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A R T I C L E   I N F O

Article title: In vivo activity and the transcriptional regulatory mechanism of the antimicrobial peptide SpHyastatin in Scylla paramamosain

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A B S T R A C T

A new gene homologous to the reported antimicrobial peptide (AMP) hyastatin from Hyas araneus was screened in the SSH library constructed from the hemocytes of Scylla paramamosain, and named SpHyastatin. In vivo study showed that SpHyastatin was predominantly expressed in hemocytes of S. paramamosain. With the challenge of either Vibrio parae-haemolyticus or lipopolysaccharide (LPS), SpHyastatin showed a positive response, meaning that it was probably involved in the immune reaction against bacterial infection in vivo. A distinctive feature of SpHyastatin in comparison with six other known AMPs tested was that SpHyastatin could maintain a higher transcription level from megalopas to the adult crab, indicating a potential consistent resistance against pathogens conferred by this peptide existing in the blood circulation of crabs. RNA interference assay was performed to inhibit SpHyastatin transcription in vivo and the result demonstrated that silencing SpHyastatin mRNA transcripts could decrease the survival rate of crabs challenged with V. parahaemolyticus. To further understand the molecular mechanisms that regulate SpHyastatin expression, a 576 bp 5′-flanking sequence of SpHyastatin was obtained using genome walking. Here, we focused our experiments on investigating the roles of the putative NF-κB binding site in LPS-mediated transcriptional regulation of the SpHyastatin gene using endothelial progenitor cells and Hela cells. Luciferase reporter analyses demonstrated that the putative NF-κB element acted as a positive regulatory element and was essential for the induction of SpHyastatin promoter by LPS. These results should shed light on the in vivo functional property and the molecular mechanism of regulation for the crab AMP SpHyastatin.

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1. Introduction

As is known, the marine invertebrate Scylla paramamosain lacks the specific adaptive immune system that commonly exists in vertebrates, and its protection against invading pathogens is primarily dependent on innate immune defense. Among the various efficient immune-associated components, the antimicrobial peptides (AMPs) from crustaceans are of most concern, owing to their significant roles in innate immunity, since they serve as effective defense weapons against bacterial, fungal and viral infections [1]. The antibacterial property of AMPs is based on their positively charged amphipathic nature, which enables these peptides to bind to the negatively charged bacterial surfaces, thus disrupting the membrane integrity of microbes [2,3]. AMPs are also found that can penetrate the cytoplasm of bacteria and then interfere with DNA synthesis, protein production, cell wall synthesis and enzyme activity, causing death of the microbial cells [3,4]. Aside from their direct antimicrobial activity, AMPs are demonstrated to be multifunctional molecules with other significant biological roles, such as anti-tumor effects, apoptosis, chemotaxis, wound healing, cytokine production and modulating signal transduction [5], even participating in iron metabolism (hepcidin) [6]. Although there are not yet many AMPs identiﬁed in freshwater or marine crabs, it is still interesting to note that those AMPs reported in crabs are found to

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be present in various tissues or organs, suggesting their potential resistant roles against pathogens widely spread in the body of crabs.

The first AMP was isolated from *Carcinus maenas* in 1996, and is a proline-rich protein which shares homology to bovine bactercin 7 [7]. Over the past two decades, many types of AMPs have been identified and characterized from different crab species, such as single-domain AMPs syclin [8], Callinectin [9], antilipopolysaccharide factor (ALF) [10], Scygonadin [11] and SCY2 [12]; multi-domain AMPs Crustin [13], arasin [14], Hyastatin [15] and GRP96 [16]; and the unconventional Histone derived AMP Sphistin [17]. These AMPs, except for the ejaculatory duct derived Scygonadin and SCY2, are usually produced and stored in hemocytes and are responsive to microbial challenge. For example, transcript abundance of SpALF4 [18], arasin-likeSp [19], GRP96 [16] in *S. paramamosain*, and Es-DWD1 in *Eriechir sinensis* [19] are significantly enhanced during the challenge course with some crustacean pathogenic bacteria or pathogen-associated molecular patterns. However, after challenge with *Vibrio alginolyticus*, PtALF5 or PtALF7 transcript changes in a time-dependent manner with obvious down-regulated expression at 6 h and significant up-regulated expression at 24 h of infection [20,21]. Thus, it is clearly seen that expression pattern varies among different AMPs which may contribute to form an effective defense network improving crab antimicrobial defenses in a complex environment.

In some invertebrates, such as insects, many AMPs can be induced by bacterial infection at transcriptional level and their transcriptional regulations have been studied [22]. When bacterial infection occurs, the Toll and IMD (immune deficiency) pathways may be activated by the pathogen signals, and three transcription factors (Relish, Dorsal and Dif) bind to κB-like motifs and can transactivate some of the AMP genes [23,24]. In comparison with those involving insects, crab AMP studies are just at the initial stage. However, the transcriptional regulation of crab AMP genes has attracted much attention because a thorough understanding of the immune defense mechanisms of crabs at the molecular level would provide constructive guidance for disease control in crab aquaculture. In recent years, some possible Toll and IMD pathway components are described in several crab species, e.g., Toll, MyD88, Pelle, Cactus, IMD, Dorsal and Relish [25–27], suggesting that these two signaling pathways may exist in crabs. As previously reported, the shrimp ALFFc and penaeidin promoter regions have several critical regulatory elements, such as the NF-xB and GATA response elements [28,29], which are suggested to be involved in the immune inducibility of *Drosophila* AMP genes [30]. Although how AMPs are governed by Toll and IMD pathways in crustaceans has not been adequately elucidated, these preliminary studies still provide an initial step towards a detailed understanding of the regulation of crab and other crustacean AMP gene expression.

Previously, an AMP gene, SpHyastatin, was screened from the SSH library constructed in the hemocytes of *S. paramamosain* [31]. The recombinant SpHyastatin exhibits potent antimicrobial activities against the aquatic animal pathogens *Aeromonas hydrophila* and *Pseudomonas fluorescens*, and confers immune protection against *V. parahaemolyticus* infection in *S. paramamosain* [32]. In the present study, the *in vivo* expression pattern of the SpHyastatin gene in normal or bacterially challenged *S. paramamosain* was measured using quantitative real-time PCR (qPCR). The potential immune function of SpHyastatin was evaluated using RNA interference (RNAi) and *V. parahaemolyticus* challenge experiments. To understand the regulation mechanisms for the activation of SpHyastatin transcription in response to lipopolysaccharide (LPS), the 5′-upstream region of this gene was cloned and a comprehensive luciferase activity analysis of the SpHyastatin promoters in a heterogeneous cell culture system was carried out.

### 2. Materials and methods

#### 2.1. Animals, bacterial challenge and sample preparation

All animal procedures were carried out in strict compliance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the animal welfare and ethics committee of Xiamen University. All of the mud crabs were obtained from a local crab farm in Xiamen (Fujian, China). The animals were acclimated in running aerated seawater at ambient temperature (25 ± 2 °C) in the laboratory for one week before processing. They were fed fresh clams daily during the acclimation period.

Tissue samples taken from both adult male and female crabs (300 ± 30 g each) were used for analysis of the tissue distribution of SpHyastatin mRNA transcripts. The hemolymph of each crab was collected from the last walking leg using a syringe, and immediately mixed with an equal volume of crab anticoagulant solution (NaCl 510 mM, glucose 100 mM, citric acid 200 mM, Na-citrate 30 mM, EDTA-Na2 10 mM, pH 7.3) [33]. The diluted hemolymph was centrifuged at 500 g for 10 min at 4 °C to separate the hemocytes. Tissues, including subcuticular epithelia, gills, hepatopancreas, heart, muscle, nerve and mid-gut, were then prepared from all the crabs. Sex-specific tissue containing testis or ovary was dissected out separately. To investigate the expression of SpHyastatin at different developmental stages, the whole embryo (I, II, III and V), zoea (I) and megalopa were sampled. For comparing the transcription levels of AMP genes at different developmental stages, megalopa, juvenile crab (10–20 mg), and hemocytes from juvenile crab (40 ± 5 g) and adult crab (300 ± 30 g) were sampled.

LPS from *Escherichia coli* (L2880, Sigma) was dissolved with mixed crab saline solution (NaCl, 496 mM; KCl, 9.52 mM; MgSO4, 12.8 mM; CaCl2, 16.2 mM; MgCl2, 0.84 mM; NaHCO3, 5.95 mM; HEPES, 20 mM; pH 7.4) [34] to a concentration of 5 mg/mL for crab injection. Fifty crabs in the LPS group received LPS injection at a dosage of 0.5 mg/kg LPS. Another 50 individuals in the control group were injected with an equal volume of crab saline solution. Tissue samples from five individuals in each group were randomly sampled at 0 h (from the normal crab), and at 3, 6, 12, 24, 48 and 96 h post-injection. Animals in the *V. parahaemolyticus* challenge experiment group were injected with 100 μL live bacteria (approximately 2.5 × 10⁷ cfu/mL) and an equal volume of crab saline solution as control treatments. The sample gathering process was in accordance with the LPS injection experiment. All samples collected above were frozen at −80 °C for later RNA extraction.

#### 2.2. RNA isolation, reverse transcription and qPCR

Total RNA was isolated from different samples using TRIzol Reagent (Invitrogen). RNA quality and quantity was assessed using 1% denaturing agarose gel electrophoresis and spectrophotometry, respectively. Reverse transcription was performed using a PrimeScript™ RT Reagent Kit (Takara). qPCR was adopted to analyze the relative expression levels of SpHyastatin and other AMPs using a 7500 Real-Time PCR System (Applied Biosystems) in a reaction volume of 20 μL following the manufacturer’s instructions of Power SYBR Green PCR Master Mix (Roche). The amplification procedure was as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. β-actin was amplified as an internal control for qPCR. The specificity of the qPCR products was confirmed using melting curve analysis. The primers with amplification efficiencies calculated within 95–105% are listed in...
Supplementary Table S1 and were used to determine the relative expression levels of the target genes. qPCR data were calculated using the 2 \(^{-\Delta\Delta Ct}\) method [35].

2.3. Determination of the genomic structure of SpHyastatin

Total genomic DNA prepared from the muscle was purified using the Universal Genomic DNA Extraction kit (Takara). A pair of gene-specific primers (SpHyas intron F and SpHyas intron R) (Supplementary Table S1) designed based on the full-length cDNA sequence was employed to amplify the genomic sequence. For the amplifications, a 20 μL reaction volume containing 2 μL 10 \(\times\) LA PCR Buffer II (Mg\(^{2+}\) Plus), 3.2 μL dNTP Mixture (2.5 mM each), 0.4 μL of each primer (10 mM), 12.8 μL of PCR grade water, 0.2 μL LA Taq\(^{®}\) (5 U/μL) (Takara) and 1 μL of DNA template (approximately 2.5 ng) was used. The PCR program was as follows: 94 °C for 1 min; 30 cycles of 2-step amplification profile of 10 s at 98 °C and 10 min at 68 °C; and 10 min at 72 °C for a final extension. The expected DNA fragments were then ligated to pMD18-T vector (Takara) and sequenced by the Invitrogen Biotechnology Co., Ltd.

2.4. Phylogenetic analysis of SpHyastatin

SpHyastatin was aligned with hyastatins and penaeidins using the Clustal X 1.83 program. A phylogenetic tree of hyastatins was constructed based on the Cys-containing C-terminal domain using the neighbor-joining (NJ) method. The tree was constructed using MAGA v4.0 software with 1000 bootstrap replicates.

2.5. Double-stranded RNA (dsRNA) preparation and dsRNAi in vivo

The dsRNAs were prepared using in vitro transcription with the T7 RiboMAX express kit (Promega) as described in the manufacturer's protocol. Briefly, gene-specific primers (Supplementary Table S1) with T7 promoter sequences at the 5’ ends were employed to amplify SpHyastatin region (414 bp) from S. paramamosain hemocytes cDNA and EGF region (502 bp) from pEGFP-1 vector (Clontech). The resulting templates were used to produce sense and antisense RNA strands, which were then annealed to generate dsRNA. S. paramamosain (averaging 40 ± 5 g each) were used for dsRNA-mediated RNAi experiments. In brief, animals were injected with either dsSpHyastatin (1 μg/g crab) or dsGFP at the base of the last walking leg. The second injection was performed after 24 h. The hemocyte samples were collected at 24 h after the second injection and used for total RNA extraction. The gene-silencing efficiency was detected using qPCR as mentioned above.

2.6. Survival rate assay of SpHyastatin-silenced crab after V. parahaemolyticus infection

To study the role of SpHyastatin in vivo further, we performed a V. parahaemolyticus infection experiment and a survival rate assay. A total of 60 crabs were divided into two groups of 30 crabs each. V. parahaemolyticus (2 \(\times\) 10\(^{7}\) cfu per crab) was injected into crabs at 48 h following the first injection with dsSpHyastatin or dsGFP. The survival rates of the two groups were recorded hourly.

2.7. SpHyastatin promoter cloning and recombinant plasmid constructions

To investigate the regulation of SpHyastatin expression further, we cloned its upstream region using genome walking with the Genome Walking Kit (Takara). Briefly, three gene-specific primers (Supplementary Table S1) as well as four shorter arbitrary degenerates (AP1, AP2, AP3 and AP4) were used to amplify the promoter region via nested PCR.

The SpHyastatin promoter sequence was amplified from S. paramamosain genomic DNA using the specific primers with Sac1 and Pst1 restriction sites, and was then inserted into the promoter-less vector, pEGFP-L. The constructed plasmid was named pEGFP-SpHyas-P. Nine serial deletion PCR fragments were acquired using PCR and were respectively subcloned into the Sac1/XhoI site of pGL3-Basic vector (Clontech), containing a firefly luciferase gene. Then, the mutants of the putative NF-κB element designed based on the SpHyastatin promoter were constructed through overlap extension PCR to confirm the regulating roles of the putative NF-κB element. All the constructed recombinant plasmids were verified by sequencing.

2.8. Cell culture, transfection and luciferase assay

Endothelial progenitor cells (EPC, Cyprinus carpio) were grown in L-15 medium (Hyclone) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 μg/mL penicillin (Gibco), and 100 μg/mL streptomycin (Gibco) at 26 °C. Hela cells were routinely cultured in Dulbecco’s modified Eagles medium (Hyclone) containing 10% FBS, 100 μg/mL penicillin, and 100 μg/mL streptomycin at 37 °C in 5% CO\(_2\).

One day before transfection, different cells were seeded on a 48-well plate at a concentration of 80–90% confluence. The recombinant pEGFP-SpHyas-P plasmid was transfected into EPC cells with Lipofectamine 2000 at the optimal ratio of 0.4 μg: 1 μL (plasmid: transfection reagent), while pEGFP-L was used as a negative control. Twenty-four hours post-transfection, images of the transfected EPC cells were collected with fluorescence microscopy (Nikon AZ100) to detect the EGFP expression. For activity assay of luciferase plasmid, EPC and Hela cells were transfected with 0.4 μg series plasmid or pGL3-basic (negative control) using Lipofectamine 2000. 2 ng per well of internal control plasmid pRL-TK were then added to co-transfect the cells for normalization of transfection efficiency. After 24 h of transfection, firefly luciferase activity was normalized to renilla luciferase activity measured using the Dual-Luciferase® Reporter Assay System (Promega) on a GloMax™ 20/20 luminometer (Promega).

To study the immunological function of SpHyastatin promoter, LPS at a final concentration of 100 μg/mL was added to EPC or Hela medium at 6 h post-transfection of reporter plasmids. After 18 h of treatment, luciferase activities were detected using the method described above. The results were obtained from more than three independent experiments.

2.9. Statistical analysis

Data were shown as mean ± S.E. Statistical analysis was performed using SPSS software (version 11.5). One-way analysis of variance (ANOVA) and the independent-samples t-test were used to determine the expression difference within or between groups. Difference was accepted at \(p < 0.05\) or \(p < 0.01\).

3. Results

3.1. Genomic DNA sequence and phylogenetic analysis of SpHyastatin

The genomic DNA sequence of SpHyastatin was approximately 1488 bp and composed of two exons and one intron (Supplementary Fig. S1A). The 5’ UTR, with the signal peptide and the entire proline-rich region covered the first exon (322 bp) followed by the unique intron (741 bp). The second exon (425 bp)
contained the Cys-containing region and the 3′ UTR of SpHyastatin. The classical canonical splicing recognition sequence GT/AG was identified at the exon-intron junctions. In addition, tri-nucleotide repeats (TAG and GAA), which matched two of the microsatellite sequences of S. paramamosain, were found in the intron sequence.

An NJ tree was constructed to perform an evolutionary relationship analysis of SpHyastatin with hyastatins from crabs and penaeidins from shrimps. As shown in Supplementary Fig. S1B, the phylogenetic tree was divided into three groups. Cluster I included mainly the subfamilies of penaeidin-3 and penaeidin-5. Cluster II consisted of the members belonging to the penaeidin-2 subfamilies. Finally, the hyastatins together with penaeidin-4 constituted Cluster III. Moreover, the NJ tree showed that SpHyastatin possessed a close evolutionary relationship to penaeidin-4.

3.2. Expression of the SpHyastatin transcript in adult tissues and different developmental stages

The qPCR results showed that the transcript distribution of SpHyastatin varied among the examined tissues. In both male and female crabs (Fig. 1A and B), SpHyastatin showed the highest expression level in the hemocytes, followed by the subcuticular epidermis, while other adult tissues exhibited poor expression. SpHyastatin mRNA transcripts were also measured in selected developmental stages (Fig. 1C). The level of SpHyastatin expression was at a low level in embryo I, II and III, but increased gradually from embryo V to the megalopa stage, indicating that it may play a more important role at later developmental stages than at earlier embryo stages.

3.3. Comparison of the expression levels of AMP genes in different developmental stages

Results of qPCR revealed different transcript abundances of the AMP genes including AFL2, GRPSp, Crustin, arasin-like Sp, lysozyme and Scygnonadin in megalopa, juvenile crab (10–20 mg), and hemocytes from the juvenile (40 ± 5 g) and adult crab (300 ± 30 g). Among them, SpHyastatin maintained a high and stable expression level at all four developmental stages tested (Fig. 2).

3.4. Temporal expression of SpHyastatin transcripts post LPS or V. parahaemolyticus challenge

The potential immunological functions of SpHyastatin after stimulation by LPS or V. parahaemolyticus were revealed using qPCR. As shown in Fig. 3A–C, injection of LPS induced significant decrease of transcript abundance for SpHyastatin in tissues associated with immune reactions (hemocytes and gill) and digestion (mid-gut) at 12 h post-infection (0.36, 0.18, 0.35-fold the control, \( P < 0.05 \)). As time progressed, the transcript expression increased gradually and reached the maximal level at 96 h post-injection in the hemocytes and mid-gut, while at 48 h post-injection in the gill. As with stimulation by LPS, a similar expression profile was found for SpHyastatin after V. parahaemolyticus challenge (Fig. 3D and E). The expression level of SpHyastatin in hemocytes and mid-gut were at lower levels than in the control group at 24 h post-injection (0.38, and 0.17-fold the control, \( P < 0.05 \)), followed by a significant increase at 96 h post-injection to 6.07-fold (\( P < 0.05 \)) and 6.10-fold (\( P < 0.01 \)) that in the control group. However, a different tendency of expression profile of SpHyastatin was found in the gill (Fig. 3F). Contrary to the control group, the expression of SpHyastatin increased during the first 6 h post bacterial injection. Then the mRNA level was down-regulated at 12–24 h, and finally was significantly up-regulated at 96 h post-injection, which was 6.48-fold that in the control group (\( P < 0.01 \)). Together, these results clearly indicated that tissue- and time-course changes in SpHyastatin mRNA levels occurred in response to LPS or V. parahaemolyticus challenge.

3.5. Suppression of SpHyastatin renders crab susceptible to V. parahaemolyticus infection

The efficiency of dsRNA-mediated RNAi for SpHyastatin in S. paramamosain was confirmed via qPCR assays, which showed that the mRNA transcript of SpHyastatin in the hemocytes at 48 h was significantly reduced to 42.58% in the dsSpHyastatin group (Fig. 4A). Subsequently, SpHyastatin-silenced animals were challenged by V. parahaemolyticus to analyze the effect on crab survival. The results showed that the survival time for SpHyastatin-silenced crab was rather shorter than that of the control group. None of the SpHyastatin-silenced crabs survived at 60 h, while the survival rate at this time was 13% for the dsGFP injected group (Fig. 4B). This study implied that SpHyastatin played an important role in the S. paramamosain immune defense against the pathogenic bacterium V. parahaemolyticus.

3.6. Sequence characterization and transcriptional activity analysis of the SpHyastatin promoter sequence

To characterize the SpHyastatin gene further, a 609 bp

![Fig. 1](image-url)
SpHyastatin upstream sequence, consisting of a 33 bp non-coding region and a 576 bp putative promoter, was obtained using genome walking. As shown in Fig. 5A, the putative core promoter region (TATA box) appeared at position –32 bp. Many clear immune-regulatory motifs, such as Oct-1, CEBP beta, HNF1, GATA, NF-κB, PAX, twist and STAT3 binding sites, were predicted in the isolated SpHyastatin promoter region using genomatix software. These motifs may function in transcriptional regulation of the SpHyastatin gene.

The recombinant plasmid pEGFP-SpHya-P was then constructed and transfected into EPC cells to explore the transcriptional activity of the putative promoter. As expected, the promoter region of SpHyastatin could effectively drive expression of EGFP protein in EPC cells, indicating its promoter activity (Fig. 5B).

3.7. Responsible regulatory element of SpHyastatin promoter

After confirming the promoter activity of SpHyastatin, nine serial 5’ deletion plasmids were constructed based on the pGL3-basic vector, and used to transiently transfected EPC cells to identify the regions responsible for SpHyastatin promoter activity. As shown in Fig. 6A, the nine plasmids exhibited positive but variable promoter activities. However, the luciferase activity of the plasmid pGL-69/+33-luc decreased intensively (28% of that of pGL-107/+33-luc). Compared with the pGL-69/+33 luc, one putative NF-κB element (CTTGACTTCCTTT, –107 to –93) appearing in the extra fragment in pGL-107/+33-luc was found through bio-informatic methods. Subsequently, a deletion mutant and five 3-base-pair substitution mutants, signatured as pGL-107/+33d-luc, pGL-107/-105s-luc, pGL-104/-102s-luc, pGL-101/-99s-luc, pGL-98/-96s-luc or pGL-95/-93s-luc, were constructed to analyze the potential function of the putative NF-κB element in SpHyastatin. Our data showed that the luciferase activities of the mutants exhibited apparently reduced promoter activities compared with pGL-107/+33-luc (P < 0.5) (Fig. 6B).

3.8. The regulatory element for activation of SpHyastatin promoter activity after LPS stimulation

To investigate whether the presence of LPS immune stimulation changes the transcriptional activity of the SpHyastatin promoter, EPC and Hela cells were transfected with the plasmid pGL3-567/+33-luc and incubated with LPS. The results showed a dramatic increase of the luciferase activity in the pGL3-567/+33-luc construct at 18 h post-LPS stimulation compared with unstimulated cells (P < 0.01) (Fig. 7B and C). Subsequently, we constructed a 3-base-pair substitution mutant (pGL3-567/+33-mut-luc) and a deletion mutant (pGL3-567/+33-del-luc) (Fig. 7A), based on the pGL3-567/+33-luc construct, to characterize the putative NF-κB element for activation in the SpHyastatin promoter region after LPS stimulation.
stimulation. Promoter activity analysis showed that the mutants exhibited significantly decreased promoter activities compared with pGL3-567/+-33-luc in both the EPC and Hela cell. Surprisingly, there was no obvious induction in promoter activity after LPS stimulation in cells transfected with the mutants (Fig. 7B and C).

4. Discussion

In our previous study, a new AMP (SpHyastatin) identified from S. paramamosain was characterized based on the SSH library constructed from the hemocytes of S. paramamosain. The overall structure of the SpHyastatin gene was composed of a signal peptide, a proline- and a cysteine-rich domain and showed certain similarities with shrimp penaeidins [32]. In the present study, we found that the genomic organization of SpHyastatin was similar to most of the classes of penaeidins from Litopenaeus vannamei and P. monodon [28,36], albeit the intron length was different among them. The entire coding region in both SpHyastatin and penaeidin genes was separated by only one unique intron. However, it was interesting to note that this intron was located in a position that divides the proline- and cysteine-rich regions in most of peptides except for PEN3 gene from L. vannamei without this typical intronic sequence [36]. A microsatellite region which is found in Scygonadin [37] and PtALF7 [21] was also present in the intronic sequence of SpHyastatin. Microsatellites, distributed throughout the UTRs protein-coding regions and introns, can cause transcription slippage and gene silencing, and can affect gene transcription, mRNA splicing, and eventually lead to phenotypic changes [38].

Fig. 3. Expression patterns of SpHyastatin transcripts after LPS and V. parahaemolyticus challenge. "N": crab without any treatment. A, B and C show the temporal expressions of SpHyastatin in hemocytes, gill and mid-gut after LPS challenge. D, E and F show the temporal expressions of SpHyastatin in hemocytes, gill and mid-gut after V. parahaemolyticus challenge. Each bar represents the mean ± SD (n = 5). These results were analyzed using an independent-samples t-test. * and ** denote significant differences (P < 0.05) and very significant differences (P < 0.01) compared with the control.
Phylogenetic analysis revealed that a close evolutionary relationship existed between SpHyastatin and penaeidins. The high similarity indicated that SpHyastatin might originate from the same ancestor protein as the penaeidins.

Crustacean hemocytes are thought to be the storage reservoir of many immune effectors and to play one of the major roles related to immune responses. Fig. 4 shows the effect of SpHyastatin depletion on crab mortality following V. parahaemolyticus infection. (A) The mRNA level of SpHyastatin was detected after injection with dsSpHyastatin and dsGFP at 48 h using qPCR analysis. Significant difference across the dsGFP group is indicated with an asterisk at P < 0.05. (B) The survival rate of SpHyastatin-silenced crab infected with V. parahaemolyticus. After 48 h of dsSpHyastatin injection, V. parahaemolyticus (2 × 10^7) was injected. Crab survival rate was recorded hourly (n = 30/treatment group) after bacterial injection.

Fig. 5. (A) Predicted promoter region of SpHyastatin. The putative transcription start site is indicated as +1. The start codon ATG is bold. Potential transcription factor binding sites for Oct-1, CEBPbeta, HNF1, NF-kB, TATA-box, CAAT-box, PAX, twist and STAT3 are underlined. EPC cells were transiently transfected with 0.4 μg pEGFP-SpHya-P (B) and 0.4 μg pEGFP-1(C) and were imaged under a fluorescence microscope at 48 h post transfection. Scale bar = 50 μm.
Fig. 6. Promoter responsible activity region studies for the SpHyastatin gene. (A) Serial deletion and luciferase activity analysis of SpHyastatin promoter in EPC cells. Variable promoter activities (P < 0.05) were calculated using one way ANOVA and denoted by letters (a, b and c). (B) Functional analysis of the mutants of the putative NF-kB element. The sequences of the putative NF-kB element and designed mutant sequences based on pGL-107/+33-luc are shown on the left. The results were calculated using an independent-samples t-test. * and ** denote significant differences (P < 0.05) and very significant differences (P < 0.01) compared with the luciferase activity of the plasmid pGL-107/+33-luc. Each bar represents the mean ± SD (n = 4).

the immune defense of the body [39]. Similar to the reported decapod AMPs which mostly originate from hemocytes [40], SpHyastatin was dominantly expressed in crab hemocytes. Similar transcription results are also reported in other decapods, such as Crustin and ALFs from S. paramamosain [13,41], and penaeidins from P. monodon [42,43]. In our study, seven AMPs were simultaneously expressed in an individual crab but showed different expression patterns. This result was reasonable to support the hypothesis that some peptides might play an in vivo synergistic role in antimicrobial defense against invading pathogens [44]. Such a synergic effect is observed between Lysozyme and lactoferrin, or antimicrobial defense against invading pathogens [44]. Interestingly, SpHyastatin was expressed continuously and steadily from megalops to the adult crab, showing its significantly wide role in the circulatory system.

The present study demonstrated that the hemocyte-derived AMP SpHyastatin was detected at low levels at the embryo stages. This suggested that this peptide might have less of a role in immune defense at the early development of crabs. The increasing trend of SpHyastatin mRNA expression seen from embryo V to the megalop stage was likely due to the high numbers of hemocytes generated, suggesting, as previously reported [45], that the innate immune response is gradually formed or even is already functional prior to embryo hatching. As observed previously, a crab AMP, Scygonandin, was more highly expressed at the embryo than the larval stage, indicating that it might be involved in protecting crabs from infection at the early developmental stages [46]. Different expression patterns among different AMPs could be important for yielding a multidimensional strategy against pathogen infection in different developmental stages of the crab.

V. parahaemolyticus is an important pathogen to both marine invertebrate and vertebrate organisms, while LPS, a cell wall component of Gram-negative bacteria, can induce immune response in S. paramamosain [31,47]. Our in vivo study showed that the mRNA transcript abundance of SpHyastatin decreased at the initial phase of V. parahaemolyticus or LPS challenge but was significantly enhanced in the later challenge phase. Similar expression profiles are also observed in other crustaceans, such as ALFs in Portunus trituberculatus [20,21], penaeidins, and Crustins and Lysozymes in L. stylirostris or L. vannamei [48,49]. Such a tendency is in agreement with a previous study which shows that the immune response of crustacean AMPs against microbial challenge can be distinguished into two distinct immune phases [50]. The decreased expression appearing in the first phase was probably due to the decrease of SpHyastatin-producing hemocytes in blood circulation. Such modifications could be associated with cell migration towards injured tissues, with hemocyte lysis or with nodule formation, as clarified previously [51–53]. Meanwhile, microbial stimulation could induce hemocyte degranulation, as a rapid haemocytic reaction, in crustaceans [52]. Consequently, a decrease of SpHyastatin mRNA concentration may occur accompanied by degranulation events. SpHyastatin un-regulation in the second phase of the immune response may represent the activation of haematopoiesis together with the proliferation process in circulating hemocytes as evidenced previously in other studies [51,54]. Concomitantly, an intense proliferation process may lead to increased SpHyastatin-producing young hemocytes, resulting in an increased transcript for SpHyastatin in circulating hemocytes. From our study, it was noticeable that the time-course changes of SpHyastatin to LPS or to V. parahaemolyticus challenge were similar on the whole, suggesting that these two immune responses of SpHyastatin could be modulated by a Gram-negative bacteria recognition system. The up-regulated expression of SpHyastatin in the gill and the mid-gut was probably due to the infiltrated hemocytes. In addition, the similar expression patterns of SpHyastatin
together with other decapod crustacean AMPs may be the result of the similar regulatory mechanism they employed to mediate their mRNA expression upon in vivo microbial challenge. Therefore, we can speculate that SpHyastatin might appear to be a useful indicator for monitoring the immune phase of crabs to microbial challenge. Therefore, we investigated LPS-mediated transcriptional regulation of the SpHyastatin gene by focusing on the roles of the putative NF-κB element, which confers immune inducibility [58]. Promoter activity analysis showed that the putative NF-κB element located in the upstream region of the SpHyastatin promoter was a positive regulator which could increase transcriptional activity of the promoter. Such a transcriptional mode is described in insects [59]. Further study revealed that SpHyastatin promoter activity was apparently increased after LPS stimulation, whereas no significant change in promoter activities was observed in the putative NF-κB element mutants. These findings strongly suggested that the putative NF-κB element was required for the activation of the SpHyastatin promoter by LPS. This was consistent with the previous report that the NF-κB element is known as an LPS-responsive element of Drosophila cecropin A and Metchnikowin [30,60]. In medaka (Oryzias latipes), the NF-κB element is crucial for up-regulation of the β-defensin gene by LPS stimulation [61], and this element is also confirmed to be responsible for inducing human β-defensin-2 by LPS [62]. These data demonstrated that the regulatory activity of the NF-κB element seemed to be a common mechanism for AMP gene expressions of both vertebrates and invertebrates. This was reminiscent of reports that the transcription factor NF-κB plays a significant role in the transcription of many AMPs. A related study finds that the Rel/NF-κB-related transcription factors, Relish, Dorsal and Dif are critical for Toll and Imd pathways to regulate Drosophila melanogaster AMP gene expression [23,63]. In addition, several studies prove that Relish and Dorsal contribute to penaeidin transcriptions in shrimp [64–67]. Thus, taking into consideration the role of the putative NF-κB element in the regulation of SpHyastatin gene promoter activity, it is likely that, at least partly, this peptide might be regulated by NF-κB-related transcription factors or a regulatory pathway in crabs under environmental stress and pathogen infection.

In summary, we further characterized the presence of AMP SpHyastatin in S. paramamosain based on our previous results. It was found that SpHyastatin was mainly localized in the hemocytes and positively responded to bacterial and LPS challenge. Moreover, silencing of SpHyastatin mRNA would render crab susceptible to V. parahaemolyticus infection. These findings strongly suggested that SpHyastatin is involved in antimicrobial immune responses. Further studies in the transcriptional regulatory mechanisms showed that the putative NF-κB element contributed to activating the transcriptional activity of the SpHyastatin promoter by LPS. Overall, this study provided new insights into the interaction between the crab AMP regulation and bacterial infection.

Acknowledgment

This work was supported by a grant (U1205123) from the National Natural Science Foundation of China (NSFC), a grant (2014N2004) from the Fujian Science and Technology Department with dsMjALF-E2 and infected with V. anguillarum shows a high and rapid mortality compared with dsGFP injected shrimp [55], and the silencing of Crustin in L. vannamei causes a marked increase in mortality in response to V. penaeicida infection [56]. Suppressed LvALF in L. vannamei results in a similar increase in mortality with the challenge of the shrimp pathogen, V. penaeicida [57]. All of these results indicated that AMPs are essential for crustacean survival and protection from pathogenic infection. However, the clear in vivo mechanism of SpHyastatin needs further investigation.

There is overwhelming evidence that crab AMPs are responsive to bacterial and LPS stimulation [19–21], but less information exists regarding the mechanism of their transcriptional regulation. Therefore, it is important to reveal the molecular mechanisms of SpHyastatin gene regulation in order to understand the antimicrobial immune responses in crab. In our study, we investigated LPS-mediated transcriptional regulation of the SpHyastatin gene by focusing on the roles of the putative NF-κB element, which confers immune inducibility [58]. Promoter activity analysis showed that the putative NF-κB element located in the upstream region of the SpHyastatin promoter was a positive regulator which could increase transcriptional activity of the promoter. Such a transcriptional mode is described in insects [59]. Further study revealed that SpHyastatin promoter activity was apparently increased after LPS stimulation, whereas no significant change in promoter activities was observed in the putative NF-κB element mutants. These findings strongly suggested that the putative NF-κB element was required for the activation of the SpHyastatin promoter by LPS. This was consistent with the previous report that the NF-κB element is known as an LPS-responsive element of Drosophila cecropin A and Metchnikowin [30,60]. In medaka (Oryzias latipes), the NF-κB element is crucial for up-regulation of the β-defensin gene by LPS stimulation [61], and this element is also confirmed to be responsible for inducing human β-defensin-2 by LPS [62]. These data demonstrated that the regulatory activity of the NF-κB element seemed to be a common mechanism for AMP gene expressions of both vertebrates and invertebrates. This was reminiscent of reports that the transcription factor NF-κB plays a significant role in the transcription of many AMPs. A related study finds that the Rel/NF-κB-related transcription factors, Relish, Dorsal and Dif are critical for Toll and Imd pathways to regulate Drosophila melanogaster AMP gene expression [23,63]. In addition, several studies prove that Relish and Dorsal contribute to penaeidin transcriptions in shrimp [64–67]. Thus, taking into consideration the role of the putative NF-κB element in the regulation of SpHyastatin gene promoter activity, it is likely that, at least partly, this peptide might be regulated by NF-κB-related transcription factors or a regulatory pathway in crabs under environmental stress and pathogen infection.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2016.10.023.


