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Identification and functional analysis of a Hemolin like protein from *Litopenaeus vannamei*

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ABSTRACT

Hemolin is a specific immune protein belonging to immunoglobulin superfamily and firstly identified in insects. Growing evidences suggest that Hemolin can be activated by bacterial and viral infections and may play an important role in antimicrobial immunity. In this paper, we firstly identified a Hemolin-like protein from *Litopenaeus vannamei* (LvHemolin). Sequence analysis showed that LvHemolin shares high similarity with insect Hemolins and is mainly composed of seven immunoglobulin (Ig) domains which form a 'horseshoe' tertiary structure. Tissue distribution analysis demonstrated that LvHemolin mainly expressed in stomach, gill, epithelium and pyloric cecum of *L. vannamei*. After challenge with pathogens or stimulants, expression of LvHemolin was significantly up-regulated in both gill and stomach. Agglutination analysis demonstrated that recombinant LvHemolin protein purified from *Escherichia coli* could accelerate the agglutination of *Vibrio parahaemolyticus*, *E. coli*, *Staphylococcus aureus*, and *Bacillus subtilis* in the presence of Ca^{2+}. To verify the immune function of LvHemolin *in vivo*, shrimps were injected with gene-specific dsRNA, followed by challenge with white spot syndrome virus (WSSV) or *V. parahaemolyticus*. The results revealed that silence of LvHemolin could increase the cumulative mortalities of shrimps challenged by pathogens and increase the WSSV copies in shrimp tissues. These suggested that Hemolin could play an important role in shrimp innate immune defense against bacterial and viral infections.

1. Introduction

Litopenaeus vannamei, known as white pacific shrimp and belonging to Penaeidae family of decapod crustaceans, is a crucial aquaculture shrimp in the world [1]. *L. vannamei* can be infected by a wide range of pathogens, such as white spot syndrome virus (WSSV), *Vibrio parahaemolyticus* and *Staphylococcus aureus*, which cause tremendous economic losses in shrimp industry. Studies on *L. vannamei* immunity could help improve the health of shrimp farming [2,3].

Hemolin known as a kind of insect specific immune protein belongs to immunoglobulin (Ig) superfamily. It was firstly identified in *Hyalophora cecropia* and proved to be activated during immune responses [4,5]. Since then, more and more homologs of Hemolin have been cloned in Lepidopterans [6–10]. Although the lengths of Hemolin genes are diverse in different insects, they typically share the same gene structure which mainly contains transcriptional regulatory sequences, promoter, 6 exons and 5 introns [11,12]. The mature proteins of Hemolin are composed of 390–400 residues and share 47–62% homology among insects [13,14]. Most of the Hemolins have a signal peptide composed of 18 amino acids [13]. Hemolins from insects are composed of 4 immunoglobulin domains which form a horseshoe shape in tertiary

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structure [15,16]. Homology analysis demonstrated that all the immunoglobulin domains belong to the immunoglobulin superfamily and share high homology with adhesion proteins [14,16,17]. Interestingly, the promoter and intron regions of Hemolin harbor several NF-κB binding motifs, which can up-regulate Hemolin expression through binding with Rel factor [11,18]. As the only member of the immunoglobulin superfamily identified in invertebrates so far, Hemolin may play an important role in Lepidoptera immunity. It is expressed in all developmental stages of Lepidoptera and can be activated after pathogen challenge, especially in fat body, an important immune and defensive organ of insects [6,7,10,19].

Similar to opsonin, Hemolin can accelerate the phagocytosis of pathogens via adhering to the membrane of hemocytes and promote hemocytes agglutination in the presence of Ca^{2+} [5,11,14,20]. In most Lepidopterans, expression of Hemolin is induced by bacterial and viral infections [21], suggesting that Hemolin may have protective function against microbial invasion. While in some insects such as *Heliothis virescens* and *Helicoverpa zea*, the expression of Hemolin can not be induced by virus infection [22]. It may imply that the function of Hemolins is different among various insects. In this paper, we identified a Hemolin-like gene from *L. vannamei*, which is, to our knowledge, the first reported Hemolin gene in crustaceans. We verified its agglutination activity against gram-negative and -positive bacteria *in vitro* and further demonstrated that it plays a role in the immunity of *L. vannamei*.

2. Materials and methods

2.1. Experimental shrimp

Juvenile and healthy *L. vannamei* (~5 g) were raised in a shrimp farm in Zhuhai City, Guangdong Province, China. Shrimps were acclimated in a re-circulating water tank system filled with air-pumped seawater (2.5% salinity) at ~27 °C and cultured for at least a week for acclimation before processing.

2.2. Cloning of LvHemolin cDNA

Total RNA was extracted from mixed sample which contains gill, stomach and hepatopancreas of *L. vannamei* using RNeasy Plus Mini Kit (QIAGEN, Germany) as described in the manufacturer's protocol. The first-strand cDNA synthesis was carried out by PrimeScript Reverse Transcriptase (Takara, Japan) with the DNase (QIAGEN) treated total RNA as template and Oligo d(T)18 as primer.

Based on a Hemolin homologous partial sequence retrieved from the transcriptome data of *L. vannamei* [23], the primers LvHem3R1, LvHem3R2, LvHem5R1 and LvHem5R2 were designed for the 3′ and 5′ rapid amplification of cDNA ends (RACE). Briefly, RACE cDNA templates were produced using the SMARTer™ RACE cDNA Amplification kit (Clontech, Japan) according to the manufacturer's protocol. The 5′- and 3′-untranslated region (UTR) were amplified using primers LvHem3R1/LvHem3R2 and LvHem5R1/LvHem5R2, respectively. The first round PCR were performed by primers of LvHem3R1 or LvHem5R1 together with Universal Primer Mix (UPM), The PCR protocol was: 3 min of initial preheating at 94 °C, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 3 min, and a final extension at 72 °C for 10 min. Nested PCR was carried out with the first PCR products as templates and LvHem3R2 or LvHem5R2 together with Nested Universal Primer (NUP) A as primers, respectively, and the PCR protocol was the same as the first round PCR. The RACE products were purified, cloned and sequenced.

2.3. Bioinformatics analysis

Sequence similarity and conserved domains of LvHemolin were searched using BLAST program on National Center for Biotechnology Information (NCBI). The cDNA sequences and deduced amino acid sequences were analyzed using DNASTAR 7.0. Prediction of signal peptide and transmembrane domains were performed using MEMSAT3 & MEMSAT-SVM (http://bioinf.cs.ucl.ac.uk/psipred/). Protein domains were predicted using the SMART program (http://smart.embl-heidelberg.de/). Tertiary structure was modeled by Protein Homology/analogy Recognition Engine (Phyre 2.0) (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). Multiple sequence alignments were performed by Multalin version 5.4.1 (http://multalin.toulouse.inra.fr/multalin/multalin.html). Phylogenetic tree was constructed using MEGA 5.05 software with the neighbor joining method and reconstructed with 1000 replicate bootstrap analysis [24].

2.4. Quantitative real-time PCR (real-time RT-PCR)

For tissue distribution analysis, the hemocyte, gill, hepatopancreas, heart, stomach, intestine, pyloric cecum, nerve, epidermis, muscle, eyestalk and scape were sampled from 9 healthy and acclimated *L. vannamei*. Total RNA of these samples were isolated using RNeasy Plus Mini Kit (QIAGEN) as described above. The first-strand cDNA was reverse transcribed using PrimeScript RT reagent Kit with gDNA Eraser (Takara) from 1 μg total RNA according to the manufacturer's instructions. Sequences of real-time RT-PCR primers for Lv-Hemolin (LvHem-qRTF and LvHem-qRTR) and internal control gene elongation factor 1 alpha (EF1-α, Genbank accession No. GU136229) (LvEF-1α-qRTF and LvEF-1α-qRTR) were listed in Table 1. Real-time RT-PCR was performed on a LightCycle 480 System (Roche, Germany) at a final volume of 10 μL comprised of 1 μL cDNA, 5 μL 2 × SYBR Premix Ex Taq™ II (Takara), and 500 nM of each primer. The optimized thermal cycling parameters were 95 °C for 2 min to activate the polymerase, followed by 40 cycles of 95 °C for 15 s, 62 °C for 15 s and 72 °C for 10 s. After the cycling protocol, melting curves were obtained by increasing the temperature from 72 °C to 95 °C (0.5 °C/s) to denature the double-stranded DNA. The expression level of LvHemolin was calculated using $2^{-\Delta\Delta Ct}$ method after normalization to LvEF-1α [25].

Table 1
Summary of primers in this study.

Primer	Sequence (5′-3′)
LvHem-3R1	CTTCTCGCTCGAGGACGGCA
LvHem-3R2	GCCTACAACGAGCTCGGCAC
LvHem-5R1	CTCCTCGACGACGACCTCAGG
LvHem-5R2	CAACGGGATGTCCAGTTGGTTGG
LvHem-qRTF	TATTTCACGGACGATTTCACCAA
LvHem-qRTR	CCCGCCATAGTAAGGCAGTTC
LvEF-1α-qRTF	TATGCTCCTTTTGGACGTTTTGC
LvEF-1α-qRTR	CCTTTTCTGCGGCCTTGGTAG
LvHem-dsT7F	TAATACGACTCACTATAGGCACAGCCGAAAATCACTTACAGC
LvHem-dsT7R	TAATACGACTCACTATAGGGGTCCGTGTTGTCCTTGTTGTTG
LvHem-dsF	CACAGCCGAAAATCACTTACAGC
LvHem-dsR	GGTCCGTGTTGTCCTTGTTGTTG
GFP-dsT7F	TAATACGACTCACTATAGGATGGTGAGCAAGGGCGAGGAG
GFP-dsT7R	TAATACGACTCACTATAGGTTACTTGTACAGCTCGTCCATGCC
GFP-dsF	ATGGTGAGCAAGGGCGAGGAG
GFP-dsR	TTACTTGTACAGCTCGTCCATGCC
WSSV32678-qRTF	TGTTTTCTGTATGTAATGCGTGTAGGT
WSSV32753-qRTR	CCCACTCCATGGCCTTCA
TaqMan probe-WSSV32706	CAAGTACCCAGGCCCAGTGTCATACGTT
LvHem-ORFF	TAGGGATCCATGTCCCGGCTGACTCTGATTTC
LvHem-ORFR	ATGGTCGACTTATTAAGCGCTGGCGGTTTCCTCC