Proteomic identification of the related immune-enhancing proteins in shrimp *Litopenaeus vannamei* stimulated with vitamin C and Chinese herbs

Jie Qiao¹, Zhiheng Du¹, Yueling Zhang d, Hong Du¹, Lingling Guo¹, Mingqi Zhong¹, Jingsong Ca³, Xiuying Wanga

¹Department of Biology and Guangdong Provincial Key laboratory of Marine Biotechnology, Shantou University, Shantou, Guangdong 515063, China
³Shantou Huaxun Aquatic Product Corporation, Shantou, Guangdong 515061, China

**A R T I C L E   I N F O**

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**A B S T R A C T**

Recently, strong interest has been focused on immunostimulants to reducing the diseases in shrimp aquaculture. However, information regarding to the related immune-enhancing proteins in shrimps is not available yet. In this study, vitamin C (Vc), Chinese herbs (CH), and the mixture of vitamin C and Chinese herbs (Mix) were tested for their enhancement on shrimp’s immune activity. Compared with those in the control group, values of phenoloxidase (PO), superoxide dismutase (SOD) and antibacterial (Ua) activity in the Mix-treated group were improved significantly 12 or 24 days after the treatment. The cumulative mortality was also lower in the Mix-treated group after infection with *Vibrio parahaemolyticus*. Furthermore, comparative proteomic approach was used to assess the protein expression profile in shrimps. Approximately 220–290 and 300–400 protein spots were observed in the 2-DE gels. Among them, 29 and 28 altered proteins from hemocytes and hepatopancreas, respectively, were subjected to matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) analysis. The results revealed that the main altered proteins showed high homologies with *Litopenaeus vannamei* hemocyanin, hemolymph clottable protein, hemoglobin beta, cytosolic MnSOD, trypsin, cathepsin I(L) and zinc proteinase Mpc1. Together, these studies found Vc and CH were suitable immunostimulants to shrimp *L. vannamei*, and 7 altered proteins could be involved in the enhanced immune activities.

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1. Introduction

Within the past twenty years, the global shrimp industry has suffered from a great economic loss due to viral, bacterial and fungal diseases such as the infection of white spot syndrome virus (WSSV), Taura syndrome virus (TSV), *Vibrio parahaemolyticus* and *Vibrio harveyi* [1–4]. Understanding of the shrimp immunology will be critically important to establishing strategies for disease control and the development of a sustainable aquaculture. Recently, a large variety of immunostimulants have been applied to control shrimp diseases. Lee and Shiau found that the weight gain of shrimp was improved as the level of vitamin E was increased in the diet [5]. Huang et al. documented that the vibriosis resistance and general immune activity in shrimp were improved 14 days after the oral administration of *Sargassum fusiforme* polysaccharide extract (SFPSE) [6]. In addition, vitamin C (Vc), various polysaccharides and some Chinese herbs (CH) could also enhance the resistance against pathogens by improving the shrimp’s immune activity [7–10]. However, the underlying mechanism of various immunostimulants to activate or boost the innate immune system is still not clear.

Two-dimensional polyacrylamide gel electrophoresis (2-DE) followed by mass spectrometry (MS) has been widely used in identification of protein variations and the protein repertoire of specific tissues [11,12]. Similarly, numerous studies on the immune response of aquatic animals to virus infection, bacteria infection and stress were performed by using proteomic strategies [13–15].

In this study, we evaluated the effects of Vc and CH in shrimp *Litopenaeus vannamei* on its cellular (phenoloxidase, superoxide dismutase, peroxidase) and humoral defenses (antibacterial). Furthermore, differentially expressed proteins in hemocytes and hepatopancreas after feeding with Vc and CH were investigated through a proteomic approach. To our knowledge, this is the first application of proteomic approach to identify altered protein responses to immunostimulants in shrimp *L. vannamei*. 

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¹ Corresponding author. Department of Biology, School of Science, Shantou University, No.243 Daxue Road, Shantou, Guangdong 515063, China. Tel.: +86 754 82902708; fax: +86 754 82902767.

E-mail address: zhangyl@stu.edu.cn (Y. Zhang).

¹ Contribution is equal.

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2. Materials and methods

2.1. Experimental animals and rearing conditions

Healthy *P. vannamei* with average size of 10–12 cm in length and 8–10 g by weight from Shantou Huaxun Aquatic Product Corporation were obtained and immediately transferred to indoor ponds. 680 shrimps were divided randomly into four groups: Control, Vc (vitamin C treated), CH (Chinese herbs treated) and Mix (Vc and CH-treated). The water was changed once a week and the air was continuously supplied using an electric pump. During the experiments, the water temperature was maintained at 25–35 °C, and salinity at 8–10‰. Ponds were placed in a light-regulated (12-h light/dark cycle) containers (400 cm × 400 cm × 75 cm). Prior to the experiment, animals were allowed to acclimatize for at least 7 days.

2.2. Preparation and use of immunostimulants containing diets

Vitamin C was purchased from Hpsion-Health (Shenzhen, China). Chinese herbs including *Licorice* root, *Astragalus membranaceus*, *poris cocos*, Chinese Angelica, *Rhizoma Atractylodis Macrocephala* and *Codonopsis pilosula* were purchased from a local Tuopo pharmacy. The dose ratio of Chinese herbs was 6:30:10:10:30:10, which was modified from Wang [16]. The dry ingredients were minced and mixed thoroughly. Water and a commercial diet produced by Longhai Bole Animal Husbandry (Shantou, China) were added until the mixture was broken up and sieved to obtain pellets in a convenient size. The content of vitamin C and Chinese herbs was 0.2% and 0.5%, respectively.

2.3. Preparation of the supernatant of hemolymph and hemocytes

Hemolymph was taken from the ventral sinus using a 1 ml sterile syringe and immediately mixed with an equal volume of anticoagulant (26 mM citrate, 30 mM sodium citrate, 0.45 M sodium chloride, 10 mM EDTA, 0.1 M glucose, pH 7.6) at day 12 and 24 during a 24-day feeding period. The hemolymph was centrifuged immediately at 600 g for 15 min (4 °C) to separate the hemocytes from plasma. The supernatants of hemolymph and hemocytes were kept at −80 °C for further assays.

2.4. Extraction of proteins and protein concentration determination

Hemocytic protein extraction was performed using a lysis buffer containing 8 M urea, 2 M thiourea, 4% v/v CHAPS, 50 mM DTT, 1 mM PMSF, and 1 mM benzamidine. After lysing, the cell lysate was centrifuged at 12,000 rpm and 4 °C for 15 min, and the supernatant was dialyzed overnight in pre-cooled 0.01 M PBS. The protein solution was used immediately for isoelectric focusing (IEF) or stored at −20 °C until posterior use for no more than 2 weeks.

The extraction of hepatopancreas proteins was performed according to Lee et al. [17] with some modifications. Briefly, 500 mg hepatopancreas were removed from shrimp at day 12 and 24 during the feeding period. The sample was mixed with 2 ml of Trizol reagent and ground thoroughly with a pestle in a 5 ml microcentrifuge tube. Subsequently, 400 μl of chloroform was added to the cell lysate and shaken vigorously for 15 s. The mixture was allowed to stand for 5 min at room temperature and then centrifuged at 12,000 rpm for 15 min at 4 °C. The pellet was resuspended with 600 μl of ethanol and then centrifuged at 4500 rpm for 5 min at 4 °C. Supernatant was transferred to a new tube and 3 ml of isopropanol was added. The mixture was allowed to stand for at least 30 min at room temperature. The precipitated proteins were then centrifuged at 12,000 rpm for 15 min at 4 °C. Pellet obtained was briefly washed with 95% ethanol before being air dried and then kept at −80 °C.

Protein concentration was determined by a modified Bradford assay (Bio-Rad, USA) using BSA as a standard protein [18].

2.5. Immune parameter assay

The phenoloxidase (PO) activity was assayed spectrophotometrically by recording the formation of dopachrome produced from 1-dihydroxyphenylalanine (1-DOPA), which was modified from the method reported previously by Hernández-López et al. [19]. 10 μl of supernatant of hemolymph (50 mg/ml) or hepatopancreas (5 mg/ml) were added to 200 μl of phosphate buffer solution (KH₂PO₄/ KH₂PO₄, 0.1 M, pH 6.0). 10 μl of 1-DOPA 0.01 M were then added as the substrate. The mixture was incubated at 25 °C for 2 min. The optical density at 490 nm was measured every 3 min using a microplate reader. An increment of 0.001 absorbance every min under this condition was defined as one unit of PO activity. The assay was conducted in triplicate.

The SOD (superoxide dismutase) activity was measured using with some slight modifications [20]. Briefly, the reaction buffer containing 50 mM Tris–HCl, 0.1 mM EDTA and 10 mM guaiacol was incubated at 25 °C. 1 μl of 30% hydrogen peroxide and 50 μl of aliquot supernatant of hemolymph (50 mg/ml) or hepatopancreas (5 mg/ml) were added to 2.5 ml of the reaction buffer. The reaction was allowed to proceed and the optical density at 470 nm was measured every 30 s using a spectrophotometer. An increment of 0.001 absorbance between 0.5 min and 3.5 min under this condition was defined as one unit of POD activity. The assay was performed in triplicate.

The antibacterial activity (Ua) was measured using the method reported by Hultmark et al. with some slight modifications [21]. *Escherichia coli* K12 was inoculated in LB solid medium and cultured overnight at 37 °C, washed and resuspended in 0.1 M phosphate buffer solution (pH 6.4) to give an absorbance between 0.3 and 0.5 at 570 nm. The tube loaded with 0.3 ml of *E. coli* K12 suspension was first laid in ice-water for 5 min, then 50 μl of aliquot supernatant of hemolymph (50 mg/ml) or hepatopancreas (5 mg/ml) were added. The optical density (A₀) at 570 nm was measured using a spectrophotometer. The mixture was then immediately transferred into a tube at 37 °C for 30 min followed by at 0 °C for 10 min to terminate the reaction, and then the absorbance (A) was measured at 570 nm. The assay was carried out in triplicate and the Ua activity was calculated as follows: Ua = [(A₀−A)/A]²/².

2.6. Experimental challenge test

To further evaluate the effect of immunostimulants, the *Vibrio parahemolyticus* challenge test was performed in the Mix-treated group. In brief, *V. parahemolyticus* was cultured overnight in broth medium at 37 °C before injection. The challenge test was conducted in replicate (30 shrimps per group) after the shrimps had been stimulated by the immunostimulants for 24 days. Each shrimp was injected intramuscularly with 100 μl of *V. parahemolyticus* suspension at a density of 1.0 × 10⁸ CFU/ml according to previous LC₅₀ experiments (data not shown). The shrimps from each group were then cultured in a square tank with 150 L of filtered water at 28–32 °C. The mortality of each replicate was recorded continuously for 48 h.
2.7. Two-dimensional gel electrophoresis (2-DE)

A total of 450 μg of hemocytic proteins or 600 μg of hepatopancreas proteins in rehydration buffer (containing 7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT and 3.4 μl of IPG buffer, pH 3–10) was used to rehydrate the IPG strip (7 cm, pH 4–7, Bio-Rad, USA) for 16 h. The IEF was performed at a constant temperature of 20 °C using a continuous increase in voltage (up to 4000 V) until reaching 32,000 Vh for hemocytic proteins or 35,000 Vh for hepatopancreas proteins. Prior to the second dimension, the focused IEP was incubated for 15 min in an equilibration buffer containing 20% w/v glycerol, 2% SDS, 0.375 M Tris–HCl (pH 8.8), 2% DTT, then further equilibrated for 15 min in a similar buffer which replaced 2% DTT with 2.5% of iodoacetamide. The strip was placed onto the top of a 12% SDS-PAGE gel. Molecular weight markers were loaded onto with a 12% SDS-PAGE gel. Molecular weight markers were loaded onto a filter paper and placed next to the IPG strip. Low-melting point agarose was used to cover the IPG strip and filter paper. The gel was run in a Mini Protean Tetra cell (Bio-Rad) at 70 V for 30 min and subsequently at 150 V for 1.5 h. After SDS-PAGE, the gel was stained with Coomassie G-250.

2.8. Imaging analysis

The 2-DE gel images were analyzed with PDQuest software version 8.0 (Bio-Rad, USA). Comparative analysis of protein spots was performed by matching corresponding spots across different gels. Each of the matched protein spots was rechecked manually. Intensity volumes of individual spots were normalized with total intensity volume of all spots present in each gel and were subjected to statistical analysis to compare the normalized intensity volumes of individual spots of the control group to those of the group fed with immunostimulants. Only differentially expressed proteins were excised and subjected to subsequent identification by MALDI-TOF mass spectrometry. Proteins were considered to be differentially expressed between the two groups on the following criteria: (1) p values must be <0.05, (2) means of both groups using the unpaired Student’s t-test; must be either ≥1.5-fold (significant increase) or ≤0.75-fold (significant decrease) or 0.75–1.49-fold of controls, (3) the change was consistent in all replicate analysis for each group of shrimps.

2.9. Mass spectrometry analysis

Differential expressed proteins spots were excised from 2-DE gels for MALDI-TOF mass spectrometry analysis. Briefly, the gel plug was digested with trypsin and 0.5 μl of the peptide mixture was mixed with the matrix z-cyano-4-hydroxycinnamic acid (1:1) and spotted onto a stainless steel MALDI plate. MS spectra were obtained using the ABI 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA) operating in a result-dependent acquisition mode. Peptide mass maps were acquired in reflectron mode (1-keV accelerating voltage) with 1000 laser shots per spectrum. Six external standards (mass standard kit for the 4700 Proteomics Analyzer calibration mixture, Part Number 4333604, Applied Biosystems, Foster City, CA) were used to calibrate each spectrum to a mass accuracy within 50 ppm. Selected peptide masses were identified: hemocyanin (spots 2, 6, 10, 12, 13, 20, 25 and 26), hemolymph or hepatopancreas increased highly significantly (p < 0.01) in shrimps fed with immunostimulants as compared to those in the control groups on the 12th and 24th day. The mean PO values in hemolymph and hepatopancreas of Mix-treated group on the 24th day was about 3.3- and 2-fold, respectively, to that of control group. And the value of PO in Mix-treated group was significantly higher (p < 0.05) than those in Vc-treated and CH-treated group. At the same time, SOD activity was selected to further determine. As shown in Fig. 1-A(b) and Fig. 1-B(b), after feeding immunostimulants for 12 and 24 days, Vc-treated, CH-treated and Mix-treated groups also exhibited very significant increase (p < 0.01) in SOD activity compared to those of the control groups. The SOD activity in hemolymph and hepatopancreas of Mix-treated group on the 12th day was about 1.6- and 1.5-fold, respectively, to that of control group. Among the three groups, Mix-treated group showed noticeable increase (p < 0.01) in SOD values relative to those of the Vc-treated and CH-treated groups. In addition, Fig. 1-A(c) and Fig. 1-B(c) indicated that the values of POD in Vc-treated were very significantly higher (p < 0.01) than those in the control group after feeding immunostimulants for 24 and 12 days, respectively. Significant differences (p < 0.05) in CH-treated and Mix-treated groups were also observed in comparison with those of control groups. And POD activity in hepatopancreas showed no significant difference (p > 0.05) among the experimental groups on the 12th day. Thus, these data suggested that stimulation with vitamin C and Chinese herbs could effectively enhance immunity in shrimp Litopenaeus vannamei.

3.2. Effect of immunostimulants on Ua activity and the resistance of shrimp to vibriosis

To further evaluate the immune-enhancing effect, the Ua activity was measured and the experimental challenge test was applied. As shown in Fig. 1-A(d) and Fig. 1-B(d), values of Ua activity in hemolymph and hepatopancreas of shrimp fed with immunostimulants were significantly higher (p < 0.05) than those in the control group on the 12th and 24th day. The mean Ua values in hemolymph of Mix-treated group and control group on 24th day were 0.724 and 0.276, respectively, which was about a 2.6-fold increase. Furthermore, the cumulative mortality of Mix-treated group was significantly less than that of the control group after injection with V. parahemolyticus for 6–48 h. During the 18 h challenge test, the mortality of Mix-treated and control groups was 43.3% and 70%, respectively (Table 1). Thus, the finding suggested that dietary supplementation with the compound of vitamin C and Chinese herbs could improve shrimp’s defence against the pathogen.

3.3. 2-DE and identification of altered proteins in the hemocytes

Shrimp hemocyte proteins were isolated and analyzed by 2-DE. Fig. 2A shows the maps of shrimp hemocyte proteins of control group and Mix-treated group after a 12- and 24-day feeding. Approximately 220–290 spots on each gel were distinguished by PDQuest software, mainly ranging from 10 to 100 kDa. Comparative analysis of the four gels indicated that 29 altered expression spots were shown in Fig. 2B. The 29 altered spots were excised from gels and subjected to MALDI-TOF analysis. Only the peptide mass fingerprint (PMF) spectra of spots 6 and 9 were shown in Fig. 2C. Together, 3 uniquely expressed proteins within the 10 differentially expressed protein spots were identified: hemocyanin (spots 2, 6, 10, 12, 13, 20, 25 and 26),...
hemoglobin (spot 9) and hemolymph clottable protein (spot 14) (Table 2). These results suggested that the 29 altered expression spots including the 3 uniquely expressed proteins could be related to the enhancement of immunity in shrimp hemocytes.

3.4. 2-DE and identification of altered proteins in the hepatopancreas

To further identify possible immune-enhancing proteins in shrimp hepatopancreas, the same strategy was performed as above. The soluble shrimp hepatopancreas proteins of control group and Mix-treated group after a 12- and 24-day feeding were separated by 2-DE (pH range 4–7). After staining with Coomassie G-250, automatic detection of the protein profiles revealed approximately 300–400 protein spots (Fig. 3A). A total of 28 altered expression spots had significantly increased or decreased levels upon the Mix-treated group. Of these, spots 1–6, 8–20, 22 and 26–28 were up-regulated while spots 7, 21 and 23–25 were down-regulated. The enlarged partial two-dimensional gels showing altered expression spots and the peptide mass fingerprint (PMF) spectra of spots 16 and 26 were shown in Fig. 3B and C, respectively. All of the differential spots were subjected to mass spectrometry analysis and 4 uniquely expressed proteins were identified, which were cytosolic MnSOD (spot 28), trypsin (spots 16, 17), Cathepsin I(L) (spot 26) and zinc proteinase Mpc1 (spot 27). These results suggested that the 28 altered expression spots including the 4 uniquely expressed proteins could be related to the enhancement of immunity in shrimp hepatopancreas.

Table 1
Comparison of the cumulative mortalities between the control and Mix-treated group after being infected by Vibrio parahemolyticus.

<table>
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<th>Time (h)</th>
<th>Control group (%)</th>
<th>Mix-treated group (%)</th>
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<tr>
<td>18</td>
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Fig. 1. Four immune parameters in hemolymph (A) and hepatopancreas (B) of shrimp when Litopenaeus vannamei were fed with diets containing immunostimulants. (a) Phenoloxidase; (b) Superoxide dismutase; (c) Peroxidase; (d) Antibacterial activity. Data (mean ± S.D.) in the same administration time with different letters (a, b, c, d) are significantly (p < 0.05) among the treatments.

4. Discussion

In present study, administration of vitamin C and Chinese herbs was found to have significant effects on the improvement of non-specific immunity in shrimp L. vannamei. Furthermore, 2-DE reference maps were established and 7 uniquely expressed proteins were found in hemocytes and hepatopancreas of Mix-treated group, indicating their potential activities in enhancing
Fig. 2. 2-DE protein profiles of the shrimp hemocytes. A, (a) Control group hemocytes at 12d; (b) Mix-treated group hemocytes at 12d; (c) Control group hemocytes at 24d; (d) Mix-treated group hemocytes at 24d. Differentially expressed proteins are labeled with numbers, which correspond to the number present in Table 2. B, (a) and (c) segments of 2-DE gel map, showing high magnification views of the identified up-regulated or down-regulated protein spots in Litopenaeus vannamei hemocytes after 12d or 24d immunostimulants feeding; (b) and (d) histogram displays the changes in spot intensity of them between Control group (white) and Mix-treated group (black), and bars represent spot intensity with relative volume divided by the total volume over the whole image, according to PDQuest software version 8.0 description. C, (a)–(b) MALDI-TOF-MS peptide mass fingerprint (PMF) spectra of spot 6 and spot 9, respectively.
the immunity of shrimps. It will be interesting to investigate the molecular mechanism underlying these observations in the future.

Immunostimulants typically affect the non-specific immune system, which provides protection against a wide range of pathogens [22]. Purivirojkul et al. reported that phenoloxidase, superoxide anion, bactericidal activity, and clearance ability were significantly increased in shrimp fed on diets containing peptidoglycan (PG) 0.18 g/kg [23]. Dietary administration of β-1,3-glucan has also been documented to improve immunological responses and resistances against WSSV infection in Penaeus monodon [24,25]. Here, we found that feeding

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Significantly up-regulated proteins (expression levels were ≥1.5-fold of controls)

Significantly down-regulated proteins (expression levels were ≤0.75-fold of controls)
Fig. 3. 2-DE protein profiles of the shrimp hepatopancreas. A, (a) Control group hepatopancreas at 12d; (b) Mix-treated group hepatopancreas at 12d; (c) Control group hepatopancreas at 24d; (d) Mix-treated group hepatopancreas at 24d. Differentially expressed proteins are labeled with numbers, which correspond to the number present in Table 3. B, (a) and (c) segments of 2-DE gel map, showing high magnification views of the identified up-regulated protein spots in Litopenaeus vannamei hepatopancreas after 12d or 24d immunostimulants feeding. (b) and (d) histogram displays the changes in spot intensity of them between Control group (white) and Mix-treated group (black), and bars represent spot intensity with relative volume divided by the total volume over the whole image, according to PDQuest software version 8.0 description. C, (a)–(b) MALDI-TOF-MS peptide mass fingerprint (PMF) spectra of spot 16 and spot 26, respectively.
with vitamin C, or Chinese herbs, or a mixture of them could significantly enhance the PO, SOD, POD and UA activities of shrimp *L. vannamei*. Particularly, shrimps in Mix-treated group not only showed the highest level of non-specific immunity indices in all experiment groups, but also possessed a certain degree of resistance to pathogens. Therefore, these results suggested that the mixture of vitamin C and Chinese herbs could be a suitable immunostimulant for penaeid shrimp farming.

Shrimp innate immunity consists of both cellular and humoral responses. Hemocytes in the hemolymph are the major component of the cellular immune responses in crustaceans [13]. To identify the possible immune-enhancing proteins, the proteomic approach including 2-DE and MALDI-TOF-MS was applied for the analysis of hemocytes from the Mix-treated group. 29 altered protein spots and 3 uniquely expressed proteins (hemocyanin, hemolymph clottable protein and hemoglobin beta) were successfully identified in the hemocytes.

Hemocyanin has been reported as a novel and important defense molecule in mollusks and arthropods. Besides its primary function as a respiratory protein, it has been suggested that hemocyanin could be functionally converted into phenoloxidase-like enzyme, antiviral agent, antimicrobial protein, agglutinin and agglutinin-like protein.

Table 3

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</table>

Fig. 3. (continued).
hemolysin [26--32]. In this study, hemocyanin was found in 8 altered protein spots (spots 2, 6, 10, 12, 13, 20, 25 and 26) in the hemocytes. Interestingly, the observed mass of six hemocyanin spots (spots 6, 10, 12, 13, 25 and 26) was under 25 kDa instead of the speculated 75 kDa. In addition, seven hemocyanin spots (spots 2, 6, 10, 12, 13, 25 and 26) were up-regulated, while one hemocyanin spot (spot 20) were down-regulated. In combination of the reports that crustacean hemocyanin could generate some fragments with obvious antibacterial and antifungal activities [29,30], and the findings that three-quarters of C-terminal hemocyanin fragments showed up-regulation, but two-thirds of N-terminal fragments displayed down-regulation during Taura syndrome virus (TSV) infection in hemocytes of Penaeus vannamei [13, the results from this study suggested that hemocyanin might play an important role in improvement of the immune activity of shrimp by generating multiple fragments with different functions, which probably could act as a molecular marker of immune improvement in shrimp.

Hemolymph clottable protein functions mainly as a substrate for transglutaminase to form stable clots. It has been shown that polymerization of clottable proteins by the hemocyte transglutaminase can trigger hemolymph clotting in crustaceans [33]. Furthermore, the expressing level of clottable protein is regulated to suit the growth status of shrimp. As noted by Chen et al., the concentration of penaeid shrimp clottable protein in summer was higher than that in winter, and was varied by the molting cycle, e.g. its plasma levels increased by 2-fold before molting and decreased to normal levels after molting [34]. Here, we found that hemolymph clottable protein could also response to the stimulation of immunostimulants, and might mediate the improvement of shrimp immunity.

Hemoglobin play an important role in the evolutionary balance between nutrient availability and the regulation of aerobic metabolism [35]. Sveinsdottir et al. reported that two 29 kDa isoforms of hemoglobin in early Atlantic cod (Gadus morhua) larvae were shown to be up-regulated after administration of probiotic bacteria [36]. Based on previous investigations and our current observation, the up-regulated hemoglobin in hemolymph might be related to the innate immune response by supplying oxygen for energy production.

The hepatopancreas is one of the most important organs for both humoral and cellular immune responses of shrimp [37]. In this study, 28 altered protein spots and 4 uniquely expressed proteins (cytosolic MnSOD, trypsin, Cathepsin I(L), zinc proteinase Mpc1) were found using proteomic analysis. Numerous publications demonstrated that these four up-regulated enzymes played important roles in the immune defense system of shrimps [15,38--40]. Wang et al. reported that mRNA levels of cMnSOD were up-regulated swiftly in the hepatopancreas of L. vannamei after feeding of β-1,3-glucan for 12 h [38]. It was also reported that the translation level of trypsin in the hepatopancreas of Fenneropenaeus japonicus showed up-regulation after hypoxia treatment [15]. Robalino et al. indicated that the transcription of cathepsin L was apparently up-regulated in WSSV challenged shrimps [39]. As noted by Zhao et al., zinc proteinase mRNA expression levels were increased in WSSV resistant shrimp L. vannamei compared with susceptible ones [40]. Consequently, it is suggested that hepatopancreas including the 4 enzymes might contribute to the response to the stimulation of immunostimulant in shrimp.

In conclusion, the present study documented that the immune activity and vibriosis resistant capability were effectively improved in shrimp L. vannamei after oral administration of vitamin C and Chinese herbs. To our knowledge, this was the first report showing that 7 uniquely expressed proteins in hemolymph and hepatopancreas could be related to the enhancement of immune response by immunostimulants.

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