Fish & Shellfish Immunology 46 (2015) 398–405

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Cloning and characterization of a novel hemocyanin variant LvHMCV4 from shrimp *Litopenaeus vannamei*

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ARTICLE INFO

Article history:
Received 10 February 2015
Received in revised form
15 June 2015
Accepted 18 June 2015
Available online 23 June 2015

Keywords:
Litopenaeus vannamei
Hemocyanin
Variant
LvHMCV4
Resistance
Bacteria

ABSTRACT

Recently, we found 3 variants of hemocyanin subunit with higher molecular weight in shrimp *Litopenaeus vannamei* (Named as LvHMCV1-3). In this study, a novel *L. vannamei* hemocyanin variant (Named as LvHMCV4) was further cloned and characterized. Bioinformatic analysis predicted that LvHMCV4 contains one open reading frame of 2137 bp and encodes a polypeptide of 678 amino acids. It shares 84–99% cDNA sequences identity to that of the classical form of *L. vannamei* hemocyanin (LvHMC, AJ250830.1) and LvHMCV1-3. LvHMCV4 possesses a conserved structure characteristic of the hemocyanin family and can be clustered into one branch along with other arthropod hemocyanins in a phylogenetic tree. Further, the full-length DNA of LvHMCV4 contains 2660 bp and two introns, which are located at the 80–538 bp and 2063–2227 bp regions, respectively. In addition, the mRNA transcript of LvHMCV4 was expressed highly in the hepatopancreas, lymphoid, brain and hemocytes, and weakly in the heart, intestine and gill, while no expression was found in the muscle, stomach and gut. Infection by *Escherichia coli* K12, *Vibrio parahaemolyticus, Vibrio alginolyticus, Vibrio fluvialis, Streptococcus pyogenes* or *Staphylococcus aureus* up-regulated significantly LvHMCV4 mRNA expression in the hepatopancreas. Furthermore, the recombinant protein of LvHMCV4 (rLvHMCV4) was prepared, which showed agglutination activities against six pathogenic bacteria at concentrations ranging from 15.6 to 125 μg/ml. When co-injected with *V. parahaemolyticus* in *L.vannamei*, rLvHMCV4 significantly increased the survival rate after 48 h injection. Together, these studies suggested that hemocyanin variant, LvHMCV4, might be involved in shrimp resistance to pathogenic infection.

1. Introduction

Hemocyanin, a multifunctional protein mainly found in the hemolymph of arthropods and mollusks, has recently been reported to play a significant role in oxygen transportation, molting regulation, and the antigen non-specific immune defence [1]. Decker et al. and Coates et al. documented that hemocyanin of *Limulus polyphemus, Eurypelma californicum,* and *Cancer magister* could be functionally converted into a phenoloxidase-like enzyme after being treated with sodium dodecyl sulfate (SDS) and phosphatidylserine [2,3]. Moreover, hemocyanin from *Rapana venosa* and *Helix Lucorum* possesses antiviral activities aganist Epstein-Barr virus (EBV) [4,5]. Besides, Jiang et al. found that horse crab *Tachypleus tridentatus* hemocyanin, when treated with exogenous protease, showed obvious antibacterial activities after producing a large number of reactive oxygen species (ROS) [6]. Further, Dolashka et al. found that hemocyanin from *Rapana venosa*, *Helix lucorum* and *Keyhole limpet* showed an antitumor effect against the ascites tumor [7]. Particularly, our previous findings indicated that hemocyanin from shrimp *Litopenaeus vannamei* or crab *Scylla serrata* possessed antigenic, agglutinative, hemolytic, and immune-enhancing activities [8–13]. However, so far information regarding to the molecular basis underlying hemocyanin multifunctionality is not available yet.

Interestingly, our previous evidences demonstrated that *L. vannamei* hemocyanin displayed obvious molecular diversity including single nucleotide polymorphisms (SNPs) and alternative

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[1] Contribution is equal.

http://dx.doi.org/10.1016/j.fsi.2015.06.022
1050-4648/

splicing variants, which might contribute to the functional diversity of hemocyanin [14–16]. Importantly, we recently found 3 variants of hemocyanin subunit with higher molecular weight in shrimp (Named as LvHMCV1-3, data unpublished).

In the present study, a novel hemocyanin variant LvHMCV4 gene from shrimp *L. vannamei* was cloned and characterized. Furthermore, its resistance to shrimp pathogens *in vitro* and *in vivo* were also investigated. The findings will assist in the study 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*, length 8–12 cm, were obtained from Shantou Huaxun Aquatic Product Corporation (Shantou, 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. RNA extraction and cDNA synthesis

Total RNA was extracted from *L. vannamei* tissues using RNAiso Plus (Takara, Dalian, China) according to manufacturer instructions. The extracted RNA was treated with RNase-Free DNase (Takara, Dalian, China) to remove contaminating DNA, and cDNA was synthesized using the M-MLV RTase cDNA Synthesis Kit or the 3′-Full RACE Core Set Ver.2.0 (Takara, Dalian, China) following manufacturer instructions.

2.3. Rapid amplification of cDNA ends (RACE) and LvHMCV4 cloning

The EST sequences of LvHMCV4 was obtained from Marine Genomics Project (URL: http://www.marinegenomics.org/organisms) (MGID518082). cDNA ends of LvHMCV4 were acquired using the 3′-Full RACE Core Set Ver.2.0 (Takara, Dalian, China). The universal primers 3′ RACE Outer/Inner were provided by the kit, and the gene specific primers 5′ Outer/Inner-LvHMCV4F were designed according to the EST sequences of LvHMCV4 (Table 1). The nested PCR reaction conditions were set according to the kit protocol. The PCR reactions were performed with an initial denaturation at 94 °C for 3 min, then 30 cycles of 94 °C for 30 s and 55 °C for 30 s and 72 °C for 2 min, and a final elongation 72 °C for 10 min. The PCR products were extracted and inserted into pMD-19T vector (Takara, Dalian, China) and then transformed into *Escherichia coli* DH5α (Promega, Madison, WI). Recombinant clones grown on MacConkey agar (Sigma, St. Louis, MO) were identified using blue-white screening. Positive clones were picked and confirmed by sequencing. Finally, the LvHMCV4 complete cDNA was cloned by RT-PCR with primers PLvHMCV4-F and PLvHMCV4-R (Table 1).

Table 1
Nucleotide sequences of primers in this article.

Primer	Sequence (5′–3′)
3′ RACE	
5′ Outer-LvHMCV4F	TAACATGAAGGTCCTCGTACTGCTC
5′ Inner-LvHMCV4F	GAGTTTGTCTATGCCCTGTATGTTG
Clone of DNA sequences	
gLvHMCV4-F	ATGAAGGTCCTCGTACTGCTCGC
gLvHMCV4-R	AAGGCGGCATTTGTTCATATTCC
Clone of cDNA sequences and prokaryotic expression	
PLvHMCV4-F	cccaagcttgcATGAAGGTCCTCGTACTGCTCGC
PLvHMCV4-R	ccgctcgagcATGGTGATGGATATGT
PLvHMC-F	cccaagcttgcATGAAGGTCCTGCTGCT
PLvHMC-R	ccgctcgagcATGATGGATATGCTCGCC
mRNA expression in different tissues	
SLvHMCV4-F	TCGGTTTCAAGGCGGATGG
SLvHMCV4-R	AGGCTGGAATTTAGCAGGGGTC
Sbeta-actin-F	CCGAGCGAGAAATCGTTCGTGAC
Sbeta-actin-R	GGAGTTGTAGGTGGTCTCGTGGAT
Quantitative real-time PCR	
QLvHMCV4-F	AGTGAAGCCATTGAAGCAGCG
QLvHMCV4-R	GGTCCGGATGACGACTGTAACTG
QEF-F	CATCTCATCTACAAATGCG
QEF-R	ATGAAATCACGATGGCCTG

2.4. DNA extraction and LvHMCV4 genomic DNA cloning

DNA was obtained using Universal Genomic DNA Extraction Kit Ver.3.0 (Takara, Dalian, China) following the recommended protocols. A pair of primers gLvHMCV4-F and gLvHMCV4-R (Table 1) was designed in the ORF region of LvHMCV4 cDNA. PCR reactions were performed with 1 cycle of denaturation at 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 2 min, followed by a 10 min extension at 72 °C. The PCR products were identified by sequencing as described in Section 2.3.

2.5. Bioinformatic analysis

The nucleic acid and deduced amino acid sequences of LvHMCV4 were analyzed using both the BLAST algorithm (NCBI, http://www.ncbi.nlm.nih.gov/BLAST/) and Bioedit. Structures within LvHMCV4 were predicted by the PSIPRED Protein Structure Prediction Server (http://bioinf.cs.ucl.ac.uk/psipred/) and the SWISS-MODEL Workspace (http://swissmodel.expasy.org/). Phylogenic tree was constructed using MEGA software version 5.0.

2.6. mRNA transcript levels of LvHMCV4

Total RNA was extracted from the *L. vannamei* muscle, stomach, intestine, heart, gut, gill, lymphoid, hepatopancreas, brain and hemocytes. Two micrograms of the total RNA was then used for the synthesis of cDNA as described in Section 2.2. The LvHMCV4 cDNA fragment was amplified using primers SLvHMCV4-F and SLvHMCV4-R (Table 1) under the following conditions: predenaturation at 94 °C for 2 min, 30 cycles of 94 °C for 30 s and 55 °C for 30 s and 72 °C for 30 s, followed by a final elongation at 72 °C for 10 min. As an internal loading control, the shrimp beta-actin cDNA fragment was amplified with primers Sbeta-actin-F/R (Table 1) using the same PCR amplification conditions.

2.7. Immune challenge tests

For immune challenge, six experimental groups and one control group were set up with each group having 30 shrimps. Each individual was injected with 50 μl of 0.8% NaCl solution containing either *Escherichia coli* K12, *Vibrio parahaemolyticus, Vibrio alginolyticus, Vibrio fluvialis, Streptococcus pyogenes* or *Staphylococcus aureus* (10^7 CFU/ml), or bacteria-free 0.8% NaCl solution. Six shrimp hepatopancreases were collected randomly and mixed completely from each group at 0, 9, 24, 48, 72 h injection for RNA extraction. After cDNA synthesis, a quantitative real-time RT-PCR assay was performed using an ABI PRISM-7300 Sequence Detection System (Applied Biosystems, Foster City, CA). Primer sequences of the target gene (QLvHMCV4-F and QLvHMCV4-R) and internal control gene (elongation factor-1α, QEF-F and QEF-R) were shown in Table 1. Data from the quantitative real-time RT-PCR analysis were subjected to the one-way analysis (one-way ANOVA) followed by an unpaired, two-tailed t-test.