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SNPs of hemocyanin C-terminal fragment in shrimp *Litopenaeus vannamei*

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ABSTRACT

In this study, we identified a variable region in the C-terminus of hemocyanin from the shrimp *Litopenaeus vannamei* (2288–2503 bp, HcSC) by sequence alignments. A total of 13 SNPs were identified by PCR-SSCP and HcSC clone sequencing. The SSCP patterns of HcSC could be modulated in *Vibro parahaemolyticus*-treated shrimps. A novel SSCP band with four SNP sites was identified in *V. parahaemolyticus*-resistant shrimps. More importantly, three of these four SNPs introduced variations in amino acid sequence and possibly secondary structure of the HcSC polypeptide and resulted in a higher agglutinative activity against seven pathogenic bacteria. These results suggest that the C-terminus of shrimp *L. vannamei* hemocyanin possesses SNPs, which may be related to shrimp resistance to different pathogens.

1. Introduction

Hemocyanin is a multifunctional protein mainly found in the hemolymph of arthropods and mollusks that has recently been reported to play roles in oxygen transport, molting regulation, and the antigen non-specific immune defense [1–3]. Nagai et al. documented that hemocyanin of horseshoe crab *Tachypleus tridentatus* could be functionally converted into a phenoloxidase-like enzyme by the clotting enzyme and by chitin-binding antimicrobial peptides [4,5]. Treating hemocyanins from crustacean with SDS resulted in an opening of the entrance to the active site of the protein for bulkyphenolic compounds [6]. Becker et al. showed that both of *Concholepas* hemocyanin subunits have an antitumor effect in the bladder carcinoma cell line MBT-2 [7]. Moreover, hemocyanin from shrimp *Penaeus monodon* and horseshoe crab *Carcinoscorpius rotundicauda* possess antiviral and antibacterial activities, respectively [8,9]. Our previous studies showed that hemocyanin from *Litopenaeus vannamei* could react with human Ig as an antigen, bind to bacteria as an agglutinin, interact with erythrocytes as a hemolysin, and act as an immune-enhancing protein [10–14]. However, the molecular basis underlying hemocyanin multifunctionality remains unknown.

Interestingly, it has been demonstrated that hemocyanin C-terminus could generate immune-related peptides with antifungal and antibacterial activities [15,16]. Four out of eight up-regulated proteins in *Penaeus vannamei* shrimp infected with Taura syndrome virus (TSV) were identified as C-terminal hemocyanin fragments [17]. Our previous research also found that shrimp *L. vannamei* hemocyanin C-terminus shared four conserved regions with the human Ig heavy chain and one conserved region with the Ig kappa light chain variable region [10]. These results imply that the hemocyanin C-terminus may act as the Ig variable region and possess single nucleotide polymorphisms (SNPs).

In this study, we identified 13 SNPs in the C-terminus of *L. vannamei* hemocyanin (HcSC). This polymorphic profile could be modulated by environmental stresses such as temperature and pathogen infection. The SNP mutations in the HcSC domain were associated with shrimp resistance to pathogens. The results will assist in the investigation of the molecular basis of hemocyanin multifunctionality and help to establish strategies for shrimp disease control.

2. Materials and methods

2.1. Animals

Adult penaeid shrimps *L. vannamei* (Guolian shrimp 1# bred by Zhanjian Guolian Aquatic Products Co. Ltd., China), length 8–12 cm, were obtained from Shantou Huaxun Aquatic Product Corporation

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(Shantou, Guangdong, China) and maintained in 25 L open-circuit filtered seawater tanks at room temperature with aeration. Shrimps were acclimatized to laboratory conditions for 2 days before experiments.

2.2. Sequence alignment of hemocyanin C-terminus and SNPs prediction

For hemocyanin C-terminal fragment variable region prediction, the amino acid and nucleotide sequences of the C-terminus of arthropoda hemocyanins from the NCBI database were aligned using Clustal X and BioEdit. Marine Genomics Project (URL: http://www.marinegenomics.org/organisms) was selected to acquire shrimp *L. vannamei* hemocyanin C-terminus EST sequences. The EST sequences were aligned using Clustal X and BioEdit, and SNPs were identified. SNP was designated as a nucleotide that had been substituted no less than three times in comparison to the *L. vannamei* hemocyanin C-terminal sequence (Accession No. X82502, 2288–2503 bp).

2.3. RNA extraction, reverse transcription (RT) and DNA extraction

Hepatopancreas from individual shrimps were collected and frozen in liquid nitrogen before being homogenized in 1 ml of Trizol Reagent (Invitrogen) for total RNA extraction. The precipitated RNA pellet was dissolved in RNase-free water. RNA concentration was measured by UV spectrophotometry (Eppendorf Bio photometer) and stored at −80 °C until use.

First strand cDNA was generated in a 50 μl reaction volume containing 5 μg total RNA, 5 μl 10 × RT buffer, 2.5 μl 10 mM dNTP, 10 μl 25 mM $MgCl_2$, 5 μl 0.1 M DTT, 40 U RNaseOUT Recombinant RNase Inhibitor and 50 U SuperScript II RNAase H-Reverse Transcriptase (Invitrogen, Calsbad, CA). The reaction was conducted at 50 °C for 50 min. DNA was obtained using the Universal Genomic DNA Extraction Kit Ver.3.0 (Takara, Japan) following manufacturer's instructions.

2.4. RT-PCR-SSCP

For the RT-PCR-SSCP analysis, the C-terminal fragment of hemocyanin (X82502.1, 2287–2528 bp) containing HcSC region (2288–2503 bp) and the β-actin control were amplified by RT-PCR using specific primers: HcSC-F (5′-TGTGGTGGCGGTGACTGA-3′) and HcSC-R (5′-GAAGAGTTGTAAGCTTGTATC-3′); Actin-F (5′-CCGAGC GAGAAATCGTTCGTGAC-3′) and Actin-R (5′-GGAGTTGTAGGTGG TCTCGTGGAT-3′), respectively. PCR was performed in a volume of 50 μl containing 1.25 U of high fidelity ExTaq (Takara, Japan), 10 × ExTaq Buffer (1.5 mM $MgCl_2$ plus), 200 μM of each deoxynucleoside triphosphate, 20 μM of each primer, and 1 μl of cDNA. The PCR program included: 94 °C for 3 min, 30 cycles of 94 °C for 30 s and 60 °C for 30 s and 72 °C for 30 s, followed by a final extension at 72 °C for 5 min. PCR product was analyzed by 1.5% agarose gel electrophoresis.

Single-strand conformation polymorphism (SSCP) analysis was carried out essentially as described by Hayashi et al. [18]. In brief, 10 μl of each RT-PCR product was mixed with 10 μl SSCP buffer (95% formamide, 10 mM sodium hydroxide, 0.25% bromophenol blue and 0.25% xylene cyanole). After denaturation at 94 °C for 5 min and snap-cooling on ice for 2 min, the denatured samples were analyzed on a 8% polyacrylamide gel, 120 V constant voltage for 5 h at 4 °C. Gels were stained with ethidium bromide (0.5 μg/ml), and a scanned image was obtained using a bioimage system (Gene genius, Syngene).

2.5. Cloning, sequencing and alignment

To determine the nucleotide variability amongst the different bands in a SSCP profile, predominant SSCP bands were excised with a sterile razor blade and eluted into 50 ml sterilized milliQ water at 4 °C overnight. The bands were re-amplified by PCR with the same conditions as described in Section 2.4. The PCR products were extracted and cloned into pMD-19T (Takara, Japan) and then transformed into *Escherichia coli* DH5α (Promega, Madison, WI). Positive colonies were selected on LB agar plates containing 40 mg/ml 5-bromo-4-chloro-3-indoly-L-β-D-galactoside and 100 μg/ml ampicillin (Sangon, Shanghai). Three positive colonies from each band were selected and sequenced by Huada Genomic Center (Shenzhen, China). The corresponding sequence alignment and SNPs prediction were carried out as described in Section 2.2.

2.6. SNPs analysis from genomic DNA and cDNA

For determination of the SNP sites in the HcSC domain at the individual level, total DNA and RNA from an individual shrimp hepatopancreas were isolated as described in Section 2.3. PCR products from genomic DNA and cDNA were ligated into pMD-19T (Takara, Japan) and sequenced as described in Section 2.5.

2.7. Evaluation of temperature and pathogen stimulation on SNPs variation

To study the possible variability of hemocyanin C-terminal SNPs, stimulations with temperature and pathogens were performed. For temperature treatments, shrimps were cultured at 16, 25 or 30 °C for 2 days. For pathogen treatments, two representative bacterial, namely *Vibro parahaemolyticus* (gram-negative bacterium) and *beta streptococcus* (gram-positive bacterium), were selected. The shrimps were inoculated intramuscularly by using 1-ml insulin syringes in the third abdominal segment with 50 μl of bacterial inoculum (2×10^7 CFU/ml) or filtered seawater (FSW). The treated animals were then returned to the tanks at room temperature. Hepatopancreas were harvested at 0, 3, 6, 9, 12 and 24 h after injection for RNA isolation. The procedures for total RNA extraction, cDNA synthesis and RT-PCR-SSCP were performed as described in Sections 2.3 and 2.4.

2.8. Analysis of SNPs variation between susceptible and resistant shrimps

To compare the SNPs between susceptible and resistant shrimps, approximately 40 *L. vannamei* were injected individually with 100 μl *V. parahaemolyticus* (2×10^7 CFU/ml), and transferred to 25 L tanks at environmental temperature for 24 h. Of these, the 20 survivors were collected as resistant shrimps, 15 shrimps near to death between 6 and 12 h were designated as the susceptible group. Hepatopancreas from a random susceptible and resistant shrimps were used for RNA isolation. The procedure of RNA extraction, cDNA synthesis and RT-PCR-SSCP were carried out as described in Sections 2.3 and 2.4. An extra band in resistant shrimps was excised, cloned and sequenced as previously performed in Section 2.5 for subsequent sequence analysis. Discovery Studio 2.5 (Accelrys, San Diego, CA) was used to predict the effect of SNPs on the structure variation of the HcSC domain in hemocyanin [19].

2.9. Purification of HcSC recombinant proteins

To further characterize an extra band in the resistant shrimps, the band was excised and sequenced as previously performed in