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A male-specific expression gene, encodes a novel anionic antimicrobial peptide, scygonadin, in *Scylla serrata*

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Abstract

Scygonadin is a novel antimicrobial peptide, which was originally isolated from the seminal plasma of the mud crab, *Scylla serrata*. Based on the partial 20-residue NH_2-terminal sequence of the peptide, H-Gly-Gln-Ala-Leu-Asn-Lys-Leu-Met-Pro-Lys-Ile-Val-Ser-Ala-Ile-Ile-Tyr-Met-Val-Gly-OH, scygonadin was cloned from the gonads of *S. serrata* using a degenerated reverse transcriptase (RT)-PCR and rapid amplification of cDNA ends (RACE). The full-length cDNA sequence contains an open reading frame of 539 bases (excluding polyA) with a coding capacity of 126 amino acids, which constitutes a putative NH_2-terminal signal sequence (1–24) and a mature peptide (25–126). Analysis of the genomic DNA sequence revealed that scygonadin consists of 2300 bp containing two introns (1569 and 120 bp) and three exons (187, 131 and 218 bp) and this sequence is different from any other reported antimicrobial peptide. The theoretical p*I* of the mature peptide is 6.09, which suggests that it is an anionic molecule. The sex and tissue-specific expression of the scygonadin gene was revealed using RT-PCR and Northern-blot analysis of multiple tissues of *S. serrata* males and females and this demonstrated that the scygonadin gene was predominantly expressed in the male reproductive tract of *S. serrata* and was restricted to the ejaculatory duct. This suggests that scygonadin might be one of the antibacterial peptides responsible for protection of the male crab reproductive tract from invading pathogenic microorganisms, so as to maintain a sterile environment leading to successful fertilization.

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Keywords: *Scylla serrata*; Scygonadin; Antimicrobial peptide; Male-specific; Gene expression; Genomic organization

1. Introduction

As a major part of the immediately effective, non-specific and natural defenses of most living organisms against invading pathogens, antimicrobial peptides (AMPs) play a crucial role in the innate immune system. AMPs can be classified into five groups according to their biochemical properties and chemical structures: cationic peptides, anionic peptides, aromatic dipeptides, peptides derived from oxygen-binding proteins and peptides processed from neuropeptide precursors (Salzet, 2002). The first anionic antimicrobial peptides discovered in sheep (Brogden et al., 1996) contain homopolymeric regions of aspartate and require zinc as a cofactor for their antimicrobial activity against both Gram-positive and Gram-negative organisms. Recently another anionic antimicrobial peptide with a broad spectrum of antimicrobial activity, dermcidin, was characterized by Schittek et al. (2001) in mammals. It was specifically and constitutively expressed in human sweat glands, secreted into the sweat and transported to the epidermal surface (Schittek et al., 2001). In the amphibian, *Bombina maxima*, an anionic peptide, Maximin H5, with antimicrobial activity only against Gram-positive bacteria (*Staphylococcus aureus*) was identified. However, it differed from the anionic peptides from sheep in that the metallic ions, Zn^{2+} and Mg^{2+}, did not increase its antimicrobial potency (Lai et al., 2002). More recently, an 86-amino acid residue linear anionic antimicrobial peptide, theromyzin, has been isolated and characterized in annelids (*Theromyzon tessulatum*) and it exhibited bacteriostatic activity against a

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Gram-positive bacterium (*Micrococcus luteus*) (Tasiemski et al., 2004). The mode of action of the anionic peptides is still not fully understood.

Several sex-specific antimicrobial peptides have been characterized in earlier publications (Samakovlis et al., 1991; Marchini et al., 1995; Li et al., 2001). Andropin was the first gene to be identified as male-specific in *Drosophila melanogaster* and its expression is exclusively restricted to the ejaculatory duct in the male reproductive tract (Samakovlis et al., 1991). Ceratotoxins A, isolated from the female reproductory system of *Ceratitis capitata*, is expressed specifically in the accessory glands of sexually mature females where the production of the ceratotoxin peptide occurs (Marchini et al., 1995). Scygonadin is a novel anionic antibacterial peptide, which was originally isolated from the seminal plasma of the mud crab, *Scylla serrata* (Huang et al., 2006). However, it was not clear from the previous data whether the peptide was male-specific or produced dominantly at the site of peptide isolation. The present study is aimed at revealing the complete cDNA and genomic DNA of this novel antimicrobial peptide and, also, to elucidate its expression in multiple tissues.

2. Materials and methods

2.1. PCR cloning

S. serrata were obtained from a local aquarist and maintained in an aerated glass aquarium at 25 °C and fed twice a day with commercial food. Crabs were sacrificed and tissues, including exoskeleton, subcuticular epithelia, gills, heart, hepatopancreas, stomach, gonad, muscle, hemocytes and eyes were carefully dissected, for RNA extraction.

For cloning the full-length sequence of scygonadin, the total RNA was extracted from the gonads of male crabs using Trizol (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized using a 3′-Full RACE Core Set (TaKaRa) with Adaptor-dT as a primer. Based on the partial N-terminal peptide sequence"H-Gly-Gln-Ala-Leu-Asn-Lys-Leu-Met-Pro-Lys-Ile-Val-Ser-Ala-Ile-Ile-Tyr-Met-Val-Gly-OH" of scygonadin obtained by Edman degradation in our previous study (Huang et al., 2006), two forward degenerate oligonucleotide primers F1 and F2 (Table 1) were designed from Asn(5) to Val(12) and Ser(13) to Met(18), respectively. The 3′ end region of scygonadin was amplified by semi-nested PCR with two primer pairs of Adaptor/F1 (first round PCR) and Adaptor/F2 (second round).

PCR was performed in a total volume of 25 μL including 2.5 μL 10× Ex Taq Buffer (Mg^{2+} plus, supplied with TaKaRa Ex Taq^{TM}), 4 μL 2.5 mM dNTP Mixture, 1U TaKaRa Ex Taq^{TM}, 1 μL 20 μM PCR primer F1, 1 μL 20 μM primer Adaptor, 5 μL RT reactant and 11 μL sterile distilled water. The first PCR scheme was: 94 °C, 2 min, followed by 31cycles of 94 °C for 30 s; 47 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 10 min. The second PCR scheme was similar to the first one except that the annealing temperature was changed to 51 °C, the forward PCR primer was F2 and the template was 1 μL product of the first PCR product. PCR products were analyzed by electrophoresis in a 2.0% agarose gel in TAE buffer (Tris–Acetic acid–EDTA). The band of second PCR products was excised from the gel, extracted using a QIA quick gel extraction kit (Qiagen), cloned into the pMD_{18}-T vector (TaKaRa) and sequenced (Invitrogen).

To amplify the 5′ end of scygonadin, the SMART^{TM} RACE cDNA Amplification Kit (BD Biosciences Clontech) was used according to the manufacturer's instructions. The male gonad cDNA was synthesized using a modified lock-docking Oligo (dT) primer [termed the 5′-RACE CDS Primer (5′-CDS)] and the SMART II A Oligonucleotide. Two gene-specific primers SR1 and SR2 (Table 1) were designed following the manufacturer's instructions. The 5′ end region of scygonadin was amplified by nested PCR with two primer pairs of UMP/SR2 (first round PCR) and NUMP/SR2 (second round). The PCR scheme was: 94 °C, 1 min, followed by 29 cycles of 94 °C for 30 s, 68 °C for 1 min and a final extension at 72 °C for 10 min. The PCR products were cloned into the pMD_{18}-T vector (TaKaRa) and sequenced (Invitrogen).

Table 1

	Sequence (5′ → 3′)	Use
SR1	GCACACGACATCTTTGCGGTGTAGAAC	5′RACE
SR2	GAAGCAATCCAGTCCTCGACCTCATCTATG	5′RACE
5′-CDS	$(T)_{25}N_{-1}N$ (N = A, C or T; N-1 = A, G or C)	Reverse transcription
SMART II A Oligonucleotide	AAGCAGTGGTATCAACGCAGAGTACGCGGG	Reverse transcription
UPM	CTAATACGACTCACTATAGGGCAACGCAGAGT	5′RACE
NUPM	AAGCAGTGGTATCAACGCAGAGT	5′RACE
Adaptor-dT	CTGATCTAGAGGTACCGGATCC-dT(17)	Reverse transcription
Adaptor	CTGATCTAGAGGTACCGGATCC	3′RACE
F1	AA(T/C)AA(A/G)(T/C)T(A/C/T/G)ATGCC(A/C/T/G)AA(A/G)AT(A/C/T)GT	3′RACE (degenerate)
F2	GCGCGGAATTCGC(A/C/T/G)AT(A/C/T)AT(A/C/T)TA(T/C)ATGGT	3′RACE (degenerate)
SF	CACTCGCCTCCAGACCCTCACAATG	Sequence confirm
SR3	TAGCGGCCGCGTAAGAAGCAATCCAGTCCT	Expression and probe labeling
SF2	CGGAATTCGGCCAGGCACTCAACAAAC	Expression and probe labeling
18S-F	GGACTGGCGCTCTTGGAT	Expression and probe labeling (control)
18S-R	TTCACCTACGGAAACCTTGT	Expression and probe labeling control

2.2. Genomic DNA amplification, PCR and sequencing

Two hundred milligrams of muscle tissue was collected from freshly dissected *S. serrata* and ground into a fine powder in liquid nitrogen. The cold powder was added to a lysis buffer (50 mM Tris–HCl pH 8.0, 50 mM EDTA, 1% SDS, 10 mM NaCl), followed by addition of proteinase K (20 mg mL^{-1}) for 3 h at 55 °C. RNase (10 mg mL^{-1}) was then added and the mixture incubated for 1 h at 37 °C. After two phenol–chloroform extractions and one chloroform extraction, the muscle DNA was precipitated by addition of 0.5 mL ethanol. The DNA pellet was washed twice with 70% ethanol, air dried for 10 min at room temperature and finally re-dissolved in 25 μL MillQ H_2O.

Fifty micrograms of the extracted DNA was used as a template in 100 μL PCR mixtures in combination with 0.1 mM final concentrations of the forward primer SF and reverse complementary primer SR2. An LA Taq system (TaKaRa) was used for amplification of the scygonadin genomic DNA in MJ Research PTC200 with the programmed temperature profile (94 °C, 5 min; 30 cycles of 94 °C, 30 s; 62 °C, 2 min; 72 °C, 2 min). Any PCR amplification product was detected after agarose gel electrophoresis and excised from the gel, extracted using QIA quick gel extraction kit (Qiagen) and cloned into the pMD_{18}-T vector (TaKaRa). Several clones from each transformation were picked out to be sequenced and analyzed. Intron positions were identified by comparison with the cDNA sequence.

2.3. Expression study

The mature fragment of scygonadin cDNA was amplified from a recombination plasmid (a construct of our laboratory) with an insert of the complete cDNA of scygonadin using a pair of primers SF2 and SR3 (Table 1), as a template for making a specific scygonadin cDNA probe. The PCR products were purified, sequenced and labeled with digoxigenin using the Dig High Prime DNA-labeling and Detection Starter Kit I (Roche Applied Science). A pair of primers 18S-F and 18S-R (Table 1), which was designed to amplify the conserved region of 18S rRNA cDNA, was used to evaluate each sample tissue of *S. serrata* for cDNA yield and quality. The PCR products were purified, sequenced and labeled with digoxigenin to produce an 18S rRNA cDNA probe to be used as an internal control for Southern-blot analysis.

To examine the gene expression of scygonadin in tissues, three healthy crabs were carefully dissected. Tissues (including exoskeleton, subcuticular epithelia, gills, heart, hepatopancreas, stomach, reproductive tract, muscle, hemocytes and eyes) were collected for RNA extraction. Total RNA was extracted using Trizol as described above.

2.3.1. RT-PCR

For RT-PCR, the first strand cDNA was synthesized using oligo (dT) primer and Avian myeloblastosis virus reverse transcriptase (AMV-RT). 18S primers (Table 1) were used as a positive control for PCR to verify the cDNA templates. Primers specific to mature fragments of scygonadin genes (SR3 and SF2) were synthesized and their sequences are shown in Table 1. The PCR program was as follows: one cycle of 94 °C for 3 min, 32 cycles of 94 °C for 40s, 55 °C for 40s (for scygonadin gene, the temperature is 60 °C) and 72 °C for 40 s, followed by a cycle of 72 °C for 5 min.

2.3.2. Southern-blot

Southern-blot was used to confirm the PCR products using one specific scygonadin cDNA probe and one probe for 18S rRNA cDNA. The PCR products (10 μl) obtained from *S. serrata* reproductive tract total RNA were transferred from the 2% agarose gel to the membrane by vacuum blotting. The membrane was prehybridized at 50 °C for 30 min and subsequently hybridized at 50 °C for 12 h with both probes. The membrane was washed in 0.5× salt–sodium citrate (SSC) at 68 °C and signals detected using the Dig High Prime DNA-labeling and Detection Starter Kit I(Roche Applied Science).

2.3.3. Northern-blot

For hybridization, 10 μg RNA from each tissue was denatured in formaldehyde-containing buffer and incubated at 68 °C for 15 min, then transferred onto a Hybond N+ membrane (Amersham Biosciences) using a vacuum-filter and UV-crosslinking. Samples of tissues involved were from the ejaculatory ducts. Hybridization was carried out for 20 h at 50 °C for the specific scygonadin cDNA probe. After a stringency wash, immunological detection was performed on the membrane following the kit protocol.

2.4. Sequence analysis

Sequence data were analyzed using DNAStar software. PCR primers were designed using Primer 5. A homology search was performed with BLASTN2.1.3 and BLASTP 2.1.2 by NCBI Net WWW Server (http://www.ncbi.nlm.nih.gov/blast). The amino-terminal signal sequence was predicted using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/signalP). Both mass and p*I* of the putative peptide was calculated separately using the ProtParam tool by ExPASy (http://www.expasy.org/tool/protparam). The ORF was predicted using ORF Finder (http://www.ncbi.nih.gov/gorf/gorf.html).

3. Results

3.1. Cloning and sequencing of scygonadin cDNA

The degenerate RT-PCR and Rapid Amplification of cDNA Ends (RACE) methods were used to fully identify the complete cDNA sequence of scygonadin. A 396 bp cDNA fragment was first obtained by degenerate PCR (primers F1 and Adaptor), followed by semi-nested PCR (primers F2 and Adaptor) and identified by sequencing (Fig. 1). Its deduced amino acid sequence contained "H-Ile-Ile-Tyr-Met-Val-Gly-OH", which was identical to the partial sequence of N-terminal amino acids (15–20) of scygonadin previously obtained from Edman degradation (Huang et al., 2006). The 5′ end of scygonadin was then amplified using the SMART RACE method. Sequences of these cDNA

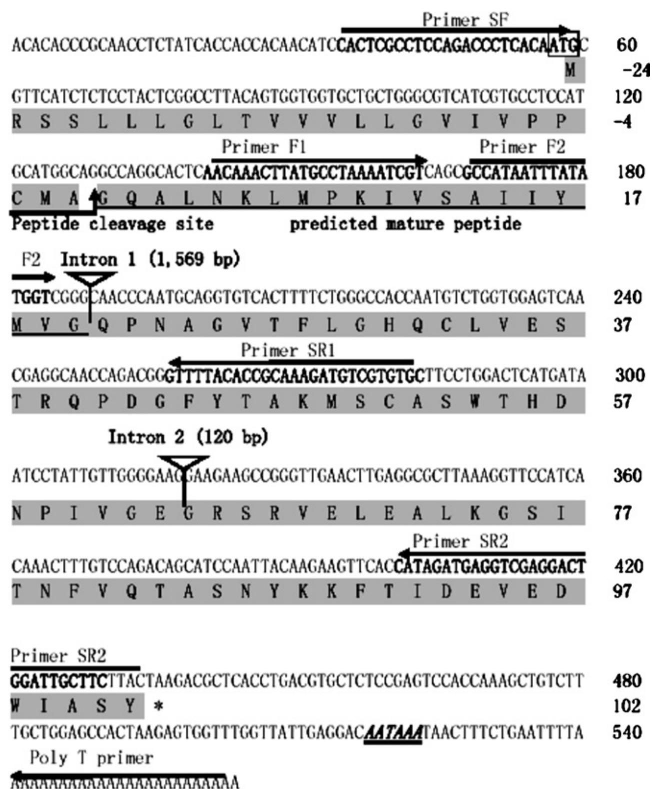

Fig. 1. Nucleotide sequence of mud crab scygonadin cDNA and predicted amino acid sequence. The numbers on the right of the sequence give the positions of the last nucleotide and amino acid on each line, respectively. Binding sites for primers are shown with arrows (5′ → 3′). Polyadenylation signal is underlined in the 3′UTR. The predicted organization of the peptide domains (signal peptide and mature peptide) is shown by gray boxes. The stop codon is indicated with an asterisk (*). Splicing sites for introns 1 and 2 are indicated with triangles. The partial N-terminal amino acids obtained using Edman degradation are underlined in the mature peptide. The GenBank Accession number is AY 864802.

fragments were confirmed by sequencing of RT-PCR products with primers SF and SR2. Finally, the full-length cDNA sequence of scygonadin was determined from the reproductive tract RNA of the unchallenged *S. serrata* (Fig. 1).

The full-length cDNA of scygonadin (GenBank accession no. **AY 864802**) is 539 bp including a 5′ UTR of 56 bp and a 3′ UTR of 127 bp with the putative polyadenylation consensus signal (AATAAA) and 16 additional nucleotides before the poly(A) tail. The complete cDNA of scygonadin contains an ORF of 381 bases, encoding a 126-amino acid protein, including a start codon (Met) and a stop codon (TAA). Its propeptide was predicted to be composed of two domains with a hydrophobic signal peptide (24 amino acids) and a mature peptide (102 amino acids). The deduced amino acid sequence begins with a 24-residue signal peptide rich in hydrophobic amino acids (H-Met-Arg-Ser-Ser-Leu-Leu-Leu-Gly-Leu-Thr-Val-Val-Val-Leu-Leu-Gly-Val-Ile-Val-Pro-Pro-Cys-Met-Ala-OH). The putative cleavage site for signal peptidase is probably located after the Ala_{24} residue, which was predicted using SignalP 3.0. The predicted cleavage site for the hydrophobic signal peptide coincided with the amino terminus of the mature peptide reported previously (Huang et al., 2006). This suggests that the mature peptide may be generated through conventional processing mechanisms and is possibly secreted to the extra-cellular medium (Tasiemski et al., 2004).

The deduced mature peptide constitutes 31 polar amino acids and 35 apolar amino acids. The theoretical p*I* of mature scygonadin is 6.09 (10 negative charge amino acids, 9 positive charge amino acids) and thus it is suggested to be an anoinic molecule. Searches in the SWISS-PROT protein data-base revealed no significant sequence homology to other antimicrobial peptides and showed low identity with other crustacean antimicrobial peptides, such as penaedin (Destoumieux et al., 1997; Cuthbertson et al., 2004; Chiou et al., 2005; O'Leary and Gross, 2006), chelonianin (Chen et al., 2005), carcinin (Brockton et al., 2007) and callinectin (Khoo et al., 1999).

3.2. Genomic DNA determination of scygonadin

Primer SF and SR2 were used to obtain a 2.1 kb amplification product from *S. serrata* genomic DNA (GenBank accession no. **DQ059303**, Fig. 2). In comparison to the scygonadin cDNA sequence, the gene is composed of two introns and three exons. The scygonadin gene appears to contain typical spliceosomal introns with donor and acceptor (GT and AG) dinucleotide sequences found in most vertebrate and invertebrate splice sites (Brunak et al., 1991). The three exons of 187, 131 and 218 bases are separated by two introns. Intron 1 is longer and contains 1569 bases with a repeat sequence (CT) of 108 nucleotides, which matched one of the microsatellite sequences of *S. serrata* (**AF508132** and **AF508131**), while intron 2 is much shorter with only 120 bases. Exon 1 encodes for the signal peptide and the partially mature peptide.

3.3. Expression analysis of scygonadin gene in naturally raised *S. serrata*

The sex-specific transcript and expression restriction of the scygonadin gene was investigated by RT-PCR and Northern-blot analysis of total RNA isolated from multiple tissues, including exoskeleton, subcuticular epithelia, gills, heart, hepatopancreas, stomach, female/male reproductive tract, muscle, hemocytes and eyes of naturally raised male and female *S. serrata* (∼200 g/crab). RT-PCR assay was performed using a primer pair specifically designed for amplification of the mature fragment of scygonadin cDNA. The results from RT-PCR analysis showed that scygonadin gene expression was not detectable in any tested tissues of mature female *S. serrata* (Fig. 3), while a high level of scygonadin gene expression was observed in the male reproductive tract (Fig. 4). As an internal control, a clear and strong 18S product was amplified in each sample analyzed, confirming good quality of the cDNA templates. Because scygonadin was originally isolated from the gonad of the mature male crab, we wondered if the scygonadin gene is exclusively expressed in the mature male crab rather than immature crabs. Considering that the gonad maturity of a healthy *S. serrata* is normally related to its body weight, three different weights (45–240 g/crab) of male crabs purchased from a local aquarist were chosen to investigate scygonadin transcripts by RT-PCR. One group of three smaller crabs (46.33 ± 1.53 g), whose gonads

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                                            Primer SF
ACACACCCGCAACCTCTATCACCACCACAACATCCACTCGCCTCCAGACCCTCACAATGC   60
                                                          M   -24
GTTCATCTCTCCTACTCGGCCTTACAGTGGTGGTGCTGCTGGGCGTCATCGTGCCTCCAT   120
R  S  S  L  L  L  G  L  T  V  V  V  L  L  G  V  I  V  P  P    - 4
GCATGGCAGGCCAGGCACTCAACAAACTTATGCCTAAAATCGTCAGCGCCATAATTTATA   180
C  M  A  G  Q  A  L  N  K  L  M  P  K  I  V  S  A  I  I  Y    17
TGGTCGGGTAAGTTGAGAATAATAGACTTATTTGAATCCCCAGTATCAGTTTGTAGCAAT   240
M  V  G                                                       20
CAAAAACGCCGAAAGGAGAATAGGAATGCCTATGAAAGCCATCTAATGGAGAGTTACTGC   300
CGAGGAAGAGATGAGTAAATTTATAAAAGGCTTTCTAAATGCTTATAATCAGAAAAAAAA   360
ATGCAAGTAATGCAGGCAAAAAAAGTAGAGCAAATGATAATAATCTGATATTTCCACAAC   420
AAACGAGATAGTGGCTGAGAGATATTTCCAAAACTATTTAGATAGTGCAATAGGAGATGG   480
GTAGGCTGTAGAATTTAAGTCGCCAGGACAAAATTAAATATATTCCTCATTAATGAAAGA   540
ATATAATTTATTTATTTATTTTTTTAATAAAATGCTAGTTAATAAAACTAGGAAATCGTT   600
AGATTCAGGAGAGAAGCCGGCGATGTGGAGGCAAGCTAAGTGCAACAGTGATAAATTTTG   660
GACCATTATTATCTTTTTATTTACATTAATGATCTGGATAATGAGATTAGTAACGATGTT   720
AGTTAATTTGCGGACGATACAAAGATAGATATATTAATTAGGTGGATTTCGAATGCAGTC   780
TCCCTGCAGGCACACTTAGATAAGACGATTGAATAGACTAAGAGACGGCAAATCAAAATT   840
AAATTAACAAGCGTACAGTACTTAATGTAGGTACAAGTCCATACAATACGTACACATTAT   900
GAAGAATGGGGCTCTGGTAAGATCAGAAAACAAAAAAACAAAAAAAAAATCGTAAGTTGT   960
AGTTAGCTATGATCTTCGCCAGAAAACTAACAATGTACAGAGACCAGAAATATGGCGGCA   1020
ATTCATGCATTAAAATTCATCTTTAGGAATACTAAAAGTAGAAGTTTCTAAGTAATATTA   1080
AACTTGTATTTCTCGCTAGACAAACCTCATCCATGAAATGATGTGTAGTTTTTGTCTCTA   1140
TGTTACAGCAGAGACATAGCCTTGTTTGAATCAGTACAAAGGAGACTAACTAAAAATATA   1200

CAAGGGATGAAAGATATTCCTTACGAAAATGAATTGAAGCTTTTATATTTACATTCCTTA   1260
GAGAAGCGTATTTTAAGAGATACATATTTAAACGGTATAGGGAATATAACAAGGGTGCCA   1320
GAAGGAAAGTCCCCAGGGTCAGAAATCAACAAAGGGCAAGAGTAATGGGTTCAGTCTTCA   1380
TACATTAAGACTTCATACATTCTTCAATAGAATGGTTGATAAATGGAATAGATTCAGTAA   1440
GCAAGCTGTTAGTGCACAACCATCAGTGAGCTTAAAACGGTTAGGCAAATAATGGATGAG   1500
CATAATAGGTAAGTATGTTTCAAGCAAGGACTCCAATTTGTAAGACAGACGGCTTCTTGA   1560
AGATTCCATTATTTCTTATGCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT   1620
TCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC   1680
TCTCTCTCTGTATACGTGAGACCGCCAAGGGTGTCAGATACGTAGTGATACTAACATGCA   1740
GGTATTTGTGATTCAGCAACCCAATGCAGGTGTCACTTTTCTGGGCCACCAATGTCTGGT   1800
                 Q  P  N  A  G  V  T  F  L  G  H  Q  C  L  V  35
                            Primer SR1
GGAGTCAACGAGGCAACCAGACGGGTTTTACACCGCAAAGATGTCGTGTGCTTCCTGGAC   1860
 E  S  T  R  Q  P  D  G  F  Y  T  A  K  M  S  C  A  S  W  T   55
TCATGATAATCCTATTGTTGGGGAAGGTGACAAAATCGTTCTGCTTCAACAAAATATATA   1920
 H  D  N  P  I  V  G  E                                        63
TATTCATTATACTTACGTACATAATACTTTCATGTTCTTACGTTGTATGTTACATTCATT   1980
CATCTGCCTCTATATTCCTCCTGCAGGAAGAAGCCGGGTTGAACTTGAGGCGCTTAAAGG   2040
                           G  R  S  R  V  E  L  E  A  L  K  G  75
                                                   Primer SR2
TTCCATCACAAACTTTGTCCAGACAGCATCCAATTACAAGAAGTTCACCATAGATGAGGT   2100
 S  I  T  N  F  V  Q  T  A  S  N  Y  K  K  F  T  I  D  E  V   95
Primer SR2
CGAGGACTGGATTGCTTCTTACTAAGACGCTCACCTGACGTGCTCTCCGAGTCCACCAAA   2160
 E  D  W  I  A  S  Y  *                                        102
GCTGTCTTTGCTGGAGCCACTAAGAGTGGTTTGGTTATTGAGGACAATAAATAACTTTCT   2220
GAATTTTAAAAAAAAAAAAAAAAAAAAAAAAA                                2227
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Fig. 2. Genomic DNA organization of scygonadin. The numbers on the right of the sequence give the positions of the last nucleotide and amino acid on each line, respectively. Binding sites for primers are shown with arrows (5′ → 3′). Polyadenylation signal is underlined in the 3′UTR. The predicted organization of the peptide domains (signal peptide and mature peptide) is shown by gray boxes. Bold italic letters indicate the intron dinucleotide acceptor and donor sites for RNA splicing. The genomic DNA sequence has been deposited in the GenBank nucleotide sequence database and given the accession number DQ059303.

were shown to be underdeveloped through dissection, one group of three medium-weight crabs (112.67 ± 10.02 g), whose gonads were also underdeveloped and one group of three mature crabs (216.33 ± 34.39 g), whose gonads were completely developed, were taken as samples. Results demonstrated that scygonadin transcripts were not detected in the underdeveloped gonads of five of the smaller/medium weight crabs but only in the largest of these (123.01 g) and then weakly detectable (Fig. 5). The clear and strong signals of scygonadin mRNA were detected in all three mature crabs (Fig. 5). This suggested that the scygonadin transcripts might be present at the stage of gonad maturity. This suggestion needs to be more widely investigated in other crabs in future. To determine whether the scygonadin gene is widely expressed or restricted to specific parts of the gonad of the mature male crab, the reproductive apparatus was dissected into four parts: ejaculatory duct, testis, seminal vesicle and vas deferens and analyzed using RT-PCR assay. Observation showed that scygonadin mRNA could only be detected in the ejaculatory ducts of male *S. serrata* (Fig. 6). This indicated that scygonadin was male-specific and expressed predominantly in the ejaculatory duct of *S. serrata*. In order to verify the specificity of the RT-PCR products, a Southern-blot approach was used to analyze the PCR products of male ejaculatory ducts and the positive hybridization signals were obtained corresponding to the fragments of all RT-PCR products (Fig. 7). Meanwhile, a Northern-blot assay was carried out and strong hybridization signals were only presented in the male ejaculatory ducts, showing the fragment was about 900 bp (Fig. 8).

4. Discussion

The full length cDNA sequence for scygonadin and its corresponding genomic DNA sequence were determined based on the partial 20-residue NH_2-terminal sequence of the peptide previously reported. The deduced propeptide sequence contained one putative signal peptide (24 amino acids), indicating that mature peptide corresponds to secreted molecules (Tasiemski et al., 2004). The mature peptide comprised 102 amino acids with a calculated iso-electric point of 6.09, thus it is probably an anionic antibacterial peptide. The hydrophilic index plot of scygonadin propeptide was analyzed using the method of Kyte and Doolittle (Kyte and Doolittle, 1982). The result showed that the mature scygonadin contained some discernable hydrophilic domains and hydrophobic domains (Fig. 9), which indicated that the mature scygonadin may be an amphipathic molecule. This was consistent with the RP-HPLC profile in that scygonadin could not be eluted until the acetonitrile concentration was

scygonadin
18S
HP HP HP FR FR FR E M EX SE H G S M HC P Marker
500 400 300 200 100

Fig. 3. RT-PCR assay of scygonadin gene expression in multiple tissues of female *S. serrata*. Tissues assayed were hepatopancreas (HP); female reproductive tract (FR); eyes (E); exoskeleton (EX); subcuticular epithelia (SE); heart (H); gills (G); stomach (S); muscle (M); hemocytes (HC); male reproductive tract as positive control (P). Amplification products from reactions using gene-specific primers for the mature peptide sequences of scygonadin (305 bp) and for 18S rRNA (147 bp) were resolved by electrophoresis on a 2% agarose gel. Marker (M) is the 100 bp ladder (TaKaRa).

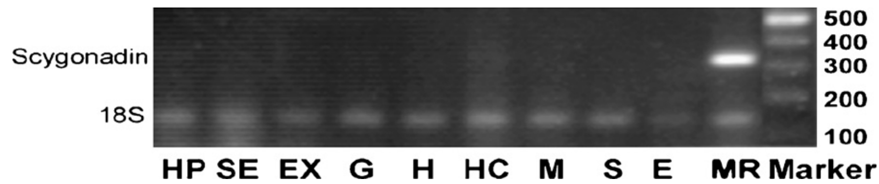

Fig. 4. RT-PCR assay of scygonadin gene expression in multiple tissues of male *S. serrata* ($n = 3$). Tissues assayed were hepatopancreas (HP); exoskeleton (EX); subcuticular epithelia (SE); gills (G); heart (H); stomach (S); muscle (M); hemocytes (HC); eyes (E); male reproductive tract (MR). Product from MR was only amplified using gene-specific primers for the mature peptide sequences of scygonadin (305 bp). Products for 18S rRNA (147 bp) were amplified from all tested tissues. Marker (M) is the 100 bp ladder (TaKaRa).

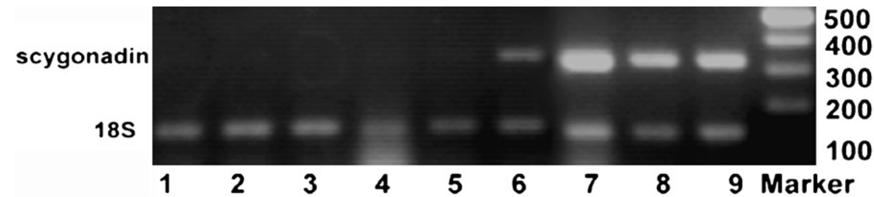

Fig. 5. RT-PCR analysis of scygonadin gene expression in the reproductive tracts among three groups of male *S. serrata*. Lanes 1–3 are samples from smaller crabs (46.33 ± 1.53 g); lanes 4–6 are samples from medium weight crabs (112.67 ± 10.02 g); lanes 7–9 are samples from mature crabs (216.33 ± 34.39 g).

50% (Huang et al., 2006). The pH for the analysis of antimicrobial activity is 6.8. Therefore, the antimicrobial activity of scygonadin may be dependent on its anionic property, being probably different to that of the cationic antimicrobial peptides. Although there are several antimicrobial peptides which have been characterized previously in crustaceans, no anionic antibacterial peptide has been discovered within our knowledge.

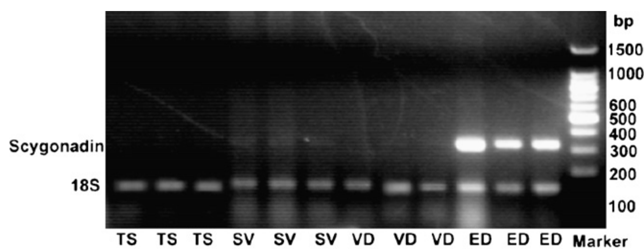

Fig. 6. RT-PCR analysis of scygonadin gene expression in different parts of the reproductive tract from mature male *S. serrata*. The different parts of the reproductive tract involved were ejaculatory duct (ED); testis (TS); seminal vesicle (SV); vas deferens (VD). Products from ED were only amplified using gene-specific primers for the mature peptide sequences of scygonadin (305 bp). Products for 18S rRNA (147 bp) were amplified from all tested parts. Marker (M) is the 100 bp ladder (TaKaRa).

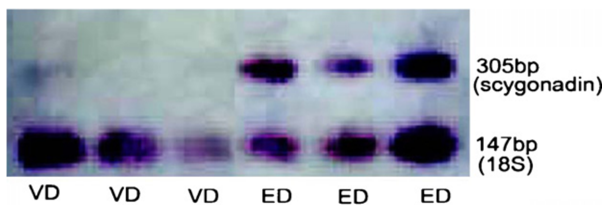

Fig. 7. Southern-blot analysis was used to identify the PCR products of scygonadin transcripts (305 bp) and 18S rRNA (147 bp) amplified from different parts of the reproductive tract of mature male *S. serrata*. The different parts of the reproductive tract involved were vas deferens (VD) and ejaculatory duct (ED). Products were hybridized with one specific scygonadin cDNA probe and one probe for 18S rRNA cDNA. Marker (M) is the 100 bp ladder (TaKaRa).

The full 3′UTR and partial 5′UTR of the transcript has been identified on the genomic DNA sequence. The genomic organi-

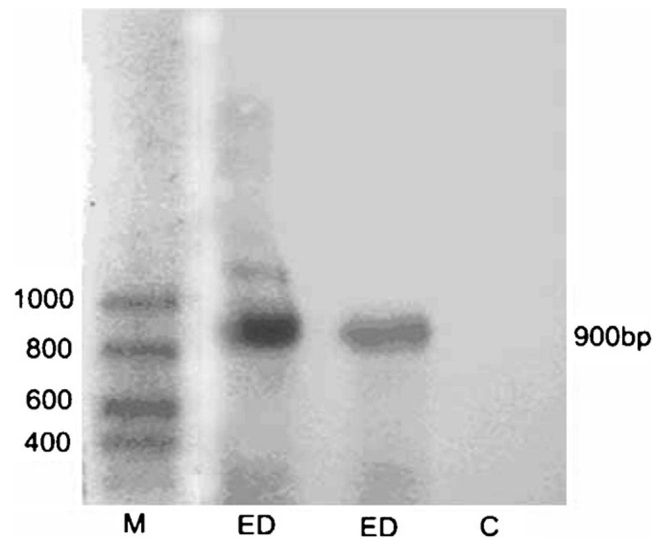

Fig. 8. Northern-blot analysis of scygonadin mRNA in the ejaculatory duct (ED) of mature male *S. serrata*. From left to right (M) RNA marker RL1000 (TaKaRa) is indicated on the left side; (ED and C) No RNA was added as a control.

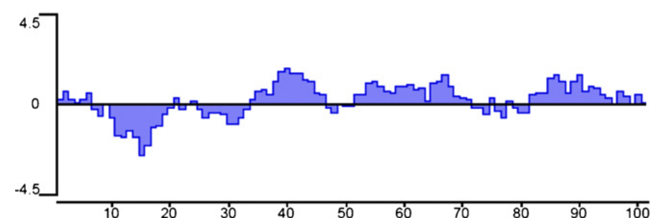

Fig. 9. The hydropathic index plot of scygonadin. The propeptide sequence of scygonadin was analyzed using the method of Kyte and Doolittle (nine residues to average). Hydrophilic domains are above zero and hydrophobic domains below zero.

zation reveals that scygonadin is transcribed from an encoding gene that comprises of at least three exons and two introns, which is similar to that of human α-defensins (1, 3 and 4), human β-defensins (5 and 6) (Chen et al., 2006). To our knowledge, the genomic organization is different from any other antimicrobial peptide in crustacean. An example in penaeidins, there exhibit four classes of genomic organizations. Each penaeidin class is encoded by a separate specific gene. The overall genomic organizations of PEN2 and PEN4 were similar in that the entire coding region of each gene is composed of two exons separated by a single intron. In contrast to PEN2 and PEN4, PEN3 was encoded by a gene lacking introns (O'Leary and Gross, 2006). Moreover, another antimicrobial peptide, carcinin, is transcribed from a multi-exon encoding gene that comprises of at least 4 exons and three introns (Brockton et al., 2007). Therefore, it is the first report on the characterization of an anionic antimicrobial peptide from the seminal plasma of crustaceans.

Considering that almost all of the anionic antimicrobial peptides reported previously were hydrophilic molecules (Brogden et al., 1996; Schittek et al., 2001; Lai et al., 2002; Tasiemski et al., 2004), scygonadin might have a distinct action mode against microorganisms, but this is still to be evaluated. However, there may be two hypotheses to explain the action mode of scygonadin. Firstly, because there is a distinct alpha amphipathic region (1–13) with p*I* of 10.01 in the N-terminal of mature scygonadin, its action mode may be similar to other reported cationic peptides which can bind to anionic components of the target membrane and kill the microorganisms by pore formation and permeabilization of the cell membrane (Brogden, 2005). Secondly, the action mode of scygonadin may be similar to the anionic antimicrobial peptides (Brogden et al., 1996; Schittek et al., 2001; Lai et al., 2002; Tasiemski et al., 2004), because the hydrophilic anionic molecules and some regions with anionic net charge have been found in scygonadin, especially in the C-terminal.

Sex-specific antimicrobial peptides have been characterized previously. Andropin was the first gene to be identified as ejaculatory duct specific in *D. melanogaster* (Samakovlis et al., 1991), while ceratotoxin was the first female-specific gene demonstrated to be related to the reproductive apparatus of the medfly (Marchini et al., 1995). Andropin gene expression is restricted exclusively to the male reproductive tract, unresponsive to bacterial injections but induced by mating (Samakovlis et al., 1991). Ceratotoxin A, isolated from the female reproductory system, is expressed specifically in the accessory glands of sexually mature females, which is also stimulated by mating (Marchini et al., 1995). Both andropin and ceratotoxin are sex-specific peptides. Scygonadin was originally identified as an antibacterial peptide, which could be purified from the seminal plasma of the mature male *S. serrata* (Huang et al., 2006). However, it was not clear from the previous data whether the peptide was male-specific or produced dominantly in the site of peptide isolation. To answer these questions, the mRNA expression of scygonadin was analyzed using RT-PCR and Northern-blot assays. The male-specific transcription of the scygonadin gene and restriction of expression has been clarified in this study. Scygonadin was predominantly expressed in the male reproductive tract of *S. serrata* and was restricted to the ejaculatory duct, possessing the characterization of tissue-specific and male-specific expression. This observation was in agreement with the original isolation source for the peptide. Therefore, it is hard to avoid speculating in this study that scygonadin might be responsible for providing resistance to invading bacteria or other pathogenic microorganisms, so as to maintain sterility in the reproductive tract.

In summary, it can be concluded from our study that scygonadin is a male-specific antimicrobial peptide gene demonstrated to be related to the reproductive apparatus of *S. serrata*. Analysis of this antimicrobial peptide will allow us better understanding of how the innate immune system is involved with maintaining sterility in the reproductive tract of *S. serrata* and thus allows successful fertilization of gametes in microorganism laden seawater. However, much is still left to be explored, such as, whether the possible mechanism of induction of scygonadin is related to bacterial challenge or induced by mating and whether scygonadin could be transferred from male to female *S. serrata* in the seminal fluid for the purpose of protecting the female and the sperm from mating-introduced bacteria as observed with the antibacterial peptide andropin (Lung et al., 2001).

Acknowledgements

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