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Effects of *Vibro harveyi* and *Staphyloccocus aureus* infection on hemocyanin synthesis and innate immune responses in white shrimp *Litopenaeus vannamei*

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ABSTRACT

Hemocyanin, a multifunctional oxygen-carrying protein, has critical effects on immune defense in crustaceans. To explore the role of hemocyanin in anti-pathogen mechanism, effects of *Vibrio harveyi* (*V. harvey*) and *Staphyloccocus aureus* (*S. aureus*) on hemocyanin synthesis and innate immune responses were investigated in *Litopenaeus vannamei* (*L. vannamei*) during infection *in vivo*. Results showed that 10^5 and 10^6 cells mL^{-1} *V. harveyi* and 10^6 cells mL^{-1} *S. aureus* significantly affected plasma hemocyanin concentration, hepatopancreas hemocyanin mRNA and subunits expressions, plasma phenol oxidase (PO), hemocyanin-derived PO (Hd-PO), antibacterial, and bacteriolytic activities during the experiment under bacterial stress, while these parameters did not change remarkably in control group. The concentration of hemocyanin in plasma fluctuated, with a minimum at 12 h and a maximum at 24 h. Moreover, the expression of hemocyanin mRNA peaked at 12 h, while the level of hemocyanin p75 and p77 subunits reached maximum at 24 h. Besides, plasma PO and Hd-PO activities peaked at 24 h, and antimicrobial and bacteriolytic activities peaked at 12 h and 24 h, respectively. In addition, 10^5 cells mL^{-1} *S. aureus* had no significant effect on the synthesis of hemocyanin and prophenoloxidase activating (pro-PO) system, but significantly increased antimicrobial activity at 12 h and bacteriolytic activity at 24 h. Therefore, these results suggest that the hemocyanin synthesis was initiated after invasion of pathogen, and the newly synthesized hemocyanin, acted as an immune molecule, can exerts PO activity to regulate the immune defense in *L. vannamei in vivo*.

1. Introduction

As a result of invertebrates lack an adaptive immune system, they mainly rely on innate immunity to resist the invasion of pathogenic microorganisms [1,2]. It is generally cognized that there are two distinct pathways for innate immunity in invertebrates: cellular defense pathway and humoral defense pathway [3,4]. Hemocyanin, a critical non-specific immune molecule in humoral defense pathway, is one of the three respiratory functional proteins (hemerythrins, hemoglobins and hemocyanins) that can bind with metal ions and act as affinity oxygen carriers, which has a variety of physiological functions including oxygen transportation, metal carrier, storage protein, regulation melanin synthesis, and immune functions [5–9]. Recently, more and more scholars have begun to focus on the immune function of hemocyanin.

Invertebrate hemocyanin, which accounts for more than 90% of total hemolymph protein, is a copper-containing respiratory protein that has an important effect on immune function [10–12]. Studies have shown that hemocyanin can exert immune function by influencing phenoloxidase (PO) activity [13,14], antimicrobial activity [7,15], antiviral activity [16], agglutination activity [17], and hemolytic activity [18]. For example, several reports showed that hemocyanin exhibits PO activity both *in vivo* and *in vitro* in crustaceans, such as Kuruma prawns (*Penaeus Japonicus*) [19], *Limulus Polyphemus* [20,21], *Panulirus Argus* [22], *Erimacrus isenbeckii* [23], and *Litopenaeus vannamei* (*L. vannamei*) [13]. Besides, crustacean hemocyanin subunits including p73, p75, and p77 subunits have been shown to function in anti-pathogenic bacteria [16,24,25]. Zhang et al. reported that p73 and p75 subunits of hemocyanin from *Penaeus monodon* have antibacterial activity [16]. In addition, they also found that hemocyanin p75 and p77 subunits in the serum of *L. vannamei* could be directly combined with pathogenic bacteria *in vitro* [24]. However, the information on how hemocyanin subunits of crustaceans exert their immune functions *in vivo* under bacterial stress is still limited.

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There have been many reports on the immune response and virulence in crustaceans under the pressure of pathogens, especially regarding the prophenoloxidase activating system (pro-PO system) [26–29]. It is well known that pro-PO system is an important innate immune defense cascade system in invertebrates of non-self-recognition, which can be activated by slight components of microorganism cell walls, such as LPS and β-1,3-glucans, and are mediated by pattern recognition protein [30–35]. Therefore, pro-PO system is very important to defend against pathogen invasion, especially for pathogenic microorganisms in crustaceans. Besides, as mentioned earlier, hemocyanin is a vital immune protein that is related to the anti-pathogen process under bacterial stress and also exhibits PO activity induced by endogenous or exogenous substances in crustaceans [36–40]. However, the relationship between hemocyanin and pro-PO system and how they regulate the immune defense of crustaceans remain unclear. In addition, many studies are relatively simple, generally focusing on the change in hemocyanin gene expression and hardly on its comprehensive synthesis including gene, subunit and protein level under bacterial stress. Thus it is necessary to further investigate the synthesis and immune function of hemocyanin under pathogen invasion.

White shrimp, *L. vannamei*, has been a worldwide aquatic animal with important economic benefits in the Asia-Pacific area [41]. In recent years, however, the large-scale massive outbreaks of diseases have seriously affected the shrimp farming [42]. At present, the major pathogens of *L. vannamei* included *Vibrio alginolyticus*, *Vibrio harveyi* (*V. harvey*), *Vibrio parahaemolyticus* and so on, causing significant losses in aquaculture industry [43–45]. Therefore, increasing attention has been paid to the regulation of immune defense in shrimp. To the best of our knowledge, studies on the immune defense mechanism of hemocyanin under bacterial stress are limited. In the present study, we investigated effects of different bacteria (Gram-negative pathogen *V. harveyi* and Gram-positive non-pathogen *Staphyloccocus aureus*, *S. aureus*) on hemocyanin synthesis and immune response of white shrimp *L. vannamei*, in order to: (1) acquire a better understanding of how did hemocyanin synthesis involve in the white shrimp immune defense against pathogen aggression; (2) lay a theoretical foundation for the innate immunity of shrimp to the pathogenic mechanism of *V. harveyi* infection.

2. Materials and methods

2.1. Experimental animals

The *L. vannamei* were purchased from a shrimp breeding farm in Shazikou Town, Qingdao. The shrimps including males and females had average body length of 10.8 ± 1.2 cm and an average body weight of 15.7 ± 2.3 g, acclimated one week in tanks (50 cm × 60 cm × 40 cm), containing aerated filtered sea water (salinity 34, pH 8.0) with an air pump at 22.9 ± 0.5 °C before formal experiment. Apparently healthy (physical integrity without injury, normal color and good viability) and intermolt stage shrimps were used during the experiment, which were identified by examining the uropod in which partial retraction of the epidermis could be distinguished. Water was changed twice a day about 3^{-1} volume of the tank and the shrimp-oriented compound feed was given during the acclimation period until 48 h before the experiment. All the experiments were following the guidelines on animal experiments of Ocean University of China.

2.2. Preparation of stress bacteria

Bacterial suspensions for *V. harveyi* and *S. aureus* (obtained from the Laboratory of Pathology and Immunology of Aquatic Animals, Ocean University of China) were prepared with conventional method. They were cultivated on trypsin soy broth (TSB) solid medium and soy broth solid nutritional medium overnight at 37 and 28 °C. The suspensions were obtained by dip washing the bacteria gently with shrimp saline solution (SSS, 50 mM NaCl, 10 mM KCl, 10 mM HEPES, pH 7.3), and then concentrations of the two bacterial suspensions were adjusted to 10^5 cells mL^{-1} and 10^6 cells mL^{-1}, respectively.

2.3. Experimental design and sampling

The experiment had five treatment groups: SSS injection group (control group), 10^5 cells mL^{-1} *V. harveyi* injection group (group I), 10^6 cells mL^{-1} *V. harveyi* injection group (group II), 10^5 cells mL^{-1} *S. aureus* injection group (group III) and 10^6 cells mL^{-1} *S. aureus* (group IV), with each treatment group having 3 parallel tanks. 375 shrimps including males and females were randomly allocated to 15 tanks (25 shrimps per tank). The injection was carried out on the telson muscle of the shrimp with bacterial suspension volume of 50 μL shrimp^{-1}. The shrimps were no death during the experiment and were sampled at 0, 12, 24, and 48 h after injection, accordingly. Hemolymph and hepatopancreas were sampled from 5 shrimps at every sampling time-point in each tank (15 shrimps/group).

Hemolymph was collected from the cardiocoelom at the posterior margin of carapace from *L. vannamei* with a medical sterile 5th needle and syringe of 1 mL. Before the hemolymph drawing, 0.3 mL improved pre-cooling anticoagulant of *L. vannamei* (0.34 M NaCl, 0.01 M KCl, 0.01 M EDTA-Na_2 and 0.01 M HEPES, pH 7.45 and 780 mOsm. kg^{-1}) was pumped into the syringe [46]. The hemolymph was collected with a 1:1 ratio to the anticoagulant. 1.0 mL hemolymph was pipetted in Eppendorf tube and centrifuged at 800 *g* for 10min (4 °C), and then the blue supernatant was collected into a new tube and stored as plasma sample at −80 °C. Hepatopancreas were flash frozen in liquid nitrogen and stored at −80 °C.

2.4. Plasma hemocyanin concentration assay

The concentration of hemocyanin was investigated as previously described with a little modification [47,48]. Briefly, 30 μL of the thawed plasma sample was pipetted into a 96-well ELISA plate, and then 270 μL of distilled water was added into the same well with the microplate reader shaking for a while to avoid the formation of little bubbles. Afterwards, the $OD_{335\ nm}$ value of the plasma sample was read and recorded, and hemocyanin concentration could be calculated by the following formula: $E_{335\ nm}$ (mg mL^{-1}) = 2.3 × $OD_{335\ nm}$ (E represents hemocyanin concentration, 2.3 is the extinction coefficient of hemocyanin for mg mL^{-1}).

2.5. Hemocyanin mRNA expression in hepatopancreas

Total hepatopancreas RNA was extracted following instructions of Trizol® (Invitrogen™). The quantity of RNA was determined using electrophoresis with 1% agarose and the optical density absorption ratio at wavelengths of 260 nm and 280 nm were assayed by nucleic acid and protein detector (Ultrospec 2100 pro). The high-quantity RNA with $A_{260\ nm}$•$A_{280\ nm}{}^{-1}$ equaling 1.8–2.0 and $A_{260\ nm}$•$A_{230\ nm}{}^{-1}$ > 2.0 was used for cDNA synthesis. Then, the cDNA was synthesised using the PrimeScript™ RT Reagent Kit (Takara, Dalian, China).

Relative expression of *hemocyanin* gene (GenBank: X82502) was studied with *β-actin* gene (GenBank: AF300705) as the internal reference. The forward and reverse primers of *hemocyanin* and *β-actin* genes were designed by Primer premier 5.0 and Oligo 6, and synthesized by Sangon Biotech Ltd (Shanghai). Primers sequences were listed in Table 1 and PCR procedures were used as previously described [49]. The annealing temperature T_m was 53.0 °C for *hemocyanin* gene or 60.3 °C for *β*-actin gene. After PCR, the product was electrophoresed with 1% agarose. Then the gel was observed and photographed under UV with the electrophoresis imager (Peiqing JS-680D) and then the gray levels of PCR product bands were determined semi-quantitatively using the bands analyzing software AlphaEaseFC with the relative gray value of *hemocyanin* gene, *i.e.* the ratio of gray value from *hemocyanin* gene fragment to gray value of *β-actin* gene fragment designated as the

Table 1
The sequences of primers of *hemocyanin* and *β-actin* gene.

Primer name	Primer sequence (5′ to 3′)	Product length (bp)
hemocyanin-F	CCCTTCTGGTGGAATGAT	445
hemocyanin-R	CAATATGGGCAGTGTTATGT	
β-actin-F	GCCCAGAGCAAGCGAGGTAT	439
β-actin-R	CGGTGGTCGTGAAGGTGTAG	
hemocyanin-qF	TTTGCTCCCCACACCACTT	172
hemocyanin-qR	TGTGTTTGCCCTCACTGTCA	
β-actin-qF	TGGACTTCGAGCAGGAGATG	138
β-actin-qR	GGAATGAGGGCTGGAACAGG	

expression level of *hemocyanin* mRNA. Moreover, the expression of *hemocyanin* gene in hepatopancreas was also detected by real-time quantitative PCR (qPCR), and the primers are shown in Table 1 and the PCR procedures were used as Zhang et al. described [50]. This study used β-actin (Primers: *β-actin*-qF, *β-actin*-qR; Table 1) as the reference gene. For all standard curves, the primer amplification efficiencies of genes were 98.3 and 99.1%, and R^2 were 0.975 and 0.992, respectively. The *hemocyanin* gene were normalized to the reference gene and expression level was compared with the relative Ct method [51].

2.6. Hemocyanin p75-subunit and p77-subunit levels in hepatopancreas

The total hepatopancreas protein extraction was also strictly carried out according to instructions of Trizol® (Invitrogen™). The concentration of total hepatopancreas protein was assayed according to dye binding method from previous investigation (Bradford 1976) and then the levels of subunits from hemocyanin (p75 and p77) were determined by SDS-PAGE using the same buffer system as investigated [52]. Protein samples (60 μg total protein of hepatopancreas) were electrophoresed with 3% stacking gel and separated with 12% gel. After SDS-PAGE, the gel was dyed with 0.025% Coomassie Brilliant Blue (CBB) R-250 and then destained until the background was transparent and the target protein bands (p75 and p77) were clear enough in the gel. Eventually, the gel was photographed and saved under the electrophoresis imager (Peiqing JS-680D) and then the gray levels of target protein bands were determined by using the bands analyzing software AlphaEaseFC.

2.7. Hemocyanin-derived PO activity assay

Hemocyanin is a hexamer composed of 70–80 kDa heterologous subunits with the approximate molecular weight of 450 kDa [53]. The assay of hemocyanin-derived PO (Hd-PO) activity in plasma was determined and modified as preciously described [22]. Native-PAGE method was adopted and 4-methyl catecholamine was used as the substrate PO activity. Protein concentrations of plasma samples were assayed by Bradford's method. Plasma samples were taken for Native-PAGE electrophoresis (3% stacking gel; 5% separating gel). After the electrophoresis, the gel was dyed with 10 mM 4-methyl catecholamine (0.3% MBTH and 25% ethanol) in the dark overnight. When the gel was colored to the fullest, the gel was scanned and photographed with Gel Imagining System (JS-680D, Peiqing). Then, the gel was dyed with 0.025% CBB R-250, decolored with 10% acetic acid till the gel background became transparent and the protein bands became clear enough. Similarly, the gel was scanned and photographed with Gel Imagining System. The gray value of target protein bands in the electrophoretogram were analyzed by the software AlphaEaseFC and the change of Hd-PO activity was recorded as the change of the gray value of target protein bands (about 450 kDa) in the electrophoretogram.

2.8. Plasma immune parameters assay

PO activity was assayed spectrophotometrically on the formation of dopachrome from L-DOPA as previously described and modified further [54]. 100 μL of plasma sample and 100 μL of L-DOPA (3 mg mL^{-1}) were added into a 96-well ELISA plate and the plate was put into the microplate reader and shaken for several seconds, and next $OD_{490\ nm}$ was read and recorded at an interval of 110 s 15 times altogether. The absorbance of blank control was assayed with 100 μL SSS instead of 100 μL plasma sample. One unit of PO activity was defined by the increase of 0.001 in $OD_{490\ nm}$ $minute^{-1}$ mL^{-1} plasma.

Antibacterial and bacteriolytic activities of plasma were assayed as previously described [32,55–57]. Shrimp pathogenic bacteria *V. harveyi* were used for antibacterial activity, and the bacteria *Micrococcus lysoleikticus* for bacteriolytic activity. In brief, antibacterial and bacteriolytic activities were determined as follows: 300 μL bacterial suspension and 10 μL plasma sample were pipetted into 96-well ELISA plate and the plate was put into microplate reader and shaken for a little while, and $OD_{570\ nm}$ was read and recorded as A_0. Then the plate was incubated in the microplate reader in dark at 37 °C for 30 min and $OD_{570\ nm}$ was read and recorded as A. In order to eliminate the interference of hemocyanin, the absorbance of blank plasma control was assayed with 10 μL 0.1 M PSB (pH 6.4) instead of 10 μL plasma sample to revise A_0 and A. Antibacterial (U_a) and bacteriolytic (U_L) activities were calculated from the following equations respectively:

$$U_a = \sqrt{(A0 - A)/A}$$

$$U_L = (A_0\text{-}A)\cdot A^{-1}$$

2.9. Statistical analysis

All data were expressed as mean ± SD and carried out via ANOVA. Supposing that significance differences were figured out at $P < 0.05$ level, the Duncan Multiple Range test were employed to recognize significant differences among the different treatment groups at the same time point. All measurements were made in triplicate and all data were analyzed using SPSS software, Version 21.0 (Chicago, IL, USA).

3. Results

3.1. Effects of bacterial stress on the concentration of hemocyanin in plasma of L. vannamei

The changes in plasma hemocyanin concentration of experimental infected shrimps are shown in Fig. 1. The results showed that hemocyanin concentration was significantly decreased in 10^5 and 10^6 cells mL^{-1} *V. harveyi* groups (Group I and Group II) at 12 h in a dose-dependent manner, but those changes were reversed at 24 h and recovered to the control level at 48 h ($P < 0.05$, Fig. 1). Similarly, 10^6 cells mL^{-1} *S. aureus* (Group IV) obviously reduced the concentration of hemocyanin in plasma after 12 h stress, while remarkably upregulated after 24 h injection and returned to the control level ($P < 0.05$, Fig. 1). There was no significant change in the concentration of hemocyanin in 10^5 cells mL^{-1} *S. aureus* group (Group III) compared to the control group (Fig. 1).

3.2. Effects of bacterial stress on the expression of hemocyanin mRNA in hepatopancreas of L. vannamei

The effect of bacterial stress on the expression of *hemocyanin* mRNA in *L. vannamei* hepatopancreas was detected by semi-quantitative method. The results showed that 10^5 and 10^6 cells mL^{-1} *V. harveyi* (Group I and Group II) and 10^6 cells mL^{-1} *S. aureus* (Group IV) remarkably increased the mRNA level of *hemocyanin* at 12 h and 24 h as a dose-dependent pattern in hepatopancreas of *L. vannamei*, and then recovered to stable level at 48 h ($P < 0.05$, Fig. 2A–C). In addition, 10^5 cells mL^{-1} *S. aureus* group (Group III) had no significantly effect on

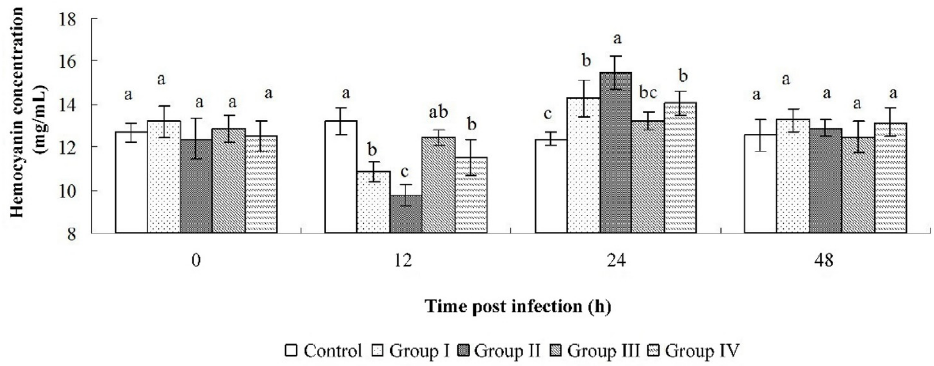

Fig. 1. Effects of *V. harveyi* and *S. aureus* infection on hemocyanin concentration in infected *L. vannamei* plasma. Comparisons were made among the control group (uninfected), Group I (10^5 cells mL^{-1} *V. harveyi* challenged), Group II (10^6 cells mL^{-1} *V. harveyi* challenged), Group III (10^5 cells mL^{-1} *S. aureus* challenged) and Group IV (10^6 cells mL^{-1} *S. aureus* challenged). Data (mean ± SD) at the same exposure time marked with different letters are significantly different ($P < 0.05$).

the expression of *hemocyanin* compared to the control group during the experiment (Fig. 2A–C). Moreover, the results of relative expression of *hemocyanin* by qPCR showed similar pattern with that of semi-quantitative results. *V. harveyi* significantly increased the expression of *hemocyanin* at 12 h and 24 h in a dose-dependent pattern (Fig. 2 D). Furthermore, *S. aureus* remarkably increased the expression of hemocyanin mRNA at 12 h and 24 h on the concentration at 10^6 cells mL^{-1}, which has no significant difference compared to 10^5 cells mL^{-1} *V.*

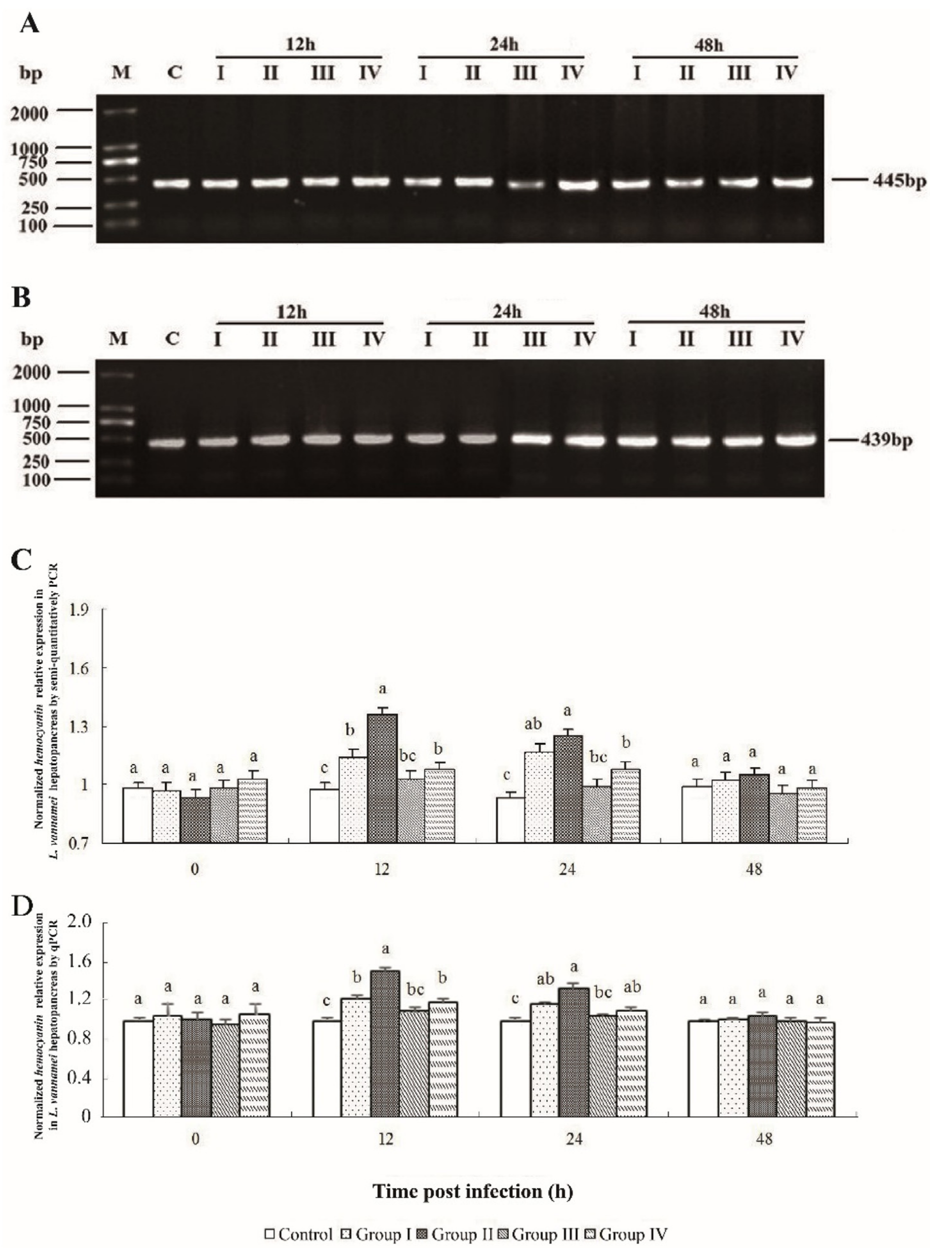

Fig. 2. Agarose gel electrophoresis (AGE) analysis of *hemocyanin* and *β-actin* genes fragments in infected *L. vannamei* hepatopancreas (A: AGE analysis for *hemocyanin* gene; B: AGE analysis for *β-actin* gene). M: Molecular weight marker of DNA; C: Control group (uninfected); I, II, III, IV: Group I (10^5 cells mL^{-1} *V. harveyi* challenged), Group II (10^6 cells mL^{-1} *V. harveyi* challenged), Group III (10^5 cells mL^{-1} *S. aureus* challenged), Group IV (10^6 cells mL^{-1} *S. aureus* challenged); 0, 12, 24, and 48 h: Time post infection. C: Effects of *V. harveyi* and *S. aureus* infection on relative expression of *hemocyanin* mRNA in infected *L. vannamei* hepatopancreas by semi-quantitatively PCR. D: Relative expression of *hemocyanin* mRNA in infected *L. vannamei* hepatopancreas by qPCR. Data (mean ± SD) at the same exposure time marked with different letters are significantly different ($P < 0.05$).

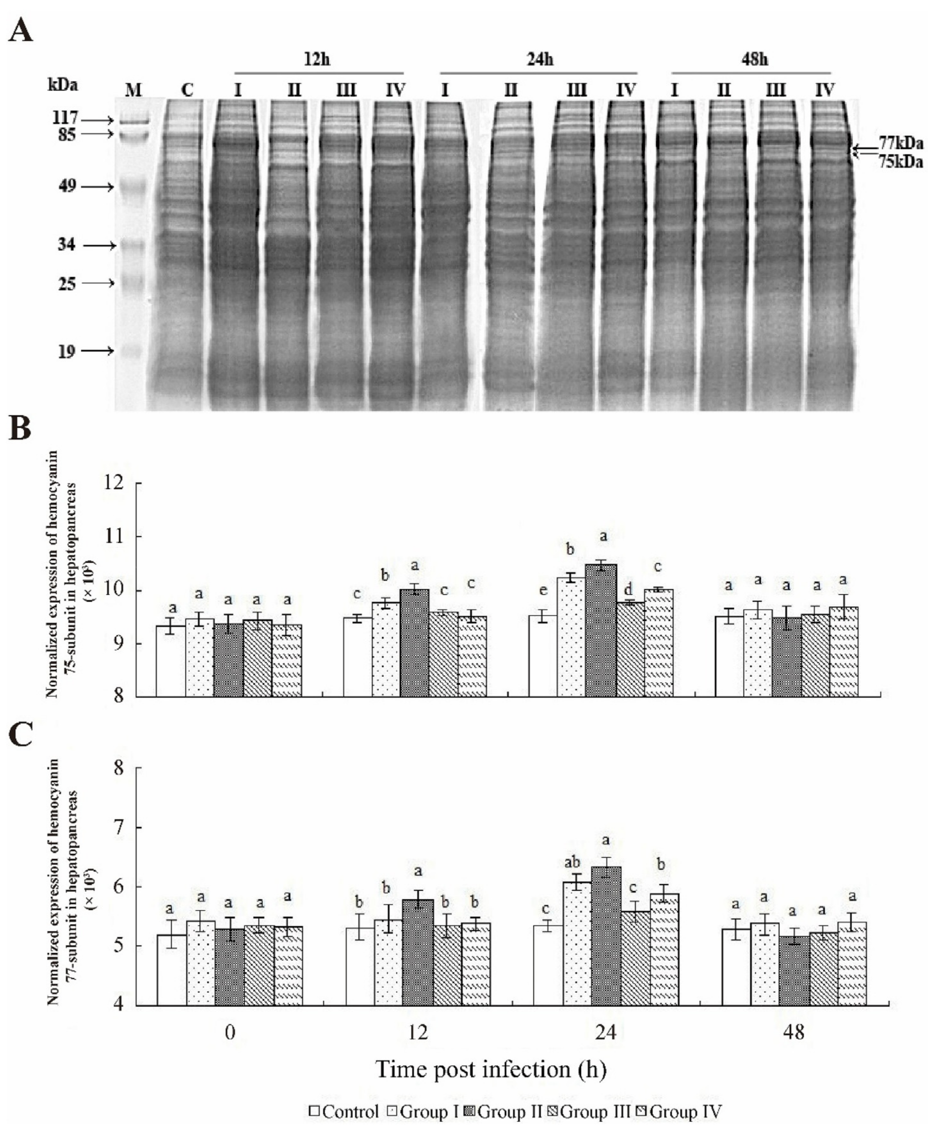

Fig. 3. SDS-PAGE analysis of total hepatopancreas protein obtained (A). Hemocyanin p75-subunit and p77 subunit were marked out by their molecular weights. M: Molecular weight marker of protein; C: Control group (uninfected); I, II, III, IV: Group I (10^5 cells mL^{-1} *V. harveyi* challenged), Group II (10^6 cells mL^{-1} *V. harveyi* challenged), Group III (10^5 cells mL^{-1} *S. aureus* challenged), Group IV (10^6 cells mL^{-1} *S. aureus* challenged). 0, 12, 24, and 48 h: Time post infection. Effects of *V. harveyi* and *S. aureus* infection on levels of hemocyanin p75-subunit and p77-subunit in infected *L. vannamei* hepatopancreas (B, C). Data (mean ± SD) at the same exposure time marked with different letters are significantly different ($P < 0.05$).

harveyi group (Fig. 2 D).

3.3. Effects of bacterial stress on the protein expression of hemocyanin subunits in hepatopancreas of L. vannamei

In this study, *V. harveyi* and high dose of *S. aureus* significantly affected the expression of hemocyanin subunits (p75-subunit and p77-subunit) in *L. vannamei* hepatopancreas. The protein level of hemocyanin p75-subunit increased significantly in a concentration-dependent pattern after 12 h and 24 h of *V. harveyi* injection (Group I and Group II), and it returned to the control group level at 48 h ($P < 0.05$, Fig. 3 A, B). However, the level of hemocyanin p75-subunit only increased remarkably at 24 h after *S. aureus* stress (Group III and Group IV) in a dose-dependent manner, but did not fluctuate significantly at 12 h and 48 h ($P < 0.05$, Fig. 3 A, B). In contrast, 10^5 and 10^6 cells mL^{-1} *V. harveyi* and *S. aureus* significantly upregulated the protein level of hemocyanin p77-subunit at 24 h, while only 10^6 cells mL^{-1} *V. harveyi* (Group II) significantly increased hemocyanin p77-subunit at 12 h ($P < 0.05$, Fig. 3 A, C). The protein level of hemocyanin p77-subunit returned to the stable level at 48h in each treatment group (Fig. 3 A, C). In addition, the expression of hemocyanin p75-subunit and p77-subunit protein did not change significantly throughout the experiment in the control group (Fig. 3A–C).

3.4. Effects of bacterial stress on plasma PO and hemocyanin-derived PO (Hd-PO) activities in L. vannamei

To explore the relationship between hemocyanin and pro-PO system under bacterial stress, this study investigates the plasma PO activity and Hd-PO activity after 10^5 and 10^6 cells mL^{-1} *V. harveyi* and *S. aureus* injection. The results showed that 10^6 cells mL^{-1} *V. harveyi* (Group II) significantly increased plasma PO activity at 12 h, while plasma PO activity enhanced significantly in dose-dependent manner after 24 h of *V. harveyi* (Group I and Group II) injection ($P < 0.05$, Fig. 4). In addition, plasma PO activity was also remarkably upregulated at 24 h when *L. vannamei* was stressed by 10^6 cells mL^{-1} *S. aureus* (Group IV), but it was lower than that of *V. harveyi* groups (Group I and Group II; $P < 0.05$, Fig. 4). Finally, the PO activity of each group in *L. vannamei* plasma returned to the control group level at 48 h (Fig. 4).

The change pattern of Hd-PO activity was similar to that of plasma PO activity during the experiment. *V. harveyi* significantly upregulated the activity of Hd-PO in a dose-dependent pattern at 12 h and 24 h (Group I and Group II), and then returned to the stable level at 48 h ($P < 0.05$, Fig. 5). Furthermore, *S. aureus* obviously increased Hd-PO activity only at 24 h when the injection dose was 10^6 cells mL^{-1} (Group IV; $P < 0.05$, Fig. 5). There was no significant change in plasma PO activity and Hd-PO activity in 10^5 cells mL^{-1} *S. aureus* group and control group during the experiment (Figs. 4 and 5).

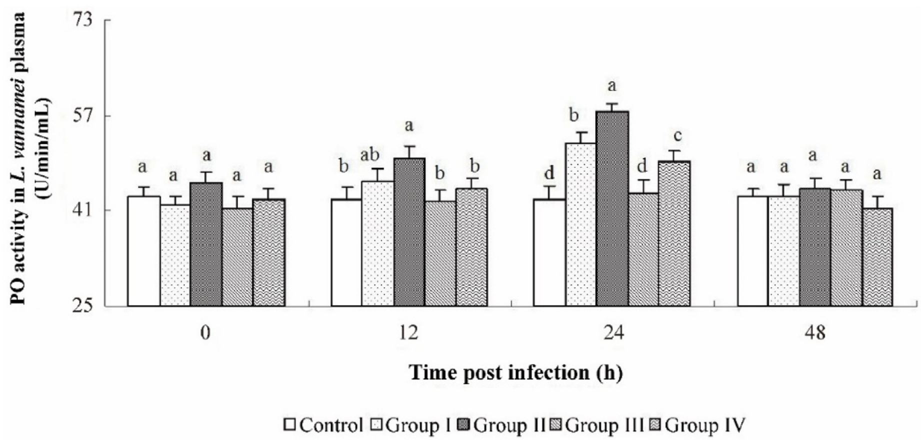

Fig. 4. Effects of *V. harveyi* and *S. aureus* infection on PO activity in infected *L. vannamei* plasma. Comparisons were made among the control group (uninfected), Group I (10^5 cells mL^{-1} *V. harveyi* challenged), Group II (10^6 cells mL^{-1} *V. harveyi* challenged), Group III (10^5 cells mL^{-1} *S. aureus* challenged) and Group IV (10^6 cells mL^{-1} *S. aureus* challenged). Data (mean ± SD) at the same exposure time marked with different letters are significantly different ($P < 0.05$).

3.5. Effects of bacterial stress on the antibacterial activity and bacteriolytic activity of plasma in L. vannamei

This study examined the antimicrobial and bacteriolytic activities of *L. vannamei* plasma under bacterial stress to explore the immune regulatory function of hemocyanin. The results showed that antimicrobial activity of plasma enhanced significantly in a dose-dependent manner at 12 h and 24 h after *V. harveyi* injection and stabilized at 48 h that has no significant difference among *V. harveyi* injection groups and control group (Group I and Group II; $P < 0.05$, Fig. 6 A). Similarly, *S. aureus* remarkably upregulated the antimicrobial activity in a dose-dependent pattern at 12 h, but it returned to the control level at 24 h (Group III and Group IV; $P < 0.05$, Fig. 6 A). The results of bacteriolytic activity showed that it was significantly increased in a dose-dependent manner

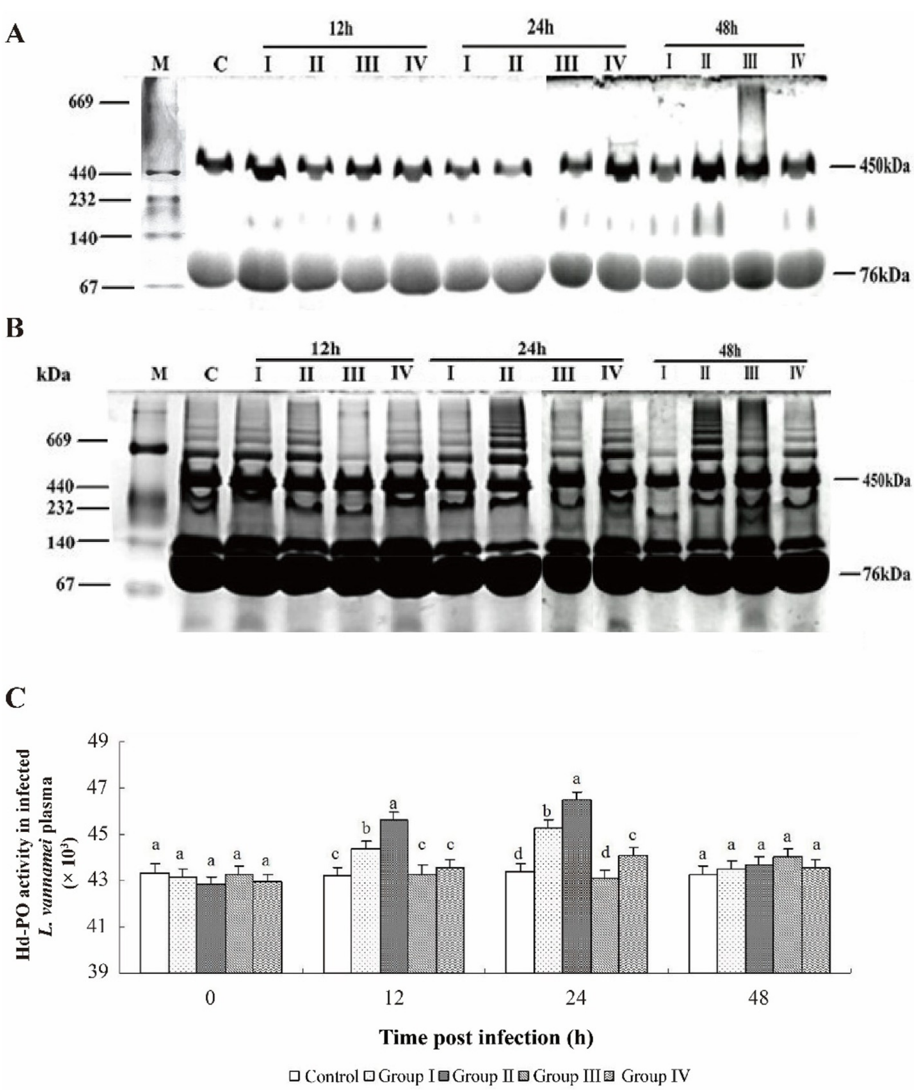

Fig. 5. Native-PAGE analysis of infected *L. vannamei* plasma protein. PO-like protein bands were stained with 4-methyl catecholamine (A), while common protein bands were stained with Coomassie Brilliant Blue G-250 (B). Hd-PO and PO were marked out by their molecular weights. M: Molecular weight marker of protein; C: Control group (uninfected); I, II, III, IV: Group I (10^5 cells mL^{-1} *V. harveyi* challenged), Group II (10^6 cells mL^{-1} *V. harveyi* challenged), Group III (10^5 cells mL^{-1} *S. aureus* challenged), Group IV (10^6 cells mL^{-1} *S. aureus* challenged). 0, 12, 24, and 48 h: Time post infection. Effects of *V. harveyi* and *S. aureus* infection on gray value of Hd-PO activity in infected *L. vannamei* plasma (C).

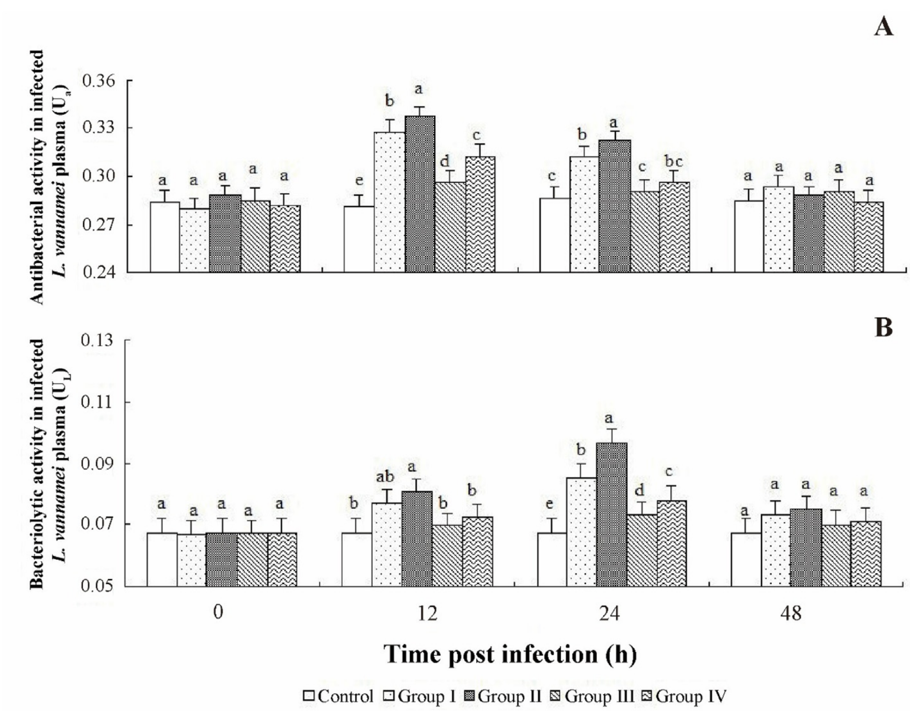

Fig. 6. Effects of *V. harveyi* and *S. aureus* infection on antibacterial (A) and bacteriolytic (B) activities in infected *L. vannamei* plasma. Comparisons were made among the control group (uninfected), Group I (10^5 cells mL^{-1} *V. harveyi* challenged), Group II (10^6 cells mL^{-1} *V. harveyi* challenged), Group III (10^5 cells mL^{-1} *S. aureus* challenged) and Group IV (10^6 cells mL^{-1} *S. aureus* challenged). Data (mean ± SD) at the same exposure time marked with different letters are significantly different ($P < 0.05$).

at 24 h induced by *V. harveyi* and *S. aureus* and recovered to the control group level at 48 h, while it obviously enhanced at 12 h only after 10^6 cells mL^{-1} *V. harveyi* injection ($P < 0.05$, Fig. 6 B). During the experiment, there was no significant change in plasma antimicrobial activity and bacteriolytic activity in the control group (Fig. 6).

4. Discussion

4.1. Effects of V. harveyi and S. aureus stress on hemocyanin synthesis in L. vannamei

Hemocyanin, a respiratory protein mainly exist in arthropods and mollusks, has been an important immune molecule in invertebrate innate humoral immunity. Ample evidences have been reported that crustacean hemocyanin could be converted into PO-like molecule under the stimulation of a variety of substances [38–40], and split out its C terminus or agglutinate pathogens to show its antimicrobial activity under pathogen infection or viral stress [7,58,59]. Moreover, it was reported that hemocyanin would be up-regulated transcriptional and translational levels in shrimp immune tissues under pathogen infection [36,37,60–63]. Although hemocyanin synthetic site in crustaceans has previously been confirmed in hepatopancreas [64,65], comprehensive hemocyanin synthesis from gene expression to post-transcriptional processing to protein expression under pathogen invasion have not been studied. Therefore, effects of pathogen *V. harveyi* and non-pathogen *S. aureus* stress on hemocyanin synthesis in *L. vannamei* were investigated in the present study, including *hemocyanin* gene and subunits expressions in hepatopancreas and hemocyanin concentration in plasma.

The results showed that different bacterial injection had different degrees of effects on the synthesis of hemocyanin. This study showed that two bands can be seen in the regions of 75 and 77 kDa, which were changed under bacterial stress. Previous studies have reported that these two bands are hemocyanin subunits (p75 -subunit and p77-subunit) [16,24,25]. Therefore, these two bands are considered to be two subunits of hemocyanin. Under pathogen stress, hemocyanin concentration in plasma reached the lowest value at 24 h, while hemocyanin subunits expression was highest at 24 h and *hemocyanin* mRNA expression was highest at 12 h in hepatopancreas post-infection. Similarly, it was also reported that the expression of *hemocyanin* mRNA was significantly increased in hepatopancreas of *L. vannamei* under the stress of *Vibrio fluvialis*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, and *S. aureus* [66,67]. The results of this study demonstrated that hemocyanin synthesis had different temporal variations under pathogen invasion, indicating that *hemocyanin* mRNA was rapidly synthesized at the early stage of infection, following translated into hemocyanin subunits, and finally hemocyanin subunits were folded into active hemocyanin secreted into plasma. However, non-pathogen *S. aureus* with high concentration had the same effect on pathogen with weaker influence than pathogen *V. harveyi* stress, but low concentration non-pathogen did not have significant effect on hemocyanin synthesis. Consistent with the results of present study, high concentration of *S. aureus* (10^7 or 3×10^8 cells mL^{-1}) significantly increased the expression of *hemocyanin* mRNA in Chinese mitten crab (*Eriocheir sinensis*) [68], red swamp crayfish (*Procambarus clarkii*) [69], *Rapana venosa* [6], and *Helix aspersa* [6]. It seems that hemocyanin plays a key role in nonspecific humoral immunity of *L. vannamei* under bacterial stress. The nonspecific immune function of hemocyanin has also been reported in Mariculture keyhole limpet (*Megathura crenulata*) [70], horseshoe crab (*Carcinoscorpius rotundicauda*) [71], Concholepas [72], and *Fissurella latimarginata* [73]. In addition, Hd-PO was significantly affected by 10^6 cells mL^{-1} *V. Harveyi* and *S. aureus* stress in this study, suggesting that *V. harveyi* and *S. aureus* with high concentration triggered immune function of hemocyanin as foreign matters in shrimp, further improving the significant hemocyanin synthesis. Therefore, these above results indicate that pathogen and non-pathogenic bacteria with high concentration can stimulate the synthesis of hemocyanin, and the new synthetic hemocyanin may regulate shrimp immunity by its PO-like and antibacterial activities.

4.2. Effects of V. harveyi and S. aureus stress on immune responses in L. vannamei

In recent years, some studies have focused on the immune response of white shrimp and its susceptibility to *Vibrio alginolyticus* under different environmental stresses [44,74–76]. Many researchers have been closely monitoring the application of antibacterial agents and immune stimulants into shrimp aquaculture to investigate their effectiveness against potential pathogens and role in shrimp growth [45,77,78]. Meanwhile, it is believed that PO activity was directly related to pathogen infection in crustaceans [79–81]. However, there have been no studies on the relationship between Hd-PO activity and pathogen infection or the relative regulatory function of antibacterial and bacteriolytic activities. In addition, we all know that most PO activity in plasma is derived from pro-PO system. Compared with pro-PO system, the PO activity of hemocyanin is indeed weak, so researchers often ignore the PO activity of hemocyanin. Therefore, this study detected the plasma PO, Hd-PO, antibacterial, and bacteriolytic activities under bacterial stress in *L. vannamei*.

The present study showed that high concentration of pathogen (*V. harveyi*) and non-pathogen (*S. aureus*) significantly improved PO, Hd-PO, antibacterial and bacteriolytic activities, while low concentration of non-pathogen could only stimulate the change of antibacterial and bacteriolytic activities. In this study, Native-PAGE was used to detect the Hd-PO activity, which showed significant bands in the reign around the weight of hemocyanin (450 kDa) and pro-PO (76 kDa). Although several researchers believe that the majority of PO activity is produced by pro-PO system (76 kDa), the present study showed that the band at 450 kDa can obviously display the PO activity of hemocyanin. Similar to this study, several researchers also used this method to explore Hd-PO [82,83]. Therefore, the results of this study showed obviously Hd-PO activity, but the function of Hd-PO needs further study. Similarly, Coates and Talbot reported that Hd-PO reaction products had antimicrobial properties involving in the innate immunity of horseshoe crab (*Limulus polyphemus*) [20]. Moreover, hemocyanin with potent PO activity has also been reported in giant freshwater prawn (*Macrobrachium rosenbergii*) [83] and Japanese spiny lobster (*Panulirus japonicus*) [84]. Besides, several reviews have described the regulation of PO in invertebrates' immune defense, such as crustaceans and bivalves [5,85,86]. In addition, recent reports showed that hemocyanin has critical antimicrobial and bacteriolytic activities to resist the invasion of external microorganisms, which was reported in *Fenneropenaeus chinensis* [87], *Haliotis tuberculate* [88], *Rapana venosa* [6], *Helix aspersa* [6], *Scylla paramamosain* [89,90], and *L. vannamei* [91–93]. Therefore, combining these reports with the results of this study indicate that pathogen can induce an increase in the relevant humoral immune parameters in shrimp, whereas the non-pathogen may be treated as foreign matters with quick different responses in antibacterial and bacteriolytic activities, revealing different interactions between microorganisms and antibacterial or bacteriolytic substances in shrimp plasma. It was concluded that pathogen invasion caused significant changes in immune responses of shrimp plasma and non-pathogen was basically internalized into foreign matters. Besides, abnormal changes in immune parameters probably indicate specific interaction patterns between immune molecules and foreign microorganisms, which requires further probe into immune regulation mechanism. Overall, this study demonstrates that the immune defense response is regulated by plasma PO and Hd-PO activities as well as antimicrobial and bacteriolytic activities in *L. vannamei* under bacterial stress.

5. Conclusion

In conclusion, the present study documented that the initiation of hemocyanin synthesis and induction of *hemocyanin* gene expression in hepatopancreas of *L. vannamei* under bacterial stress (pathogen or non-pathogen), while the new synthetic hemocyanin showed its immune function like Hd-PO taking part in immune defense in the hemolymph of shrimp. Moreover, high concentration of non-pathogen had the same effects as the pathogen. This study reports the combination of hemocyanin synthesis (mRNA and protein), plasma PO and Hd-PO, and immune defense parameters of antimicrobial and bacteriolytic activities of *L. vannamei* after bacterial infection *in vivo*. It provides a theoretical basis for further investigating the regulation mechanism of hemocyanin in innate immune of crustaceans *in vivo*. Future studies need to explore the mechanism of hemocyanin exerting PO activity and the signal transduction mechanism of PO under bacterial stress in crustaceans, in order to have a better understanding of their immune defense function.

Disclosure summary

The authors have nothing to disclose.

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References

[1] E.S. Loker, C.M. Adema, S.M. Zhang, T.B. Kepler, Invertebrate immune systems-not homogeneous, not simple, not well understood, Immunol. Rev. 198 (1) (2004) 10–24.
[2] N.G. Van, S.M. Hattingh, A.M. Engelbrecht, Invertebrates: why no adaptive immune system? Scand. J. Immunol. 83 (2) (2016) 160–161.
[3] C. Hauton, The scope of the crustacean immune system for disease control, J. Invertebr. Pathol. 110 (2) (2012) 251–260.
[4] G. Chiara, S. Filippo, B. Loriano, Insight on cellular and humoral components of innate immunity in *Squilla mantis* (Crustacea, Stomatopoda), Fish Shellfish Immunol. 31 (3) (2011) 423–431.
[5] C.J. Coates, H. Decker, Immunological properties of oxygen-transport proteins: hemoglobin, hemocyanin and hemerythrin, Cell. Mol. Life Sci. 74 (2) (2017) 293–317.
[6] P. Dolashka, A. Dolashki, J.V. Beeumen, M. Floetenmeyerd, L. Velkova, S. Stevanovic, W. Voelter, Antimicrobial activity of Molluscan hemocyanins from *Helix* and *Rapana Snails*, Curr. Pharmaceut. Biotechnol. 17 (3) (2016) 1–8.
[7] L. So Young, L. Bok Luel, S.D.L. Kenneth, Processing of an antibacterial peptide from hemocyanin of the freshwater crayfish *Pacifastacus leniusculus*, J. Biol. Chem. 278 (10) (2003) 7927–7933.
[8] K. Adachi, K. Wakamatsu, S.,N. Miyamoto, T. Kokubo, T. Nishioka, T. Hirata, An oxygen transporter hemocyanin can act on the late pathway of melanin synthesis, Pigment Cell Res. 18 (3) (2010) 214–219.
[9] C.J. Coates, J. Nairn, Diverse immune functions of hemocyanins, Dev. Comp. Immunol. 45 (1) (2014) 43–55.
[10] W. Decleir, A. Richard, A study of the blood proteins in *Sepia officinalis* L. with special reference to embryonic hemocyanin, Comp. Biochem. Physiol. 34 (1) (1970) 203–211.
[11] J.S. Djangmah, The effects of feeding and starvation on copper in the blood and hepatopancreas, and on blood proteins of *crangon vulgaris* (fabricius), Comp. Biochem. Physiol. 32 (4) (1970) 709–718.
[12] T.Y. Zhong, S. Arancibia, R. Born, R. Tampe, J. Villar, C.M. Del, A. Manubens, M.I. Becker, Hemocyanins stimulate innate immunity by inducing different temporal patterns of proinflammatory cytokine expression in macrophages, J. Immunol. 196 (11) (2016) 4650–4662.
[13] K.Z. Wang, L.Y. Wen, Z.C. Ye, H.G. Wu, J.Y. Pan, Hemocyanin-derived phenoloxidase activity is dependent on dodecameric structure in shrimp *Litopenaeus vannamei*, Arch. Biol. Sci. 67 (1) (2015) 19–23.
[14] T. Zlateva, P. Muro, Di, B. Salvato, M. Beltramini, The o-diphenol oxidase activity of arthropod hemocyanin, FEBS Lett. 384 (3) (1996) 251–254.
[15] J. Zhuang, C.J. Coates, H. Zhu, P. Zhu, Z. Wu, L. Xie, Identification of candidate antimicrobial peptides derived from abalone hemocyanin, Dev. Comp. Immunol. 49 (1) (2015) 96–102.
[16] X. Zhang, C. Huang, Q. Qin, Antiviral properties of hemocyanin isolated from shrimp *Penaeus monodon*, Antivir. Res. 61 (2) (2004) 93–99.
[17] J.Y. Pan, W.U. Hai-Gang, H.B. Wang, Differences in the agglutination activity of two oligomers of hemocyanin from *Litopenaeus vannamei*, J. Pathog. Biol. 6 (1) (2011) 26–37.
[18] Y. Zhang, F. Yan, Z. Hu, X. Zhao, S. Min, Z. Du, S. Zhao, X. Ye, Y. Li, Hemocyanin from shrimp *Litopenaeus vannamei* shows hemolytic activity, Fish Shellfish Immunol. 27 (2) (2009) 330–335.
[19] K. Adachi, T. Hirata, T. Nishioka, M. Sakaguchi, Hemocyte components in crustaceans convert hemocyanin into a phenoloxidase-like enzyme, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 134 (1) (2003) 135–141.
[20] C.J. Coates, J. Talbot, Hemocyanin-derived phenoloxidase reaction products display anti-infective properties, Dev. Comp. Immunol. 86 (2018) 47–51.
[21] C.J. Coates, S.M. Kelly, J. Nairn, Possible role of phosphatidylserine–hemocyanin interaction in the innate immune response of *Limulus polyphemus*, Dev. Comp. Immunol. 35 (2) (2011) 155–163.
[22] R. Perdomo-Morales, V. Montero-Alejo, E. Perera, Z. Pardo-Ruiz, E. Alonso-Jiménez, Phenoloxidase activity in the hemolymph of the spiny lobster *Panulirus argus*, Fish Shellfish Immunol. 23 (6) (2007) 1187–1195.

[23] G.K. Sang, B.W. Jung, H.H. Kim, Hemocyanin-derived phenoloxidase activity with broad temperature stability extending into the cold environment in hemocytes of the hair crab *Erimacrus isenbeckii*, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 159 (2) (2011) 103–108.
[24] Y. Zhang, Identification of the main proteins binding with pathogen directly in *Litopenaeus vannamei* serum, J. Fish. China 32 (1) (2008) 105–110.
[25] Y.L. Zhang, Y.M. Zhuo, Y.F. Zhu, S.Y. Wang, T.W. Huang, Identification of two types of main differential proteinsin the hepatopancreas in whiteleg shrimp *Litopenaeus vannamei* infected by pathogenic bacteria Vibrio alginolyticus, Fish. Sci. 24 (6) (2005) 19–23.
[26] P. Amparyup, W. Charoensapsri, A. Tassanakajon, Two prophenoloxidases are important for the survival of *Vibrio harveyi* challenged shrimp *Penaeus monodon*, Dev. Comp. Immunol. 33 (2) (2009) 247–256.
[27] W. Charoensapsri, P. Amparyup, I. Hirono, T. Aoki, A. Tassanakajon, Gene silencing of a prophenoloxidase activating enzyme in the shrimp, *Penaeus monodon*, increases susceptibility to *Vibrio harveyi* infection, Dev. Comp. Immunol. 33 (7) (2009) 811–820.
[28] C. Zhaoxia, L. Yuan, W. Danhua, L. Weisha, W. Shuangyan, L. Qianqian, S. Chengwen, Molecular cloning and characterization of a serine proteinase homolog prophenoloxidase-activating factor in the swimming crab *Portunus trituberculatus*, Fish Shellfish Immunol. 29 (4) (2010) 679–686.
[29] S. Radha, P. Mullainadhan, M. Arumugam, Detection of two distinct types of hemolymphatic prophenoloxidase and their differential responses in the black tiger shrimp, *Penaeus monodon*, upon infection by white spot syndrome virus, Aquaculture 376 (3) (2013) 76–84.
[30] K. Söderhäll, L. Cerenius, Role of the prophenoloxidase-activating system in invertebrate immunity, Curr. Opin. Immunol. 10 (1) (1998) 23–28.
[31] Z.Y. Fang, L. Dan, X.J. Li, Z. Xing, Y.T. Zhu, W.W. Li, Q. Wang, A single CRD C-type lectin from *Eriocheir sinensis* (EsLecB) with microbial-binding, antibacterial prophenoloxidase activation and hem-encapsulation activities, Fish Shellfish Immunol. 50 (2016) 175–190.
[32] X. Zhang, L. Pan, J. Yu, H. Huang, One recombinant C-type lectin (LvLec) from white shrimp *Litopenaeus vannamei* affected the haemocyte immune response *in vitro*, Fish Shellfish Immunol. 89 (2019) 35–42.
[33] L.M. Perazzolo, M.A. Barracco, The prophenoloxidase activating system of the shrimp *Penaeus paulensis* and associated factors, Dev. Comp. Immunol. 21 (5) (1997) 385–395.
[34] A. Piti, S. Jantiwan, C. Walaiporn, T. Anchalee, Pattern recognition protein binds to lipopolysaccharide and β-1,3-glucan and activates shrimp prophenoloxidase system, J. Biol. Chem. 287 (13) (2012) 10060–10069.
[35] G. Arumugam, B. Sreeramulu, R. Paulchamy, S. Thangavel, J. Sundaram, Purification and functional characterization of lectin with phenoloxidase activity from the hemolymph of cockroach, Periplaneta americana, Arch. Insect Biochem. Physiol. 95 (2) (2017) 1–20.
[36] K. Somboonwiwat, V. Chaikeeratisak, H.C. Wang, F.L. Chu, A. Tassanakajon, Proteomic analysis of differentially expressed proteins in *Penaeus monodon* hemocytes after *Vibrio harveyi* infection, Proteome Sci. 8 (1: 39) (2010) 1–11.
[37] S. Nayak, K.M. Ajay, N. Ramaiah, R.M. Meena, R.A. Sreepada, Profiling of a few immune responsive genes expressed in postlarvae of *Fenneropenaeus indicus* challenged with *Vibrio harveyi* D3, J. Invertebr. Pathol. 107 (2) (2011) 168–172.
[38] R. Perdomo-Morales, V. Montero-Alejo, E. Perera, Z. Pardo-Ruiz, E. Alonso-Jiménez, Hemocyanin-derived phenoloxidase activity in the spiny lobster *Panulirus argus* (Latreille, 1804), Biochim. Biophys. Acta Gen. Subj. 1780 (4) (2008) 652–658.
[39] T. Fan, Y. Zhang, L. Yang, X. Yang, G. Jiang, M. Yu, R. Cong, Identification and characterization of a hemocyanin-derived phenoloxidase from the crab *Charybdis japonica*, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 152 (2) (2009) 144–149.
[40] K. Idakieva, Y. Raynova, F. Meersman, C. Gielens, Phenoloxidase activity and thermostability of *Cancer pagurus* and *Limulus polyphemus* hemocyanin, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 164 (3) (2013) 201–209.
[41] Q. Zhao, L. Pan, Q. Ren, L. Wang, J. Miao, Effect of salinity on regulation mechanism of neuroendocrine-immunoregulatory network in *Litopenaeus vannamei*, Fish Shellfish Immunol. 49 (2016) 396–406.
[42] L.M. Perazzolo, R. Gargioni, P. Ogliari, M.A.A. Barracco, Evaluation of some hemato-immunological parameters in shrimp *Farfantepenaeus paulensis* submitted to environmental and physiological stress, Aquaculture 214 (1) (2002) 19–33.
[43] J.L. Balcázar, T. Rojas-Luna, D.P. Cunningham, Effect of the addition of four potential probiotic strains on the survival of pacific white shrimp (*Litopenaeus vannamei*) following immersion challenge with *Vibrio parahaemolyticus*, J. Invertebr. Pathol. 96 (2) (2007) 147–150.
[44] C.C. Li, J.C. Chen, The immune response of white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio alginolyticus* under low and high pH stress, Fish Shellfish Immunol. 25 (6) (2008) 701–709.
[45] K. Kongnum, T. Hongpattarakere, Effect of *Lactobacillus plantarum* isolated from digestive tract of wild shrimp on growth and survival of white shrimp (*Litopenaeus vannamei*) challenged with *Vibrio harveyi*, Fish Shellfish Immunol. 32 (1) (2012) 170–177.
[46] F. Vargas-Albores, M.A. Guzmán, J.L. Ochoa, An anticoagulant solution for haemolymph collection and prophenoloxidase studies of penaeid shrimp (*Penaeus californiensis*), Comp. Biochem. Physiol. Part A Physiology 106 (2) (1993) 299–303.
[47] K.W. Nickerson, K.E.V. Holde, A comparison of molluscan and arthropod hemocyanin—I. Circular dichroism and absorption spectra, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 39 (4) (2015) 855–872.
[48] S.P. Baden, S.P. Eriksson, J.M. Weeks, Uptake, accumulation and regulation of managanese during experimental hypoxia and normoxia by the decapod *Nephrops norvegicus* (L.), Mar. Pollut. Bull. 31 (3) (1995) 93–102.
[49] W. Yu-Chi, C. Poh-Shing, C. Houng-Yung, Tissue expressions of nine genes important to immune defence of the Pacific white shrimp *Litopenaeus vannamei*, Fish Shellfish Immunol. 23 (6) (2007) 1161–1177.
[50] L. Zhang, L. Pan, L. Xu, L. Si, Effects of ammonia-N exposure on the concentrations of neurotransmitters, hemocyte intracellular signaling pathways and immune responses in white shrimp *Litopenaeus vannamei*, Fish Shellfish Immunol. 75 (2018) 48–57.
[51] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method, Methods 25 (4) (2001) 402–408.
[52] U. Laemmli, Cleavage of structure proteins during the assembly of the head of bacteriophage T4, Nature 40 (1970) 734–744.
[53] T. Burmester, Evolutionary history and diversity of arthropod hemocyanins, Micron 35 (1–2) (2004) 121–122.
[54] M. Ashida, Purification and characterization of pre-phenoloxidase from hemolymph of the silkworm *Bombyx mori*, Arch. Biochem. Biophys. 144 (2) (1971) 749–762.
[55] D. Hultmark, H. Steiner, T. Rasmuson, H.G. Boman, Insect immunity. Purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of *Hyalophora cecropia*, Eur. J. Biochem. 106 (1) (1980) 7–16.
[56] H.G. Boman, I. Nilsson-Faye, K. Paul, T. Rasmuson, Insect immunity I. Characteristics of an inducible cell-free antibacterial reaction in hemolymph of *Samia cynthia* pupae, Infect. Immun. 10 (1) (1974) 136–145.
[57] L. Wang, G. Li, Y. Mao, Studies on the activities and characteristics of the anti-bacteria, bacteriolysis and phenoloxidase in the haemolymph of *Penaeus chinensis*, Oceanol. Limnol. Sinica 26 (2) (1995) 179–185.
[58] D. Destoumieux-Garzon, D. Saulnier, J. Garnier, C. Jouffrey, P. Bulet, E. Bachere, Crustacean immunity. Antifungal peptides are generated from the C terminus of shrimp hemocyanin in response to microbial challenge, J. Biol. Chem. 276 (50) (2001) 47070–47077.
[59] F. Yan, Y. Zhang, R. Jiang, M. Zhong, Z. Hu, H. Du, J. Lun, J. Chen, Y. Li, Identification and agglutination properties of hemocyanin from the mud crab (*Scylla serrata*), Fish Shellfish Immunol. 30 (1) (2011) 354–360.
[60] S. Nayak, S.K. Singh, N. Ramaiah, R.A. Sreepada, Identification of upregulated immune-related genes in *Vibrio harveyi* challenged *Penaeus monodon* postlarvae, Fish Shellfish Immunol. 29 (3) (2010) 544–549.
[61] A.M. Tarrant, J.J. Stegeman, V. Tim, Altered gene expression associated with epizootic shell disease in the American lobster, *Homarus americanus*, Fish Shellfish Immunol. 29 (6) (2010) 1003–1009.
[62] V. Chaikeeratisak, K. Somboonwiwat, H.C. Wang, F.L. Chu, A. Tassanakajon, Proteomic analysis of differentially expressed proteins in the lymphoid organ of *Vibrio harveyi*-infected *Penaeus monodon*, Mol. Biol. Rep. 39 (5) (2012) 6367–6377.
[63] L. Xiang, S. Xin-Tao, Z. Qing-Feng, L. Hui, P. Xuan-Xian, Heterogeneous interactome between *Litopenaeus vannamei* plasma proteins and *Vibrio parahaemolyticus* outer membrane proteins, Fish Shellfish Immunol. 34 (1) (2013) 192–198.
[64] J. Rainer, M. Brouwer, Hemocyanin synthesis in the blue crab *Callinectes sapidus*, Comp. Biochem. Physiol. Part B Comparative Biochemistry 104 (1) (1993) 69–73.
[65] M. Khayat, B. Funkenstein, A. Tietz, E. Lubzens, *In vivo, in vitro* and cell-free synthesis of hemocyanin in the shrimp *Penaeus semisulcatus* (de Haan), Comp. Biochem. Physiol. B Biochem. Mol. Biol. 112 (1) (1995) 31–38.
[66] L. Xin, L. Hui, L. Guo, Z. Zhang, X. Zhao, M. Zhong, S. Li, Y. Zhang, Cloning and characterization of a novel hemocyanin variant LvHMCV4 from shrimp *Litopenaeus vannamei*, Fish Shellfish Immunol. 46 (2) (2015) 398–405.
[67] X. Zhao, L. Guo, X. Lu, H. Lu, F. Wang, M. Zhong, J. Chen, Y. Zhang, Evidences of abundant hemocyanin variants in shrimp *Litopenaeus vannamei*, Mol. Immunol. 77 (2016) 103–112.
[68] Y. Huang, X. Huang, L. Hou, L. An, K.M. Hui, Q. Ren, W. Wang, Molecular cloning and characterization of three novel Hemocyanins from Chinese mitten crab, *Eriocheir sinensis*, Aquaculture 434 (2014) 385–396.
[69] Z. Qin, V.S. Babu, Q. Wan, A. Muhammad, J. Li, J. Lan, L. Lin, Antibacterial activity of hemocyanin from red swamp crayfish (*Procambarus clarkii*), Fish Shellfish Immunol. 75 (2018) 391–399.
[70] J. Pizarrobauerle, I. Maldonado, E. Sosoniukroche, G. Vallejos, M.N. López, F. Salazaronfray, L. Aguilarguzmán, C. Valck, A. Ferreira, M.I. Becker, Molluskan hemocyanins activate the classical pathway of the human complement system through natural antibodies, Front. Immunol. 8 (2017) 188.
[71] N. Jiang, N. Tan, B.,J. Ding, Respiratory protein-generated reactive oxygen species as an antimicrobial strategy, Nat. Immunol. 8 (10) (2007) 1114–1122.
[72] S. Arancibia, M.D. Campo, E. Nova, F. Salazar, M.I. Becker, Enhanced structural stability of Concholepas hemocyanin increases its immunogenicity and maintains its non-specific immunostimulatory effects, Eur. J. Immunol. 42 (3) (2012) 688–699.
[73] M. Becker, S. Arancibia, E. Cecilia, F. Salazar, M. Del Campo, R. Born, J. Ferreira, A. Manubens, A. De Ioannes, Novel hemocyanin from the *Fissurella latimarginata* exhibits an outstanding immunogenicity and non-specific immunomodulatory effects in a melanoma model, J. Immunol. 188 (5) (2012) 277.
[74] W. Cheng, L.U. Wang, J.C. Chen, Effect of water temperature on the immune response of white shrimp *Litopenaeus vannamei* to *Vibrio alginolyticus*, Aquaculture 250 (3) (2005) 592–601.
[75] L.U. Wang, J.C. Chen, The immune response of white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio alginolyticus* at different salinity levels, Fish Shellfish Immunol. 18 (4) (2005) 269–278.
[76] S.W. Hsu, J.C. Chen, The immune response of white shrimp *Penaeus vannamei* and its susceptibility to *Vibrio alginolyticus* under sulfide stress, Aquaculture 271 (1–4) (2007) 61–69.
[77] S. Soto-Rodríguez, M. Armenta, B. Gomez-Gil, Effects of enrofloxacin and florfenicol on survival and bacterial population in an experimental infection with luminescent *Vibrio campbellii* in shrimp larvae of *Litopenaeus vannamei*, Aquaculture 255 (1–4) (2006) 48–54.

[78] M.D.C. Flores-Miranda, A. Luna-González, Á.I. Campa-Córdova, H.A. González-Ocampo, J.A. Fierro-Coronado, B.O. Partida-Arangure, Microbial immunostimulants reduce mortality in whiteleg shrimp (*Litopenaeus vannamei*) challenged with *Vibrio sinaloensis* strains, Aquaculture 320 (1–2) (2011) 51–55.
[79] S.L. Hsieh, Y.H. Ruan, Y.C. Li, P.S. Hsieh, C.H. Hu, C.M. Kuo, Immune and physiological responses in Pacific white shrimp (*Penaeus vannamei*) to *Vibrio alginolyticus*, Aquaculture 275 (1–4) (2008) 335–341.
[80] C.C. Li, S.T. Yeh, J.C. Chen, The immune response of white shrimp *Litopenaeus vannamei* following *Vibrio alginolyticus* injection, Fish Shellfish Immunol. 25 (6) (2008) 853–860.
[81] S.L. Xu, D.L. Wang, C.Y. Jia, S. Jin, C.L. Wang, X. Zou, Effects of *Vibrio alginolyticus* infection on immune-related enzyme activities and ultrastructure of *Charybdis japonica* gills, Aquaculture 396–399 (6) (2013) 82–88.
[82] F. Yan, Y.L. Zhang, H.Q. Luo, Z. Hu, T.W. Huang, X.Q. Ye, The phenoloxidase activity of hemocyanin from white leg shrimp *Litopenaeus vannamei*, Fish. Sci. 27 (1) (2008) 5–8.
[83] S.M. Ramasamy, M. Denis, S. Sivakumar, A. Munusamy, Phenoloxidase activity in humoral plasma, hemocyanin and hemocyanin separated proteins of the giant freshwater prawn *Macrobrachium rosenbergii*, Int. J. Biol. Macromol. 102 (2017) 977–985.
[84] T. Masuda, T. Kawauchi, Y. Yata, Y. Matoba, H. Toyohara, Two types of phenoloxidases contribute to hemolymph PO activity in spiny lobster, Food Chem. 260 (2018) 166–173.
[85] A. Luna-Acosta, M. Breitwieser, T. Renault, H. Thomas-Guyon, Recent findings on phenoloxidases in bivalves, Mar. Pollut. Bull. 122 (1–2) (2017) 5–16.
[86] L. Cerenius, B.L. Lee, K. Söderhäll, The proPO-system: pros and cons for its role in invertebrate immunity, Trends Immunol. 29 (6) (2008) 263–271.
[87] C. Qiu, J. Sun, M. Liu, B. Wang, K. Jiang, S. Sun, X. Meng, Z. Luo, L. Wang, Molecular cloning of hemocyanin cDNA from *Fenneropenaeus chinensis* and antimicrobial analysis of two C-terminal fragments, Mar. Biotechnol. 16 (1) (2014) 46–53.
[88] Z.N. Talaei, M. Miranda-Saksena, P. Valtchev, R.J. Diefenbach, L. Hueston, E. Diefenbach, F. Sairi, V.G. Gomes, A.L. Cunningham, F. Dehghani, Abalone hemocyanin blocks the entry of herpes simplex virus 1 into cells: a potential new antiviral strategy, Antimicrob. Agents Chemother. 60 (2) (2016) 1003–1012.
[89] J. Wang, F.Y. Zhang, W. Song, Y.B. Fang, J.H. Hu, M. Zhao, K.J. Jiang, L.B. Ma, Characterization of hemocyanin from the mud crab *Scylla paramamosain* and its expression analysis in different tissues, at various stages, and under *Vibrio parahaemolyticus* infection, Genet. Mol. Res. Gmr 14 (4) (2015) 16639–16651.
[90] J. Cao, Z. Wang, Y. Zhang, F. Qu, L. Guo, M. Zhong, S. Li, H. Zou, J. Chen, X. Wang, Identification and characterization of the related immune-enhancing proteins in crab *Scylla paramamosain* stimulated with rhubarb polysaccharides, Mol. Immunol. 57 (2) (2014) 263–273.
[91] Z. Zhang, F. Wang, C. Chen, Z. Zheng, J.J. Aweya, Y. Zhang, Glycosylation of hemocyanin in *Litopenaeus vannamei* is an antibacterial response feature, Immunol. Lett. 192 (2017) 42–47.
[92] Y. Wen, S. Zhan, H. Huang, M. Zhong, J. Chen, C. You, F. Wang, Y. Zhang, Identification and characterization of an 18.4kDa antimicrobial truncation from shrimp *Litopenaeus vannamei* hemocyanin upon *Vibrio parahaemolyticus* infection, Fish Shellfish Immunol. 56 (2016) 450–458.
[93] S. Yang, H. Huang, F. Wang, J.J. Aweya, Z. Zheng, Y. Zhang, Prediction and characterization of a novel hemocyanin-derived antimicrobial peptide from shrimp *Litopenaeus vannamei*, Amino Acids 50 (8) (2018) 995–1005.