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Biochemical and Genetic Characterization of Arazyme, an Extracellular Metalloprotease Produced from *Serratia proteamaculans* HY-3

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Abstract *Serratia proteamaculans* HY-3 isolated from the digestive tract of a spider produces an extracellular protease named arazyme, with an estimated molecular mass of 51.5 kDa. The purified enzyme was characterized as having high activities at wide pH and temperature ranges. We further characterized biochemical features of the enzymatic reactions under various reaction conditions. The protease efficiently hydrolyzed a broad range of protein substrates including albumin, keratin, and collagen. The dependence of enzymatic activities on the presence of metal ions such as calcium and zinc indicated that the enzyme is a metalloprotease, together with the previous observation that the proteolytic activity of the enzyme was not inhibited by aspartate, cysteine, or serine protease inhibitors, but strongly inhibited by 1,10-phenanthroline and EDTA. The *araA* gene encoding the exoprotease was isolated as a 5.6 kb BamHI fragment after PCR amplification using degenerate primers and subsequent Southern hybridization. The nucleotide sequence revealed that the deduced amino acid sequences shared extensive similarity with those of the serralysin family of metalloproteases from other enteric bacteria. A gene (*inh*) encoding a putative protease inhibitor was also identified immediately adjacent to the *araA* structural gene.

Keywords: Arazyme, metalloprotease, *Serratia proteamaculans*, HY-3, serralysin, enzymatic kinetics

Serratia is a gram-negative enteric bacterium that is an opportunistic pathogen responsible for infections in immunocompromised and surgical patients [30]. *Serratia* is also an attractive host, because it establishes a pathogenic or mutualistic relationship with many insects. *Serratia marcescens* strains have been isolated from hemolymphs of a boll weevil [5] and the intestinal canals of larval silkworms [32]. *Serratia entomophila* and *Serratia proteamaculans* have been identified as the causative agents of amber disease in larvae of a grass grub [38].

Serratia excretes a variety of degradative enzymes into surrounding media, such as proteases, chitinases, and lipases [17]. One of the major extracellular enzymes is the serralysin subfamily of metalloproteases, which exhibits broad specificities in the cleaveage of oxidized insulin B and shows caseinolytic activities in broad pH and temperature ranges [28]. The enzymes contain one atom of zinc per molecule as an essential component [31] and are inhibited by EDTA and 1,10-phenanthroline [28], indicating that they are zinc-containing metalloproteases. Some of the serralysins are widely used as anti-inflamatory agents because of their ability to hydrolyze inflammatory peptides such as bradykinin and histamine [39]. The genes encoding serralysins are present in many enteric bacteria including *S. marcescens* [6, 24, 34], *Pseudomonas aeruginosa* [12], *Pseudomonas fluorescens* [26], *Pseudomonas brassicacearum* [7], *Erwinia chrysanthemi* [24], *Erwinia carotovora* [29], and *P. luminescens* [4].

We previously isolated an enteric bacterium from the midgut of a spider, *Nephila clavata*, which excretes an extracellular protease with a powerful digestive activity on the basis of its ability to form a clear zone on a skim milk medium. Phylogenetic analysis of 16S rDNA sequence determination and numerical taxonomy with many physiological characterizations demonstrated that the bacterium belongs to a strain of *Serratia proteamaculans* HY-3, also called *Aranicola proteolyticus* HY-3, [3, 20]. The exoprotease in the culture supernatant was purified to near electrophoretic homogeneity after filtration and ion-exchange and size exclusion chromatographies [23].

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Activity staining of the extracellular protein fractions using a zymogram demonstrated that the major protein contained a high proteolytic activity. The purified protease showed high relative activities at wide pH and temperature ranges [23]. In this research, we investigated the biochemical and genetic characteristics of the extracellular protease. Several pieces of evidence demonstrated that the enzyme is a zinc-containing metalloprotease. The deduced amino acid sequence was highly homologous to those of serralysins from other enteric bacteria.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The *S. proteamaculans* HY-3 (KCTC2390) previously isolated from a spider, *Nephila clavata*, was used in this work [33]. This bacterium was routinely grown at 30°C in Luria-Bertani (LB), skim milk, or TY media [33]. The *E. coli* strain DH5α (MCR) [11] was used as a transformation host for cloning DNA fragments and was grown at 37°C in LB broth supplemented with ampicillin (100 μg/ml) as needed. The plasmid pIJ2926 [15] was used for cloning genomic DNA fragments of *S. proteamaculans* HY-3.

Enzyme Purification

All experiments were performed at 4°C. Extracellular fractions were obtained by centrifugation of the culture medium at 5,000 ×*g* for 10 min, or by filtration using a 0.2 μl membrane filter (Microza Hollow fiber, Pall Life Sciences, U.S.A.). Chromatography was performed on a DEAE-cellulose column, which had been equilibrated with 50 mM potassium phosphate buffer (pH 7.6). Bound proteins were eluted with a concentration gradient of sodium chloride ranging from 0.1 to 0.5 M at a flow rate of 400 ml/h, and collected fractions were concentrated with a 10 kD Cassette membrane (Microza Hollow fiber, Pall Life Sciences, U.S.A.). Then, the protein solution was loaded at a flow rate of 20 ml/h onto a Sephadex G-75 column previously equilibrated with 50 mM potassium phosphate buffer, pH 7.8. Fractions containing proteolytic activity were concentrated with the 10 kD Cassette membrane and stored at −20°C.

Characterization of Enzyme Activities

The standard solution for all enzyme reactions contained 3 μg of purified enzyme and 1% azocasein in 0.3 ml of 50 mM phosphate buffer, pH 7.8, and was incubated for 30 min at 37°C. The proteolytic activity was determined spectrophotometrically by measuring the increase of absorbance at 405 nm according to Braun and Schmitz [5] with minor modifications. Nondigested azocasein was precipitated by adding 0.3 ml of 10% trichloroacetic acid (TCA), incubated for 1 h at 4°C, and centrifuged at 13,000 ×*g* for 5 min. The supernatants were added to 15 μl of 10% NaOH. The absorbance of the resulting supernatant was measured at 405 nm, and one unit was defined as the increase of 1.0 in absorbance at 405 nm for 30 min under the assay condition used. The protein concentration was determined according to the method proposed by Bradford using BSA as a standard. SDS-PAGE (10%) was performed according to the method of Laemmli [22].

To test the effect of metal ions on the proteolytic activity, 1, 5, 10, and 20 mM of various metal ions ($CaCl_2$, $CoCl_2$, $CuCl_2$, $FeCl_2$, $MgSO_4$, $MnCl_2$, and $ZnCl_2$) were added in the standard reactions. To determine the enzymatic specificity against various protein substrates, 1% of either albumin, casein, collagen, hemoglobin, elastin, keratin, or gellatin was added to the standard reactions, instead of azocasein. Enzymatic hydrolysis was measured by the increase in TCA soluble products released to the supernatant, using the Bradford method, with bovine serum albumin as the standard.

Hydrolytic Activities with Aminoacyl-nitroanilides (pNA) Derivatives

To determine relative activities of the purified enzyme, 1.0 mM of synthetic aminoacyl-p-nitroanilides (purchased from Sigma, U.S.A.) were incubated at 37°C for 5 min in 1.0 ml of reaction mixtures containing 3 μg of the enzyme in 50 mM phosphate buffer, pH 7.8. The absorbance increase of p-NA was measured at 405 nm.

Determination of Amino Acid Sequences

The N-terminal sequence and the internal amino acid sequence of the purified enzyme were obtained according to Kwak *et al.* [21]. To obtain the N-terminal sequence, the purified enzyme was subjected to SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) in a semi-dry blotter (Hoefer Scientific) in 10 mM CAPS buffer (pH 11.0) containing 10% methanol. For internal amino acid sequence, the purified enzyme was digested with V8 protease (Sigma, U.S.A.) and fractionated by SDS-PAGE. The two fragmented peptides were transferred to PVDF membranes. The PVDF membrane was stained with Coomassie blue R250. The amino acid sequence was determined by automated Edman degradation with a MilliGen/Bioresearch 6600 Presequencer System (Millipore).

DNA Analysis

Standard or previously published methods [21] were used for analysis and manipulation of DNA fragments for ligation, PCR, plasmid minipreparations, and transformation. The Genius II system was used for Southern hybridization after transfer of DNA to a membrane (Nylon Membrane, positively charged, Roche), and *Taq* DNA polymerase (Promega) was used for PCR amplification according to the manufacturer's recommendations. The DNA sequence was determined by Genotech Incorporation (Daejeon, Korea).

RESULTS

Substrate Specificity of AraA

The previous study by zymogram experiment using gelatin as a substrate showed that *S. proteamaculans* HY-3 produces an extracellular protein containing a relatively high proteolytic activity [23]. The protease, named AraA, showed maximum activity between pH 8.0 to 8.5 and 60% of relative activities between pH 6 to 10. The highest activity was observed at 37°C and relatively high activities were retained at broad temperature [23].

The protease was purified from the extracellular extracts of *S. proteamaculans* HY-3 through filtration, and DEAE-cellulose and Sephadex G-75 chromatographies. The purity of the final preparation was confirmed to be 95%, after staining with Coomassie Brilliant Blue R-250, by means of densitometry. The molecular mass of 51.5 kDa was also confirmed by fractionation with SDS-PAGE. Various proteins were used as substrates to determine the specificity of the extracellular protease, arazyme. As shown in Table 1, the enzyme hydrolyzed albumin with the highest efficiency, and elastin, keratin, and gelatin with approximately 40% relative activities. These results indicated that AraA shows broad substrate specificity. To further characterize the specificity of peptide bond cleavage of the protease, enzymatic hydrolysis was examined by using various synthetic peptide substrates. AraA showed the highest cleavage efficiency on the synthetic substrates containing the peptide bonds after Arg, N-benzoyl-Phe-Val-Arg-pNA, and N-benzoyl-Arg-pNA, and hydrolyzed N-benzoyl-D,L-Arg-pNA and Gly-Arg-pNA with moderate efficiency (Table 2). The substrates containing the peptide bonds after Pro, Phe, and Tyr were poorly cleaved (Table 2).

Effect of Metal Ions

In a previous set of experiments to investigate the effects of metal ions on the enzymatic activity, most metal ions, Ca^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}, recovered enzyme activities to the pre-incubated enzyme reactions, and addition of 1 and 5 mM Zn^{2+} markedly recovered the activities, among various metal ions tested [23]. To further confirm the effect of metal ions on the enzymatic activity, we added 1, 5, or 10 mM of various metal ions (Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}) to the reactions. All metal ions except Cu^{2+} increased the protease activities, and Ca^{2+} and Zn^{2+} showed approximately 40% increases in relative activities when 10 mM metal ions were added to the reaction mixtures (Table 3).

Table 1. Hydrolysis activities of various protein substrates.

Substrate	Relative activity (%)		
	0.5 h	2.0 h	24 h
Albumin	11	28	100
Casein	6	22	63
Collagen	17	23	55
Elastin	12	23	41
Keratin	10	24	41
Gellatin	2	22	40
Hemoglobin	7	10	42

The relative activity was determined at pH 7.8 and at different temperatures in the standard reaction conditions using azocasein. The value obtained with albumin was taken as 100%. The concentration of TCA-soluble protein released to the supernatant was measured using the Bradford method, with bovine serum albumin as the standard.

Kinetic Analysis of Arazyme

To elucidate the catalytic mechanisms of the protease, we measured the kinetic parameters with azocasein as a substrate. When the values were extrapolated with initial velocities by using Lineweaver-Burk and Hanes-Woolf equations, the K_m values were 7.147 mg/ml and 5.954 mg/ml and V_{max} values were 1.322 mg/ml·min and 1.022 mg/ml·min, respectively (Fig. 1).

Table 2. Hydrolytic cleavage of peptidyl-pNA by AraA.

Substrate	Enzyme	Relative activity (%)
L-Gly-pNA	Aminopeptidase	31
L-Leu-pNA		25
Gly-Arg-pNA	Urokinase	28
N-Benzoyl-D,L-Arg-pNA	Cathepsin	56
N-Benzoyl-L-Arg-pNA	Papain	98
N-Benzoyl-Phe-Val-Arg-pNA	Plasmin	100
L-Pro-pNA	Proline arylamidase	6
N-Benzoyl-L-Tyr-pNA	Chymotrypsin	2
N-Succinyl-Ala-Ala-Pro-Phe-pNA		12
N-Methoxysuccinyl-Ala-Ala-Pro-Val-pNA	Elastase	4
N-Succinyl-Ala-Ala-Pro-Leu-pNA		11
N-succinyl-Ala-Ala-Val-pNA		10
N-Cbz-Gly-Gly-Leu-pNA	Subtilisin	5

The enzyme activity with N-benzoyl-Phe-Val-Arg-pNA was taken as 100% activity. The enzymes show the previously characterized proteases that hydrolyze the substrates at the highest efficiency.

Table 3. Effect of metal ions on the enzyme activity.

Metal salts	Concentration (mM)	Relative activity (%)
$CaCl_2$	1	118
	5	142
	10	117
$CoCl_2$	1	128
	5	133
	10	102
$CuCl_2$	1	94
	5	81
	10	69
$FeCl_2$	1	115
	5	114
	10	113
$MgSO_4$	1	116
	5	121
	10	112
$MnCl_2$	1	114
	5	120
	10	129
$ZnCl_2$	1	110
	5	112
	10	135

Effect of metal ions on the protease activity. The reactions with the purified protease were incubated with the metal ions for 30 min at 37°C and the activities were measured in the presence of metal ions by the standard method using azocasein.

Isolation and Nucleotide Sequence Analysis of the *araA* Gene

We were not able to obtain the amino acid sequence of the purified enzyme from the N-terminus, due perhaps to N-terminal blocking. The internal amino acid sequences isolated from the digested peptides were identified as EQQQQQKLXLQ and EIGHALGASYP. Two degenerate oligonucleotide primers (upstream primer, 5'-GARCARC-ARCARCARCARAA-3'; downstream primer, 5'-ATRCT-NGCNCCNARNGCRTG-3') designed from the internal amino acid sequences were used for PCR reactions to amplify the gene fragment from the genomic DNA. The resulting 0.3 kb DNA fragment was cloned into pIJ2926 generating pKL101, and the DNA sequence of the insert cloned in pKL101 was determined. We isolated and cloned a 5.6-kb BamHI fragment of genomic DNA containing the *proA* open reading frame into pIJ2926 (generating pKL102) by Southern and colony hybridizations using the 0.3 kb insert as a probe.

Nucleotide sequence determination showed that two putative open reading frames (ORFs) were present in the DNA fragment (Figs. 2 and 3; GenBank Accession Number AY818193). The first open reading frame (*araA*) encoded a polypeptide of 487 amino acids with a molecular mass of approximately 50 kDa. There was a putative promoter including -35 (TGTGCA) and -10 (TATAAT) regions with a gap of 16 nt, resembling the consensus sequence recognized by the *E. coli* housekeeping sigma factor. A putative ribosome binding site (AGGAG) was present 10 bp upstream of the putative translation start site. The calculated molecular mass of the deduced amino acid sequence was almost the same as that observed in PAGE. There were one and two mismatches of amino acid residues between each internal amino acid sequence of mature protein and the deduced amino acid sequence, due perhaps to poor accuracy of the amino acid sequencing. Despite several rounds of Southern hybridization in low stringency, we were not able to identify another fragment from the

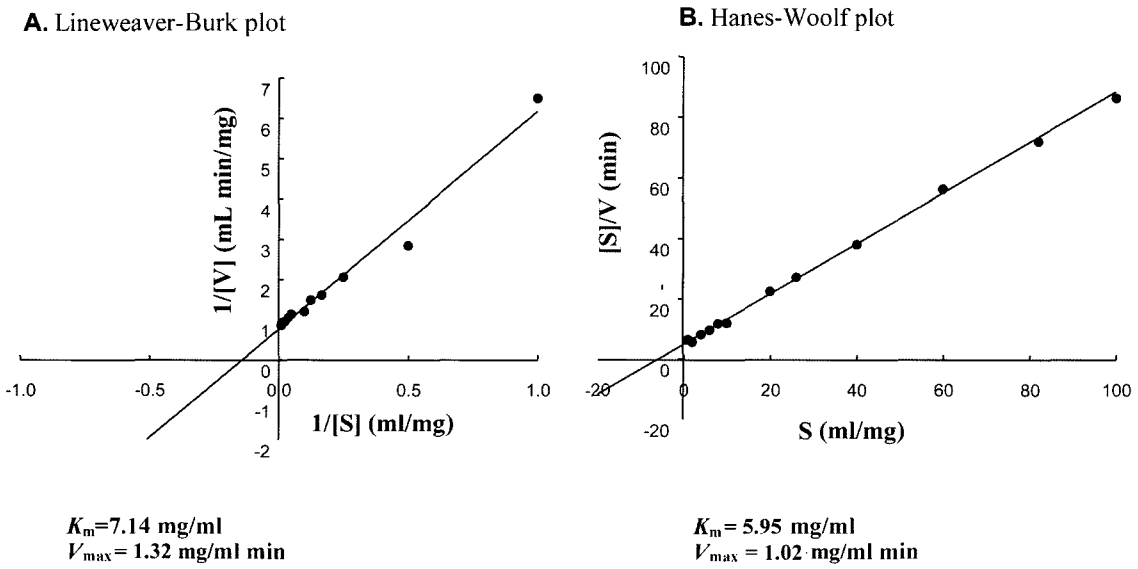

Fig. 1. Kinetic parameters of AraA. Azocasein was used as a substrate to measure the kinetic parameters.
The values were extrapolated by using (**A**) Lineweaver-Burk and (**B**) Hanes-Woolf equations. K_m values were 7.147 mg/ml and 5.954 mg/ml and V_{max} values were 1.322 mg/ml/min and 1.022 mg/ml/min, respectively

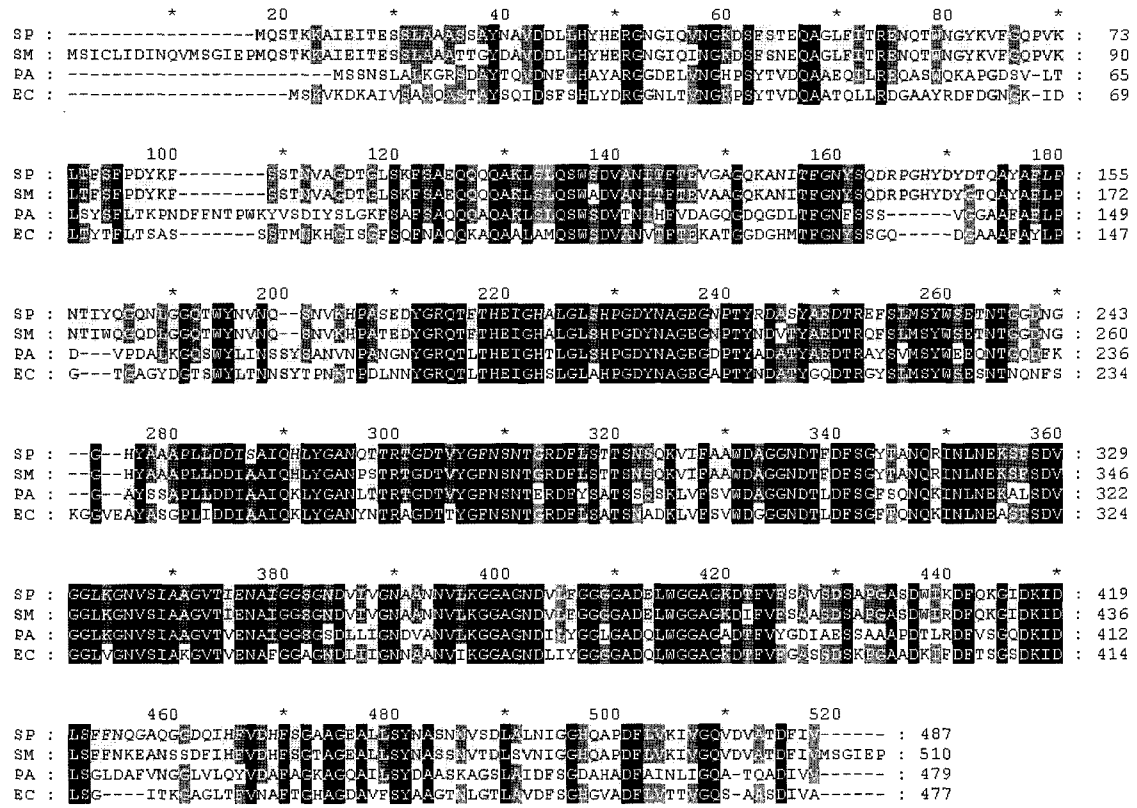

Fig. 2. Alignment of the deduced amino acid sequence of AraA with known proteins.
SM, metalloprotease from *S. marcescens* [6]; PA, alkaline protease from *P. aeruginosa* PAO1 [12]; EC, extracellular protease from *E. chrysanthemi* [24]. The amino acids in filled regions show identical amino acids. The shaded amino acids in rectangles represent amino acid similarity. The dotted underlines mark the amino acid sequences acquired by internal amino acid sequences from the purified AraA. The box represents the consensus amino acid sequence for zinc binding. The thick underlines mark the consensus amino acid sequence GGXGXD (where X is an arbitrary amino acid) for calcium binding. The thin underlines show two putative amino acid sequences that fit for DXXX (where X is a hydrophobic residue) recognized by ABC transporters [3].

genomic DNA that hybridizes with the PCR fragment cloned in pKL101 (data not shown).

Computer analysis showed that the deduced amino acid sequence shared 90% identity and 94% similarity with that of the metalloprotease from *S. marcescens* [6, 24, 34] and approximately 50% identity and 80% similarity with those of the alkaline protease from *P. aeruginosa* [12] and the extracellular protease from *E. chrysanthemi* [24]. There was a strong zinc-binding consensus sequence, HEXXHXUGUXH (where X represents an arbitrary residue and U is a bulky hydrophobic amino acid), in the N-terminal region [28] (Fig. 2). The deduced amino acid sequence showed the existence of 4 obvious tandem repeats of GGXGXD motifs, the putative calcium-binding sites in the C-terminal region [2]. The DFLV and DFIV sequences were found in the last 20 amino acids of the C-terminal region of the deduced AraA, which is consistent with the motif DXXX sequence (where Xs are hydrophobic residues) recognized by the

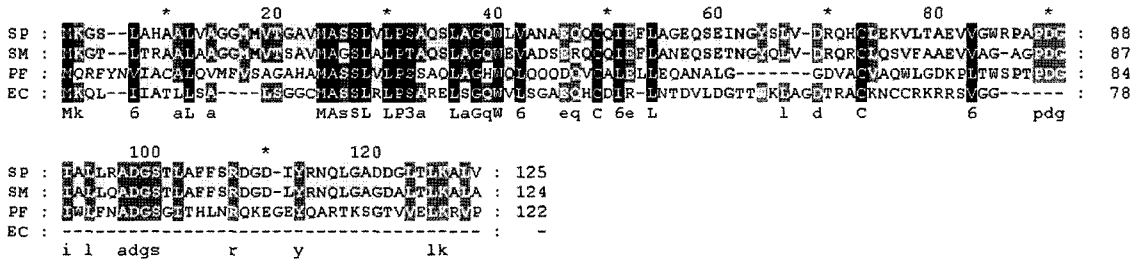

Fig. 3. Alignment of the deduced amino acid sequence of the putative protease inhibitor (*inh*) with known protease inhibitors.
EC, *E. chrysanthemi* [24]; PF, *P. fluorescens* [26]; SM, *S. marcescens* [1]. The amino acids in filled regions show identical amino acids. The shaded amino acids in rectangles represent amino acid similarity.

ABC transport system [25]. The second ORF (*inh*) was present downstream of *araA*. The predicted amino acid sequence exhibited 50 to 90% similarity to metalloprotease inhibitor proteins found in *S. marcescens* [6], *E. chrysanthemi* [24], and *P. aeruginosa* [12] (Fig. 3).

DISCUSSION

In the previous study [23], we isolated an extracellular protease, AraA with an estimated molecular mass of approximately 51.5 kDa from *S. proteamaculans* HY-3, which was isolated from the digestive tract of the spider *Nephila clavata*. The protease has many biochemical characteristics of commercial importance such as high activities at wide pH and temperature ranges [23]. The enzyme contains almost three times higher specific activities than that of the zinc metalloprotease from *Yersinia ruckeri*, which was measured with the same substrate and under similar reaction conditions [37]. This study also showed that the enzyme hydrolyzed broad ranges of protein substrates including keratin, elastin, collagen, and albumin.

Many pieces of biochemical and genetic evidence demonstrated that the enzyme belongs to the serralysin family of zinc metalloproteases. AraA exhibited typical biochemical properties of the serralysins, such as high relative activities at wide pH and temperature ranges, when tested with azocasein as substrate [5, 26]. To further characterize the specificity of peptide bond cleavage of the protease, enzymatic hydrolysis was examined by using various synthetic peptide substrates. AraA efficiently hydrolyzed the peptide bonds of synthetic substrates containing an Arg residue at the N-terminal side, as in the case of other metalloproteases from *P. aeruginosa* and *S. marcescens* [28]. EDTA, a metal chelating agent, and phenanthroline, a specific metalloprotease inhibitor, strongly inhibit the enzyme activity, whereas addition of many inhibitors for aspartate, cysteine, and serine proteases did not change the enzyme activity [23]. The enzyme is also insensitive to phosphoramidon, a specific inhibitor of the thermolysin family of metalloproteases. The protease was activated by incubation with several cations including Zn^{2+} and Ca^{2+}. The deduced amino acid sequences of *araA* shared strong similarity with those of other serralysins from enteric bacteria including *S. marcescens* [6, 24, 34], *P. aeruginosa* [12], and *E. chrysanthemi* [24]. The strong zinc-binding consensus sequence is also present in the N-terminal region of the deduced amino acid sequence of *araA* [28].The activity was moderately inhibited by EGTA, which entraps Ca^{2+} more preferentially than EDTA, indicating that Ca^{2+} is also necessary for the activity of the enzymes [23].

The deduced amino acid sequence showed that ProA contained four tandem repeats of calcium ion-binding motifs, GGXGXD in the C-terminal region [2]. Ca^{2+} ions are known to bind to the domain by electrostatic interactions with the aspartic acid residues and by octahedral coordination with the carbonyl oxygen atoms of the glycine residues [2]. The serralysins are secreted extracellularly as an inactive zymogen, and the N-terminal 15 to 17 amino acids are removed during generation of the active enzyme in the presence of divalent cations, especially Zn^{2+} and Ca^{2+} [10]. It was also shown that the presence of Ca^{2+} in the solution could enhance the stability, integrity, production, and heat resistance of the metalloprotease from *P. fluorescens* [26]. The deduced amino acid contained a zinc-binding domain for its activity and calcium-binding site for its possible autoprocessing.The AraA from *S. proteamaculans* HY-3 may be secreted to the extracellular location *via* a Type I secretion pathway, an ATP-binding cassette (ABC) pathway. The signal peptide-independent transport system that requires three specific components, an ABC protein, a membrane fusion protein, and an outer membrane protein [1, 17], bypasses the periplasm and exports a variety of substrates, such as antibiotics, sugars, peptides, and proteins [14]. Neither identifiable signal peptides nor significant stretches of hydrophobic amino acids have been observed in the deduced amino acid sequence of AraA. All three serralysin metalloproteases so far tested, PrtB, C, and G from *E. chrysanthemi*, are secreted to the external medium by an ABC transport system. There were two consensus sequences, DXXX sequences (where Xs are hydrophobic residues) in the extreme C-terminal region for recognition of ABC transport systems.

Bacteria produce many extracellular enzymes to satisfy their diverse physiological demands. *Streptomyces griseus* excretes a metalloprotease, SgmA, required for degradation of substrate mycelia during morphological differentiation [16], and trypsin-like proteases, SprA, SprB, SprD, SprT, and SprU, during the stationary phase of growth [18, 40]. *Bacillus subtilis* secrets subtilisin and several neutral proteases into the medium at the end of exponential growth, which may be necessary for nutrient supply and endospore formation [8, 9]. We do not quite understand the role of AraA in the relationship with spiders infected with the *Serratia* strain. However, we do not exclude the possibility that AraA functions as a pathogenic determinant in the infected host, since many bacteria producing metalloproteases function as pathogens in their hosts. Zinc metalloproteases are required for the pathogenic factor, phospholipase C maturation in *Listeria monocytogenes* [36], and direct activation of toxin activities in the cases of enterotoxin in *Bacteroides fragilis* [19] and *Bacillus anthracis* lethal toxin [13]. *P. aeruginosa* metalloprotease plays a role in pathogenesis by facilitating bacterial degradation of host connective tissues and by providing peptides as nutrients for the microorganism [35]. A metalloprotease has been shown to be involved in the pathogenesis of *S. marcescens* in experimental pneumonia in guinea pigs and mice [27].

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