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Purification and part characterization of a novel antibacterial protein Scygonadin, isolated from the seminal plasma of mud crab, *Scylla serrata* (Forskål, 1775)

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Abstract

A novel protein was isolated from the seminal plasma of the mud crab, *Scylla serrata* (Forskål, 1775). It exhibited an antibacterial activity against the Gram-positive bacterium *Micrococcus leteus* with IC90 of 0.125 mg/ml. The extraction procedure for the protein included techniques of acid extraction, ion-exchange chromatography on SP-Sepharose Fast Flow and reverse-phase liquid chromatography on Source 5R RPC. It showed a molecular mass of 10.8 kDa by SDS-PAGE. A partial 20 residue NH_2-terminal sequence was determined by Edman degradation and MS-fingerprint of the protein was conducted. Similarity search in protein databases (BLAST) revealed that the protein exhibited no significant homology to any other reported antimicrobial peptides. We propose the name Scygonadin (from the gonad of *S. serrata*) for this antibacterial protein.

Keywords: Antibacterial protein; Scygonadin; *Scylla serrata*; Seminal plasma

1. Introduction

Since male and female genital orifices are exposed to the external environment containing a host of microorganisms, they are potential ports of entry for microorganisms deleterious to fertility (Lung et al., 2001). A number of papers postulated that the male genital tract is a potential source of novel antimicrobial molecules involved in host defense (Hall et al., 2002). In invertebrates, some antimicrobial peptides have been isolated from both the male and female reproductive tracts. One of them, andropin, is expressed exclusively in the male reproductive tract and has been characterized in *Drosophila melanogaster* (Samakovlis et al., 1991). More recently, antibacterial peptides, ceratotoxin (forms A and B), have been identified from the female reproductive tract of *Ceratitis capitata* (Marchini et al., 1993). In crustaceans, Jayasankar and Subramoniam (1999) reported that a 20 kDa protein from the seminal fluid of *Scylla serrata* was found to have antimicrobial activity against commonly occurring marine bacteria. This suggests that there might be some components in the seminal plasma of crabs responsible for resistance to invading bacteria or other pathogenic microorganisms. Analysis of the antimicrobial activities of proteins and peptides present in the seminal fluid of the mud crab will allow us better understanding of

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how the innate immune system is involved with maintaining sterility of the reproductive tracts of these organisms and thus allows successful fertilization of gametes in microorganism laden seawater.

We report here the isolation, biochemical characterization and antimicrobial activities of a small protein from the seminal plasma of *S. serrata*. The protein, which is distinct from other groups of antimicrobial peptides hitherto described, was named scygonadin, after the genus *Scylla*.

2. Materials and methods

2.1. Seminal plasma preparation

S. serrata (Forskål, 1775), was purchased from a local farm in Xiamen, Fujian province, P.R.China. The seminal contents from the *vas deferens* of adult males with carapaces ranging from 11 to 14 cm in width were teased out. A total of about 50 g seminal plasma was ground into fine powder in liquid nitrogen. The cold powder was brought up to half of its original volume with trifluoroacetic acid/acetic acid/water (0.1:15:84.9, by vol.) and stirred for 2 h at 4 °C. The mixture was centrifuged (Sigma 1–15 K refrigerated centrifuge) at 15,000 ×*g* for 45 min at 4 °C, and then the supernatant was collected and lyophilized (Freezone 6L Freeze Dry System, Labconco, USA).

2.2. Ionic-exchange chromatography purification

The lyophilized powder was reconstituted with 50 mM sodium acetate (pH 5.0) and loaded onto 26×150 mm SP-Sepharose Fast Flow cartridges (Pharmacia LBK Biotechnology, Sweden) connected to an ÄKTA Purifier 100 (Amersham Bioscience, Sweden). Bound material was eluted with a linear gradient of 0–0.75 M ammonium acetate. The eluant was monitored at 280 nm. Fractions (about 50 ml each) were manually collected, lyophilized, reconstituted in 2–5 ml MilliQ

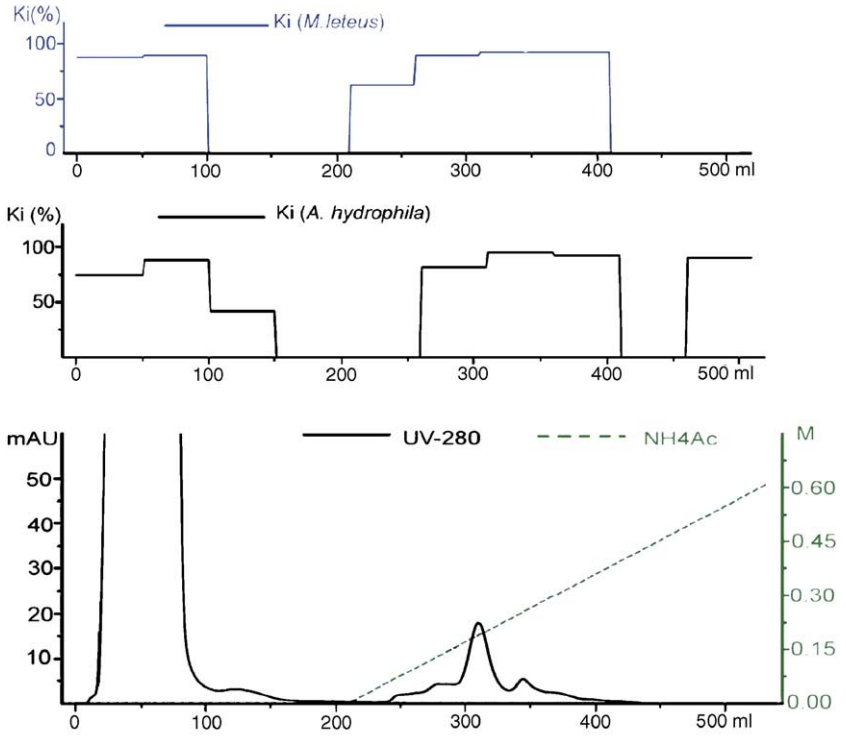

Fig. 1. Elution profile for the ion-exchange chromatography of acid extracts of seminal plasma from *S. serrata* (Forskål, 1775). SP-Sepharose column (26 mm×150 mm, Pharmacia LBK Biotechnology) was used. One column volume (CV) was 67.64 ml. The column was pre-equilibrated in 50 mM sodium acetate (pH 5.0). Unbound material was eluted by extensive washing at a flow rate of 5 ml/min for 2 CV. Bound material was eluted with a linear gradient from 0 to 0.75 M ammonium acetate for 5 CV. Absorbance was monitored at 280 nm (solid line). Fractions (about 50 ml each) were manually collected and respectively assayed for antibacterial activity against *A. hydrophila* and *M. leteus* by liquid growth inhibition assays. Fractions from 210 to 405 ml, corresponding to a concentration of ammonium acetate between 0.12 M and 0.45 M, had antibacterial activity against both the test bacteria and were used for further study.

water (MilliQ synthesis A10), and then subjected to the antimicrobial assay. The quantities of proteins and peptides were estimated by the method of Bradford using bovine serum albumin (Pierce, USA) as a standard.

2.3. Reverse-phase chromatography purification

Fractions with ammonium acetate concentration between 0.12 and 0.45 M were pooled and applied onto Source 5R RPC-ST-4.6/150 column (Amersham Bioscience, Sweden). Elution was performed at 1 ml/min using a gradient from 2% acetonitrile in 0.0065% TFA to 80% acetonitrile in 0.005% TFA. The absorbance was monitored at 215 nm. Fractions were manually collected in polypropylene tube, lyophilized, and reconstituted in 100 μl MilliQ water before antimicrobial activity was tested.

2.4. Tricine-SDS-PAGE analysis

Tricine-SDS-PAGE of purified proteins was done in a 16% (w/v) separation gel. The gel was stained with Coomassie Brilliant Blue R-250. The relative molecular weight of the protein band was determined using peptide molecular weight markers (Amersham Biosciences, Sweden) and Low molecular weight markers (Fermentas, USA).

2.5. Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry

Native peptide was dissolved in 1% ammonium bicarbonate (pH 8.5). Trypsin protease (porcine, EC 3.4.21.4) was added to the solution which was incubated at 37 °C for 16 h in a substrate: enzyme ratio of 50:1 (W:W). Digestion was quenched by storing the sample at −70 °C. The sample was lyophilized and reconstituted in 50 μl 0.5% TFA before use. 5 μl of sample solution was added to a tube containing 10 μl of the MALDI matrix α-cyano-4-hydroxycinnamic acid (CHCA) (Sigma, St, Louis, USA) prepared as a 10 mg/ml solution in methanol/acetonitrile/water(1:1:1, by vol.). The solution was stirred in a vortex mixer; a 1 μl aliquot of this solution was applied on the MALDI target and allowed to evaporate at room temperature prior to MALDI-TOF-MS spectral analysis. The mass of the digested fragments were analyzed by Genecorn Co. Ltd (Shanghai, China) using a Voyager DE-Pro MALDI mass spectrometer (ABI, USA), which was equipped with a nitrogen laser (337 nm, 3 nm pulse) for desorbing ions from the sample specimen.

2.6. Amino acid sequencing

Sequencing was carried out on an Applied Biosystems ABI492cLC protein sequencer (ABI, USA) at Genecorn Co. Ltd (Shanghai, China).

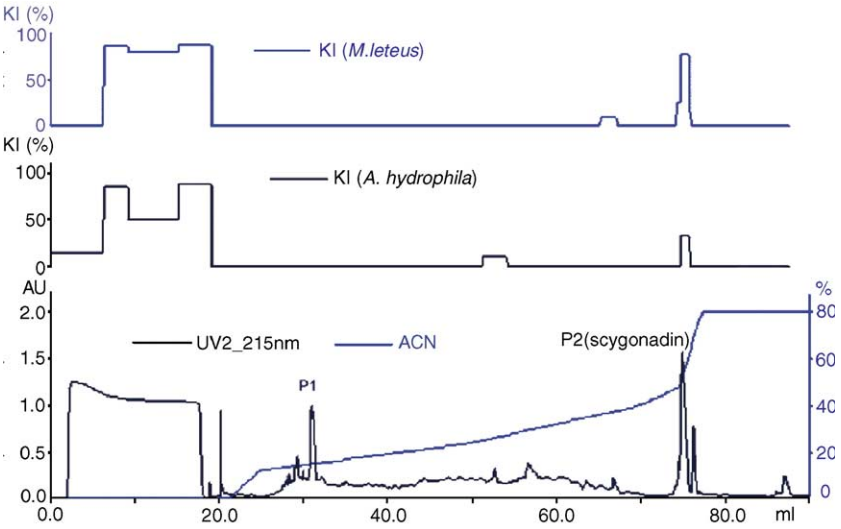

Fig. 2. Elution profile for Source 5R RPC-HPLC (Amersham Bioscience, Sweden) of the sample obtained from the ion-exchange column as described in Materials and methods section. Bound material was eluted at a flow rate of 1 ml/min using a gradient from 2% acetonitrile in 0.065% TFA to 80% acetonitrile in 0.05% TFA as follows: 2–12% (1.5 CV), 12–24% (10 CV), 24–40% (8 CV), 40–64% (3 CV) and finally 64–80% (1 CV). Column effluent was monitored by its UV absorption at 215 nm. Normally, 3–5 ml per fraction was manually collected in polypropylene tube, lyophilized (about 24 h), and reconstituted in 100 μl MilliQ water before antibacterial activity was tested. Antibacterial activity was present mainly in fractions 0–19 ml and in fractions eluted at an acetonitrile concentration between 40% and 65%. Fraction P2 was named scygonadin. The result showed that P2 (named scygonadin) could kill the test bacteria to some extent.

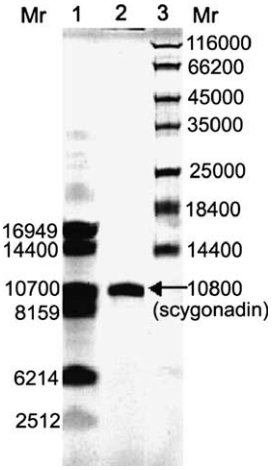

Fig. 3. The Tricine-SDS-PAGE of scygonadin was performed using mini-slab gel system. Lane 1 contained peptide Markers from Amersham Pharmacia (Mr 2512, 6214, 8159, 10700, 14404, 16949, from Horse myoglobin peptides) and lane 3 contained low molecular weight markers from Fermenta (Beta-galactosidase from *E. coli*, Mr 116000; Bovine serum albumin from bovine plasma, Mr 66200; ovalbumin from chicken egg white, Mr 45000; lactate dehydrogenase from porcine muscle, Mr 35000; REase BSP 981 from *E. coli*, Mr 25000; Beta-lactoglobulin from bovine milk, Mr 18400; lysozyme from chicken egg white, Mr 14400), lane 2 contained P2 purified by RP-HPLC. The result showed that P2 was a single-chain polypeptide with molecular weight of about 10.8 kDa.

2.7. Antimicrobial assay

After each step of purification, an aliquot of each eluted fraction was reconstituted in MilliQ water and was tested by the liquid growth inhibition assay as described by Anderson and Beaven (2001). *Aeromonas hydrophila*, a Gram-negative bacterium that is pathogenic for fish was a kind gift from Professor RZ Guan (Fisheries College of Jimei University, P.R.China), and *Micrococcus leteus* ATCC 4698, a Gram-positive bacterium was purchased from Sigma. A bacterial suspension in 50 mM HEPES (pH 6.8) was prepared (10^6 cells·ml^{-1}; OD_{620}=0.100). Experimental and control sets from each sample were carried out in four replicate wells in a standard 96-well microtiter plate. In one set (bacteria), 25 μl bacterial suspension was added to 25 μl 50 mM HEPES; another set (bacteria+sample) contained 25 μl bacterial suspension and 25 μl sample; a blank set (blank) contained 25 μl HEPES and 25 μl sample. The microtiter plate was incubated in a saturated humidity chamber for 3 h at 30 °C. A grow-out period for the surviving bacteria was initiated by adding 50 μl sterile Muller–Hinton broth (MHB) to all wells and incubating 2 h at 30 °C. Finally, 10 μl 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium and phenazine methosulfate (MTS-PMS reagent, Promega Corp., Madison, WI) was added to the wells and incubated for 1 h at 30 °C. The numbers of viable bacteria were determined colorimetrically by measuring the production of reduced MTS formazan at 492 nm on an ELISA plate reader (CliniBio 128C ELISA plate reader, Bio-Rad, USA). Absorbance values were corrected by subtraction of the blank. A killing index (percentage killed) was calculated with corrected absorbance values using the following equation where Ki is killing index, corrAMP=A_{492} of bacteria+sample corrected by subtraction of blank value, and corrAB=A_{492} of bacteria alone corrected by subtraction of blank value.

$$\mathrm{Ki} = [1-(\mathrm{corrAMP}/\mathrm{corrAB})] \times 100\%$$

The concentration of the antibacterial protein required for Ki ≥ 90% was expressed as IC90.

3. Results

3.1. Isolation of Antimicrobial Proteins from seminal plasma of S. serrata

In ion-exchange chromatography, fractions eluting from 210 to 405 ml, corresponding to a concentration of

Table 1
Protein identification of scygonadin on the ExPASy WWW server was shown

Rank	Protein name	Species	Acc. #	MOWSE	#hit/ submitted	MW/p*I*	Intensity matched	Avg/SD (Δppm)
1	Regulator of transfer gene Art A	*Staphylococcus aureus*	3676434	3.12E+ 02	3/18	7181/8.0	12	43±42
2	Growth hormone	*Silurus Asotus*	26891554	3.08E+ 02	4/18	22653/6.2	21	43±55
3	Major urinary protein 5 precursor	*Mus Musculus*	33859841	2.72E+ 02	4/18	21006/4.9	13	43±53
4	Chain A. Sodium channel Iia inactivation gate	*Rattus Uorvegicus*	6435540	2.18E+ 02	3/18	6254/9.9	30	52±17
5	Periplasmic divalent cation tolerance protein	*Thermotoga maritima*	15643814	2.04E+ 02	3/18	12177/5.3	21	45±13
6	Hypothetical protein MTH1875	*Methanothermobacter thermautotrophicus*	1579863	1.98E+ 02	3/18	7714/6.7	14	37±46

The result showed that scygonadin had little similarity to other proteins in databases and is most likely a novel protein.

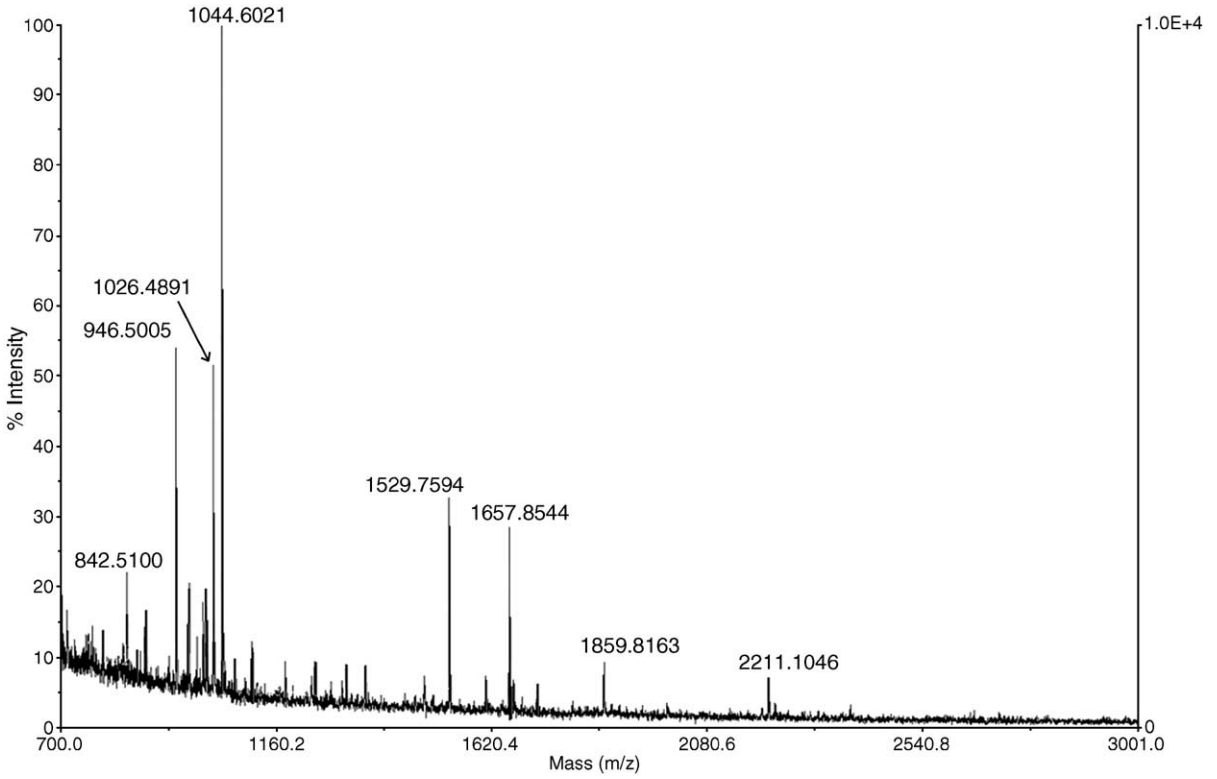

Fig. 4. MS-fingerprint of scygonadin was shown. Scygonadin was digested by Trypsin protease (porcine, EC 3.4.21.4; Promega, USA) and then mixed with MALDI matrix α-cyano-4-hydroxycinnamic acid (CHCA) (Sigma, USA) in methanol/acetonitrile/water (1:1:1). The sample was determined using a Voyager DE-Pro MALDI mass spectrometer (ABI, USA).

ammonium acetate between 0.12 M and 0.45 M, had antibacterial activity against both *M. leteus* and *A. hydrophila* (Fig. 1). These fractions were pooled, adjusted with TFA to 0.065% and applied to a Source 5R RPC-ST-4.6/150 column. In this column, fractions containing significant antimicrobial activity were predominantly seen in the flow through fractions (2–18 ml; Fig. 2) and fractions eluting between 40 and 65% acetonitrile (65–75 ml; Fig. 2). Only peak No. 2 (P2) was submitted for further chemical characterization (Fig. 2).

3.2. Characterization and similarity search of AMP

The purity and molecular weight of material eluting in the P2 peak were evaluated by Tricine SDS-PAGE (Fig. 3). Result showed that it was a single-chain polypeptide with molecular weight of about 10.8 kDa. MS-fingerprint of P2 (Fig. 4) was used in a similarity search and the results showed that P2 has little similarity to other proteins in the databases and is most likely a novel protein (Table 1). The N-terminal amino acid sequence of P2 was identified to be H-GQALNKLMPKIVSAIIYMVG-OH by Edman degradation. So far, no protein was found to match this sequence in protein databases around the world. The fragment P2 was temporarily named as scygonadin after genus of *Scylla*.

3.3. Antimicrobial activity

During preliminary HPLC purification steps, the extracts of the seminal plasma from *S. serrata* were shown to display several fractions with bactericidal activities (Fig. 2), which will be subjected to further analysis. In liquid growth inhibition assays, the purified P2 had antibacterial activity against Gram-positive bacterium *M. leteus* with IC90 of 125 μg/ml.

4. Discussion

Although there have been several reports on antibacterial activity in seminal plasma or in plasma (Chattopadhyay and Chatterjee, 1993, 1997; Chattopadhyay et al., 1996; Hoq et al., 2003; Jayasankar and Subramoniam, 1999; Majumder et al., 1997), few antibacterial peptides have been characterized in *S. serrata*. From hemolymph, Chattopadhyay and Chatterjee (1993)

purified and partially characterized a low molecular weight (4.8–5 kDa) bactericidal lectin named scyllin by affinity chromatography on GalNAc–Separon affinity adsorbent, which could agglutinate both Gram-positive and Gram-negative bacteria and inhibit microbial growth (Chattopadhyay et al., 1996). The chemical composition of scyllin was also analyzed and found to be rich in acidic and neutral amino acids and contained high levels of mannose (Majumder et al., 1997). Recently Hoq et al. (2003) investigated the difference in the antibacterial activity of hemolymph obtained from bacterial-induced and naïve mud crab. The result revealed that the induced hemolymph had antibacterial activity against some bacteria whereas the non-induced hemolymph had none. They also found that the components responsible for antibacterial activity were proteins with molecular weights of 64, 61, 56.5, 49 and 36 kDa, respectively (Hoq et al., 2003). From seminal plasma, Jayasankar and Subramoniam (1999) originally reported the presence of antibacterial activity against some of the commonly occurring marine bacteria. They partially purified seminal plasma proteins by gel permeation chromatography on Sephadex G200, and found that the component responsible for antibacterial activity was a protein. This had a molecular weight of 20 kDa estimated by gel permeation chromatography using Sephadex G200 (Jayasankar and Subramoniam, 1999). Our present study reveals a novel antibacterial protein with a mass of 10.8 kDa, which is distinct in molecular weight from any other peptides/proteins discovered in this organism, as mentioned above. Through MALDI-TOF-MS similarity search and by BLAST analysis of the N-terminal sequence obtained by Edman degradation, the result showed that scygonadin has little similarity to other proteins in databases, and therefore it is most likely a novel protein (Table 1).

Based on the elution profile from the Source 5R RPC chromatography as well as the partial N-terminal amino acid sequence of scygonadin, it can be deduced that the protein is either hydrophobic or may be an amphipathic molecule. In cationic antimicrobial peptides, the amphipathic property may be crucial to their function as antibacterial peptides by allowing them to interact with bacterial membrane phospholipids (Khoo et al., 1999). For further characterization of scygonadin, the cDNA and genomic DNA sequences of the protein need to be elucidated based on the N-terminal amino acid sequence of H-GQALNKLMPKIVSAIIYMVG-OH.

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