-[Gly/Ala]-Xaa-Ser-[Leu/Ile/Val/Met]-Arg-Xaa-Pro-[Gly/Ser/Ala/Cys] (where Xaa represents an arbitrary amino acid residue). This sequence is completely conserved in EstA8 (residues: 150–181). Recently, the three-dimensional structures of two members of the amidase signature family, peptide amidase from *Stenotrophomonas maltophilia* [18]and malonamidase E2 from *Bradyrhizobium japonicum* [17], were determined. The structures of these proteins were different from the α/β hydrolase fold and any other known protein fold. The proteins of the amidase signature family are now regarded as non-classical serine hydrolases. Interestingly, the catalytic triad of this class of the enzymes is not the classical Ser–His–Asp, but a novel triad, Ser–Ser–Lys [17,18]. The triad was conserved as Ser176, Ser152, and Lys74 in EstA8, where Ser176 is probably the catalytic nucleophile. Ser152 probably donates its side-chain proton to the carbonyl oxygen of the substrate and accepts the side-chain proton of Ser176 instead.

3.2. Purification of EstA8 and determination of its molecular weight

EstA8 was purified 3.0-fold to homogeneity with a 15% yield from the crude extract of *E. coli* JM109 harboring pEstA8 (Table 1). The N-terminal amino acid sequence of the purified enzyme was Met-Asn-Phe-Leu-Glu-Tyr-Leu-Gln-Tyr-Asp-Gly-Leu-Gly-Leu-Ala-, which is identical to that deduced from the nucleotide sequence of the gene (Fig. 2). The molecular weight of the subunit was estimated to be about 54,000 by SDS–polyacrylamide gel electrophoresis (Fig. 4), which agrees well with that deduced from the nucleotide sequence of the gene (54,038). The native molecular weight of the enzyme was determined to be about 52,000 by gel filtration, suggesting that EstA8 is a monomeric protein.

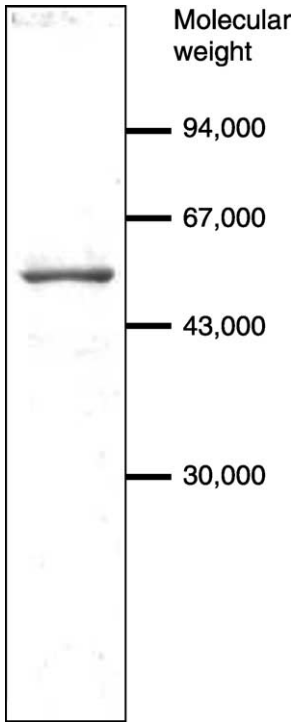

Fig. 4. SDS–polyacrylamide gel electrophoresis of EstA8. The positions of molecular weight markers are indicated.

3.3. Kinetic parameters for EstA8

The specific activity of the purified enzyme toward *p*-nitrophenyl butyrate was about 0.97 units/mg. EstA8 followed the Michaelis–Menten kinetics when *p*-nitrophenyl butyrate was used as the substrate, and the K_m and V_{max} values were 0.1 mM and 1.0 units/mg, respectively.

3.4. Effects of temperature and pH on EstA8

The maximum activity of EstA8 was found at 50 °C (Fig. 5A). The activity drastically dropped when the

Table 1
Purification of EstA8

Step	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	Yield (%)	Fold
Crude extract	58	180	0.32	100	1
Ammonium sulfate	50	90	0.56	86	1.8
Gigapite	8.7	9	0.97	15	3.0

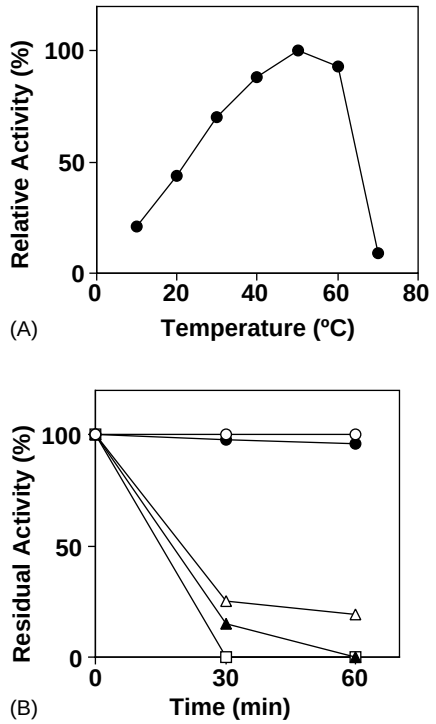

Fig. 5. Effects of temperature on EstA8. (A) Temperature–activity profile of EstA8. (B) Stability of EstA8 at 4 (○), 30 (●), 40 (△), 50 (▲), and 60 °C (□).

reaction was carried out at temperatures higher than 60 °C. Prolonged incubation caused inactivation of the enzyme even at moderate temperatures: about 75% of the original activity was lost by incubation at 40 °C for 30 min (Fig. 5B). Such instability is regarded as the characteristic of cold-active enzymes from cold-adapted bacteria.

The effect of pH on the stability of EstA8 was examined. The enzyme was incubated at various pHs ranging from 4.5 to 10.5 at 4 °C for 24 h. The enzyme was stable between pH 7.3 and pH 8.6. The optimum pH of the reaction was about 7.8.

3.5. Effects of organic solvents, salts, and various reagents on EstA8

EstA8 was stable in methanol and ethanol (up to 30%) (Table 2). In contrast, the enzyme was almost completely inactivated by 30-min incubation in 30% acetonitrile and partially inactivated in 30% dimethyl sulfoxide. Methanol and ethanol would be useful to increase the solubility of water-insoluble substrates of EstA8.

Table 2
Stability of EstA8 in organic solvents

Organic solvent	Remaining activity (%)	
	10[a]	30[a]
Acetonitrile	89	2
Methanol	139	123
Ethanol	128	94
Dimethyl sulfoxide	81	63

[a] Concentration of organic solvent (%).

During purification of EstA8, we noticed that the enzyme is very sensitive to NaCl and KCl. The remaining activities after 48-h incubation in a 10 mM KPB (pH 7.3) containing NaCl and KCl were as follows: 0.1 M NaCl, 28%; 0.2 M NaCl, 14%; 0.1 M KCl, 22%; 0.2 M KCl, 15%. Thus, the use of these salts had to be avoided in the purification procedure. EstA8 was more stable in a KPB: 94% activity remained after 48-h incubation in a 0.4 M KPB (pH7.3).

We examined the effect of various chemicals on the activity of EstA8. The following reagents affected the enzyme activity little (activity remaining: 80–110%): 1 mM EDTA, 1mM $MgCl_2$, 1 mM $FeCl_2$, 1 mM $FeCl_3$, 1 mM mercaptoethanol, 1 mM dithiothreitol, and 10% sorbitol. The enzyme was totally inactivated by 100 μg/ml phenylmethylsulfonyl fluoride, an inhibitor of serine hydrolases. Ser176, which is supposed to be the catalytic nucleophile of EstA8, was probably modified with this reagent.

3.6. Substrate specificity of EstA8

The activities of EstA8 toward various ester and amide compounds were tested. Among various *p*-nitrophenyl esters, the enzyme showed the highest activity toward *p*-nitrophenyl caprylate (C_8) (Table 3). The enzyme hydrolyzed the substrate with a short-chain-length acyl group, such as *p*-nitrophenyl acetate (C_2), less efficiently. Although EstA8 showed significant sequence similarity to 6-aminohexanoate-cyclic-dimer hydrolase [13], EstA8 did not hydrolyze 6-aminohexanoate cyclic dimer. None of the following amide compounds served as the substrate of EstA8: *N*-methylacetamide, *N*-ethylacetamide, *N*-methylpropionamide, *N*-methylbenzamide, *n*-butyramide, methacry-

Table 3
Activity of EstA8 toward various ester compounds

Substrate	Specific activity (unit/mg)
p-Nitrophenyl acetate (C_2)	0.40
p-Nitrophenyl butyrate (C_4)	0.51
p-Nitrophenyl valerate (C_5)	0.46
p-Nitrophenyl caproate (C_6)	0.63
p-Nitrophenyl caprylate (C_8)	0.83
p-Nitrophenyl laurate (C_{12})	0.72

Table 4
Activity of EstA8 toward various amide compounds

Substrate	Specific activity (unit/mg)
p-Nitroacetanilide	0.40×10^{-2}
N-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide	0.85×10^{-2}
N-Succinyl-Gly-Gly-Phe *p*-nitroanilide	0.43×10^{-2}
N-Succinyl-Ala-Ala-Pro-Asp *p*-nitroanilide	0.23×10^{-2}
N-Succinyl-Ala-Ala-Pro-Leu *p*-nitroanilide	0.18×10^{-2}
N-Succinyl-Ala-Ala-Val-Ala *p*-nitroanilide	0.07×10^{-2}
N-Succinyl-Gly-Gly-Gly *p*-nitroanilide	0.02×10^{-2}
N-Succinyl-Ala-Ala-Ala *p*-nitroanilide	0.02×10^{-2}
N-Succinyl-L-Phe *p*-nitroanilide	0.00×10^{-2}

lamide, 2-chloroacetamide, 2-phenylacetamide, malonamide, succinamide, benzamide, *o*-toluamide, *p*-toluamide, *o*-aminobenzamide, *m*-aminobenzamide, *p*-aminobenzamide, salicylamide, nicotinamide, isonicotinamide, pyrazinamide, methylurea, ethylurea, phenylurea, glycinamide, or L-threoninamide. EstA8 showed weak but significant activities toward various *p*-nitroanilides (Table 4). *N*-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide was the best substrate among them. EstA8 preferred to digest the amide bond at the C-terminal of an aromatic amino acid residue: *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide was a better substrate than *N*-succinyl-Ala-Ala-Pro-Asp *p*-nitroanilide and *N*-succinyl-Ala-Ala-Pro-Leu *p*-nitroanilide, and *N*-succinyl-Gly-Gly-Phe *p*-nitroanilide was a better substrate than *N*-succinyl-Gly-Gly-Gly *p*-nitroanilide. However, *N*-succinyl-L-Phe *p*-nitroanilide did not serve as the substrate. Although EstA8 belongs to the amidase signature family, its esterase activity toward *p*-nitrophenyl esters, such as *p*-nitrophenyl acetate, was much higher than its amidase activity toward *p*-nitroanilides, such as *p*-nitroacetanilide. It would be interesting to see what determines the unusual amide/ester reactivity of EstA8. Comparative studies of EstA8 and other proteins in the amidase signature family would be helpful in understanding the structural requirements for the amidase activity and the esterase activity.

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References

[1] A.F. Bradbury, D.G. Smyth, Biosci. Rep. 7 (1987) 907.
[2] A. Schwarz, C. Wandrey, D. Steinke, M. Kula, Biotechnol. Bioeng. 39 (1992) 132.
[3] U.T. Bornscheuer, FEMS Microbiol. Rev. 26 (2002) 73.
[4] T. Suzuki, T. Nakayama, T. Kurihara, T. Nishino, N. Esaki, J. Mol. Catal. B: Enzyme 16 (2002) 255.
[5] D.W. Choo, T. Kurihara, T. Suzuki, K. Soda, N. Esaki, Appl. Environ. Microbiol. 64 (1998) 486.
[6] L. Kulakova, A. Galkin, T. Kurihara, T. Yoshimura, N. Esaki, Appl. Environ. Microbiol. 65 (1999) 611.
[7] T. Suzuki, T. Nakayama, T. Kurihara, T. Nishino, N. Esaki, J. Biosci. Bioeng. 92 (2001) 144.
[8] T. Suzuki, T. Nakayama, T. Kurihara, T. Nishino, N. Esaki, Biosci. Biotechnol. Biochem. 66 (2002) 1682.
[9] J.L. Arpingny, J. Lamotte, C. Gerday, J. Mol. Catal. B: Enzyme 3 (1997) 29.
[10] C. Gerday, M. Aittaleb, M. Bentahir, J.P. Chessa, P. Claverie, T. Collins, S. D'Amico, J. Dumont, G. Garsoux, D. Georlette, A. Hoyoux, T. Lonhienne, M.A. Meuwis, G. Feller, Trends Biotechnol. 18 (2000) 103.
[11] K. Yokoigawa, Y. Okubo, H. Kawai, N. Esaki, K. Soda, J. Mol. Catal. B: Enzyme 12 (2001) 27.
[12] R. Cavicchioli, K.S. Siddiqui, D. Andrews, K.R. Sowers, Curr. Opin. Biotechnol. 13 (2002) 253.
[13] K. Tsuchiya, S. Fukuyama, N. Kanzaki, K. Kanagawa, S. Negoro, H. Okada, J. Bacteriol. 171 (1989) 3187.
[14] R. Fields, Biochem. J. 124 (1971) 581.
[15] A.W. Curnow, K. Hong, R. Yuan, S. Kim, O. Martins, W. Winkler, T.M. Henkin, D. Soll, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 11819.
[16] M.P. Patricelli, B.F. Cravatt, Biochemistry 40 (2001) 6107.
[17] S. Shin, T.H. Lee, N.C. Ha, H.M. Koo, S.Y. Kim, H.S. Lee, Y.S. Kim, B.H. Oh, EMBO J. 21 (2002) 2509.
[18] J. Labahn, S. Neumann, G. Buldt, M.R. Kula, J. Granzin, J. Mol. Biol. 322 (2002) 1053.