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An extracellular halophilic protease SptA from a halophilic archaeon *Natrinema* sp. J7: gene cloning, expression and characterization

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Abstract A gene encoding an extracellular protease, *sptA*, was cloned from the halophilic archaeon *Natrinema* sp. J7. It encoded a polypeptide of 565 amino acids containing a putative 49-amino acid signal peptide, a 103-amino acid propeptide, as well as a mature region and C-terminal extension, with a high proportion of acidic amino acid residues. The *sptA* gene was expressed in *Haloferax volcanii* WFD11, and the recombinant enzyme could be secreted into the medium as an active mature form. The *N*-terminal amino acid sequencing and MALDI-TOF mass spectrometry analysis of the purified SptA protease indicated that the 152-amino acid prepropeptide was cleaved and the C-terminal extension was not processed after secretion. The SptA protease was optimally active at 50°C in 2.5 M NaCl at pH 8.0. The NaCl removed enzyme retained 20% of its activity, and 60% of the activity could be restored by reintroducing 2.5 M NaCl into the NaCl removed enzyme. When the twin-arginine motif in the signal peptide of SptA protease was replaced with a twin-lysine motif, the enzyme was not exported from *Hfx. volcanii* WFD11, suggesting that the SptA protease was a Tat-dependent substrate.

Keywords Halophilic archaeon · Extracellular protease · *Natrinema* sp. J7

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Introduction

Halophilic archaea (extreme halophiles) generally require 15–30% NaCl, depending on the species, for optimum growth (Oren 2002). Most of the enzymes from halophilic archaea are stable at high salt concentrations, representing a model for biocatalysis in low-water activity media (Sellek and Chaudhuri 1999; Ryu et al. 1994) and an attractive example of adaptation (Lanyi 1974; Madern et al. 2000).

Many halophilic archaea possess proteolytic activity, and some of the extracellular proteases isolated from the halophilic archaea are serine proteases which enable the degradation of proteins and peptides in the natural environment (De Castro et al. 2006; Oren 2002; Gibbons 1957). Several proteases from halophilic archaea, including *Halobacterium salinarum* (*Halobacterium halobium*) (Kim and Dordick 1997; Ryu et al. 1994; Izotova et al. 1983; Norberg and Von Hofsten 1969), *Natrialba asiatica* (Kamekura et al. 1992; Kamekura and Seno 1990), *Haloferax mediterranei* (Kamekura et al. 1996; Stepanov et al. 1992), *Natronomonas pharaonis* (Stanlotter et al. 1999), *Natrialba magadii* (Giménez et al. 2000) and *Natronococcus occultus* (Elsztein et al. 2001; Studdert et al. 2001; Studdert et al. 1997) have been isolated and characterized. The halophilic protease from *Hbt. salinarum* was used in the synthesis of glycine-containing oligopeptides in yields up to 76% in aqueous/organic media (Ryu et al. 1994), showing the great potential of halophilic proteases for peptide synthesis (Sellek and Chaudhuri 1999). Thus far, the biochemical properties of many halophilic proteases have been studied extensively, while only the genes encoding halolysin 172P1 from *Nab. asiatica* (Kamekura et al. 1992) and halolysin R4 from *Hfx. Mediterranei* (Kamekura et al. 1996) have been identified. Characterization of the biochemical properties in combination with the gene information would be helpful to improve the understanding of halophilic proteases. In addition, the mechanism of secretion and activation of extracellular

haloarchaeal proteases remains to be elucidated (De Castro et al. 2006).

Natrinema sp. J7, previously named as *Halobacterium salinarum* J7, was isolated from a salt mine in Hubei province, China. It was found that this strain harbors a high copy number plasmid pHH205 (Ye et al. 2003), and possesses extracellular proteolytic activity. Recently, the analysis of its 16S rRNA gene sequence (unpublished data) revealed that this strain J7 belongs to the genus *Natrinema* (McGenity et al. 1998). In this article, we describe the cloning of an extracellular protease gene, designated *sptA*, from this halophilic archaeon. In addition, the *sptA* gene had been successfully expressed in *Hfx. volcanii* WFD11, and the recombinant SptA protease had been purified and characterized. Furthermore, the mutation analysis of the signal peptide of the SptA protease suggested that this enzyme was most likely exported via the twin-arginine translocation (Tat) pathway.

Materials and methods

Materials

The restriction enzymes and *T4* DNA ligase were purchased from New England Biolabs. The *Taq* and LA *Taq* DNA polymerase were purchased from BioStar, Canada and Takara, Dalian, respectively. Azocasein was from Sigma. The *Escherichia coli-Hfx. volcanii* shuttle plasmid pSY1 (Yang et al. 2003) was used as the vector to express the recombinant protease.

Strains and culture conditions

Natrinema sp. strain J7 was grown in a complex medium containing 2.5 g of lactalbumin hydrolysate, 2 g of yeast extract, 250 g of NaCl and 30 g of $MgCl_2 \bullet 6H_2O$ per liter at 37°C (Ye et al. 2003). *E. coli* JM110 was used as the host for the construction of the expression vector, and was grown in Luria–Bertani (LB) medium with ampicillin (50 μg/ml) at 37°C. The *Hfx. volcanii* WFD11 strain, kindly provided by Dr. W. F. Doolittle (Dalhousie University, Halifax, Canada), was used as the host for expression and was grown in Modified Growth Medium (18% MGM) containing 1 g of yeast extract, 5 g of peptone, 144 g of NaCl, 18 g of $MgCl_2 \bullet 6H_2O$, 21 g of $MgSO_4 \bullet 7H_2O$, 4.2 g of KCl, 0.5 g of $CaCl_2 \bullet 2H_2O$, 0.12 g of $NaHCO_3$ and 0.48 g of NaBr per liter at 37°C (http://www.microbiol.unimelb.edu.au/micro/staff/mds/HaloHandbook).

Cloning and sequencing of the *Natrinema* sp. J7 protease gene

Firstly, genomic DNA of *Natrinema* sp. J7 was prepared according to the method of Kamekura et al. (1992), and was used as template for PCR. Then, a partial DNA fragment of a serine protease gene (*sptA*) was amplified by PCR employing two consensus-degenerate hybrid oligonucleotide primers (CODEHOPs), primer F and primer R (Table 1), designed on the basis of two highly conserved amino acid sequences in the subtilisin-like proteases (Wu et al. 2004; Rose et al. 1998). The amplified DNA fragment was digested with *Eco*RI, and was purified by gel electrophoresis and gel extraction using the E.Z.N.A. Gel Extraction kit (Omega Bio-tek, USA). The purified DNA fragment was ligated into the *Eco*RI restriction site of pUC18 to construct a recombinant plasmid for nucleotide sequencing using the BigDye Terminator Cycle Sequencing kit (Perkin–Elmer Applied Biosystems, Foster City, CA, USA). In order to amplify the 5′ and 3′ ends of *sptA*, inverse PCR (IPCR) and thermal asymmetric interlaced-PCR (TAIL-PCR) were performed as follows: The *Sal*I digested fragments of the genomic DNA of *Natrinema* sp. J7 was subjected to self-ligation by T4 DNA ligase at 16°C for 20 h. After phenol:chloroform extraction and ethanol precipitation, the ligated product was dissolved in ddH_2O and used as the template for IPCR. The IPCR primer 1 and IPCR primer 2 (Table 1) were designed based on the confirmed partial nucleotide sequence of the *sptA*. IPCR was conducted according to the method of Martin and Mohn (1999). The amplified DNA fragment was ligated

Table 1 Oligonucleotide primers used in this study

Primer	Nucleotide sequence
Primer F	5′-CG*GAATTC*TCCGACGAGATCCAYGGNCANCAYGT-3′
Primer R	5′-CG*GAATTC*GACGACGGGCGTCGCCATNGANGTNCC-3′
IPCR primer 1	5′-AACAGTTCGAAATACCGGCATGGCC-3′
IPCR primer 2	5′-GAAACGCTGTCCGACTTCTCGAACG-3′
TP1	5′-GTCCCCTGCGAGGCGGCGTA-3′
TP2	5′-ATCGGCGATGTCGGAGAGCG-3′
TP3	5′-CCCGTCCCGTTGTCGGTGCC-3′
AD	5′- CANTCSTASTCGNAGG-3′
sptA ATG1	5′-GCG*CATATG*TTTAGGAAGAATTTAATAGCGTGCTGGA-3′
sptA ATG2	5′-GCG*CATATG*TCCGGTGACAATAACCAACACATGG-3′
sptA ATG3	5′-GCG*CATATG*GATCGAAGATCGCTTTTAC-3′
sptA primer 2	5′-GTG*CCATGG*TGCTACGGTTTCGTCACGCGA-3′
SM1	5′-CTCCGTGTCTGACGGTTCAT-3′
SM2	5′-GTAAAAGCGA**CTTCTT**ATCCATGTG-3′

Italicized sections are the restriction enzyme sites; Y = C or T; S = G or C; N = A, C, G or T; W = A or T; Bold sections are the mutant sites of the RR motif

into pMD18-T vector (TaKaRa) according to the method supplied by the manufacturer, and was sequenced as described above. In order to determine the flanking sequences of the known fragment, TAIL-PCR was performed according to the methods described by Liu and Whittier (1995). Three antisense primers (TP1, TP2 and TP3, Table 1) for 5′ end TAIL-PCR were designed according to the nucleotide sequence of the known DNA fragment (Supplementary). AD (Table 1) was used as the degenerate primer. After sequencing the DNA fragment amplified by TAIL-PCR, the *sptA* gene of *Natrinema* sp. J7 was obtained.

Expression and purification

Because there are three ATGs at the 5′ end of the *sptA* gene, we subcloned the three ORFs and ligated into the pSY1 plasmid to construct three expression vectors pSPTA1, pSPTA2 and pSPTA3, respectively. Using genomic DNA of *Natrinema* sp. J7 as template, the DNA fragments encoding the three ORFs (from Met^{-152}, Met^{-136} and Met^{-128}, respectively, Fig. 1) were individually amplified with LA *Taq* DNA polymerase by employing the following sets of primers: sptA ATG1, sptA ATG2, sptA ATG3 and sptA primer 2 (Table 1), prepared based on the nucleotide sequences of 5′ and 3′ terminal sequences of the *sptA*. Each of the 25 amplification cycles consisted of denaturation at 94°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 3 min. The amplified DNA fragments were digested with *Nde*I and *Nco*I, and ligated into the *Nde*I–*Nco*I restriction site of the pSY1 plasmid (Yang et al. 2003). The nucleotide sequences of the inserted gene fragments were confirmed by DNA sequencing, and the expression vectors were amplified in *E. coli* JM110 and then transformed into *Hfx. volcanii* WFD11, respectively, as described by Cline et al. (1989). The transformed cells were plated on 18% MGM ,1.5% agar plates containing 1% skim milk and 0.3 μg/ml novobiocin, and incubated at 37°C for 1 week. The positive transformants were detected as colonies surrounded by clear halos on the

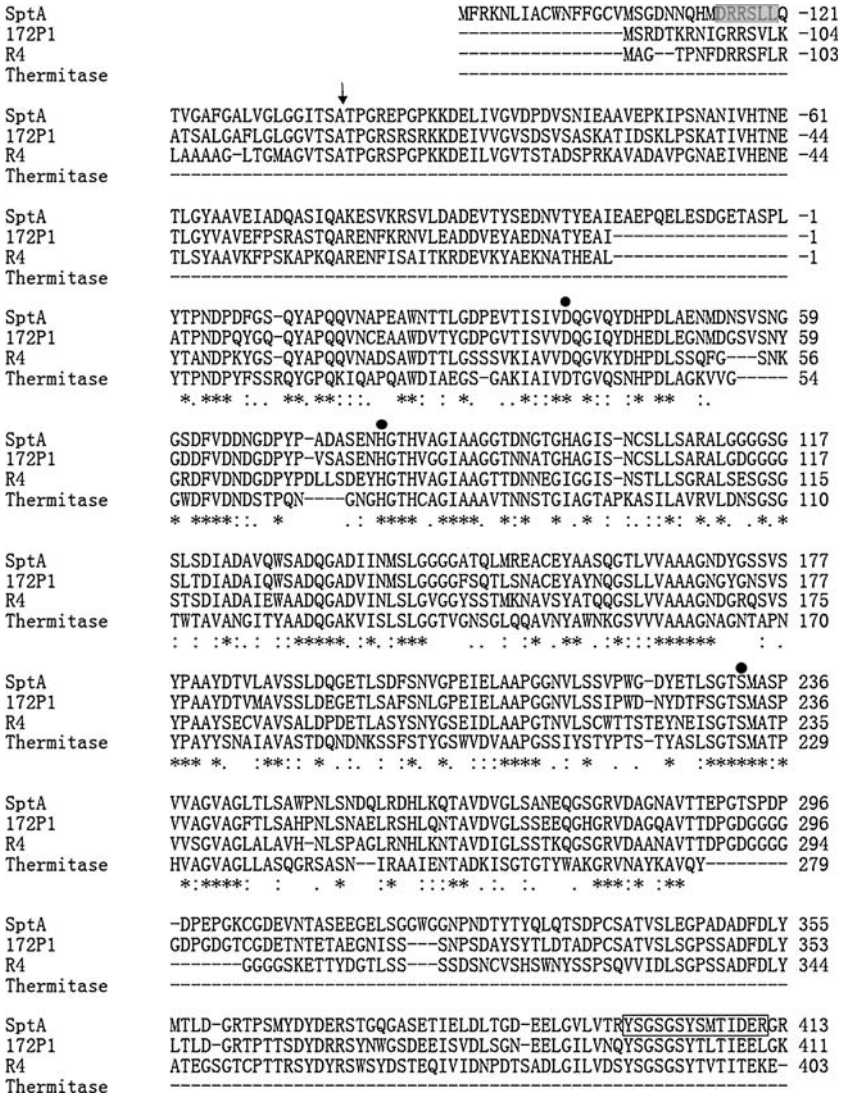

Fig. 1 Amino acid sequence comparison of SptA protease with halolysin 172P1, halolysin R4 and thermitase using CLUSTAL W software (Thompson et al. 1994). The *shaded residues* indicate the putative signal peptide motif. The *vertical arrow* indicates the putative signal peptide cleavage site. Amino acid residues of prepropeptides are coded with negative numbers. The *solid circles* mark the residues of the catalytic triad. The detected fragment by MALDI-TOF MS at C-terminal extension was *boxed*

skim milk plate. *Hfx. volcanii* WFD11/pSPTA1 was cultivated in 18% MGM containing 0.4 μg/ml novobiocin as described above. After 6 days, the cells were removed by centrifugation and the supernatant of the culture was subjected to affinity chromatography on a bacitracin–Sepharose 4B column (1.6 × 20 cm) using the method described by Izotova et al. (1983) and Stepanov and Rudenskaya. (1983). The active fractions were dialyzed against 50 mM Tris–HCl (pH 8.0) containing 2.5 M NaCl and 10 mM $CaCl_2$, and stored at 4°C until use.

Site-directed mutagenesis

The twin-arginine residues (R^{-126} and R^{-125}) localized in the signal sequence of the SptA protease were substituted by twin-lysine residues using site-directed mutagenesis (megaprimer PCR). Firstly, a 195 bp fragment encompassing the 5′ end of the *sptA* gene was amplified from plasmid pSPTA1 using a forward primer SM1 and a reverse primer SM2 (Table 1). Thereafter, a second PCR was performed using the amplified 195 bp fragment as the forward primer and sptA primer 2 as the reverse primer to amplify the gene of double mutant R-126K/R-125K. The amplified DNA fragments were digested with *Nde*I and *Nco*I, and ligated into the *Nde*I–*Nco*I restriction site of pSY1 to construct the plasmid pASM1. The plasmid pASM1 was amplified in *E. coli* JM110 and then transformed into *Hfx. volcanii* WFD11.

Proteolytic activity assay

The proteolytic activity of the SptA protease was assayed using azocasein as substrate. Unless otherwise indicated, the assay was carried out at 37°C for 30 min in 1 ml of reaction mixture containing 0.5% (w/v) azocasein, 50 mM Tris–HCl (pH 8.0), 2.5 M NaCl, 10 mM $CaCl_2$ and enzyme solution. The reaction was terminated by the addition of 1 ml of 40% trichloroacetic acid into the reaction mixture. After keeping at room temperature for 20 min, the mixture was centrifuged at 20,000*g* for 10 min, and the absorbance of the supernatant was measured at 335 nm (Giménez et al. 2000). One unit (U) of activity was defined as the amount of enzyme required to increase the absorbance by 0.001 per minute under the conditions described here.

To measure the activity of NaCl removed SptA protease, the purified enzyme solution with 2.5 M NaCl was first dialyzed against 50 mM Tris–HCl (pH 8.0) containing 10 mM $CaCl_2$ at 4°C by stirring for 9 h and the dialyzing buffer was replaced by fresh buffer every 3 h. Then, the NaCl removed SptA protease was subjected to proteolytic activity assay in the absence of NaCl. The effect of pH on the activity of the SptA protease was assayed at 37°C using 50 mM buffers of various pH values: Na_2HPO_4–NaH_2PO_4 (pH 6–7), Tris–HCl (pH 7–9) and CAPS (pH 9–11). To analyze enzyme inhibition by chemical reagents, the purified enzymes were preincubated with each reagent in 50 mM Tris–HCl (pH 8.0) containing 2.5 M NaCl and 10 mM $CaCl_2$ at 37°C for 30 min, and then added into the reaction mixture for proteolytic activity assay as described above. The residual activity was expressed as percent of the activity of the uninhibited enzyme.

SDS-PAGE

The SDS-PAGE was carried out according to the methods of King and Laemmli (1971). To prevent self-degradation of the protease during sample preparation, the protease was precipitated by trichloroacetic acid at a final concentration of 20%, and then washed with acetone before being subjected to SDS-PAGE.

Mass spectrometry

The target band on SDS-PAGE gel was excised and subjected to in-gel digestion with trypsin followed by peptide mass fingerprint analysis by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/ms) using MALDI-TOF Voyager DE PRO (Applied Biosystems) according to the instructions of the manufacturer.

N-terminal amino acid sequencing

The components separated by SDS-PAGE were transferred onto a polyvinylidene difluoride (PVDF) membrane by Western blotting. After staining with Coomassie Brilliant blue R-250, the target band was excised and subjected to *N*-terminal amino acid sequence analysis using a Procise 492 cLC peptide sequencer (Applied Biosystems).

Nucleotide sequence accession number

The nucleotide sequence of *sptA* gene reported in this article has been deposited in GenBank database under the accession number of AY800382.

Results

Cloning and sequencing of the *sptA* gene from *Natrinema* sp. J7

Natrinema sp. J7 could grow on the complex medium agar plate containing 1.5% milk, and clear halos of proteolysis were observed around the colonies. Using azocasein as substrate, proteolytic activity was detected in the culture supernatant of this halophilic archeaon, and the activity was inhibited by phenylmethylsulfonyl

fluoride (PMSF), suggesting that *Natrinema* sp. J7 secreted a serine protease to the medium during cultivation.

A 475 bp DNA fragment was amplified by PCR (see Methods), and subsequently cloned into plasmid pUC18 for DNA sequencing. The deduced amino acid sequence was found to be a part of a halolysin-like protease (SptA) by Blast search. To determine the flanking sequence, a 1,769 bp DNA fragment was amplified by IPCR. Thereafter, the unknown 5′ end of *sptA* gene was amplified by TAIL-PCR and a 1,144 bp DNA fragment was obtained. After assembling the 475, 1,769 and 1,144 bp DNA fragments together, the overall DNA sequence of *sptA* was obtained.

Amino acid sequence and homology analysis

The *sptA* gene encoded a polypeptide consisting of 565 amino acid residues with a calculated molecular mass of 58,566.0 and a pI of 3.67. Analysis of the amino acid compositions showed that the percent of acidic amino acid residues were 15.8%. SptA protease belonged to the superfamily of subtilisin-like serine proteases (Siezen and Leunissen 1997) and the mature region of the enzyme showed the greatest homology to the thermitase group, exhibiting 72 and 56% identities with halolysin 172P1 from *N. asiatica* (Kamekura et al. 1992) and halolysin R4 from *Hfx. mediterranei* R4 (Kamekura et al. 1996), respectively. When aligned with thermitase (Teplyakov et al. 1989), the mature SptA protease was found to be composed of a core region (catalytic domain) and a C-terminal extension of 126 amino acid residues (Fig. 1), resembling the cases of halolysin 172P1 and halolysin R4.

It was reported that the majority of haloarchaeal secreted proteins were predicted substrates of the twin-arginine translocation (Tat) pathway, and a signal peptide motif was proposed to be $(X^{-1})R^{0}R^{+1}(X^{+2})(X^{+3})(X^{+4})$ between residues 2 and 35 of the predicted protein, where the amino acid at position X^{-1} had a hydrophobicity score ≤ 0.26; X^{+2} had a hydrophobicity score ≤ 0.02; X^{+3} had a hydrophobicity score ≥ 0.77 and X^{+4} was I, L, V, M or F (Pohlschröder et al. 2004). Such a signal peptide motif was also observed at the *N*-terminus of the precursor of SptA (D^{-127}-R^{-126}-R^{-125}-S^{-124}-L^{-123}-L^{-122}). To determine the Tat specificity of SptA protease, we have constructed the plasmid pASM1 to express the double mutant R-126K /R-125K of SptA protease. In contrast to *Hfx. volcanii* WFD11/pSPTA1 that could secrete the recombinant SptA protease (see below), the colony of *Hfx. volcanii* WFD11/ pASM1 did not form a clear halo on 18% MGM skim milk agar plate, and no proteolytic activity was detected in the culture filtrate, implying that the SptA protease secreted from the cell through the Tat pathway. The exact cleavage site of the signal peptide of the enzyme was not determined experimentally. However, it was assumed that the cleavage site localized at Ala^{-104}-↓Thr^{-103} (Fig. 1) according to the method of Pohlschröder et al. (2004). The cleavage of the *N*-terminal propeptide of SptA precursor was identified to take place at the C-terminus of Leu^{-1} (Fig. 1) by *N*-terminal amino acid sequencing of the mature SptA protease (see below). Thus the precursor of SptA protease was determined to be composed of a putative 49 amino acid residues signal peptide, a 103 amino acid residues propeptide and a 413 amino acid residues mature region.

Expression of the *sptA* in *Hfx. volcanii* WFD11 and purification of the recombinant SptA protease

It was observed that three ATGs existed at the 5′ end of the *sptA* gene. The second and third ATGs were 48 and 75 bp downstream the first ATG, respectively (Supplementary). In order to express the *sptA* gene in *Hfx. volcanii* WFD11, we have constructed three expression vectors, pSPTA1, pSPTA2 and pSPTA3, using the three ATGs as the translation initial codons, respectively. It was found that only the colonies of *Hfx. volcanii* WFD11/pSPTA1 could form clear halos on 18% MGM skim milk agar plate. When *Hfx. volcanii*/pSPTA1 was grown in 18% MGM containing 0.4 μg/ml of novobiocin, proteolytic activity was detected in the culture filtrate using azocasine as substrate. These results indicated that the *sptA* gene had been successfully expressed in *Hfx. volcanii* WFD11, and the recombinant SptA protease could be secreted into the culture media. The recombinant SptA protease that existed in the supernatant of the culture was purified by affinity chromatography on a bacitracin-Sepharose 4B column, showing a single band on the SDS-PAGE gel (Fig. 2). The five *N*-terminal amino acid residues of the purified enzyme were determined to be YTPND by Edman degradation sequencing analysis, suggesting that a 152-amino acid residues prepropeptide had been processed from the mature SptA protease. However, the purified mature SptA protease displayed a molecular mass of 62 kDa estimated by SDS-PAGE (Fig. 2), much higher than that calculated from the amino acids composition of the mature SptA protease (42,320.9 Da). Overestimation of the molecular mass by SDS-PAGE analysis was also observed in other halophilic enzymes (acidic proteins) due to the resistance of the acidic protein toward SDS denaturation (Izotova et al. 1983). In some cases, the apparent molecular mass of halophilic proteins on the gel could be overestimated by as much as 50% (Madern et al. 2000). To check if the C-terminal extension had been processed from mature SptA protease, the tryptic digested fragments of mature SptA protease were subjected to MALDI-TOF mass spectrometry analysis, and a peptide (YSGSGSYSMTIDER) with a mass of 1,552.172 Da was detected. The detected peptide was found at the C-terminus of SptA protease (Fig. 1), suggesting that the C-terminal extension had not been cleaved from the mature enzyme.

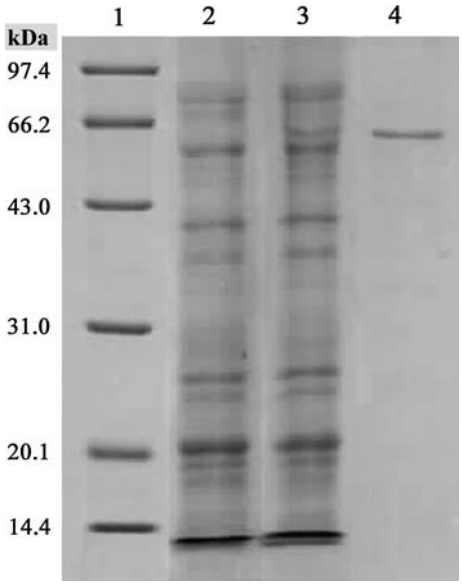

Fig. 2 SDS-PAGE analysis of the expression and purification of the SptA protease. *Lane 1* molecular mass marker, *lane 2* the free cell supernatant of *Hfx. volcanii* WFD11, *lane 3* the free cell supernatant of *Hfx. volcanii* WFD11/pSPTA1, *lane 4* the purified recombinant SptA protease

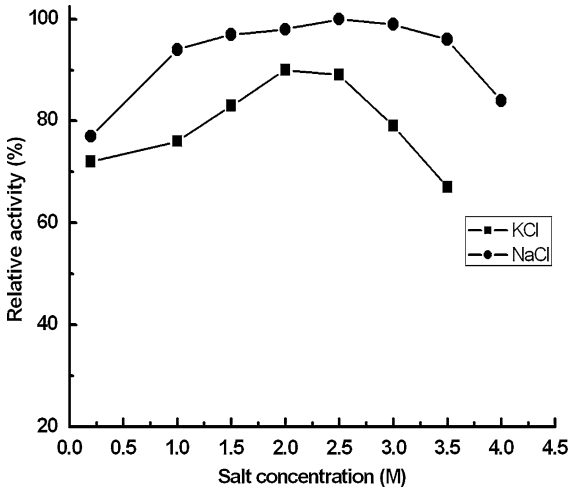

Fig. 3 Salt dependence of the proteolytic activity of the SptA protease. Azocaseinolytic activity of the SptA protease was measured at 37°C in the presence of the indicated concentrations of NaCl (*filled square*) or KCl (*filled circle*) in 50 mM Tris–HCl (pH 8.0) containing 10 mM $CaCl_2$. Results are expressed as percent of the highest activity in 2.5 M NaCl. All the experiments were performed in triplicate and the standard error was within 5%

Characterization of the recombinant SptA protease

Using azocasine as substrate, the purified SptA protease displayed temperature and pH optima of 50°C and pH 8.0, respectively. The enzyme was stable for at least for 1 month at 4°C in 50 mM Tris–HCl (pH 8.0) containing 2.5 M NaCl and 10 mM $CaCl_2$. In the presence of 2.5 M NaCl, no loss of activity was observed after incubating the enzyme at 37°C for 2 h, and the half-life of the enzyme was 90 min at 60°C. However, the enzyme was unstable at 70°C, with a half-life of 3 min. As expected, the serine protease inhibitor PMSF (1 mM) completely inhibited the enzyme activity. While activity of the enzyme was partially inhibited by 50 mM EDTA, 50 mM EGTA, 1% SDS, 1 mM DTT and 10% isopropanol, with residual activities of 55.7, 86.3, 49.6, 53.4 and 64.1%, respectively. The salt dependence of SptA protease was evaluated by measuring the enzyme activities at various NaCl (0.2–4.5 M) or KCl (0.2–3.5 M) concentrations in 50 mM Tris–HCl (pH 8.0) containing 10 mM $CaCl_2$. As shown in Fig. 3, the maximal activity was attained in 2.5 M NaCl or 2 M KCl, while 64% of the highest activity was obtained at 4.5 M NaCl. It was noted that the NaCl removed SptA protease still retained 20% of its activity in the absence of NaCl, and no detectable precipitation and degradation were observed by SDS-PAGE analysis. After reintroducing 2.5 M NaCl into the NaCl removed enzyme by dialyzing the NaCl removed enzyme against 50 mM Tris–HCl (pH 8.0) containing 2.5 M NaCl and 10 mM $CaCl_2$, the protease activity was partially restored to 60% of the initial activity, indicating that the denaturation of SptA protease in the absence of NaCl was reversible.

Discussion

General secretory (Sec) and twin-arginine-translocation (Tat) pathways for protein secretion are conserved in the haloarchaea (Ring and Eichler 2004; Pohlschröder et al. 2004). By systematic whole-genome analyses, 103 proteins with putative signal peptides were identified in *Halobacterium* sp. NRC-1, and more than 60% of these contain a twin-arginine motif. This is extremely high since in bacteria and non-halophilic archaea the majority of proteins (> 90%) appeared to be Sec-dependent. Although it has to be proven experimentally that *Halobacterium* sp. NRC-1 proteins are indeed Tat-dependent, the frequent occurrence of the twin-arginine motif in the signal peptides of haloarchaeal proteins suggests that the haloarchaeal Tat pathway plays a major role in protein translocation (Bolhuis 2002). So far, only the α-amylase of *Natronococcus amylolyticus* has been experimentally proved to be exported via Tat pathway experimentally, and the enzyme was not secreted from *Hfx. volcanii* when the twin-arginine motif in its signal peptide was replaced with a twin-lysine motif (Rose et al. 2002). It was hypothesized that, in response to extremely high-salt conditions, the Halobacteriaceae rerouted the translocation of most secreted proteins to the Tat pathway, allowing these proteins to fold in the cytoplasm before their secretion (Rose et al. 2002). Regarding the haloarchaeal extracellular proteases, little was known about the mechanism(s) of secretion (De Castro et al. 2006). The twin-arginine motif was conserved in the signal peptide of SptA protease, and the enzyme could not be secreted from *Hfx. volcanii* after the twin-arginine residues were substituted by twin-lysine residues, resembling the case of the α-amylase of

Natronococcus amylolyticus. This result suggested that the SptA protease was a Tat-dependent substrate, since conserved changes that maintain the overall charge distribution would normally not affect a Sec substrate (Hutcheon et al. 2005). Tat signal peptides are markedly longer than their Sec counterparts, reaching up to 58 amino acid residues in length in some cases (Berks 1996). The signal peptide of SptA protease was composed of 49 amino acid residues and possessed three Methionine residues. According to the homology analysis, the second one (Met^{-136}) corresponded to the *N*-terminal Met residues of 172P1 and R4, in which the twin-arginine motifs were also conserved (Fig. 1). However, colonies of *Hfx. volcanii* WFD11/pSPTA2, where the second ATG was used as the translation initial codon of the gene of SptA protease, could not form clear halos on 18% MGM skim milk agar plates, suggesting that the first 16 amino acid residues (Met^{-152} to Val^{-137}, Fig. 1) of the signal peptide of SptA protease were also an important functional region necessary for the enzyme translocation.

The recombinant SptA protease exhibited the feature of a halophilic enzyme with a maximal activity at 2.5 M NaCl and 64% activity at 4.5 M NaCl. In contrast to many reported halophilic proteases including halolysin R4 (Kamekura et al. 1996) and the serine proteases from *Hbt. salinarum* (*Hbt. halobium*) (Izotova et al. 1983) and *Hfx. mediterranei* (Stepanov et al. 1992), where the NaCl removed enzymes suffered irreversible inactivation due to fast autodigestion, the NaCl removed SptA protease retained 20% of its activity. It was found that halolysin 172P1, which is closely related to SptA protease with identities of 72%, remained active at 1.3% NaCl at 50°C (Kamekura et al. 1992). In addition, it was reported that the halophilic chymotrypsinogen B-like protease from *Natronomonas pharaonis* retained 50% of its activity in buffers with as low as 3 mM NaCl (Stan-lotter et al. 1999). It is interesting that 60% of its initial activity could be restored by reintroducing 2.5 M NaCl into the NaCl removed SptA protease sample, a feature not observed in other extracellular halophilic proteases so far, but reported for the 20 S proteasome from *Hfx. volcanii* where the proteasome dissociated into monomers and lost its activity after removal of salt and recovered almost 70% of its initial activity after addition of 2 M NaCl (Wilson et al. 1999). The reversible denaturation of SptA protease enables us to further investigate the unfolding and refolding mechanism of this halophilic enzyme in low and high salt concentration environments.

Although the genes of haloarchaeal proteases, such as halolysin 172P1 and SptA protease, could be expressed in *Hfx. volcanii* WFD11, the expression levels of the recombinant proteins and the growth rate of the haloarchaeal host were very low. Attempt to express the halolysin 172P1 in an osmophilic yeast was unsuccessful because the plasmid vector containing *hly* gene was quite unstable (Kamekura et al. 1992). The development of strategies for fast overexpression of stable haloarchaeal proteases will benefit enzymological studies and potential applications of these enzymes (De Castro et al. 2006). Several halophilic enzymes were successfully expressed using *E. coli* as the host, and the expressed inactive products could be reactivated through a refolding process (Pire et al. 2001; Connaris et al. 1999; Diaz et al. 2006). Recently, we have succeeded in the overexpression of the *sptA* gene in *E. coli* BL21(DE3), and the inactive recombinant protein could convert into the active mature enzyme after renaturation in the presence of NaCl. The investigation of the refolding and processing mechanism of SptA protease is in progress.

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