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ORIGINAL PAPER

Expression and characterization of a novel mesophilic protease from metagenomic library derived from Antarctic coastal sediment

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Abstract A metagenomic cosmid library was constructed, in which the insert DNA was derived from the coastal sediment near Antarctic China Zhongshan Station. One clone (ACPRO001) expressing protease activity was isolated from the library using milk agar plates. Sequencing of the clone revealed a novel protease gene. The amino acid sequence comparison and phylogenetic analysis indicated that it could be classified as a subtilisin-like serine protease, though the highly conserved residue Asp was replaced by Ala. The ACPRO001 protease gene was expressed in pET-His and purified for characterization. The optimal temperature and pH for the activity of the ACPRO001 protease were 60°C and pH 9.0, respectively. The enzyme retained about 73% of residual activity after 2 h incubation at 50°C in the presence of Ca^{2+}. The presence of Ca^{2+} increased the thermostability of ACPRO001 protease obviously. The enzymatic activity was inhibited by 1 mM phenylmethyl sulfonylfluoride (PMSF) and hydrochloride 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), indicating that it was a serine protease.

Keywords Protease · Metagenomic library · Antarctic

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Introduction

Microorganisms are a very important source of biocatalysts. Living on a wide range of different environment, they have developed various highly optimized enzymes (Pace 1997; Whitman et al. 1998; Demirjian et al. 2001). Traditionally, the approaches for isolating a new enzyme from environmental microorganisms include enrichment of microorganisms from environmental samples, isolating those microorganisms in pure culture, and finally screening for the potential enzymes (Ogawa and Shimizu 1999). However, it has been reported that only less than 1% of the microorganisms in soil can be cultured by standard laboratory conditions (Amann et al. 1995). Thus, in order to overcome the inherent lose of diversity caused by the traditional cultivation so that we can search for new or reformative enzymes, the sum total genomes of soil microorganisms from a given habitat, or metagenome, can be extracted and used to construct metagenomic libraries (Ferrer et al. 2009; Steele et al. 2009). Previous studies have discovered various enzymes by metagenomic techniques, including amylase (Richardson et al. 2002; Rondon et al. 2000; Lammle et al. 2007; Yun et al. 2004), lipase (Lee et al. 2006; Jeon et al. 2009b; Hardeman and Sjoling 2007; Lee et al. 2004), esterase (Rhee et al. 2005; Park et al. 2007; Ferrer et al. 2005; Jeon et al. 2009a), protease (Acevedo et al. 2008) and chitinase (LeCleir et al. 2004; Cottrell et al. 1999).

Proteases are enzymes that hydrolyze proteins into amino acids and peptides. They act as processing enzymes taking part in regulatory or catabolic processes in the cell or as extracellular enzymes playing an important role in degrading proteinaceous substrates serving as carbon or energy sources (Godde et al. 2005). Till date, proteases have been used in a wide range of industrial fields. Whether an enzyme can be used in industrial reactions depends

on its unique characteristics, for example, its action pattern, substrate specificity, major reaction products, optimal temperature, pH, etc. Although lots of protease had been studied, the discovering of new protease is still significant for both commerce and research. In this paper, we report the functional screening, cloning, expression and characterization of a novel mesophilic protease from metagenomic library derived from Antarctic coastal sediment.

Materials and methods

Sample and construction of metagenomic library

The Antarctic sample collected from the coast near China Zhongshan Station was the subsurface sediment at depths of 10–15 cm within the treated sewage effluent to the sea. It was transferred to sterile Falcon Tubes in clean bench and kept aseptically at −20°C until back to laboratory. The microbial community genomic DNA was extracted by combining chemical lysis and enzyme digestion, and then purifying by DNA-adsorbent resin (Zeng et al. 2005). The crude DNA was checked by pulse field gel electrophoresis with low melting point agarose gel. The optimal size (30–45 kb) DNA was recovered from gel and purified with *GELase*™ *Agarose Gel-Digesting Preparation* (Epicentre), and then was ligated into the *pWEB-TNC*™ vector (Epicentre). The construction of Cosmid library was performed according to the manufacturer's instruction (*pWEB-TNC*™ Cosmid Cloning Kit). All ampicillin- and neomycin-resistant clones were grown in 96-well microtiter plates (LB medium containing 7% glycerol and antibiotic) and stored at 4°C for future research.

Screening for protease gene from metagenomic library

The clones with protease activity were screened by medium containing casein (1%) and yeast extract (0.2% w/v) at 30°C. One clone (ACPRO001) producing largest clear zone on medium plate was selected for determining the sequence of insert by shotgun sequencing method that was carried out by Takara. Open reading frames (ORFs) were predicted with the DNAMAN Program. The putative ORFs and their nucleotide sequences were blasted on NCBI, SwissProt, and EMBL database by blastN (http://www.ncbi.nlm.nih.gov/BLAST/), blastX (http://www.ncbi.nlm.nih.gov/BLAST/) and Wu-blast (http://www.ebi.ac.uk/blast2/).

Enzyme expression and purification

The protease gene was subcloned from ACPRO001 by primers designed according to the sequenced ORF of protease. The forward and reward primers were 5′-ATCTT AAGCTACTTCACGCCCCCG-3′ with an *Eco*RI site and 5′-TAGGATCCATGCACGGATGGAATT-3′ with a *Bam*HI site, respectively. The PCR product was ligated into pET-His which was digested with *Eco*RI and *Bam*HI, and the pET-His-ACPRO001 was transformed into *E. coli* BL21 (DE3) cells. The positive transformants were grown in LB medium supplemented with ampicillin (50 μg/mL) at 37°C with shaking for overnight. The culture was inoculated (at 10% v/v) into fresh LB medium containing 0.2% dextrose and was incubated at 37°C with vigorous shaking to A_{600} about 0.6. Then the culture was induced by adding 100 μg/mL isopropyl-β-D-thiogalactopyranoside (IPTG) and was incubated at 16°C to A_{600} about 1.5. The cells were harvested by centrifugation at 9,000×g for 10 min at 4°C, and resuspended with 40 mL 0.1 M Tris–HCl (pH 9.0). The preparation was submitted to ultrasonic treatment at 400 W for 4 min with the pulse cycle of 2 s on and 7 s off. Cell debris was removed by centrifugation at 12,000×*g* for 15 min at 4°C. The supernatant was then purified by Fusion Protein Purification Kit (TOYOBO).

Characterization of ACPRO001 protease

The protease activity was determined with Folin & Ciocalteu's phenol reagent using casein as substrate under the following standard conditions. The reaction mixture including 200 μL purified protease and 800 μL 2% (w/v) casein in 50 mM Tris–HCl (pH 9.0) was incubated in 10–85°C with the interval at 5°C for 20 min and the reaction was stopped by adding 270 μL 10% (v/v) trichloroacetic acid. The precipitate was removed by centrifugation at 10,000×*g* for 2 min. Then 1 mL supernatant was used to determine the tyrosine amount by adding 5 mL 0.4 M Na_2CO_3 and 0.5 mL 1 N Folin-Ciocalteu's Phenol reagent (Sigma), and measured fluorometrically at 680 nm. One unit of activity (U) was defined as the amount of the enzyme releasing 1 μg of tyrosine per minute under the conditions just described. All assays were performed in triplicate.

The effect of temperature on ACPRO001 protease was determined by measuring the activity at different temperatures. Before thermo-stability study, the enzyme was dialyzed extensively against 50 mM Tris–HCl (pH 9.0) containing 2 mM EDTA and then dialyzed twice against the same buffer without EDTA. Thermo-stability of the protease was examined by measuring the residual activities of EDTA-treated enzyme fractions which were pre-incubated in 20–80°C with the interval at 10°C in 0.1 M Tris–HCl (pH 9.0) within or without 10 mM Ca^{2+} for 7.5, 15, 30, 45, 60, 120 min, respectively.

The optimal pH for protease activity was determined at 60°C in 0.1 M acetate–NaOH (pH 4.0–5.7), phosphate–NaOH (pH 5.7–6.3), MOPS–NaOH (pH 6.5–7.8),

Tris–HCl (pH 7.5–9.0), CHES–NaOH (pH 8.7–9.5), glycine–NaOH (pH 9.5–11.0). All pHs were adjusted at room temperature.

The effects of metal ions and denaturing agents on protease were examined by determining the activities of EDTA-treated enzyme after 60 min incubation at 20°C in 0.1 M Tris–HCl (pH 9.0) buffers containing various metal ions and denaturing agents.

The enzyme activity toward synthetic substrates (1 mM final concentration) was assayed in 0.5 mL of 0.1 M potassium phosphate buffer (pH 7.5) at 60°C and followed spectrophotometrically at 405 nm. The synthetic substrates used (Bachem, Switzerland) were as follows: Suc-Ala-Ala-Pro-Phe-*p*NA (Suc, succinyl; *p*NA, *p* nitroanilide), Suc-Ala-Ala-Pro-Leu-*p*NA, Suc-Ala-Ala-Pro-Asp-*p*NA, Suc-Ala-Ala-Pro-Arg-*p*NA, Suc-Ala-Ala-Pro-Lys-*p*NA, Suc-Ala-Ala-*p*NA, Suc-Gly-Gly-Phe-*p*NA.

Nucleotide sequence accession numbers

The nucleotide and amino acid sequences of ACPRO001 protease have been deposited in GenBank under accession number FM163400 and CAQ57698, respectively.

Results

Screening of metagenomic library and sequence analysis

A total of about 7,000 clones with average 35 kb size insert were obtained from the Antarctic coastal sediment metagenomic library. Among the 5,000 clones screened, six clones showed clear zone on screening medium plate. One clone (ACPRO001) of them was picked for sequence because it not only showed protease activity but also showed antibacteria activity towards *Staphylococcus aureus*, *Bacillus subtilis*, *Candida albicans* and *Pseudomonas fluorescens*.

A 1,278-bp long ORF coding a putative protease was found among the determined sequence with the size of 41,264 bp. No signal peptide was predicted by Signal IP analysis. A homology search in protein databases showed that ACPRO001 protease were 51 and 45% identical to subtilase peptidase from *Salinibacter ruber* DSM 13855 (Mongodin et al. 2005) and subtilisin Novo from *Microscilla marina* ATCC 23134, respectively. On the phylogenetic tree based on amino acids sequences (Fig. 1), ACPRO001 and

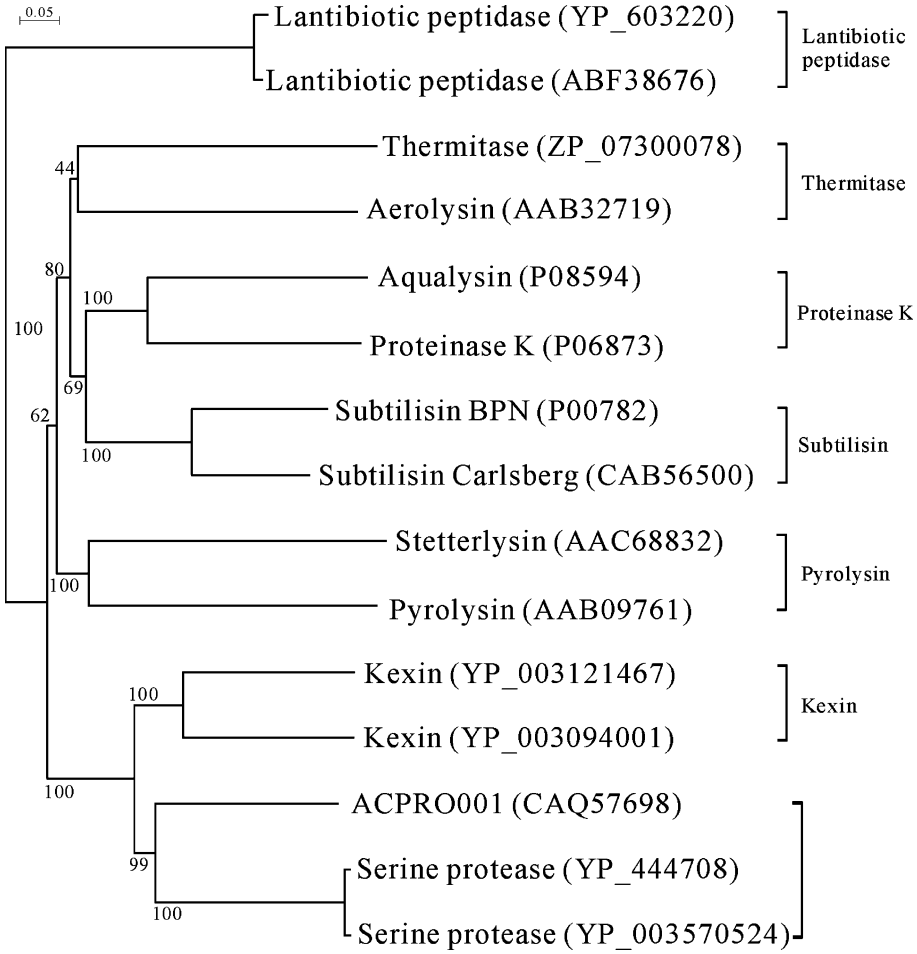

Fig. 1 Phylogenetic relation between ACPRO001 protease and other enzymes of subtilase super family. The result is based on the amino acid sequence alignment. The *number* in *brackets* is the GenBank accession number of each protease: YP_6032220 and ABF38673, from *Streptococcus pyogenes*; ZP_07300078, from *Streptomyces hygroscopicus*; AAB32719, from *Pyrobaculum aerophilum*; P08594, from *Thermus aquaticus*; P06873, from *Tritirachium album*; P00782, from *B. amyloliquefaciens*; CAB56500, from *Bacillus licheniformis*; AAC68832, from *Thermococcus stetteri*; AAB09761, from *Pyrococcus furiosus*; YP_003121467, from *Chitinophaga pinensis*; YP_003094001, from *Pedobacter heparinus*; YP_444708 and YP_003570524, from *Salinibacter rubber*

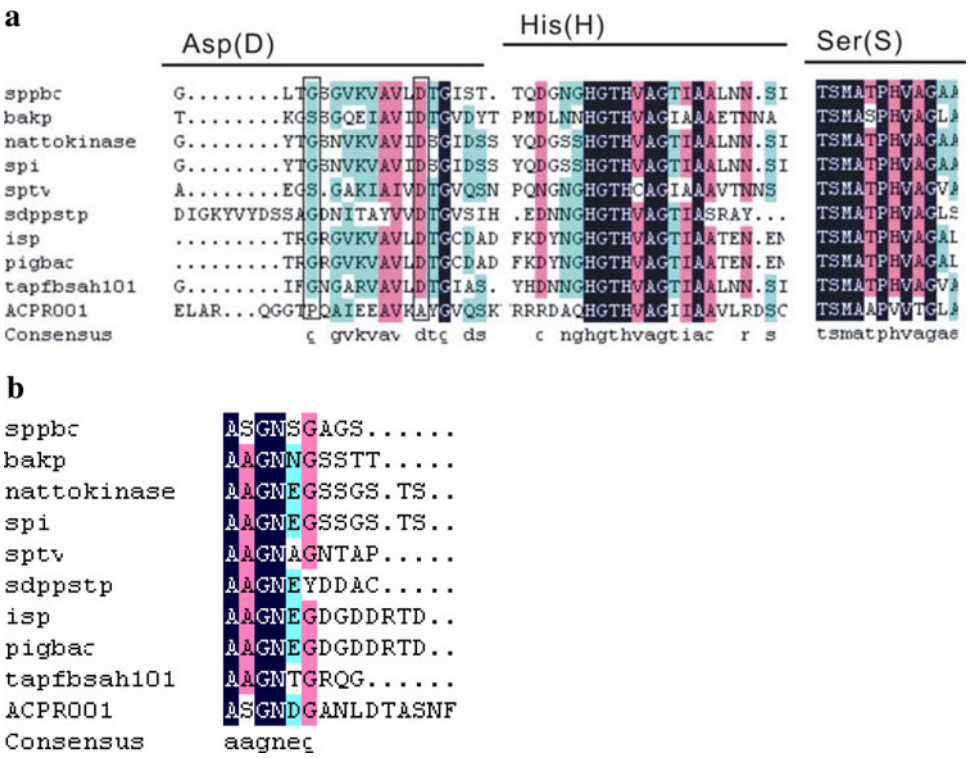

Fig. 2 Conserved regions around the three catalytic residues Asp (D)-His (H)-Ser (S) (**a**) and around the Asn (N) of the oxyanion hole (**b**). *Letters* in *white* represent the identical positions found in the ten sequences, and *letters* shaded in *gray* represent the conservative amino acids. Sppbc, serine protease pb92 from *Bacillus Alcalophilus* (1ah2); bakp, bacillus ak.1 protease from *Bacillus sp*. (1dbi_a); nattokinase, from *Bacillus subtilis* subsp. *natto*(P35835); spi, serine protease from *Bacillus pumilus* (1MEE_A); sptv, serine protease from *Thermoactinomyces vulgaris* (1THM); sdppstp, protease from *Schizosaccharomyces pombe* (P40903); isp, intracellular serine protease from *Bacillus subtilis* (P11018); pigbac, proteases from *Bacillus polymyxa* (P29139); tapfbsah101, thermostable alkaline protease from *bacillus* sp. no. *ah-101* (P41363)

two sequences showing high homology with ACPRO001 were located in an independent clade that was most closely related to the cluster of kexin, which belonged to the subtilase super family. Most enzymes of subtilase had three conserved regions (Asp, His, and Ser), which form the catalytic triad (Siezen and Leunissen 1997) (Fig. 2a). But the alignment of amino acids sequence revealed that the conserved residue Asp was replaced by a Ala residue in ACPRO001 protease (Fig. 2a). Also, we found the conserved residue of the oxyanion hole which was responsible for oxygen-binding in this super family (Fig. 2b).

Purification of ACPRO001 protease

The ACPRO001 protease was overexpressed as a His-tagged protein using pET-His (Novagen) vector. The protein was purified from the soluble fraction by a single step of Fusion Protein Purification Kit (TOYOBO). The purified protease exhibited a specific activity of 548 U/mg, corresponding to a purification factor of 21-fold and a total yield of 10.1%. The size of ACPRO001 protease, as determined by SDS-PAGE, was about 25 kDa (Fig. 3).

Characterization of ACPRO001 protease

The optimum temperature for ACPRO001 protease activity was 60°C, which was quite similar with the subtilisin BPN′ from *Bacillus amyloliquefaciens* and thermitase from *Thermoactinomyces vulgaris* (Kleine 1982; Wells et al. 1983; Voorhorst et al. 1997). The enzyme retained about 60% of residual activity at the range of 45–70°C, and about 11% of residual activity at 85°C in the presence of 10 mM Ca^{2+}. The purified ACPRO001 protease exhibited high activity in the pH range of 6.5–9.5, with an optimum pH of 9.0. The residual activity between pH 6.5 and 9.5 was more than 60%. In the absence of Ca^{2+}, the ACPRO001 protease do not lose activity after 120 min incubation at 20°C, and retained about 50% of activity after 30 min incubation at 50°C or 7.5 min incubation at 60°C, while the enzyme retained about 73% of activity after a 120-min incubation at 50°C in the presence of 10 mM Ca^{2+} (Fig. 4). The data revealed that ACPRO001 protease required Ca^{2+} for stability, as do other subtilases (Siezen and Leunissen 1997). Though at 60°C, the thermo-stability of ACPRO001 protease was improved slightly. In the presence of 10 mM

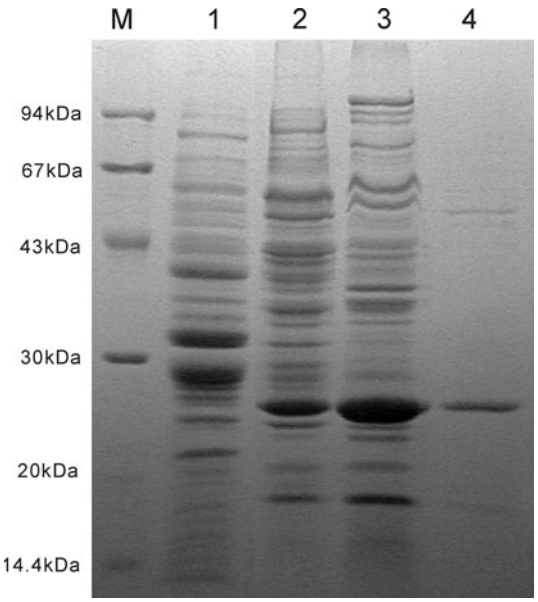

Fig. 3 SDS-PAGE analysis of ACPRO001 protease expressed in *E. coli* BL21(DE3). *Lane M*, Molecular mass standard; *lane 1*, *E. coli* BL21 (DE3) extract; *lane 2*, protein from the recombinant pet-His-ACPRO001-plasmid *E. coli* BL21(DE3) without IPTG-induce; *lane 3*, protein from the recombinant pet-His-ACPRO001-plasmid *E. coli* BL21(DE3) with IPTG-induce; *lane 4*, purified enzyme eluting from transform *E. coli* BL21(DE3)

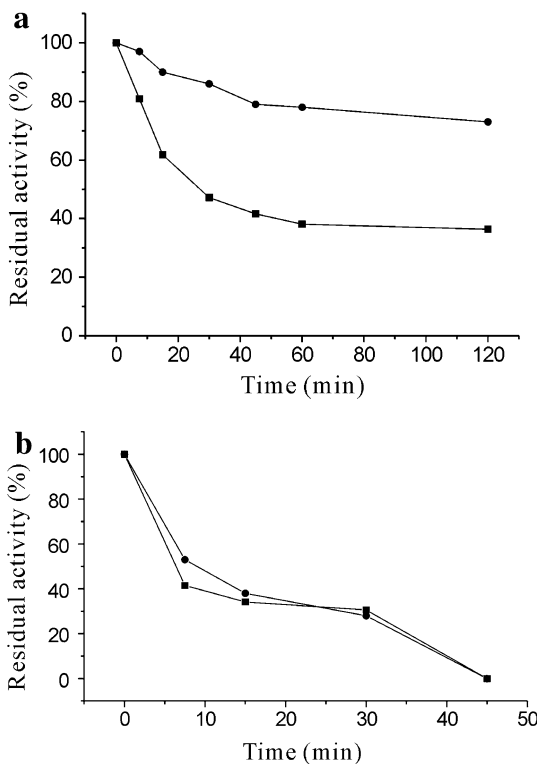

Fig. 4 Stability of purified protease at temperatures of 50°C (**a**) and 60°C (**b**) with 10 mM Ca^{2+} (*filled circle*) and without 10 mM Ca^{2+} (*filled square*) were taken as 100%, respectively

Ca^{2+}, it lost enzymatic activity with half-lives of about 9 min at 60°C (Fig. 4).

Among the synthetic substrates, the ACPRO001 protease displayed slight activity towards Suc-Ala-Ala-Pro-Phe-*p*NA (100%), Suc-Ala-Ala-Pro-Lys-*p*NA (68%) and Suc-Ala-Ala-Pro-Arg-*p*NA (52%). Their activities were all below 120 nmol/min/mg protein. The enzyme was completely inhibited by 5 mM PMSF and AEBSF, indicating that it was a typical serine protease (Table 1). The enzyme was resistant to thiol reducing agents such as dithiotreitol (DTT) (10 mM) and 2-mercaptoethanol (5%), suggesting that disulfide bonding was not involved in preserving proteolytic activity. On the other hand, the enzyme was sensitive to urea (4 M), SDS (1%) and guanidine–HCl (1 M), indicating that hydrogen bonds played an important role in maintaining enzyme activity.

The effects of metal ions on the activity of ACPRO001 protease showed that Ca^{2+} stimulated (30%) the activity significantly, and Mn^{2+} strongly inhibited the activity (Table 2). Other metal ions including Fe^{3+}, Zn^{2+}, K^{+}, Na^{+}, Fe^{2+}, and Cu^{2+} had no significant effect on the activity of the enzyme.

Table 1 Effects of protease inhibitors and denaturing agents on ACPRO001 protease activity

Inhibitor and reagent	Concentration	Residual activity (%)
None		100
EDTA	1 mM	71
AEBSF	1 mM	8
PMSF	1 mM	5
Pepstatin A	1 μM	98
SDS	1%	57
Urea	4 M	51
HCl guanidine	1 M	43
Dithiothreitol (DTT)	10 mM	81
2-Mercaptoethanol	5%	82

Discussion

Considerable focus has been placed on screening novel enzymes from metagenomic library of different environmental samples. However, little attention has been invested in Antarctic coastal sediment. It is interesting that the optimal temperature for ACPRO001 protease activity was 60°C, which was much higher than the temperature of its inhibition. To the best of our knowledge, this is the first study on expression and characterization of a mesophilic enzyme from Antarctic. In other experiments, we determined all the protease-producing bacteria isolated from the same sample, but none of their proteases had the optimal temperature higher than 40°C. Considering the sample was collected

Table 2 Effects of metal ions on ACPRO001 protease activity

Metal ion	Concentration (mM)	Relative activity (%)
None		100
Na^{+}	5	99
Ca^{2+}	10	130
Mg^{2+}	5	108
Zn^{2+}	5	99
Fe^{2+}	5	95
Cu^{2+}	5	103
K^{+}	5	106
Sr^{2+}	5	104
Mn^{2+}	5	38
Fe^{3+}	5	113

within the treated sewage effluent to the sea, the results suggested that the ACPRO001 protease came from non-indigenous microorganism, and the Antarctic environment has been affected by the activity of human being.

The ACPRO001 protease exhibited typical characters of subtilisin-like protease. Subtilisin-like serine proteases, subtilases, have been classified into six families on the basis of their amino acid sequences: subtilisin, thermitase, proteinase K, lantibiotic peptidase, pyrolysin, and kexin (Siezen and Leunissen 1997). Most of other highest conserved residues in subtilase could be found in ACPRO001 protease, except the Asp residue which was the catalytic triad residue, was replaced by the Ala in the ACPRO001 protease. On the phylogenetic tree based on amino acids sequences, two serine proteases and ACPRO001 protease formed an independent clade that was different from other six subtilases (Fig. 1). These two proteases were identified from *Salinibacter ruber* and showed high homology with ACPRO001 protease. It is interesting that they all have no catalytic Asp residue either (Mongodin et al. 2005). But we do not find evident special character related to the substitution of Asp of these proteases in our data and previous study (Mongodin et al. 2005). It was worth of further study.

The majority of the subtilases are synthesized as a precursor with a pre- and pro-sequence extension of the N terminus of the mature protein (Bryan et al. 1995). Some of them have C-terminal extension, which is less conserved than the catalytic region (Siezen and Leunissen 1997; Voorhorst et al. 1996). The size of ACPRO001 protease was determined as about 45 kDa by prediction on the basis of the amino acid sequence (433 residues). One single domain belonging to S8 peptidase superfamily was identified by searching the CDD (Conserved Domain Database) from the amino acid sequence of ACPRO001 protease. The mature protein has a length of 235 amino acid residues (146–380), and a molecular weight of about 24.7 kDa (Fig. 3). The cleavage of ACPRO001 protease probably caused by an autocatalytic event on the basis of its specificity.

When comparing to the subtilisin Carlsberg which also belonged to the subtilisin-like super family with wide application, ACPRO001 protease showed some better characteristics. For example, the subtilisin Carlsberg also has an optimum pH of 9.0, and exhibited the highest levels of activity at the temperature of 60°C. But ACPRO001 protease was more stable than subtilisin Carlsberg. It retained about 40% of activity after 15 min incubation at 60°C (Fig. 4b), and was stable under 50°C (Fig. 4a) at the presence of 10 mM Ca^{2+}, that gives ACPRO001 protease a potential for industrial applications.

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