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Cloning, purification and properties of a hyperthermophilic esterase from archaeon *Aeropyrum pernix* K1

Renjun Gao [a,b], Yan Feng [a], Kazuhiko Ishikawa [b], Hiroyasu Ishida [b], Susumu Ando [b], Yoshitsugu Kosugi [b], Shugui Cao [a,*]

[a] *Key Laboratory for Molecular Enzymology and Engineering of Ministry of Education, Jilin University, Changchunm 130023, PR China*

[b] *National Institute of Advanced Industrial Science and Technology, Tsukuba Center, Japan*

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Abstract

The gene APE1547 of the aerobic thermophilic *Aeropyrum pernix* K1 encoding 582 amino acid residues was cloned into *Escherichia coli*. BL21 (DE3) by using vector pET11a with a T7 promoter. An alignment of similarity analysis of APE1547 with protein sequences from *A. pernix* K1 databank revealed that it showed a lipase motif and low homology with the known thermophilic esterases. However, it had a high degree homology with several acyl amino acid-releasing enzymes. After purified by ion exchange chromatography and gel filtration chromatography, the recombinant protein showed both esterase activity and acylamino acid-releasing enzyme (AARE) activities. The optimum of temperature and pH of the esterase activity are 90 °C and 8.0, respectively. The recombinant protein showed the hydrolytic activity for a wide range of substrates, such as *p*-nitrophenyl alkanoate esters of varying alkyl chain lengths, pNA-labelled amino acid and peptide. The highest activity was observed for the substrate *p*-nitrophenyl caprylate. The recombinant enzyme was extremely stable and protein concentration-dependent. Its half-life at 90 °C was over 160 h. at the concentration of 2.14 mg/ml, which renders this new esterase very attractive for biotechnological applications.

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Keywords: Cloning; Esterase; Acylamino acid-releasing enzyme; *Aeropyrum pernix* K1

1. Introduction

Esterases (EC 3.1.1.1) are widely distributed in animals, plants and microorganisms. For their activities in both aqueous and nonaqueous solvent systems, esterases have developed into the most widely used class of enzymes in various industrial processes, finding use in stereospecific hydrolysis, transesterification, ester synthesis, modification of physicochemical properties of triglycerides for the fats and other organic biosynthesis reactions [1–3]. Mesophilic enzymes are often not well suited for the harsh reaction conditions (such as high temperature, exposure to organic solvents, etc.) required in industrial processes because of the lack of enzyme stability. The discovery of specific esterase working under extreme conditions is necessary, which will widely extend the range of reactions of esterase. It has been reported that the enzyme resistance to denaturation in organic solvents is correlated with their thermostability in water [4]. For this reason, thermostable enzymes are focused on [5].

* Corresponding author. Tel.: +86-431-849-9722; fax: +86-431-898-7975.

E-mail addresses: y-kosugi@aist.go.jp (Y. Kosugi), sgcao@jlu.edu.cn (S. Cao).

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Since thermophilic archaea may provide most of thermostable enzymes, esterase from archaea has gotten more attentions. Till now, five esterases coming from archaeon have been reported [6–10]. Considering esterase from different sources with varied substrate specificity, we searched esterase activity further from new reported archaea genome data. *Aeropyrum pernix* K1 has been isolated in 1993 from coastal solfotaric thermal vent in Kodakara-jima Island in Kyusyu, Japan. It grows in the range of 90–98 °C with an optimal temperature of 95 °C [11]. From the genome sequences of *A. pernix* K1, we found five genes with lipase motif; APE1547 was selected as the target protein. After the gene was cloned and expressed in *E. coli* strain, the recombinant protein turned out to show both esterase and acylamino acid-releasing enzyme (AARE) (EC 3.4.19.1) activities. AARE may catalyze the NH_2-terminal hydrolysis of N^{α}-acylpeptides to release N^{α}-acylated amino acids, which was first purified from rat liver by Tsunasawa et al. [12].

The new enzymes from hyperthermophilic bacteria could add new insights into the evolutive relationships of esterase and add new model for fundamental studies in the field of protein stability. [13]. The crystal of the recombinant protein has been revealed [14]. Here, we described the cloning and expression of the gene APE1547 from *A. pernix* K1. The recombinant protein was demonstrated to be one of the most thermostable esterase.

2. Materials and methods

2.1. Materials

Escherichia coli BL21 (DE3) and vector pET11a were obtained from Novagen (Madison, WI). Vent DNA polymerase was purchased from New England Biolabs. Restriction enzymes were purchased from Promega and Toyobo (Osaka, Japan), ultrapure deoxynucleotide solution (dNTPs) was purchased from Pharmacia Biotech (Sweden). Isopryl-β-D-thiogalactopyranoside (IPTG), *p*-nitrophenyl acetate (pNPC2), *p*-nitrophenyl propionate (pNPC3), *p*-nitrophenyl caprylate (pNPC8), *p*-nitrophenyl laurate (pNPC12), *p*-nitrophenyl palmitate (pNPC16), *p*-nitrophenyl stearate (pNPC18), DNase I, N^{α}-acetylamino acid *p*-nitroanilide derivatives (Ac-amino acid-pNA), Fast Blue RR, and β-naphtylacetate were purchased from Sigma. All other chemicals were of the highest reagent grade commercially available.

2.2. Cloning and sequencing of the gene

The gene APE1547 with lipase motif was chosen from the genome of *A. pernix* K1. The gene was amplified by PCR method using the following two primers with *Nde* I and *Bam* HI restriction sites: CTTACGAGTATCTCATATGCGCATTATAATGCCTGT (upper primer, *Nde* I cutting site as underlined); TTGGAGGCCCCTCCCGGCGGTGGATCCCTATCTCCT (lower primer, *Bam* HI cutting site as underlined). PCR was conducted using 40 ng of *A. pernix* K1 genome DNA as template and the following parameters: initial denaturation (94 °C, 4 min); followed by 35 cycles of denaturation (94 °C, 1 min), annealing (48 °C, 2 min), and extension (72 °C, 3 min) using vent DNA polymerase. After purification of the PCR product by microfuge affinity tube according to the manufacture's instruction (QIGEN, Germany), it was hydrolyzed by endonucleases and inserted in pET11a cut by the same endonucleases according to the manufacturer's instructions.

The cloned fragment was complete sequenced on both strands using the T7 DNA polymerase sequence kit by using an IRD700 labeled primer and an IRD800 labeled primer. Since this gene is quite long (1746 bp), a middle primer from position 741–760 CATCACCTGGCTAGGCTACT labeled with IRD700 was used for analyzing the middle part of the gene sequence. The sequence was assayed with a DNA sequencer ABI model 373 (Perkin-Elmer, Applied Biosystems Division, Foster City, CA).

2.3. Expression of the gene

The host *E. coli* BL21 (DE3) was transformed by the constructed plasmid. The transformant cell was grown in 2YT medium (1% yeast extract, 1.6% tryptone, and 0.5% NaCl) containing ampicillin (100 μg/ml) at 37 °C. After incubation with shaking at 37 °C until the A_{600} reached 0.6–1.0, the induction was carried out by adding IPTG at a final concentration of 1 mM and shaking for 4 h at 37 °C. The induced cells were collected by centrifugation and stored at −20 °C.

2.4. Purification of the enzyme

The frozen cells were melted and mixed with 50 mM Tris–HCl buffer (pH 8.0). After ultrasonic cell disintegration, the cell suspension was centrifuged at $4000 \times g$ for 20 min. The supernatant was incubated at 37 °C for 30 min with 0.5 mg/ml of bovine DNase I, then heated at 85 °C for 30 min. After centrifuged at $10,000 \times g$ for 20 min at 4 °C, the supernatant was applied to a HiTrap Q-Sepharose column (5 ml, Pharmacia, Uppsala, Sweden) pre-equilibrated with 50 mM Tris–HCl buffer (pH 8.0). The esterase was eluted with a linear gradient (0–1.0 M NaCl in the same buffer) at a flow rate of 60 ml/h. The fractions were collected and the esterase activity was analyzed. The active fractions were collected and concentrated to 1.0 ml by a Centricon 10 filter (Millipore, USA). The concentrated material was then applied on a HiLoad Sephacryl S-200 column (2 cm × 60 cm and eluted with 100 mM Tris–HCl buffer (pH 8.0) containing 100 mM NaCl. The active fractions were pooled, concentrated and analyzed for purity by SDS-PAGE.

2.5. Determination of molecular mass

The molecular mass of the purified enzyme was estimated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed with gels containing 12.5% acylamide using the Bio-Rad protein mini gel system (Bio-Rad Laboratories, Richmond, CA, USA) essentially as described by Laemmli [15]. Marker proteins included rabbit phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), rabbit actin (43 kDa), bovine carbonic anhydrase (31 kDa), trypsin inhibitor (20.1 kDa), and hen egg white lysozyme (14.4 kDa). SDS-PAGE gels were stained with Coomassie Brilliant Blue. The molecular mass of the denatured enzyme was obtained by interpolation on a plot of log of molecular mass against relative migrations (R_f values). Nondenaturing PAGE was performed at basic pH (separting gel, pH 8.8; running buffer, pH 8.3) with 7.5 or 10% polyacrylamide slab gels. Nondenaturing gels were stained for esterase activity as described [16]. In brief, gels were incubated in a solution (100 ml) Tris–HCl, pH 7.5, containing 5 mg of β-naphtylacetate and 25 mg of Fast Blue RR at 85 °C. Reactions were stopped after 30 min by rinsing with tap water and placing gels in 7.5% (v/v) acetic acid. To analysis N-terminal amino acid sequence, 1 mg of lyophilized homogenous sample was dissolved in 200 μl of distilled water, 4 μl of the treated sample was analyzed by HP G1005A Protein Sequencing System at Takara Holdings Inc. (Kyoto, Japan).

2.6. Enzyme assay

The time course of the esterase-catalyzed hydrolysis of pNPC8 was followed by monitoring the production of *p*-nitrophenyl at 405 nm in 1 cm pathlength cells with a double-beam HTACHI 557 ultraviolet–visible spectrophotometer equipped with a temperature controller. The substrate pNPC8 was dissolved in acetonitrile at a concentration of 10 mM. In the standard assay, 20 μl of 10 mM pNPC8 solution was added to reaction system to a final concentration of 0.2 mM in 50 mM phosphate buffer (pH 8.0) incubated at 70 °C, then the reaction was started by addition of 20 μl of the enzymatic solution. The background hydrolysis of the substrate was deducted by using a reference sample of identical composition to the incubation mixture, except that esterase was omitted. One unit of enzymatic activity was defined as the amount of protein releasing 1 μmol of *p*-nitrophenyl from pNPC8 per minute [17]. The substrate specificity of the enzyme was studied with *p*-nitrophenyl alkanoate esters of varying alkyl chain lengths.

The AARE activity of this protein was determined using Ac-amino acid-pNAs and amino acid-pNAs. The enzyme was incubated at 85 °C with 2 mM substrates in 50 mM Tris–HCl buffer (pH 8.0). After 3 min, the A_{406} was measured with HITACHI 557, and the activity was calculated using the absorption coefficient $\varepsilon_{406} = 9.91\ \text{mM}^{-1}$ for *p*-nitroaniline formation. One unit of activity corresponds to the amount of enzyme catalyzes the hydrolysis of 1 μmol Ac-amino acid-pNA per minute [18]. The activity toward the peptides was measured by the detection of the exposed α-NH_2 group with the cadmium–ninhydrin colorimetric method [19].

2.7. Effects of temperature and pH on enzyme activity

The optimal temperature for the hydrolysis of pNPC8 was determined by measuring the rate of the

reaction at temperatures ranging from 55 to 95 °C under standard assay conditions. The optimal pH for enzyme activity was determined at 70 °C from pH 6.5 to 10.0 in various buffers, in 50 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 6–8), 50 mM H_3BO_3–$Na_2B_3O_7$ buffer (pH 7.5–9) and 50 mM $NaHCO_3$–Na_2CO_3 buffer (pH 9–10). For studying the thermostability, the enzyme was incubated at 90 °C for 7 days. and residual activity was determined at 70 °C using pNPC8 as substrate.

3. Results and discussion

3.1. Sequence alignment

The APE1547 ORF encoded a protein of 582 amino acids. We found the C-terminal of this protein is highly homogenous to prolyl oligopeptidase family domain and carboxylesterase domain using NCBI Conserved Domain Search (Fig. 1A). The deduced amino acid

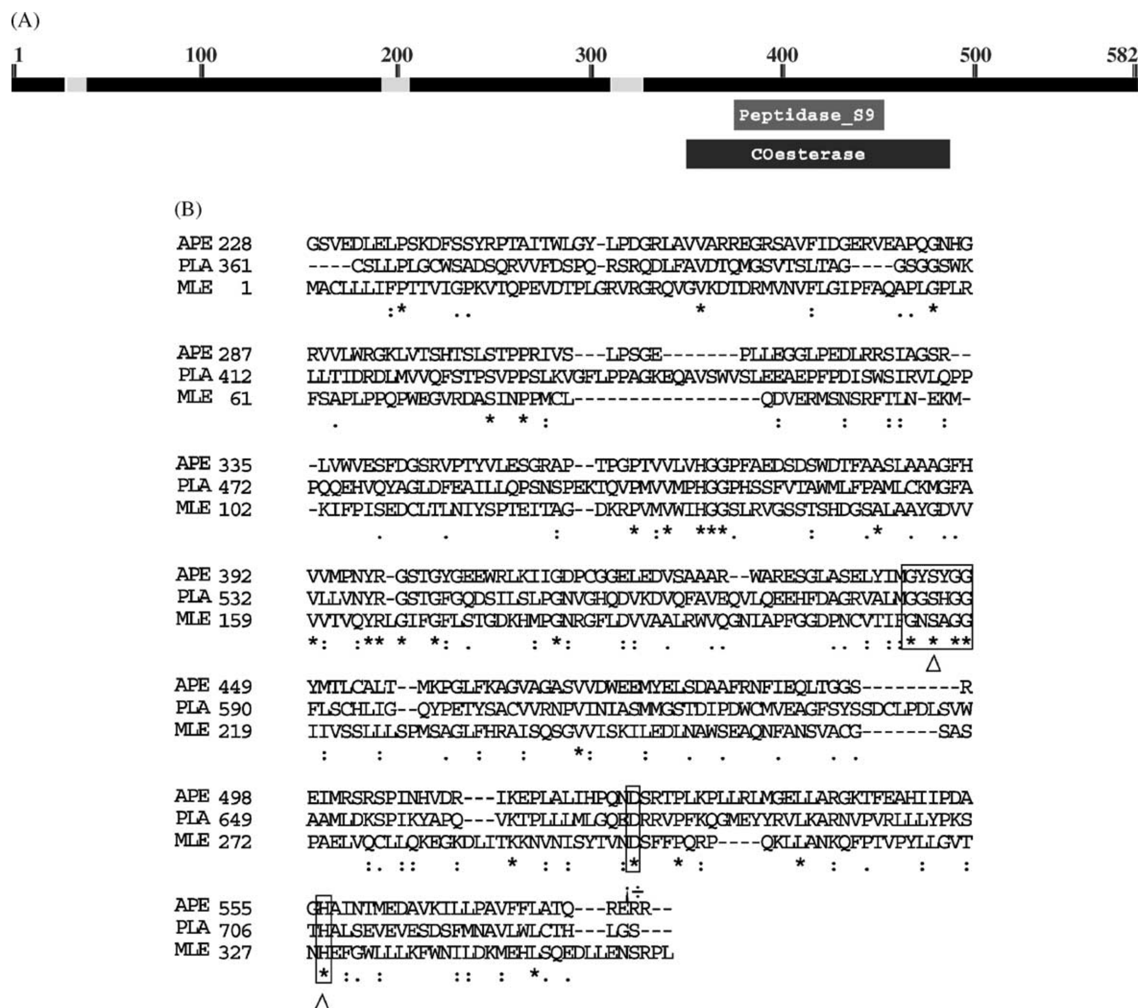

Fig. 1. (A) NCBI Conserved Domain Search, Peptidase_S9: prolyl oligopeptidase family domain and coesterase: carboxylesterase domain. (B) Alignment of the predicted amino acid sequence of esterase from *A. pernix* K1, AARE from pig liver and carboxylesterase from mouse liver. Sequence alignment was performed by Clustal X (1.8) program. The sequences have been aligned with dashes indicating gaps. Conserved residues are marked with an asterisk (*). Putative active residues (Ser, Asp, and His) are marked (△). The large boxed region indicates the area containing the GXSXG motif. Abbreviations: APE: esterase from *A. pernix* K1; PLA: AARE from pig liver; MLE: carboxylesterase from mouse liver.

derived from APE1547 ORF exhibited significant levels of similarity to the amino acid sequence of an AARE from pig liver (29% identity) and a carboxylesterase from mouse liver (26.9% identity) [20,21]. Fig 1B shows a multiple alignment of those enzymes belonging to subfamilies of serine hydrolase family. The characteristics of serine hydrolase include a tertiary structure called the α/β-hydrolase fold and a catalytic triad consisting of serine, aspartic acid and histidine residues. The alignment of the APE1547 with the amino acid sequences of AARE from pig liver and carboxylesterase from mouse liver clearly revealed the presence of the three amino acids of the catalytic triad (Ser-445, Asp-524, and His-556) and the consensus sequence around the active serine (Gly-X-Ser-X-Gly) in APE1547 (Fig. 1B). The pentapeptide is conserved throughout the lipase, esterase and serine protease superfamily, which suggests that prolyl oligopeptidase, AARE, esterase and lipase might be evolutionally related [22,23].

3.2. Cloning and expression of APE1547

The gene APE1547 contains high contents of (G + C, 61%) and cannot be amplified at predicted temperature, it was amplified by PCR using low annealing temperature at 48 °C. The sequence analysis result showed that the amplified gene was exactly matched the APE1547 gene. The amplified gene was inserted in pET11a and expressed in *E. coli* BL21 (DE3). An efficient expression system of the APE1547 gene was devised in *E. coli* and biochemical studies on the recombinant hyperthermostable enzyme were performed. The high-level expression was attained after 4 h of IPTG induction and most of proteins from *E. coli* were removed by taking advantage of its thermostability at 85 °C.

3.3. Purification of APE1547

In our attempt to purify the recombinant protein, the cell free supernatant was incubated at 85 °C for 30 min, then purified by HiTrap Q-Sepharose ion exchange chromatography and gel filtration chromatography (Figs. 2 and 3). Table 1 summarizes the purification steps involved the yield of the esterase activity at each step. Analysis of the densitometer by using Phoretix 1D Advanced software for the gel of

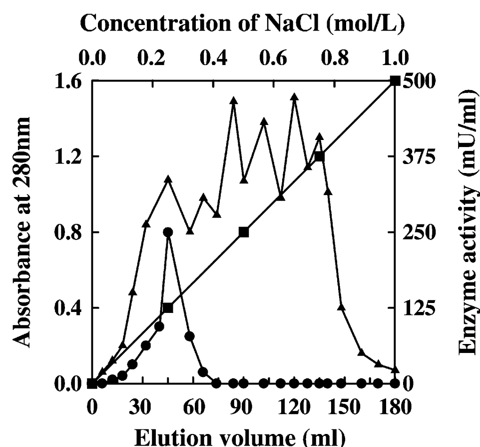

Fig. 2. Chromatography of the recombinant esterase from *A. pernix* K1 on Hitrap Q-Sepharose ion exchange chromatography. Column 1 mm × 5 mm was preequilibrated with 20 mM Tris–HCl (pH 8.0) and was eluted with a gradient of sodium chloride up to 1 M at the flow rate of 2 ml/min. (▲) Absorbance at 280 nm; (●) enzyme activity; and (■) concentration of sodium chloride.

SDS-PAGE indicated that the purity was about 99% with a predicted molecular mass of 63 kDa. With the described purification procedure, a 43-fold increase in enzyme specific activity was achieved with overall yield of 31%. Automated Edman degradation of the purified enzyme revealed seven amino acids of the NH_2-terminal as Met-Arg-Ile-Ile-Met-Pro-Val-Glu-Phe. Except the first amino acid was changed from

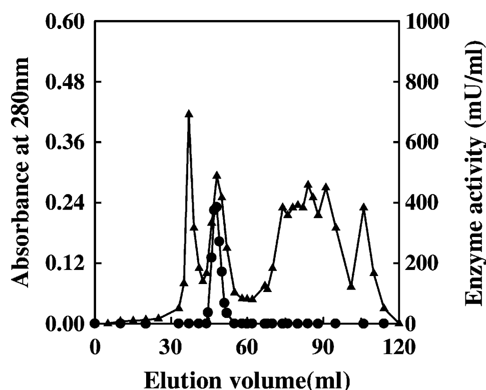

Fig. 3. Chromatography of the recombinant esterase from *A. pernix* K1 on Hitrap Sephacryl S-200 gel filtration chromatography. Column (1.6 cm × 60 cm) was equilibrated with 50 mM Tris–HCl (pH 8.0) at a flow rate of 30 ml/h. The sample was loaded and eluted with the same buffer. (▲) Absorbance at 280 nm; and (●) enzyme activity.

Table 1
Summary of purification of esterase from *A. pernix* K1

	Total (U)	Total protein (mg)	Specific activity (mU/mg)	Purification fold	Yield (%)
Crude extract	64.34	2900	21.44	1	100
Heat treatment at 85 °C	36.28	945	38.4	1.8	56
Hitrap Q-Sepharose	22.68	84	300	14	35.3
Sephacryl S-200	20.0	22	920	43	31

Val to Met during PCR reaction (GTG → ATG), the sequence was same as predicted by the genome information.

3.4. Determination of molecular mass

The molecular mass of the purified protein, as determined by SDS-PAGE (Fig. 4A, lane 4), was consistent with that calculated from the amino acid sequence (63 kDa). In a native PAGE a single band also was observed (Fig. 4B, lane 2) showed the activity (Fig. 4B, lane 1). The purified protein showed a single band corresponding 63 kDa by SDS-PAGE (Fig. 4A) and by native PAGE (Fig. 4B), indicating the recombinant protein presented as a monomer.

3.5. Effect of temperature and pH on the enzyme activity

The temperature–activity curve shows obvious esterase activity from 55 to 95 °C (Fig. 5). The enzyme exhibited maximum activity toward pNPC8 at a temperature of 90 °C at pH 8.0. The enzyme (2.14 mg/ml) retained about 60% esterase activity after incubated at 90 °C for 160 h (Fig. 6), which indicated the recombinant protein had extremely stability at optimum temperature. It was shown from Fig. 7 that the thermostability related to the protein concentration. The

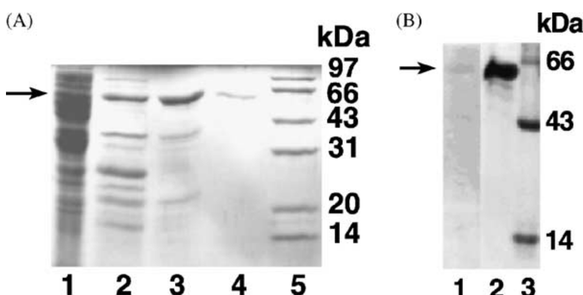

Fig. 4. (A) SDS-PAGE electrophoresis of the purified esterase from *A. pernix* K1. Lane 1: crude extract; lane 2: supernatant after heat treatment at 85 °C; lane 3: Hitrap Q-Sepharose ion change chromatography (peak 1); lane 4: HiLoad Sephacryl S-200 gel filtration chromatography (peak 2); lane 5: standard protein markers. (B) Nondenaturing polyacrylamide gel and activity staining electrophoresis of esterase APE1547. Lane 1: activity staining; lane 2: native PAGE; lane 3: standard protein marker.

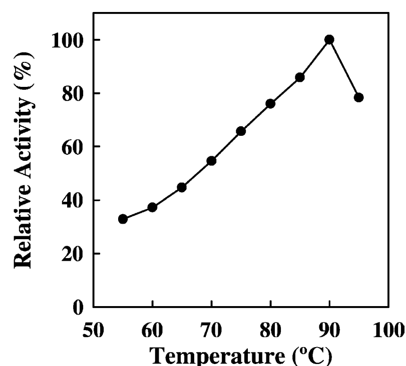

Fig. 5. Effect of temperature on esterase activity. Activity was determined at 50 mM Tris–HCl (pH 8.0) using pNPC8 as substrate.

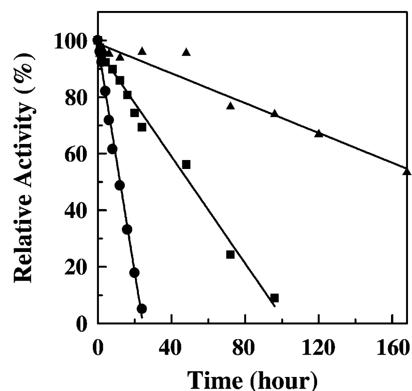

Fig. 6. Thermostability of recombinant esterase at optimum temperature. Incubation was performed at 90 °C with the protein concentration of 2.14 mg/ml (▲); 0.8 mg/ml (■); and 0.2 mg/ml (●).

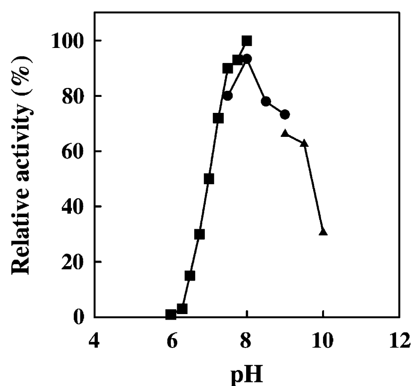

Fig. 7. Effect of pH on esterase activity. The enzyme activity was measured photometrically with pNPC8 (0.2 mM) as substrate. The reaction was carried out at 70 °C; pH 6–8: 50 mM Na_2HPO_4/NaH_2PO_4 buffer (■); pH 7.5–9: 50 mM H_3BO_3–$Na_2B_3O_7$ buffer (●); and pH 9–10: 50 mM $NaHCO_3$–Na_2CO_3 buffer (▲).

higher concentration, the more stable the protein was. The pH dependence of the esterase was determined with pNPC8 as the substrate. The pH profiles show that the esterase is active over a broad pH range with an optimum activity around pH 8.0 (Fig. 7). No assays could be done at pH higher than 10 due to a high rate of autohydrolysis of pNPC8. The purified protein showed obvious activity at pH range from 6.5 to 10, which is similar to those of most microbial esterases [24].

The purified protein showed optimum temperature at 90 °C and had high thermostability at 90 °C. These features represent important prerequisites for biotechnological applications, such as the synthesis of chiral compounds and the transesterification reactions exploited in the food and pharmaceutical industries, when high temperatures are required for the solubilization of both reagents and products of the organic synthesis.

3.6. Substrate specificity

The esterase showed the highest activity toward pNPC8 among the synthetic substrates (C2–C18 acyl groups) (Table 2). When the acyl chain length of the substrate was increased over C8 or decreased below C8, there was a decrease in the enzyme activity. In addition, no activity could be detected on hydrolysis of olive oil for a lipase assay (<0.05 U/mg), confirming the lack of lipase activity. The recombinant protein shows high activity for short chain fatty acid esters (pNPC8), but very low activity for long chain fatty acid esters (pNPC18) or undetectable (olive oil). These results suggested that the gene APE1547 encoded an esterase rather than a lipase. The sequence of the first 20 NH_2-terminal amino acids of this protein was Met-Arg-Ile-Ile-Met-Pro-Val-Glu-Phe-Ser-Arg-Ile-Val-Arg-Asp-Val-Glu-Arg-Leu-Ile. Database searches indicated that the sequence showed no significant homology with any other protein sequence in the database, suggesting that the purified enzyme may belong to a novel class of microbial esterases.

Table 2
Substrate specificity of esterase activity against *p*-nitrophenyl alkanoate esters

Substrate[a]	Relative activity[b] (% of control)
p-Nitrophenyl acetate	51
p-Nitrophenyl propionate	66
p-Nitrophenyl caprylate	100
p-Nitrophenyl laurate	28
p-Nitrophenyl palmitate	26
p-Nitrophenyl stearate	19

Hydrolysis of pNPC8 was taken as reference values (100%).
[a] All substrates were used at a final concentration of 2 mM.
[b] Esterase activity was determined by measuring free *p*-nitrophenol after 1 min incubation.

To examine the AARE activity of this protein, Leu-pNA, Ac-Leu-pNA, Ala-pNA, Ac-Ala-pNA, Ac-Phe-pNA, Ac-Phe-pNA, Ac-Glu-pNA, Ac-Lys-pNA, Ac-Arg-pNA, and Ac-Tyr-pNA were used as substrates. Table 3 shows the hydrolytic activity (releasing pNA) of the protein for them. At 85 °C and pH 8.0, the protein exhibited high hydrolytic activity for Ac-Leu-pNA and Ac-Phe-pNA and low hydrolytic activity for Ac-Ala-pNA and Ac-Lys-pNA. Compared with Ac-Leu-pNA and Ac-Ala-pNA, it showed low hydrolytic activity for Leu-pNA and Ala-pNA (Table 3). It indicated that this protein is an acylamino acid-releasing enzyme. The protein also showed hydrolytic activity for Ala-Phe and Ala-Asp (Table 4). That means the AARE activity of purified protein will lead it to be used in amino acid sequence analysis. Based on a sequence homology search, AAREs have been classified as a new serine protease subfamily. This kind of enzyme is expected to remove N^{α}-acylated resides in short peptide sequence analysis at high temperatures [25].

Table 3
Substrate specificity of purified AARE activity against amino acid-pNAs

Substrate[a]	Relative activity[b] (% of control)
Leu-pNA	7.4
Ac-Leu-pNA	100
Ala-pNA	3.8
Ac-Ala-pNA	30
Ac-Phe-pNA	115
Ac-Glu-pNA	1.7
Ac-Lys-pNA	31.6
Ac-Arg-pNA	2.3
Ac-Tyr-pNA	1.9

Hydrolysis of Ac-Leu-pNA was taken as reference values (100%).
[a] All substrates were used at a final concentration of 1 mM.
[b] AARE activity after 1 h incubation was determined by measuring free pNA.

Table 4
Substrate specificity of purified AARE activity against peptides

Substrate[a]	Relative activity[b] (% of control)
Ala-Tyr	18
Ala-Asp	67
Ala-Phe	100
Ala-Gly	66
Tyr-Ala	48
Phe-Ala	60
Arg-Ala	50
Trp-Ala	29

[a] All substrates were used at a final concentration of 2 mM.
[b] Hydrolysis of Ala-Phe was taken as reference values (100%).

In this paper, we have cloned the APE1547 gene and identify its function as esterase and AARE. The recombinant protein has high thermostability and wide substrate adapted. Studies about the crystal structure, stability in organic solvent and hydrolytic mechanism of esterase APE1547 are in progress.

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