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Purification and characterization of antibacterial proteins from granular hemocytes of Indian mud crab, *Scylla serrata*

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Marine invertebrates depend upon antimicrobial peptides (AMPs) as a major component of innate immunity, as they are rapidly synthesized and diffuse upon pathogen invasion. In this study, we report the identification and characterization of a 11 kDa antimicrobial protein, which we name SSAP (for *Scylla serrata* antimicrobial protein), from granular hemocytes of the mangrove crab *S. serrata*. The protein is highly similar to scygonadin, a male-specific AMP isolated from the ejaculatory duct of *S. serrata*. SSAP was isolated using various chromatographic techniques, viz. ion-exchange, ultra filtration and RP-HPLC, and demonstrated antibacterial activity against Gram positive and Gram negative bacteria. Full length mRNA encoding SSAP was amplified using a combination of RT-PCR and RACE. The nucleotide sequence revealed a full-length ORF of 381 bp coding for a preprotein of 126 amino acids comprising a signal peptide of 24 amino acids and a mature protein of 102 amino acids with a predicted mass of 11435 Da and pI of 5.70. Unlike scygonadin, SSAP is expressed in several tissues of both male and female crabs, as evidenced by RT-PCR, Northern and Western blot analyses. The study suggests that SSAP might be an isoform or a variant of scygonadin and might play an important role in regulating the immunity of the crab upon microbial infection.

Keywords: antibacterial protein, crab, *Scylla serrata*, hemocytes, innate immunity

INTRODUCTION

The invertebrates comprise over 95% of animal species and some live in environments rich in potentially harmful microorganisms. As a result these animals have developed various competent strategies to defend their lives against invading pathogens (Jiravanichpaisal *et al*., 2006). Earlier reports have substantiated the fact that invertebrates lack the complexity of the adaptive immune system characterized by memory and depend solely on non specific innate immune responses of amazing diversity and evolutionary success. Recent experimental data from invertebrates suggests that past exposure to pathogens in individual animals can lead to enhanced immunity and some are also known to have considerable specificity by recognizing non-self pathogen-associated molecules through a series of pattern recognition receptors that are highly conserved in evolution (Janeway & Medzhitov, 2002; Kurtz, 2005). These reports corroborate the existence of both specificity and memory in invertebrates.

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Abbreviations: AMPs, antimicrobial peptides; BSA, bovine serum albumin; BV, bacterial vaginosis; CMC, carboxymethyl cellulose; CSPD, disodium 3-[4-metoxyspirol (1,2-dioxetane-3,2′-(5′-chloro)tricyclo)decan]phenyl phosphate; LB, Luria-Bertani broth; MHB, Mueller-Hinton broth; MIC, minimal inhibitory concentration; ORF, open reading frame; PI, protease inhibitor cocktail; RP-HPLC, reversed phase high pressure liquid chromatography; RT-PCR, reverse transcriptase polymerase chain reaction; RACE, rapid amplification of cDNA ends; SDS/PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SSAP, *Scylla serrata* antimicrobial protein; STI, sexually transmitted infection; TFA, trifluroacetic acid.

Some of the known innate immune responses in invertebrates include pahogocytosis, nodulation and encapsulation, synthesis of (AMPs) and activation of proteolytic cascades that lead to melanization, blood coagulation, release of stress-responsive proteins and molecules believed to function in opsonization and iron sequestration (Lee & Soderhall, 2002; Iwanaga & Lee, 2005).

AMPs are a major component of the innate immune defense system in marine invertebrates. These molecules are the first line of host defense in various species, have a mass of ≤10 kDa and are readily synthesized and efficiently diffuse at the point of pathogen entry or infection, hence form an invaluable component of the innate immune system (Reddy *et al.*, 2004a, 2004b; Cole, 2005). AMPs have also been shown to demonstrate *in vitro* microbicidal properties against various clinical pathogens, including the sexually transmitted infection (STI)-causing *Treponema pallidum*, *Chlamydia trachomatis* and HIV (Yedery & Reddy, 2005). These peptides have been isolated from a wide variety of vertebrate and invertebrate phyla including mammals, reptiles, ascidians, insects, chelicerates, annelids and mollusks (Andreu & Rivas, 1998; Cooper, 1985; Lehrer & Ganz, 1999). In particular, several AMPs have been identified from a variety of marine invertebrate species. Tachyplesins and polyphemusins are two well characterized cationic molecules from granular hemocytes of Japanese horseshoe crab (*Tachypleus tridentatus*) and American horseshoe crab (*Limulus polyphemus*), respectively (Miyata *et al.*, 1989; Murakami *et al.*, 1991). Prominent among AMPs isolated from crustaceans are penaeidins from the shrimp *Penaeus vannamei*, while in marine mollusks, defensins, myticins and mytilins isolated from *Mytilus galloprovincialis* and *Mytilus edilus,* respectively, have been studied for their specific roles in innate immune mechanisms (Mitta *et al.*, 2000).

Scylla serrata, commonly known as the mud crab or mangrove crab, is an economically important marine invertebrate distributed throughout the West Pacific and Indian Oceans. This crab inhabits muddy bottoms of brackish water along the shoreline, mangrove areas, and river mouths, hence the name mud crab or mangrove crab. It is the most important edible crab for commercial culture in the Indo-West Pacific region and commands a high price in both the domestic and export markets (Samonte & Agbayani, 1992). The crabs are in intimate contact with an environment rich in pathogenic bacteria, and are prone to infection by microbes at various stages of growth, losses due to disease can be enormous (Hudson & Lester, 1994). Hence there is an urgent need to understand the existing defense mechanisms in such animals and find ways of enhancing their natural immunity against infectious pathogens.

Earlier, Chattopadhyay and Chatterjee (1997) described the isolation and characterization of scyllin, an antimicrobial lectin from the hemolymph of *S. serrata*, while Hoq and colleagues (2003) identified various protein fractions from the hemolymph of *S. serrata* that demonstrated antimicrobial activity against various Gram positive and Gram negative bacteria. Recently, Huang and colleagues (2006) have isolated a 10.8 kDa anionic protein named scygonadin from the male reproductive tract of *S. serrata* that inhibited growth of *Micrococcus luteus* and *Aeromonas hydrophila*. The present study demonstrates the identification of about 11 kDa antimicrobial protein from hemocytes of *S. serrata*, SSAP, which shows high sequence similarity with scygonadin. Preliminary comparisons of cDNA and translated protein sequences strongly indicate that SSAP could possibly be a variant of scygonadin. Recent reports have postulated the possibility of using AMPs as vaginal microbicidal compounds to combat the spread of STI and HIV (Cole, 2005). Hence in our present study all antimicrobial assays were carried out with vaginal bacterial isolates from women suffering from bacterial vaginosis (BV).

MATERIALS AND METHODS

Experimental animals. Specimens of the common Indian mud crab, *S. serrata*, were purchased from the local fish market and kept in a glass tank with seawater until further use. They were fed twice daily with minced fish meat. Both male and female, healthy intermoult animals (7±1 cm carapace width) were used. Each animal was subjected to a single bleed amounting to 3–4 ml of hemolymph.

Microbial strains and culture. Vaginal isolates of Gram negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*, and Gram positive bacteria *Staphylococcus aureus* and *Streptococcus pyogenes* were obtained from the Microbiology Department of King Edwards Memorial Hospital (Mumbai, India). The strains were isolated from vaginal swab cultures of women tested positive for BV, were identified by Gram staining and characterized biochemically. The organisms were grown in MHB (Hi-Media Laboratories, Mumbai, India) at 37°C for 18 h and then subcultured into fresh broth and raised to log phase for 4–6 h. Log phase bacteria were harvested by centrifuging at 2000×*g* (10 min, 4°C) and were washed twice in sterile physiological saline (0.9% NaCl) before final resuspension in fresh sterile saline. All bacterial suspensions were calibrated and routinely standardized to an absorbance of 0.4 at 570 nm, which gave a concentration of approx. 10^9 c.f.u. ml^{-1}.

Antibacterial assays. Antibacterial activity of purified fractions obtained at each step of purification was assessed by a two-layer radial diffusion method described earlier by Relf and coworkers (1999). Briefly, the modified protocol involves preparing a 14-ml bacterial underlay of 1% agarose in 10% MH broth supplemented with 0.02% Tween in a 12×12 cm Petri dish. The agar was seeded with 1×10^6 washed bacteria. Wells of 3 mm diameter were punched into the agarose and 50 µl of the test sample was pipetted into each. Negative controls comprised only sterile deionized water containing 0.1% acetic acid. The plates were incubated at 4°C for 3 h and then overlaid with 14 ml of sterile 1% agarose containing double strength LBB. They were incubated for a further 24 h at 35°C. Clear zones in the agar underlays were taken as indicating antibacterial activity. Their diameters were measured and activity was expressed as clear zone area (in mm^2) minus the area of the well.

Determination of the minimal inhibitory concentrations (MICs). Minimal inhibitory concentrations of purified SSAP were determined against Gram negative bacteria *E. coli* and *P. aeruginosa* and Gram positive bacteria *S. aureus* and *S. pyogenes* in sterilized 96-well plates (NUNC, Roskilde, Denmark) in a final volume of 100 µl composed of 50 µl of 10^6 bacteria in MH broth and 50 µl of SSAP in serial two fold dilutions (6.25–200 µg ml^{-1}) in sterile water containing 0.1% acetic acid. Wells without protein were used as a negative control. The plates were left with shaking (90 r.p.m.) at 37°C and bacterial growth was assayed by measurement of the optical density at 600 nm after 24-h incubation. The MIC values are expressed as described by Casteels *et al.* (1993).

Preparation of granular hemocyte extract. Fresh hemolymph was collected aseptically from crabs of either sex with a 21 gauge needle attached to a syringe, by puncturing into the heart from the dorsal surface of the carapace. The hemolymph was diluted with equal volume of sterile marine anticoagulant (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 36 mM citric acid and 10 mM EDTA, pH 4.6). Granular hemocyte populations were separated on a 60% continuous Percoll gradient as described elsewhere (Soderhall & Smith, 1983). The granular cell band was removed with a sterile Pasteur pipette and transferred into a 50-ml polycarbonate tube containing an equal volume of marine anticoagulant supplemented with complete protease inhibitor cocktail (PI) (Roche, Mannheim, Germany). The cells were pelleted by centrifugation at 1900×*g* (10 min, 4°C) and the supernatant discarded.

The cells were suspended in 2.5 ml of sterile saline containing PI and incubated on ice for 20 min followed by lysis of cells with an ultrasonicator (Remi Instruments, Mumbai, India) for 10 min. The sample was centrifuged at 12000×*g* (20 min at 4°C) and the supernatant stored overnight at –20°C. The pellet was resuspended in 2.5 ml of 10% acetic acid containing PI and incubated overnight at 4°C followed by centrifugation at 12000×*g*. After further centrifugation, the two supernatants were pooled and dialyzed using the Amicon ultrafiltration units with a 3 kDa cut-off membrane (Millipore, Bedford, MA, USA) with two washes with 0.2 M sodium acetate (pH 4.0). Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Purification of antimicrobial proteins. Ion exchange chromatography was performed as described by Zasloff (1987). The dialyzed extract of granular hemocytes (5 ml) was loaded onto a 36×2.8 cm glass column packed with carboxymethyl cellulose (Sigma, St. Louis, MO, USA), equilibrated with 0.2 M sodium acetate (pH 4.0) buffer. Unbound proteins were washed off with sodium acetate until the absorbance returned to baseline. Bound proteins were eluted with 0.2 M ammonium acetate buffer (pH 5.2). Fractions (2 ml) were collected and their absorbance was monitored at 280 nm using a spectrophotometer (UV-160A, Shimadzu, Japan). Protein fractions were freeze-dried, dissolved in 50 µl of sterile deionized water containing 0.1% acetic acid and tested for antibacterial activity. Fractions demonstrating antibacterial activity were pooled and loaded onto an Amicon ultrafiltration unit with a 30 kDa cut-off membrane (Millipore, Bedford, MA, USA). The unit was centrifuged at 5000×*g* for 30 min and the filtrate was lyophilized.

The lyophilized sample was acidified to a final concentration of 0.1% TFA and applied onto a 22×250 mm Vydac C_8 RP-HPLC column (Hesperia, CA, USA). Adsorbed proteins were eluted (1 ml/min) in a three-step gradient of 0.1% TFA/water and 0.1% (v/v) TFA in 50% acetonitrile: 0–100% (60 min), held at 100% for 5 min and brought back to 0% (100–0%). The absorbance of the fractions was monitored at 280 nm and they were freeze-dried. The samples were dissolved in 50 µl of sterile deionized water containing 0.1% acetic acid and assayed for antibacterial activity. Fractions with antibacterial activity were loaded further on the same column to check for homogeneity. The above purification protocol was repeated a number of times to obtain bulk amount of active protein. The active protein was termed SSAP.

SDS/polyacrylamide gel electrophoresis. One dimensional SDS/PAGE (Laemmli, 1970) was performed using a vertical slab gel apparatus (Biorad, Hercules, CA, USA) with the stacking gel containing 3% acrylamide and the resolving gel with 15% acrylamide. Briefly, the RP-HPLC-purified SSAP

fractions were diluted with reducing sample buffer (20% glycerol, 1% SDS, 0.125 M Tris/HCl, 2% β-mercaptoethanol and 0.5% bromophenol blue), heated for 5 min at 100°C and centrifuged at 10000×*g* for 5 min. The supernatant was loaded into a single well and subjected to electrophoresis. The gel was calibrated using molecular mass markers (Amersham Biosciences, Piscataway, NJ, USA) and stained with silver nitrate (Sigma, St. Louis, MO, USA).

Protein sequencing. Fractions containing the purified SSAP were subjected to trypsin digestion using 0.2 μg of modified trypsin (Pierce, Rockford, IL, USA) for 18 h at 37°C. Cysteines were modified by reducing the protein in 100 mM ammonium bicarbonate containing 0.25% (v/v) 2-mercaptoethanol for 30 min at 65°C. Then the protein was treated with iodoacetamide (final concentration 0.35 mM) and incubated for 30 min at room temp. The mixture was desalted on a 3 kDa cut-off centriprep filter (Millipore, Bedford, MA, USA) and the resulting peptides were separated on a 22 mm×250 mm Vydac C_8 RP-HPLC system. Selected peptide peaks were subjected to sequence analysis by automated Edman degradation using a Procise protein sequencing system (Applied Biosystems, Foster City, CA, USA).

RNA isolation from hemocytes. Granular hemocytes were separated from freshly drawn hemolymph as described earlier. Total RNA was extracted from 0.5 ml of undiluted granular hemocytes by homogenization with an ultrasonicator (Remi Instruments, Mumbai, India) for 5 min in 1 ml of ice cold Trizol (Roche, Mannheim, Germany). The sample was brought to room temp. and mixed with 0.2 ml of chloroform. The tube was shaken vigorously for 15 s and incubated at room temp. for 2 min before centrifugation at 12000×*g* (15 min, 4°C). The upper, aqueous phase containing RNA was transferred to a fresh tube and the nucleic acid precipitated by mixing with 0.25 ml of absolute isopropanol for 10 min at room temp. The pellet remaining after centrifugation at 12000×*g* (10 min, 4°C) was washed once with 1 ml of 75% ethanol, mixed by vortexing and re-centrifuged at 7500×*g* (5 min, 4°C). The RNA obtained was checked for purity by spectrophotometer at A_{260}/A_{280} nm (Schimadzu, Japan). The quality and integrity of RNA was further determined by 1.2% denaturing formaldehyde/agarose gel electrophoresis. The resulting RNA (A_{260}/A_{280}: 1.74, concn.: 800 μg ml^{-1}) was dried at room temp. for 5 min and then redissolved in 50 μl of DEPC water.

Design of degenerate primers and RT-PCR. Full-length cDNA of SSAP was obtained by the procedures of RT-PCR and RACE method. For this purpose degenerate primes were designed from two of the known amino-acid sequences, the forward degenerate primer DF (5′-CCNGAYGGNTTYTAYACN-3′) was designed from the peptide PDGFYT, and the reverse degenerate primer DR (5′-YTTNARNG-CYTCTARYTC-3′) was designed from the peptide ELEALK. RT-PCR was performed with 2–3 μg of hemocyte total RNA with primers DF and DR, using Qiagen one step RT-PCR kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The PCR reaction included an initial RT reaction at 50°C for 30 min followed by PCR activation step at 95°C for 15 min, followed by 35 cycles at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s and a final extension at 72°C for 10 min. PCR products were separated on a 2.0% agarose gel.

The resulting about 100 bp PCR product was excised from the gel using a QIA quick gel extraction kit (Qiagen, Valencia, CA, USA). The purified product was ligated into pMOSBlue vector (Amersham Biosciences, Piscataway, NJ, USA) and transformed into *E. coli*, DH5α competent cells and incubated overnight at 37°C. Positive clones were isolated by blue/white screening. Single white colonies were picked individually and grown over night for plasmid preparation. Plasmid DNA was purified using Qiagen Plasmid Purification Kit as per the manufacturer's instructions and sequenced using T7 and M13 promoter primers on an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

5′ and 3′ RACE PCR. Five micrograms of hemocyte total RNA was reverse transcribed to prepare 5′ and 3′ RACE Ready cDNA using SMART™ RACE cDNA Amplification kit (Clontech, Mountainview, CA, USA) according to manufacturer's recommendations. To obtain the sequence of full-length SSAP cDNA from *S. serrata*, 3′ and 5′ RACE PCR were performed using gene-specific primers based on the about 100 bp fragment that was obtained from RT-PCR analysis. For 5′ RACE PCR, a 50 μl reaction was set up containing 5 μl 5′ RACE cDNA and primers UP1 (5′-CTAATACGACTCAC-TATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′) and SR (5′-AACCCGGCTTCTTCCTTC-3′). The first PCR conditions were as follows: 5 min initial denaturation at 94°C for one cycle, then 30 cycles of denaturation at 94°C for 30 s, primer annealing at 58°C for 30 s and extension at 72°C for 1 min, and finally 10 min final extension at 72°C. The products were analyzed on agarose gels.

The nested 5′ PCR was carried out with 5 μl of 1:50 dilution of the above PCR product and primers UP2 (5′-AAGCAGTGGTATCAACGCAGAGT-3′) and SR using the same conditions as above. The 5′ nested PCR product of the predicted size was gel-separated, purified, inserted into the pMOSBlue vector, and the positive clones were sequenced.

The 3′ RACE PCR was carried out with 3′ RACE cDNA and primers UP1 and SF (5′-TGTGGT-GTACTTCCTGGA-3′), and the nested 3′ PCR was

carried out with primers UP2 and SF using the same conditions as above. The annealing temperature used in the reaction was 60°C. The products were cloned and sequenced as above.

Sequence analysis. The full-length cDNA of SSAP was compared with default settings with the complete non-redundant GenBank database using the BLAST program available at the NCBI website (http://www.ncbi.nlm.nih.gov/blast/). The full length SSAP cDNA sequence was translated to a predicted amino-acid sequence using ExPASy proteomic tool (http://www.expasy.org/tools/dna.html). The putative cleavage site of the signal peptide was predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP/). Both the mass and the pI of the putative protein were calculated using the ProtParam tool by ExPASy (http://www.expasy.org/tool/protparam). The hydropathy plot of the deduced amino-acid sequence was plotted using the method of Kyte and Doolittle (http://fasta.bioch.virginia.edu/fasta_www2/fasta_www.cgi?rm=misc1).

Expression study. Healthy crabs (both male and female) were dissected and their gills, heart, hepatopancreas, reproductive tract, muscle, hemocytes, eyes and digestive tract were carefully collected aseptically and stored at –80°C. Total RNA was extracted using Trizol as described earlier. To analyze the tissue expression pattern of SSAP, RT-PCR was performed with 1–2 μg of hemocyte total RNA with ORF-specific primers OF (5′-ATGCGTTCATCTCTCTACTC-3′) and OR (5′-CCCGACCATATAAATTATGGC-3′), using Qiagen one step RT-PCR kit. The PCR program was as follows: one cycle at 95°C for 2 min, 35 cycles of 94°C for 20 s, 55°C for 30 s and 72°C for 20 s, followed by a cycle at 72°C for 2 min. Primers for actin, CF (5′-CGTTCGTGACATCAAGGAGA-3′) and CR (5′-AGGAAGGAAGGCTGGAAGAG-3′) were used in a positive control reaction. The PCR products were analyzed on a 1.5% agarose gel, eluted and digoxigenin (DIG) labeled by DIG DNA Labeling Kit (Roche, Mannheim, Germany).

Northern blot analysis. For Northern blot analysis, 30 μg of RNA from the above-mentioned tissues, including hemocytes, was denatured in formaldehyde-containing buffer and incubated at 68°C for 15 min, then electrophoresed. The RNA was transferred onto a Hybond N+ membrane (Amersham Biosciences, Piscataway, NJ, USA) by using conventional capillary blotting in 20×SSC buffer (3 M NaCl, 0.3 M sodium citrate) overnight. After UV crosslinking, the membrane was incubated with the DIG-labeled 381 bp probe for hybridization at 50°C for approx. 16 h. After stringent washes, the transcript was visualized by chemiluminescence using CSPD (Roche, Mannheim, Germany) as a substrate. Negative control (without RNA) was also run to check the specificity of the transcript expression.

Peptide synthesis. A 31 amino-acid peptide corresponding to residues N-KLMPKIV-SAIIYMVGQPNAGVTFLGHQCLVE-C (30–61) of SSAP was selected for synthesis after evaluating for B cell epitopes (http://tools.immuneepitope.org/tools/bcell/iedb_input) using the Kolaskar and Tongaonkar method and prediction of antimicrobial properties using the AMP database (http://aps.unmc.edu/AP/prediction/prediction_main.php). Peptides were synthesized co-linearly as a single molecule by the solid phase peptide synthesis using Fmoc amino acids on a full automated peptide synthesizer (Applied Biosystems, Foster City, CA, USA). Fmoc amino acids were activated using diisopropylcarbodiimide and hydroxybenzotriazole. Coupling was monitored for completion of the substitution using conductivity test and by estimation of the released Fmoc group at 324 nm. TFA-cleaved peptides were purified on a Waters C18 reversed phase column using a Pharmacia HPLC system. The peptide was eluted with a linear gradient of 0.1% TFA in water and 80% acetonitrile in 0.1% TFA in water. The peptide collected as a single peak was lyophilized and used for immunization.

Antibody production. About 1 mg of the peptide was coupled to keyhole limpet hemocyanin (Pierce, Rockford, IL, USA) following manufacturer's protocol. Two New Zealand White rabbits were immunized intraperitoneally with 200 μl of phosphate buffered saline (PBS) containing 100 μg of the peptide emulsified in complete Freund's adjuvant. The first immunization was followed by two boosts at 2-week intervals with aliquots of peptide emulsified in incomplete Freund's adjuvant. Antiserum was collected 10 days after the last injection. The anti-sera were checked by ELISA and the titres were found to be 1:8000. The specific anti-peptide antibody was purified *via* affinity chromatography over a cyanogen bromide-activated Sepharose column conjugated with the peptide fragment used as the immunogen.

Western blotting. *S. serrata* tissues were homogenized in a buffer containing 100 mM Hepes, pH 7.0, 10 mM $MgCl_2$, 30 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and the proteinase inhibitor mix and centrifuged at 14000 r.p.m. for 15 min. About 100–150 μg of proteins from various tissue lysates were separated on a 15% gel as described earlier. A colour protein mass ladder was loaded for reference. The proteins were transferred onto a nitrocellulose membrane and were incubated with anti-SSAP peptide rabbit polyclonal antibodies at a 1:100 dilution overnight, followed by horseradish

peroxidase-conjugated anti-rabbit antibody (Sigma) at 1:1000. Bands were visualized by chemiluminescence using the ECL advance Western blotting detection kit (GE Healthcare, Buckinghamshire, UK).

RESULTS

Identification of antimicrobial proteins

Freshly prepared lysates of granular hemocytes containing about 49 mg of protein were fractionated first on a CMC cation exchange resin. Fractions (2 ml) were eluted in 0.2 M ammonium acetate, lyophilized and tested for antibacterial activity (Fig. 1a). Fractions 29–32 and 35 were found to contain detectable growth inhibiting activity against the four bacterial strains tested (*E. coli*, *P. aeruginosa*, *S. aureus* and *S. pyogenes*) as demonstrated by radial diffusion assay (Fig. 1b). The Gram negative bacteria (*E. coli* and *P. aeruginosa*) seem to be more susceptible to the fractions than Gram positive ones tested (*S. aureus* and *S. pyogenes*). The active fractions were pooled (about 7.86 mg) and low molecular mass proteins were concentrated on an Amicon ultrafiltration unit with a 30 kDa cut-off membrane. The filtrate (about 2.28 mg) was lyophilized, reconstituted with 0.1% TFA and loaded onto a C_8 RP-HPLC column. The proteins were eluted in a three-step gradient as mentioned in Materials and Methods (Fig. 1c). The peaks (P1–P6) were collected, lyophilized and tested for antibacterial activity. Peak 5 demonstrated detectable antibacterial activity (Fig. 1d) and was re-loaded on the same column to check the homogeneity. The eluted fraction, silver stained on a 15% SDS/PAGE under denaturing conditions, showed the presence of a single protein band with a mobility corresponding to a molecular mass close to about 11 kDa (Fig. 1c) and was named SSAP.

Protein sequencing of SSAP

Following trypsin digestion, cysteine stabilization and HPLC, three fractions were sequenced by automated Edman degradation. The sequence of one peptide was VELEALK and the sequences of other two peptides were QPDGFYTAK and NFVQTASNYK.

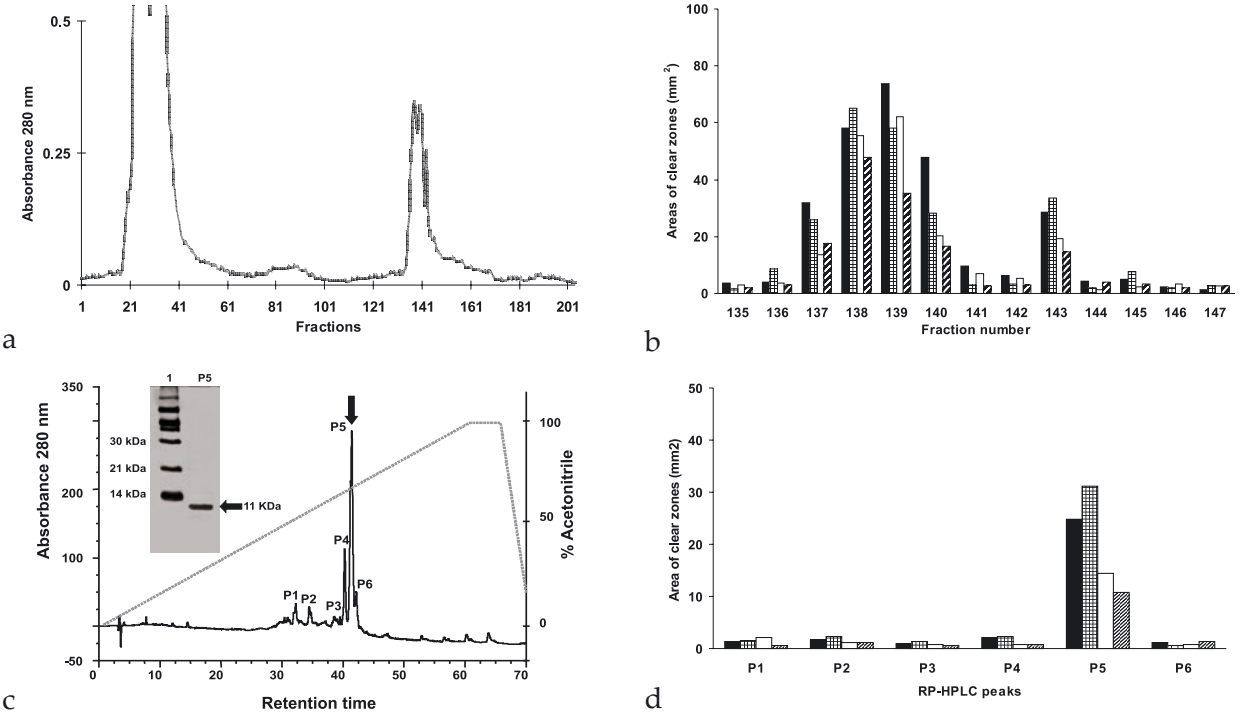

Figure 1. Purification and antimicrobial characterization of SSAP from granular hemocyte lysate of *S. serrata*.
(a) Protein profiles of dialyzed granular hemocyte lysates of *S. serrata* applied onto a CMC column. Unbound fractions were washed with 0.2 M sodium acetate (2 ml fractions) until about 214 ml of buffer passed through the column. Bound fractions were eluted with 0.2 M ammonium acetate. (b) Antimicrobial activity of fractions (2 ml) were tested against Gram negative *E. coli* (black fill) and *P. aeruginosa* (square fill), and Gram positive *S. aureus* (white fill) and *S. pyogens* (crossed fill), by the modified radial diffusion assay and expressed as area of inhibition (mm^2). (c) Elution pattern of proteins ≤30 kDa, concentrated by ultrafiltration, separated on a Vydac C_8 RP-HPLC column equilibrated with 0.1% (v/v) TFA/water. Elution (1 ml/min) was performed according to the procedure described in the experimental section. Peak P5 demonstrated a strong antimicrobial activity as tested by the modified radial diffusion assay against the bacteria described above (d). The active fraction showed a single protein band of 11 kDa on SDS/PAGE (inset of Fig. 1c.)

```
SSAP   1                                                          M  R
ssap   1 ACACACCCGCAACCTCTATCACCACCACAACATCCACTCGCCTCCAGACCCTCACAATGC
scyg   1        G            A                  G      A
SCYG   1

SSAP   3   S  S  L  L  L  G  L  T  V  V  V  L  L  G  V  I  V  P  P  C
ssap  61 GTTCATCTCTCCTACTCGGCCTTACAGTGGTGGTGCTGCTGGGCGTCATCGTGCCTCCAT
scyg  61                                  CT             C
SCYG   3                                  L              T

SSAP  23   M  A  G  Q  A  L  N  K  L  M  P  K  I  V  S  A  I  I  Y  M
ssap 121 GCATGGCAGGCCAGGCACTCAACAAACTTATGCCTAAAATCGTCAGCGCCATAATTTATA
scyg 121               T
SCYG  23

SSAP  43   V  G  Q  P  N  A  G  V  T  F  L  G  H  Q  C  L  V  E  S  T
ssap 181 TGGTCGGGCAACCCAATGCAGGTGTCACTTTTCTGGGCCACCAATGTCTGGTGGAGTCAA
scyg 181   A       G  G  C             C                          G
SCYG  43   I

SSAP  63   R  Q  P  D  G  F  Y  T  A  K  M  S  C  A  S  W  T  H  D  N
ssap 241 CGAGGCAACCAGACGGGTTTTACACCGCAAAGATGTCGTGTGCTTCCTGGACTCATGATA
scyg 241        G           C           A    G    A
SCYG  63                                   W    T

SSAP  83   P  I  V  G  E  G  R  S  R  V  E  L  E  A  L  K  G  S  I  T
ssap 301 ATCCTATTGTTGGGGAAGGAAGAAGCCGGGTTGAACTTGAGGCGCTTAAAGGTTCCATCA
scyg 301                                                 G
SCYG  83                                                          R

SSAP 103   N  F  V  Q  T  A  S  N  Y  K  K  F  T  I  D  E  V  E  D  W
ssap 361 CAAACTTTGTCCAGACAGCATCCAATTACAAGAAGTTCACCATAGATGAGGTCGAGGACT
scyg 361 G   T           G      G                    G  A
SCYG 103                        D                   F

SSAP 123   I  A  S  Y  *
ssap 421 GGATTGCTTCTTACTAAGACGCTCACCTGACGTGCTCTCCGAGTCCACCAAAGCTGTCTT
scyg 421                              C      A
SCYG 123

ssap 481 TGCTGGAGCCACTAAGAGTGGTTTGGTTATTGAGGACAATAAATAACTTTCTGAATTTTA
scyg 481      C          C      A

ssap 541 AAAAAAAAAAAAAAAAAAAAAAA
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Figure 2. The SSAP cDNA nucleotide sequence (ssap) and the predicted amino-acid sequence (SSAP) in comparison with scygonadin cDNA nucleotide (scyg) and amino-acid sequence (SCYG).
The numbers on the left of the sequence indicate the position of the first nucleotide and amino acid on each line. The nucleotide and amino acid substitutions in scygonadin compared to SSAP are denoted appropriately in grey fonts. The signal peptide sequence, polyadenylation signal and the stop codon are underlined in black. The partial amino-acid sequences obtained by Edman degradation are underlined in grey. The cDNA sequence has been deposited in the GenBank at accession number EF137865.

Cloning and sequencing of SSAP gene

From the peptides PDGFYT and ELEALK, two degenerate primers, DF and DR, were designed, respectively, and a 103 bp fragment of cDNA was amplified by RT-PCR from hemocytes of *S. serrata*. Based on the sequence obtained, primers (SF and SR) were designed and using the 5′ and 3′ RACE PCR approaches, 370 and 332 bp cDNA fragments were amplified, respectively, and the products were cloned and sequenced. The full-length *S. serrata* SSAP cDNA sequence was obtained by overlapping three cDNA fragments. The complete cDNA sequence and the deduced amino-acid sequence of SSAP of *S. serrata* are shown in Fig 2. The full-length SSAP cDNA consisted of 553 bp, containing an ORF of 381 bp, a 49 bp 5′ untranslated region, and a 123 bp 3′ untranslated region with a poly A signal. When compared with the complete non-redundant GenBank database using BLAST program, the SSAP cDNA sequence showed a high identity with scygonadin (94%) and scygonadin 2 precursor (77%). The translated protein sequence demonstrated significant homology with scygonadin (92%), scygonadin 2 precursor (64%) and anti-lipopolysaccharide factor (ALF) (29–35%) from various marine invertebrates (Table 1). Scygonadin is about 10.8 kDa anionic protein isolated from the male reproductive tract of *S. serrata* that inhibited growth of *Micrococcus luteus* and *Aeromonas hydrophila* and the gene coding for scygonadin has been well characterized (Huang *et al.*, 2006).

Based on the deduced polypeptide sequence, SSAP was found to be composed of 126 amino acids, including a 24 amino-acid signal sequence predicted by SignalP software (ExPASy) in the N-terminal region of the polypeptide chain. Therefore, the mature SSAP is composed of 102 amino-acids residues. Amino acids are numbered beginning with the first methionine (Fig. 2). The calculated molecular mass of the mature protein (102 amino acids) is 11435 Da with an estimated pI of 5.7. The SSAP cDNA sequence and deduced amino-acid sequence have been submitted to the NCBI GenBank as accession numbers EF137865 and ABM05493, respectively.

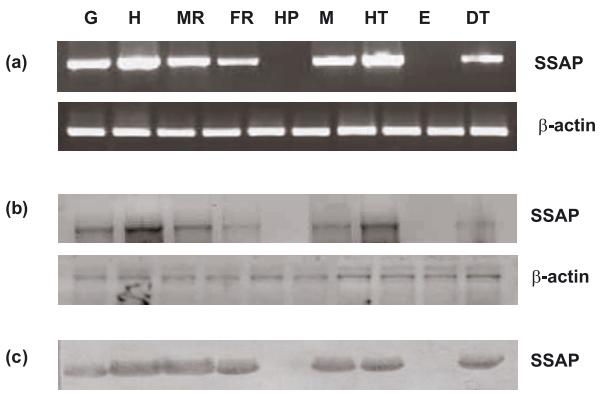

Figure 3. SSAP gene and protein expression profiles of male and female crab tissues.
(a) Tissue expression profiles of SSAP as demonstrated by RT-PCR with primers OF and OR. Visible expression was observed for the following tissues: gills (G), hemocytes (H), male reproductive tract (MR), female reproductive tract (FR), muscle (M), heart (HT) and digestive tract (DT). No expression was seen in hepatopancreas (HP) and eyes (E). A strong signal was observed for β-actin in all tissues analyzed. A similar expression pattern was observed with Northern hybridization (b), with a visible signal at about 550 bp (SSAP) and about 650 bp (β-actin), respectively. (c) Western blot analysis with a polyclonal antibody raised against a peptide corresponding to residues 30–61 of SSAP demonstrated a strong signal at about 11 kDa for G, H, MR, FR, M, HT and DT. No signal was observed for HP and E.

Expression profile by RT-PCR, Northern and Western blot

A one step RT-PCR with ORF-specific primers (OF/OR) and RNA from various tissues: gills, heart, hepatopancreas, reproductive tract, muscle, hemocytes and eyes, led to the amplification of about 381 bp expected fragment. While hemocytes, gills and reproductive tract produced a strongly expressed transcript, muscle, heart and digestive tract showed a comparatively weaker expression. No expression of SSAP gene was observed in hepatopancreas and eyes (Fig. 3a). A strong about 200 bp signal for β-actin was observed for all the tissues tested. Similar results were observed when the DIG-labeled about 380 bp PCR product was used to probe total RNA from various tissues transferred onto nylon membrane after separating on 1% formamide gel. The blot showed the presence of a strong, single transcript at about 550 bp (Fig. 3b). The detected fragment could be the complete mRNA (553 bp) coding for the SSAP gene including the 381 bp ORF sequence along with the 5′ and 3′ untranslated regions. Again, about 650 bp β-actin transcript was observed in all tissues. Nitrocellulose membrane blots of tissue lysate proteins incubated with anti-SSAP peptide antibodies expressed a clear signal at about 11 kDa for multiple tissues. Analogous to the results obtained by RT-PCR and Northern blot, no signal was obtained for heaptopancreas and eyes (Fig. 3c).

Antimicrobial characterization of the protein

The MIC assay with SSAP clearly demonstrated detectable antimicrobial activity against both Gram positive and Gram negative bacteria (Table 2). The higher susceptibility of Gram negative bacteria *E. coli* and *P. aeruginosa* as compared to the Gram positive *S. aureus* and *S. pyogenes* clearly indicates the possibility of SSAP being target-specific (e.g., against lipopolysaccharides) in respect to its antimicrobial activity. Similar experiments need to be performed with a broader panel of microorganisms including filamentous fungi and yeast.

DISCUSSION

The present study documents the presence of about 11 kDa antimicrobial protein, SSAP, in granular hemocytes of the Indian mud crab *S. serrata*. The protein demonstrated a significant sequence homology with that of scygonadin, also identified from *S. serrata*, both at the cDNA and protein level and displayed antimicrobial properties against both Gram-positive and Gram-negative bacteria. Yet there are significant differences observed at the tissue

Table 1. Homology of SSAP cDNA sequence with other sequences in the GenBank as analyzed using BLAST.

It is interesting to note that all the proteins highlighted in the BLAST search are known to have antimicrobial properties.

Protein	Organism	NCBI accession number	Number of amino acids	Homology (%)
Scygonadin precursor	*S. serrata*	AAW57403.1	126	92
Scygonadin 2 precursor	*S. serrata*	ABI96918.1	124	64
Anti-lipopolysaccharide factor (ALF)	*Litopenaeus schmitti*	ABJ90465.1	123	30
ALF	*Penaeus monodon*	ABP73291.1	120	29
ALF	*Pacifastacus leniusculus*	ABQ12866.1	120	29
ALF	*Farfantepenaeus paulensis*	ABQ96196.1	79	35
ALF	*Tachypleus tridentatus*	AAK00651	103	32
ALF	*Limulus polyphemus*	P07086	101	33
ALF	*S. paramamosain*	ABP96981	123	23

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ALFLITO      MRVSVLTSLVVAVFLVALFAPECQAQGWQAVAAAVASKIVGLWR--NEET 48
ALFPEN       MR--VLVSFLMALSLIALM-PRCQGQGVQDLLPALVEKIAGLWH--SDEV 45
ALFPAC       MRTWVLVT---VLSLVVVLQQPCQAQVPPEVVSLIISKLVNLWS--DGQV 45
ALFTACHY     ----------------------MEGGIWTQLALALVKNLATLWQ--SGDF 26
ALFLIM       ----------------------MEDGIWTQLIFTLVKNLATLWQ--SGDF 24
SSAP         MRSSLLLGLTVVLLLGVTVPPCMAGQALNKLMPKIVSAIIYMIGQPNAGV 50
SCY2PREC     MRPSFLFGLTVVVLLGVSVPACQAGLALNRLMNKAVDAIVYMVGQQDAGV 50
                                           :    . :  :    .

ALFLITO      ELLGHKCRFTVKPYIKRLQLHYKGKMWCPGWTPIT---GEARTRSHSGVA 95
ALFPEN       EFLGHSCRYSQRPSFYRWELYFNGRMWCPGWAPFT---GRSRTRSPSGAI 92
ALFPAC       EFMGHTCNYSYSPTISKFQLYYKGKMWCPGWAPFS---GNSKTKSRAGSI 92
ALFTACHY     QFLGHECHYRVNPTVKRLKWKYKGKFWCPSWTSIT---GRATKSSRSGAV 73
ALFLIM       QFLDHECHYRIKPTFRRLKWKYKGKFWCPSWTSIT---GRATKSSRSGAV 71
SSAP         TFLGHQCLVES---TRQPDGFYTAKMWCTSWTSDNPIVGEGRSRVELEAL 97
SCY2PREC     SLLGHPCLVES---AKQPEGIYTAVMSCASWTPR--FVGEGTSEVELEAL 95
              ::.* *          : .  :.. : *..*:.     *.. .

ALFLITO      GRTARDFVQKAFERGLISEQDAKRWLSS-- 123
ALFPEN       EHATRDFVQKALQSNLITEEDARIWLEH-- 120
ALFPAC       EHATRDFVTKAIDQKLITAEQASAWIKN-- 120
ALFTACHY     EHSVRDFVGQAKSSGLITEKEAQTFISQYQ 103
ALFLIM       EHSVRNFVGQAKSSGLITQRQAEQFISQYN 103
SSAP         KGSIRNFVQTASDYKKFTIEEVEDWIASY- 126
SCY2PREC     KGSIRSFIRKASDYQLLSKEDLEDWLASY- 124
               : *.*:  * .   :: .:   ::
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Figure 4. Multiple sequence alignment with Clustal W and secondary structure prediction with JPred of SSAP and related proteins.
Analyzed were: anti-lipopolysaccharide factor (ALF) *L. schmitti* (ABJ90465.1), ALF *P. monodon* (ABP73291.1), ALF *P. leniusculus* (ABQ12866.1), ALF *T. tridentatus* (AAK00651), ALF *L. polyphemus* (P07086), SSAP *S. serrata* (ABM05493.1), scygonadin 2 precursor *S. serrata* (ABI96918.1). The numbers on the right of the sequence indicate the position of the last amino acid for a protein in each line. The secretory signal sequences predicted by SiganlP are denoted in italics, the predicted α-helical domains are in bold grey (underlined) and the predicted β-sheets are highlighted in bold black.

expression level and the physical properties of the protein. Granular hemocytes were used as a source for isolation of proteins as it generated a higher yield of active protein for a given amount of starting material than with whole hemolymph. A similar approach was used earlier to isolate a cysteine-rich antibacterial protein from the shore crab *Carcinus maenas* (Schnapp *et al*., 1996).

It is interesting to note that SSAP showed 94% sequence homology with scygonadin, both at the nucleotide and amino-acid level. Scygonadin was reported as a 10.8 kDa antibacterial protein isolated from the seminal plasma of *S. serrata* (Huang *et al*., 2006). Further studies with RT-PCR and Northern blot established the expression of the scygonadin gene as male-specific, with tissue expression limited to the ejaculatory duct (Wang *et al*., 2007). Analogous to scygonadin, the full length cDNA sequence of SSAP is also composed of about 539 bp and codes for a 126 amino-acid molecule with a 24 amino-acid signal peptide and a 102 amino-acid mature protein. Full length cDNA sequence comparisons of SSAP with that of scygonadin showed 33 nucleotide substitutions of which a few led to amino acid changes at 8 residues (Fig. 2). These alterations justify the difference in the calculated mass and the pI of the two proteins. While SSAP demonstrates a mass and pI of 11435 Da and 5.77, respectively, scygonadin displayed a mass of 11271.8 Da and a pI of 6.09.

While scygonadin is expressed exclusively in the male crabs and limited to the ejaculatory duct, SSAP is expressed in multiple tissues of both male and female crabs. In crabs of either sex, hemocytes, gills and reproductive tract showed a stronger expression both at protein and RNA level as evidenced by Western and Northern blots, respectively. It has been documented that both hemocytes and gills of marine animals play a very important role in regulating the innate immune mechanisms to combat microbial invasion. Hemocytes are known to be a rich source of antimicrobial proteins and peptides, and exhibit a complex system of innate defense mechanisms involving cellular and humoral responses influenced by the presence of microbial components such as lipopolysaccharides (LPS) or β-1,3-glucans, once pathogens gain entry into the hemocoel of the host (Lee & Soderhall, 2002; Iwanaga & Lee, 2005). Similarly, gills in marine organisms are covered with only a single layer of mucus-lined fragile cells and, being constantly flushed with water, that may contain pathogens, constituting a very important site of pathogen entry. Therefore, potent AMP can be expected to be found in gills to prevent such an entry as reported in several species including fish and crabs (Smith & Ratcliffe, 1989; Iijima *et al*., 2003).

There is also strong evidence for regulated expression of AMPs in the male and female reproductive tracts of various species. Andropin from male *Drosophila melanogaster*, ceratotoxin from fe-

Table 2. The antibacterial activity of purified SSAP determined as MIC.

Serial dilutions of SSAP (6.25–200 μg ml^{-1}) were tested against bacteria. MIC are expressed as the interval *a–b*, where *a* is the highest concentration tested at which microorganisms are able to grow and *b* the lowest concentration that causes 100% growth inhibition.

Microorganism	MIC range (μg ml^{-1}) [*a–b*]
Escherichia coli	25–50
Pseudomonas aeruginosa	12.5–25
Staphylococcus aureus	50–100
Streptococcus pyogenes	25–50

male medfly *Ceratitis capitata* and β-defensins from humans and rhesus are good examples (Samakovlis *et al.,* 1991; Marchini *et al.,* 1995; Quayle *et al.,* 1998; Harder *et al.,* 2001). The expression and functions of other AMPs expressed in human reproductive tracts have also been reviewed elsewhere (Hall *et al.,* 2002; Wira & Fahey, 2004).

Wang and his group (2007) have studied the expression of scygonadin in male gonads of three groups of animals based on their body mass: small (46.33 ± 1.53 g), medium (112.67 ± 10.02 g) and large or mature (216.33 ± 34.39 g). While in the small and medium groups of animals no scygonadin transcripts were detected owing to underdevelopment of the gonads, the expression was quite strong in large animals inferred to possess mature and developed gonads. In our study, the animals of either sex weighed around 193.56 ± 27.04 g, which located them between the medium and large category as described above.

Apart from the high identity of SSAP with scygonadin, it is of interest to note that the protein shows a significant sequence homology (30–35%) to a family of proteins known as anti-lipopolysaccharide factor (ALF). ALFs are small proteins that can bind and neutralize lipopolysaccharide (LPS). The proteins described in Table 1 were aligned using CLUSTAL W (http://www.ebi.ac.uk/tools/clustalw2) and their secondary structures predicted from amino-acid sequences with the JPRED tool (http://www.compbio.dundee.ac.uk/~www-jpred). The results described in Fig. 4 clearly highlight the common features among the listed proteins, namely the presence of a fairly conserved secretory signal sequence, appropriately placed α-helical and β-sheet domains and highly conserved cysteine residues.

The first ALF, originally called LALF, a 11.8 kDa protein identified from the amebocyte of the horseshoe crab *Limulus polyphemus* was shown to bind and neutralize LPS and demonstrated a strong antibacterial effect on Gram-negative R-type bacteria (Morita *et al.,* 1985). Recently, several ALFs have been isolated and characterized from hemocytes in several species of shrimps, while some were discovered from an EST library of the shore crab *C. maenas* and the blue crab *C. sapidus* (Towle & Smith, 2006; Imjongjirak *et al.,* 2007). Recently, Imjongjirak and colleagues (2007) have been successful in isolating a full-length cDNA encoding for an 11.18 kDa ALF protein (ALFSp) from hemocytes of mud crab, *Scylla paramamosain*. Tissue distribution analysis revealed that ALFSp was abundantly expressed in hemocytes, intestine, and muscle but not in eyestalk, and a synthetic ALFSp peptide containing putative LPS binding domain revealed a strong antimicrobial activity against *Micrococcus luteus* and *Vibrio harveyi* (Imjongjirak *et al.,* 2007). It is still not known if *S. serrata* expresses a homolog of ALFSp in either of its tissues. But an interesting observation from the MIC studies was the increased susceptibility of Gram negative bacteria, *E. coli* and *P. aeruginosa* to SSAP compared to Gram positive *S. aureus* and *S. pyogenes.* Further experiments will have to be executed to analyze if SSAP has LPS binding properties.

It is unclear why SSAP and scygonadin demonstrate differential tissue expression in spite of their almost full identity at the protein and cDNA levels. One possibility is that SSAP could possibly be an isoform or a differentially expressed variant of scygonadin with the expression influenced by factors present in the tissue. Similar observations were made by Zhao and colleagues (1999) where goat defensin precursors GBD-1 and GBD-2, identical in 96.8% of their bases and 88.2% of their amino acids, exhibited differential expression in the digestive and respiratory tracts. In yet another study, Huttner and colleagues (1998) detected two distinct sheep β-defensin cDNA sequences, SBD1 and SBD2, which shared 87% identity at the nucleotide level and 78% identity at the protein level. While both the isoforms were expressed with same intensity at 130 day gestation period, the gene expression varied in adult animals, with SBD1 expression retained in multiple tissues of the digestive tract and that of SBD2 restricted to ileum and colon. Further experiments are warranted to elucidate if SSAP is a variant of scygonadin and if they demonstrate different tissue expression patterns as described above.

The partially purified protein fractions obtained from ion exchange chromatography and RP-HPLC were tested against bacterial isolates from vagina of patients tested positive for BV. BV is a common condition with numerous health problems affecting millions of women annually, characterized by an alteration of genital tract flora and associated with increased susceptibility to HIV infection (Spear *et al.,* 2007). The intention of testing the identified protein against bacteria involved in BV was to develop the molecule as a microbicide to combat the spread of sexually transmitted infection (STI)-causing pathogens, including HIV. Microbicides are anti-infective preparations for topical self-administration prior to sexual intercourse to protect against the transmission of HIV and other STIs and recently AMPs have been analyzed for their use as a microbicide. Excellent reviews suggest that AMPs can be highly effective in inhibting the growth of STI-causing pathogens and HIV (Reddy *et al.,* 2004; Cole, 2005).

SSAP is similar to many other AMPs as they are translated with a signal, mature and a propiece domain (Hughes & Yeager, 1997). The translated protein sequence of SSAP is composed of a sig-

nal peptide of 24 amino acids with a hydrophobicity of 62.5% which is an ideal characteristic of such sequences. It is followed by a mature protein sequence of 102 amino acids with a high concentration of negatively charged residues in the C-terminal, a characteristic of an anionic propiece seen in many mature AMPs. It is generally proposed that the anionic properties of the propiece help in neutralizing the cytotoxicity of the mature protein or peptide until required *in vivo* (Hughes & Yeager, 1997). The net charges of SSAP were calculated as +2 based on the number of positively (arginine, histidine and lysine) and negatively charged residues (aspartic and glutamic acids). It could be postulated that the cytotoxicity of the protein is attributed to the overall net positive charge and a high content of hydrophobic residues (51%) at the N-terminal region of the mature protein. It is likely that SSAP, like other cationic peptides, might be able to bind to anionic components of microbial membranes and kill them by formation of pores or by permeabilizing the cell membranes (Brogden, 2005).

In summary, it can be concluded from our study that SSAP is a multi-tissue expressed antimicrobial protein that demonstrates antibacterial properties *in vitro*. However, it is unclear if the protein demonstrates similar defense properties against invading microbes *in vivo* or if it has any role in the development of the animal. Further experimentation is required to understand the nature of induction of SSAP in response to microbial invasion and also to study the exact mechanism of its action. Future studies will be aimed at expressing the protein in a suitable recombinant system enabling the production of larger amounts of active protein to carry out the above-mentioned experiments.

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