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Evidences of abundant hemocyanin variants in shrimp *Litopenaeus vannamei*

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ABSTRACT

Hemocyanin (HMC) is a multifunctional immune molecule present in mollusks and arthropods and functions as an important antigen non-specific immune protein. Our previous evidences demonstrated that *Litopenaeus vannamei* HMC might display extensive molecular diversities. In this study, bioinformatics analysis showed dozens of variant sequences of the HMC subunit with higher molecular weight from *L. vannamei* (LvHMC). Three variant fragments, named as LvHMCV1-3, which shared 85–99% nucleotide identity with that of the classical form of LvHMC (AJ250830.1), were cloned and characterized. Spatial expression profiles showed that LvHMCV1-3 had different tissue-specific distribution, which were affected by stimulation with six pathogenic bacteria, including *Escherichia coli* K12, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Vibrio fluvialis*, *Streptococcus pyogenes* and *Staphylococcus aureus*, with each variant fragment showing a specific stress pattern to different bacterial pathogens. Full length cDNA of LvHMCV3 was further cloned and characterized. The deduced amino acid sequence shared 92% identity with that of LvHMC, possessed a conserved structure characteristic of the HMC family and could be clustered into one branch along with other arthropod HMC in a phylogenetic tree. In addition, the recombinant protein of LvHMCV3 (rLvHMCV3) showed obvious agglutination activities against three aquaculture pathogenic bacteria including *E. coli* K12, *V. parahaemolyticus* and *S.aureus* at concentrations ranging from 31.25–62.5 g/mL. It also showed obvious antibacterial activity against *V. parahaemolyticus* at concentrations 0.02–0.5 mg/mL, and possessed the best inhibitive effects compared with those of rLvHMCV4 and rLvHMC. Co-injection of *V. parahaemolyticus* and rLvHMCV3 in *L. vannamei* showed significant decrease of the mortality rate at 24–72 h after injection. Therefore, these studies suggested that *L. vannamei* had abundant HMC variants, which possessed obvious resistance to pathogenic infection and might specifically target on different pathogens in shrimp.

1. Introduction

Shrimp aquaculture started in 1970s as an industrial activity and expanded rapidly to become a major global industry. However, enormous losses due to relatively poor basic knowledge of the cultivated species and pathogens infection, such as white spot syndrome (WSS) and acute hepatopancreatic necrosis disease (AHPND), causing losses estimated at approximately US $1 billion per year since the early 1990s (Flegel and Sritunyalucksana, 2011; Tran et al., 2013; Soto-Rodriguez et al., 2014). Therefore, understanding of shrimp immunology will be very helpful to establish strategies for the control of diseases in shrimp aquaculture (Li and Xiang, 2013; Ng et al., 2015).

Recently, much work has been performed on the innate immune mechanisms of shrimp (Lee and Söderhäll, 2002; Liu et al., 2009; Wang and Wang, 2013c; Tassanakajon et al., 2013; Li and Xiang, 2013). A large variety of pattern recognition proteins and immune-related molecules have been discovered, such as β-1,3-glucanase-related protein (BGRP) (Sritunyalucksana et al., 2002), β-1,3-glucan-binding protein (BGBP) (Romo-Figueroa et al., 2004), lipopolysaccharide and β-1,3-glucan binding protein (LGBP) (Cheng et al., 2005), Toll receptor (Yang et al., 2007), C-type lectin (Wang and Wang, 2013b), scavenger receptor (SCR) (Areschoug and Gordon, 2009), galectin (Vasta, 2012), fibrinogen-related protein (FREP) (Chai et al., 2012), thioester-containing protein (TEP) (Ma et al., 2010), Down syndrome cell adhesion molecule (DSCAM) (Watthanasurorot et al., 2011), serine protease homologue (SPH)

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(Jitvaropas et al., 2009), trans-activation response RNA-binding protein (TRBP) (Wang et al., 2012), alpha 2-macroglobulin (A2M) (Lin et al., 2007), antimicrobial peptide, serine proteinase and inhibitor, phenoloxidase, oxidative enzyme, clottable protein, and so on. Further, several major signal transduction pathways or cascades, including Toll, IMD, JAK/STAT and RNAi pathways, prophenoloxidase and clotting cascades, have been characterized (Li and Xiang, 2013; Cerenius et al., 2010; Yan et al., 2015; Chen et al., 2012; Sivaprasad et al, 2015). Notably, shrimp hemocyanin (HMC), in addition to their primary functions as an oxygen carrier, is identified as a novel important type of non-specific innate immune defense molecule (Coates and Nairn, 2014). It can be functionally converted into a phenoloxidase-like enzyme (Coates et al., 2013) and display antiviral (Zhang et al., 2004), antimicrobial (Destoumieux-Garzon et al., 2001; Lee et al., 2003), agglutinative (Zhang et al., 2006) and hemolytic activities (Zhang et al., 2009).

Interestingly, our previous evidences demonstrated that *L. vannamei* HMC displayed molecular diversities, including single nucleotide polymorphisms (SNPs) in the C-terminal fragment and alternative splicing in the subunit with lower molecular weight (about 75 kDa) (Zhao et al., 2012; Guo et al., 2013; Zhao et al., 2013). Particularly, we recently identified certain HMC variant LvHMCV4 from *L. vannamei* which had unique expression pattern induced by certain microbe (Lu et al., 2015). This phenomenon suggest that HMC might work as a pattern recognition receptor (PRR) and its variants might be important for shrimp *L. vannamei* to against diverse environmental microbes. Intrigued by this question, we predicted a large variety of LvHMC mRNA variants using bioinformatics approach in this study, and three variant fragments were identified and characterized by biological and immunological strategies. Furthermore, a HMC variant LvHMCV3 gene was cloned and its resistance to shrimp pathogens *in vitro* and *in vivo* was also investigated. The results will provide useful information for understanding the molecular basis of HMC multifunctionality and help to formulate disease control strategies of shrimp.

2. Materials and methods

2.1. Animals

Adult shrimps *L. vannamei*, length 8–12 cm, were obtained from Shantou Huaxun Aquatic Product Corporation (Shantou, Guangdong, China) and maintained in 25 L open-circuit filtered seawater tanks at about 28 °C with aeration. Shrimps were fed with formulated diet made in Bole Animal Husbandry Corporation (Longhai, Fujian, China) and acclimatized to laboratory conditions for 2 days before experiments.

2.2. Prediction of HMC fragment variants

For HMC variants prediction, the nucleotide sequence of *L. vannamei* HMC subunit with higher molecular weight (Genbank AJ250830.1, named as LvHMC) was acquired from NCBI database. The Shrimp Gene and Protein Annotation Tool (ShrimpGPAT) (http://shrimpgpat.sc.mahidol.ac.th/ShrimpGPATV2/) was selected to acquire the homologous EST sequences of LvHMC. All EST sequences were aligned using Clustal X 1.81 and BioEdit. HMC fragment variants were designated as a different cluster of homologous sequences to distinguish from the LvHMC.

2.3. Cloning of HMC fragment variants

2.3.1. RNA isolation and cDNA synthesis

Total RNA was extracted from *L. vannamei* hepatopancreas using RNAiso Plus (Takara, Dalian, China) according to manufacturer instructions. The extracted RNA was treated with DNase I (Takara, Dalian, China) to remove contaminating DNA.

First strand cDNA was synthesized using the PrimeScript™ 1 st Strand cDNA Synthesis Kit (Takara, Dalian, China). The reaction was performed in a total volume of 10 μL, including 1 μL dNTP Mixture (10 mM each), 1 μL Oligo dT Primer (2.5 μM), 5 μg total RNA, and 3 μL RNase Free dH_2O. After incubation for 50 min at 65 °C, 4 μL 5 × PrimeScript™ Buffer, 0.5 μL RNase Inhibitor (40 U/μL), 0.5 μL PrimeScript™ RTase, and 5 μL RNase Free dH_2O were added. The reaction was conducted at 30 °C for 10 min and 42 °C for 30 min and 95 °C for 5 min.

Table 1
Nucleotide sequences of primers used in cloning and expression analysis.

Primers	Sequence (5′ to 3′)
Clone of variants and mRNA expression	
LvHMC-F	TTCCCATTCTGGTGGGACGAC
LvHMC-R	TGAAACTTTGACGGTGGGTTAG
LvHMCV1-F	TCGGTTTCAAGGCGGATGG
LvHMCV1-R	AGGCTGGAATTTAGCAGGGGTC
LvHMCV2-F	TTGAGGCTGCTGCCGTCCA
LvHMCV2-R	AGGCTGGAATTTAGCAGGGGTC
LvHMCV3-F	AATTACTTGGATCCCGTTGGT
LvHMCV3-R	GTATTTTCCATGTGGATCGGC
β-actin-F	CCGAGCGAGAAATCGTTCGTGAC
β-actin-R	GGAGTTGTAGGTGGTCTCGTGGAT
Quantitative real-time PCR	
qLvHMCV1-F	AGTGAAGCCATTGAAGCAGCG
qLvHMCV1-R	GGTCCGGATGACGACTGTAACTG
qLvHMCV2-F	TTGAGGCTGCTGCCGTCCA
qLvHMCV2-R	TCCGGGTCAAAGGAATCAGC
qLvHMCV3-F	CAGCTTATCGAGCCAAACAGACG
qLvHMCV3-R	CGAAATAGGCAACTCTTTGTCCG
3′ RACE	
O-LvHMCV3F	ACCATCAACATGAGGGTCTTCG
I-LvHMCV3F	GTCTTCGTGGTGCTCGCTCTCGTTG
Prokaryotic expression	
pLvHMCV3-F	cccaagcttgcATGAGGGTCTTCGTGGTG
pLvHMCV3-R	ccgctcgagcATTTTGTTTGATGTGTACGC
pLvHMC-F	cccaagcttgcATGAAGGTCCTGCTGCT
pLvHMC-R	ccgctcgagcATGATGGATATGCTCGCC

2.3.2. Primer design and HMC fragment variants cloning

Based on the predicted sequences of HMC fragment variants, a pair of primers LvHMC-F/LvHMC-R (Table 1) was designed to amplify potential LvHMC fragment variants (655–1184 bp). PCR was performed in a volume of 20 μL containing 10 μL 2 × PCR Mix (containing Taq DNA polymerase, dNTPs and buffer), 1 μL cDNA template, 1 μM of each primer, added ddH_2O up to 20 μL. The PCR reactions were performed with an initial denaturation at 94 °C for 5 min, then 30 cycles of 94 °C for 30 s and 60 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 10 min. The PCR products were cloned into pMD 19-T Vector (Takara, Dalian, China) and then transformed into *Escherichia coli* DH5a (Promega, Madison, WI). 142 positive clones were selected randomly on LB agar plates containing 40 mg/mL 5-bromo-4-chloro-3-indoly-L-b-D-galactoside and 100 μg/mL ampicillin (Sangon, Shanghai) and sequenced by Huada Genomic Center (Shenzhen, China).

Based on the EST and 142 clone sequences, three HMC fragment variants, viz. LvHMCV1 (518 bp), LvHMCV2 (372 bp) and LvHMCV3 (527 bp), were selected and amplified using primers LvHMCV1-F/R, LvHMCV2-F/R, LvHMCV3-F/R, respectively (Table 1). Three positive clones from each variant were confirmed by sequencing. Additionally, 69 positive clones of LvHMCV1 were sequenced for further sequence alignment using BioEdit software.