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Cloning and tissue expression of hemocyanin gene in *Cherax quadricarinatus* during white spot syndrome virus infection

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ABSTRACT

The role of hemocyanin in terms of immune defense was explored in *Cherax quadricarinatus* infected by WSSV. The cDNA of a hemocyanin gene (CqHC) was cloned from the hepatopancreas of *C. quadricarinatus* (crayfish) by the Rapid Amplification Complementary DNA Ends (RACE) method. The full-length cDNA of CqHC consisted of 2167 bp with a 2043 bp open reading frame encoding a 680 amino acid protein with a predicted signal peptide of 19 amino acids. The deduced CqHC protein sequence was found to contain two conserved copper-binding sites and to share 74%, 73%, 72%, 71% and 71% similarity with *Penaeus monodon*, *Marsupenaeus japonicus*, *Fenneropenaeus chinensis*, *Metacarcinus magister* and *Litopenaeus vannamei*, respectively. Phylogenetic analysis revealed that CqHC and HC from *F. chinensis*, *M. japonicus* and *P. monodon* clustered in the same phylogenetic branch. Real-time PCR analysis showed that CqHC was widely distributed, with the highest level in hepatopancreas, at lower amounts in hemocytes, gills and antennal gland. HC levels in hepatopancreas, hemocytes and gills from *C. quadricarinatus* were also compared after white spot syndrome virus (WSSV) infection. After 24 h of infection, CqHC levels rose significantly higher than that of the controls ($P < 0.05$) but then sharply declined over the infection period. The HC gene expression in hemocytes and gills (but not hepatopancreas) of crayfish injected with polysaccharides before infection were higher than that of the non-polysaccharide injected infected groups, showing an immunoprotective rate of 51.86% at 7 days after exposure, indicating that polysaccharide can act as immunopotentiator and could improve the antiviral ability of *C. quadricarinatus* against WSSV.

1. Introduction

Cherax quadricarinatus is currently one of the most important commercially farmed fresh crayfish in the world due to its many advantages, including polyphagia, fast growth and easy culture, as well as being rich in protein and low in cholesterol (Mu et al., 2007). The scale of *C. quadricarinatus* aquaculture has been constantly expanding in China since 1992, but the rapid development of intensive culture techniques has been accompanied by environmental and health problems due to infectious disease outbreaks. The worldwide shrimp/crayfish culture industry has been beset with diseases mainly caused by viruses, particularly white spot syndrome virus (WSSV), and has suffered significant economic losses. Since the 1990s, WSSV has become one of the most detrimental and widely disseminated viruses in shrimp (Lightner, 1996; Zhou, 1999), infecting several species of both wild and cultured shrimps and has a broad host range, including marine and freshwater crustaceans such as crabs and crayfish (Chen et al., 2000; Zhan et al., 2000). In recent years, there have been many cases of natural infection of redclaw crayfish by WSSV in Zhejiang, Jiangsu and other areas of China (Shen et al., 2007).

Hemocyanin (HC) is a copper-based respiratory protein in the hemolymph of arthropods. Studies in recent years have indicated that HC, an oxygen transporter, is a multifunctional protein related to energy storage and osmotic pressure maintenance and adjustment during the molting process (Pan and Li, 2009; Zhang et al., 2005). Additionally, HC exists in high molecular weight aggregates and is regarded as an essential immune molecule in arthropods, exhibiting phenoloxidase (PO) and antimicrobial activities (Lee et al., 2003; Pan and Li, 2009; Pascual et al., 2006; Zhang et al., 2005, 2006). Zhang et al. (2004) first reported that the HC in *Penaeus monodon* provided non-specific immunity against viruses. Thereafter, Zhao et al. (2007) and Lei et al. (2008) found that the HC gene expression in *Litopenaeus vannamei* significantly increased after WSSV infection.

Improving immunity and disease resistance in crustaceans is one of the fundamental measures for preventing and controlling disease. Lipopolysaccharide, glucan, and peptidoglycan have been shown to improve immune protection against pathogenic microbial infection in shrimp (Chen et al., 2004; Raa, 1999; Suphantharika et al., 2003). Therefore, this study aimed to: (1) acquire a better understanding

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of the changes in the HC mRNA expression levels and hemocyanin (HC) contents in the hemolymph supernatant after infection, and association with immunoprotection conferred by polysaccharide (PS) challenge; (2) and provide a theoretical foundation for the pathogenic mechanism of WSSV infection in *C. quadricarinatus*, which could help to advance strategies for control of WSSV in crayfish.

2. Materials and methods

2.1. Experimental materials

Healthy *C. quadricarinatus* (75.26 ± 6.13 g body weight; 14.65 ± 1.16 cm length; males:females ≈ 1:1) were collected from an aquafarm located at Jingshan, Shanghai, China, in 2010. They were transported to the laboratory, maintained in an aerated freshwater system (100 L) at 25 ± 1 °C with autoclaved pellet feed and acclimated for 7 days. These experimental crayfish were detected WSSV negative by PCR. The RNA extraction kit was obtained from Axygen, USA, and the fast extract kit was from Yuanpinghao Biotechnology Company Limited, Tianjing, China. Real-time-PCR was performed using the Premix Ex Taq™ (hot start version) from TaKaRa, Japan. The reverse transcription enzyme and other main reagents were also from TaKaRa. 3′ and 5′ RACE PCR amplifications were performed using the SMARTer™ RACE cDNA Amplification kit produced by Clontech, USA. PCR primers were synthesized by Shanghai Boshang Biology Company (Shanghai, China) and PCR products were sequenced by Shanghai Shenggong Biology Company (Shanghai, China). Polysaccharides (the product contains β-glucan ≥29.0%, α-mannan peptide ≥20.0%, peptide and protein ≥30.0%, chitin ≥2.0%) were purchased from Hubei Anqi Company Limited, Hubei, China.

2.2. Virus preparation

WSSV virus was obtained from the Key Laboratory of Fish Immunology and Health of Chinese Academy of Fishery Sciences, Zhejiang Institute of Freshwater Fisheries, Huzhou city, Zhejiang province, China. Hemolymph and gill samples were taken using the following procedure from a crayfish confirmed to be WSSV-infected by PCR (data not shown) before each experiment. These tissues were homogenized at a 1:10 ratio (W:V) by adding normal saline and then centrifuging at 2358 ×*g* for 5 min and 3929 ×*g* for 10 min at 4 °C. The supernatant was then passed through a 220-nm membrane filter. Filtrates were stored at −80 °C. Healthy redclaw crayfish were artificially infected with the prepared supernatant (0.14–0.20 mL per crayfish) in order to amplify the virus. By 3–5 days after infection, the hemolymph of sick crayfish (about eight crayfish) was collected and treated with repeat freezing and thawing, followed by centrifugation at 1571 ×*g* for 5 min. The supernatant was collected to inoculate healthy crayfish again using 0.14 mL per crayfish, after which they were fed and maintained at 25 °C.

2.3. Primer design

The HC gene sequences of *Fenneropenaeus chinensis* (ACM61982.1), *P. monodon* (AAL27460.1), *L. vannamei* (CAA57880.1) and *Marsupenaeus japonicus* (ABR14693.1) from GenBank were aligned, and a pair of degenerate primers, HCF and HCR, were designed from conserved regions to amplify the HC gene fragments from cDNA of *C. quadricarinatus*. Based on the fragments obtained, specific primers for 5′ RACE and 3′ RACE, HC5′ R and HC3′F, were synthesized by the Shanghai Boshang Biology Company (Shanghai,China). Real-time quantitative PCR primers also were designed based on the full-length cDNA sequence of *C. quadricarinatus* HC (CqHC). 18S rRNA primers for RT-PCR and qRT-PCR were designed based on the previously reported *C. quadricarinatus* cDNA sequences (AF235966) (see primers in Table 1).

2.4. Extraction of total RNA in hepatopancreas and obtaining HC cDNA full-length sequence

Total RNA was obtained from the hepatopancreas samples of *C. quadricarinatus* (with three crayfish i.e. three replicates) kept at −80 °C using an RNA extraction kit (Axygen). The RNA was converted to cDNA using a reverse transcription kit (PrimeScript RT Master Mix Perfect Real Time Kit, TaKaRa, Japan) with a 15 min reverse transcription step at 37 °C, followed by incubation at 85 °C for 5 s to inactivate the enzyme.

PCR amplification was performed with the HCF and HCR degenerate primers with the hepatopancreas cDNA of *C. quadricarinatus* as a template. The total PCR volume was 50 μl, and the optimized amplification conditions were: initial denaturation for 1 min at 94 °C, followed by 35 cycles of denaturation for 10 s at 98 °C, annealing for 30 s at 55 °C, extension for 5 min at 72 °C, and a final hold at 4 °C. The PCR products were delivered to the Shanghai Shenggong Biology Company (Shanghai, China) for sequencing.

The sequencing results were analyzed and compared using BlastX in the NCBI database (http://www.ncbi.nlm.nih.gov). If the sequence of the HC fragment from the experiment was homologous with HC of other species, the forward (HC5′R) and reverse (HC3′F) primers for RACE amplification were designed. 5′ and 3′ RACE amplifications were performed with the SMART™ RACE cDNA kit (Clontech) to acquire the full-length cDNA.

2.5. Analysis of HC gene expression

2.5.1. Experimental groups

The experimental crayfish were divided into four groups (with six crayfish per aquarium) and three replicates used per group (3 aquarium per group with a total of 18 animals): control group crayfish were each injected with 0.10 mL phosphate-buffered saline (PBS) buffer by intramuscular injection at the abdominal sections 1 and 2; (Group I) polysaccharide injected non-infected group crayfish were each injected with 0.10 mL of 1.0% polysaccharide solution; (Group II) non- polysaccharide injected infected group crayfish were each injected with 0.10 mL PBS buffer and then 48 h later injected with 0.14 mL of the virus solution; (Group III) polysaccharide injected infected group crayfish were each injected with 0.10 mL of 1.0% polysaccharide and then 48 h later injected with 0.14 mL of the WSSV virus solution.

2.5.2. Preparation of hepatopancreas and other tissues

Hepatopancreas, gill, stomach, intestine, antennal gland, muscle, spermary, ovary and hemolymph (adding appropriate anticoagulant, 1454 ×*g* centrifugation for 10 min at 4 °C, getting hemolymph supernatant) from crayfish of each group (three crayfish) were removed at

Table 1
Oligonucleotide primers used in the study.

Primer	Primer sequence
HCF	GGYTTTGCTCCCCACACCAC
HCR	TGAGGCCAGGCRAARATRCG
HC5′R	TGTTCCAGCACTCCAGGAGGTAAGT
HC3′F	TAGGCCGCCAAGGAGATCCACAT
UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
HC-S	GTCTTGGTGCTCTGTGCT
HC-V	AAAGGTCGTGGAAGATGC
HCRTF	TGATGTCCTTGGTGATGTCATTG
HCRTR	AATCTAAAGAATGCTGGGTCGC
18S rRNA-F	CATGCCCGTTCTTAGTTGGT
18S rRNA-R	GTGCGGCCCAGAAATATAAA

Note: Y = C or T, R = A or G.