

# Analysis of *Litopenaeus vannamei* hemocyanin interacting proteins reveals its role in hemolymph clotting

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## ABSTRACT

Hemocyanin is the main component of hemolymph plasma proteins and possesses diverse immunological properties and immunomodulatory functions. However, the interacting networks of hemocyanin in shrimp immune response remain poorly understood. In this study, 39 potential hemocyanin interacting partners were identified from *Litopenaeus vannamei* plasma by co-immunoprecipitation (Co-IP) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Gene Ontology (GO) analysis showed that most of the identified interactors were cell proteins involved in metabolic process and binding. Among these identified proteins, transglutaminase (TGase), a crucial regulator in hemolymph clotting cascade, was chosen for further studies. Far-Western blot and His-pull down assays revealed that hemocyanin directly interacted with TGase. Further analysis demonstrated that hemocyanin and TGase followed similar expression patterns upon pathogen infection. Moreover, in vivo knockdown of hemocyanin led to a significant decrease in TGase expression, as well as inhibited hemolymph clotting. Taken together, these data suggest that hemocyanin might positively regulate hemolymph clotting by modulating TGase in shrimp.

**Significance:** The interaction networks among immune-related factors is critical for the innate immune response in invertebrates. We report for the first time, proteins that potentially interact with hemocyanin, which led to the identification of 39 possible hemocyanin-proteins including the clotting-related factor TGase. Further studies demonstrated that hemocyanin directly interacted with TGase and modulated its expression, therefore affecting the formation of hemolymph clotting. These findings not only extend our knowledge of the immune interaction networks but also contribute to shrimp disease control and prevention.

## 1. Introduction

*Litopenaeus vannamei*, commonly known as Pacific white shrimp or white leg shrimp, is a prawn native to the eastern Pacific Ocean and has now become one of the dominant shrimp species cultivated worldwide. According to statistics of the Food and Agriculture Organization (FAO) of the United Nations, *L. vannamei* aquaculture is one of the most fast-growing mariculture industry, with global production reaching 4,155,827 t, and accounting for approximately 80% of the world's total shrimp production in 2016 [1]. However, shrimp aquaculture has suffered from the outbreaks of viral or bacterial diseases such as white spot disease (WSD) and acute hepatopancreatic necrosis disease (AHPND) in the past decades [2,3]. There are currently no effective preventive and control methods against infectious diseases in shrimp. Thus, there is an

urgent need to explore the immune defense mechanism of shrimp against invading pathogens so as to provide useful information for the design of effective disease control strategies.

Hemocyanin is a copper-containing respiratory protein found in many arthropods and molluscs [4,5]. It is a multimeric protein complex composed of identical subunits and freely dissolved in hemolymph, constituting the major part of plasma proteins. Aside from its role in oxygen transport, hemocyanin has been demonstrated to possess several immunological properties such as phenoloxidase [6,7], anti-virus [8–10], anti-bacteria [11–13], agglutination [14–16], hemolysis [17,18] and anti-tumor activities [19–21]. Furthermore, a growing body of studies suggests that hemocyanin is involved in immune modulation by interacting with other immune molecules or immune signaling pathways. For example, Xu et al. reported that *Penaeus*

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*japonicus* hemocyanin interacted with QM protein and myosin, thereby participating in the regulation of phenol oxidase activity [22]. In addition, Havanapan et al. demonstrated that the C-terminal of *Penaeus vannamei* hemocyanin could bind ERK1/2 and undergo serine phosphorylation during Taura syndrome virus (TSV) infection [23]. Recently, hemocyanin has been reported to function as a pattern recognition receptor (PRR) involved in the activation of innate immunity in shrimp and crayfish [12,13]. From the foregoing, it indicates that hemocyanin is a multifunctional molecule, which seem to execute its immune functions via complex interaction networks. However, these hemocyanin-mediated interaction networks remain poorly understood in *L. vannamei*.

In the present study, the interacting networks of hemocyanin were explored using Co-immunoprecipitation (Co-IP) coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. A total of 39 potential hemocyanin interacting proteins were identified in shrimp plasma. One of these identified proteins, transglutaminase (TGase), a key enzyme in hemolymph clotting [24], was chosen for further studies. These findings are the first to show that hemocyanin might positively regulate hemolymph clotting by modulating TGase in *L. vannamei*.

## 2. Materials and methods

### 2.1. Shrimp culture

Shrimp (weighing about 5–8 g) were purchased from Shantou Huaxun Aquatic Product Corporation (Shantou, China), and cultured in aerated seawater at room temperature. Shrimps were acclimatized to laboratory conditions for 3–5 days prior to all experimental procedure. All shrimp experiments were carried out in accordance with the guidelines and approval of the Animal Research and Ethics Committees of Shantou University.

### 2.2. Co-immunoprecipitation (Co-IP) analysis

The rabbit anti-hemocyanin (anti-HMC) antibody used for the Co-IP was prepared in-house by immunizing rabbits with recombinant *L. vannamei* hemocyanin (GenBank number: X82502). The Co-IP assays were performed using the Immunoprecipitation Kit (Sangon Biotech, China) according to manufacturer's protocol with some modifications. Briefly, shrimp hemolymph was withdrawn directly from the pericardial sinus using a sterile syringe into an equal volume of precooled (4 °C) anticoagulant (27 mM Na citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 7.0). Plasma was obtained after centrifugation at 800 × g for 10 min at 4 °C. For the Co-IP experiments, the plasma was further diluted by 20 times and 700 µL aliquot of the diluted plasma incubated with 20 µL protein A/G agarose beads and 5 µg anti-HMC antibody (or control rabbit IgG) overnight on a gentle shaker at 4 °C. After incubation, the agarose beads were pelleted and washed seven times with 1 × IP buffer, followed by three washes with 0.1 × IP buffer. Finally, bound proteins were eluted from the agarose beads by boiling for 10 min with 1 × protein loading buffer (50 mM Tris-HCl, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.1% bromophenol blue, pH 6.8) and then analyzed by sodium sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue staining.

### 2.3. Protein identification by LC-MS/MS analysis

To identify the interacting partners of *L. vannamei* hemocyanin, the differential gel bands, compared to the negative control, were excised from the gels and submitted to Beijing Genomics Institute (BGI) (BGI, China) for LC-MS/MS analysis as described previously [25]. In brief, for protein digestion, the gel bands were cut into small pieces and destained using 50% acetonitrile in 50 mM NH<sub>4</sub>HCO<sub>3</sub>, followed by dehydration with 100% acetonitrile. Proteins were then reduced with

10 mM dithiothreitol (DTT) at 56 °C for 1 h and alkylated using 55 mM Iodoacetamide (IAM) for 45 min in the dark at room temperature. After reduction and alkylation, proteins were digested with trypsin solution (12 ng/µL in 20 mM NH<sub>4</sub>HCO<sub>3</sub>) overnight at 37 °C. The digested peptides were extracted from the gels with 5% formic acid/50% acetonitrile and dried in a SpeedVac centrifuge (Thermo Scientific, USA). Next, the dried peptides were reconstituted in 20 µL of 0.1% formic acid/5% acetonitrile and analyzed by LC-MS/MS using a Q-Exactive mass spectrometer (Thermo Scientific, USA). The generated MS/MS spectra were searched against the Crustacea Protein Database at the National Center for Biotechnology Information (NCBI) using Mascot Server (V2.3.02). All procedures, methods and analysis used in the LC-MS/MS followed the standard commercial pipeline of BGI.

### 2.4. Gene ontology (GO) analysis

For GO analysis, the GO terms of each identified proteins were analyzed with Uniprot database (<http://www.uniprot.org>). Next, the GO terms were further subjected to GO functional classification using Web Gene Ontology Annotation Plot (WEGO) program (<http://wego.genomics.org.cn>).

### 2.5. Purification of native hemocyanin from *L. vannamei* hemolymph

The native hemocyanin was purified using chromatography on a Sephadex G-100 column (Henghuibio, China) according to a previous method with minor modifications [26]. Briefly, *L. vannamei* hemolymph was collected using sterile syringes and then transferred to centrifuge tubes. After coagulation overnight at 4 °C, shrimp serum was separated by centrifugation at 3000 × g for 20 min. Next, the serum (0.5 mL) was loaded onto Sephadex G-100 column, followed by elution using 50 mM Tris-HCl (pH 8.0) at a flow rate of 1 mL/min. The eluted fractions (1.5 mL per tube) were collected and analyzed by SDS-PAGE. The fraction containing hemocyanin was analyzed by SDS-PAGE and further confirmed by Western blot with rabbit anti-HMC antibody as described previously [27].

### 2.6. Expression and purification of *L. vannamei* TGase

To construct the plasmid expressing recombinant TGase (rTGase), the open reading frame (ORF) of *L. vannamei* TGase (GenBank number: ABN13875) was amplified by PCR using the primers rTGase-F and rTGase-R (Table 1). The PCR product was digested with *Eco*R I and *Hind* III, and then ligated into the pET-32a (+) vector (Solarbio, China) to produce the Trx and His-tagged plasmids pET-32a-TGase. After the

**Table 1**  
Sequences of primers and siRNAs used in this paper.

| Primer               | Sequence (5'–3') <sup>a</sup>                    |
|----------------------|--------------------------------------------------|
| Plasmid construction |                                                  |
| rTGase-F             | <b>GGAA</b> <u>TC</u> CATGCCCGCCGTGGACC          |
| rTGase-R             | CCC <b>AA</b> <u>GC</u> TTAGCGTTCGTTACAACGATCTTC |
| Real-time RT-PCR     |                                                  |
| qHMC-F               | CAAGGGCAACGATAGAGGC                              |
| qHMC-R               | CATGGGAACCGTAGTGATT                              |
| qTGase-F             | CCACGACCTTATCAAGAGCA                             |
| qTGase-R             | AGGACACCCTTATGGGAACA                             |
| qEF1α-F              | CATCTCATCTACAAATGCG                              |
| qEF1α-R              | ATGAAATCACGATGGCCTG                              |
| siRNA synthesis      |                                                  |
| siHMC-F              | GGCCUCAUAAAGACAACAATT                            |
| siHMC-R              | UUGUUGUCUUUAGAGGCCCTT                            |
| siNon-F              | UUCUCCGAACGUGUCACGUTT                            |
| siNon-R              | ACGUGACACGUUCGGAGAATT                            |

<sup>a</sup> Restriction sites for plasmid construction were underlined and highlighted in bold.

constructs were confirmed by DNA sequencing, the constructed plasmids were transformed into *Escherichia coli* BL21. The transformed bacteria were cultured at 37 °C in Luria-Bertani medium containing 100 µg/mL ampicillin. When the bacteria density reached an OD<sub>600</sub> of about 0.6, protein expression was induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 4 h at 37 °C.

For purification of rTGase, the induced bacteria were harvested by centrifugation at 5000 × g for 20 min at 4 °C. The pelleted bacteria were washed once with 0.9% NaCl, and then resuspended in sonication buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, pH 8.0) for sonication on ice. After sonication, the inclusion bodies containing the rTGase were pelleted by centrifugation and then subjected to protein denaturation and renaturation as follows: First, the inclusion bodies were sequentially washed three times using washing buffer A (50 mM Tris-HCl, 1 M NaCl, 5 mM EDTA, 0.05% Triton X-100, 5% glycerol, pH 8.0) and washing buffer B (50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, 0.05% Triton X-100, 5% glycerol, pH 8.0). The precipitates were then collected by centrifugation and dissolved in denaturing buffer (50 mM Tris-HCl and 8 M urea, pH 8.0). After centrifugation, the supernatants were dialyzed against Tris buffer (50 mM Tris-HCl, pH 8.0) containing a concentration gradient of urea (8 M, 6 M, 4 M, 2 M, and 0 M) at 4 °C for 4 h. Finally, the renatured rTGase was purified using Ni-NTA agarose beads (Henghuibio, China) and dialyzed against Tris buffer (50 mM Tris-HCl, pH 8.0) at 4 °C for 4 h. The purified rTGase was further confirmed by Western blot analysis using a mouse anti-TGase antibody previously prepared using recombinant Ig-like domain of *L. vannamei* TGase in our lab [25].

## 2.7. Far-Western blot

The Far-Western blot analysis was performed as described previously with slight modifications [28]. Briefly, purified proteins including native hemocyanin, rTGase and rTrx (negative control) were separated by SDS-PAGE and then transferred onto PVDF membranes. After blocking for 1 h at room temperature in 5% (w/v) skimmed milk, membranes were washed three times with TBST (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.6) and then incubated with 12 ng/µL purified native hemocyanin for 1 h at room temperature. Next, membranes were incubated with rabbit anti-HMC antibody (1:10000) and horseradish peroxidase (HRP)-linked goat anti-rabbit secondary antibody (1: 10000) (Thermo Scientific, USA) for 1 h at room temperature, respectively. Signals were detected with enhanced chemiluminescence substrates (Thermo Scientific, USA) and visualized using a Chemiluminescence (GE Healthcare, USA) detection system.

## 2.8. His-pull down

His-pull down assays were performed as described previously [29]. In brief, approximately 1.5 mg of purified rTGase and rTrx (negative control) were incubated with Ni-NTA agarose beads (Henghuibio, China) for 1 h at 4 °C, respectively. The beads were washed three times with washing buffer (50 mM Tris-HCl, pH 8.0) until the OD<sub>280</sub> was zero. Subsequently, the purified native hemocyanin (1.5 mL, 0.2 mg/mL) was added to the beads and incubated for 4 h at 4 °C. After three washes (OD<sub>280</sub> = 0), proteins were eluted with elution buffer (50 mM Tris-HCl, 200 mM imidazole, pH 8.0). The eluted proteins were then resolved by SDS-PAGE to analyze the interaction between hemocyanin and TGase.

## 2.9. Pathogen challenge and real-time quantitative PCR

In this study, pathogenic *Vibrio parahaemolyticus* (Gram-negative) and *Streptococcus iniae* (Gram-positive) were used for challenge experiments. The bacteria were prepared as described previously [15] and suspended in sterile 0.85% saline. For bacteria challenge assays, each shrimp in the experimental group was intramuscularly injected with either 100 µL of *S. iniae* or *V. parahaemolyticus* ( $1.0 \times 10^5$  CFU/mL). At

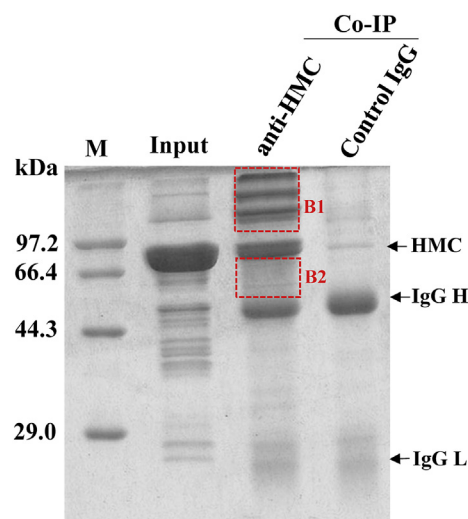


**Fig. 1.** Identification of *L. vannamei* hemocyanin interacting partners using Co-IP analysis. The hemolymph plasma was incubated with rabbit anti-HMC antibody (or equivalent control rabbit IgG) and protein A/G agarose beads. The diluted plasma without incubation with antibodies and protein A/G agarose beads was used for input sample. After SDS-PAGE analysis, the differential bands were cut from the gel for LC-MS/MS analysis. M, Protein marker.

0, 12, 24, 36, 48 and 72 h post infection, hepatopancreas tissues of four randomly selected shrimps were collected for immediate RNA extraction and cDNA synthesis. Total RNA was extracted using the RNA-Fast200 kit (FeiJie, China) and cDNA was synthesized using the PrimeScript RT reagent kit (Takara, Japan) according to the manufacturers' instructions.

To analyze the expression profiles of hemocyanin and TGase post pathogen challenge, quantitative PCR (qPCR) analysis was performed on a 7300 Real-Time PCR system (Applied Biosystems). The qPCR program was as follows: one cycle at 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The expression levels of hemocyanin and TGase genes were normalized to EF1α (internal control gene) using the  $2^{-\Delta\Delta CT}$  method. The hemocyanin, TGase and EF1α primers used are listed in Table 1.

## 2.10. RNA interference (RNAi) assays

To further study the relationship between hemocyanin and TGase, the RNAi technique was used to knockdown hemocyanin and then determined the effect of this on TGase. First, specific siRNA targeting the hemocyanin gene (siHMC) and scrambled control siRNA (siNon) were designed and chemically synthesized by GenePharma company (Suzhou, China). Thereafter, shrimps were intramuscularly injected with 100 µL of 5.0 µg siHMC or an equivalent amount of the siNon for negative control. At 72 h after siRNA injection, hepatopancreas tissues of 4 shrimps were collected and then subjected to RNA extraction and cDNA synthesis. Finally, the expression levels of hemocyanin and TGase post siRNA injection were measured by qPCR as described in Section 2.9.

## 2.11. Hemolymph clotting analysis

Hemolymph clotting was analyzed following the protocol described by Maningas et al. [30]. Briefly, shrimps were intramuscularly injected with either 5.0 µg siHMC or siNon (negative control). At 72 h after siRNA injection, hemolymph from four shrimps per group was withdrawn from the pericardial sinus using a sterile syringe into 1.5 mL tubes with or without anticoagulants. The state of hemolymph clotting was directly visualized and images taken after tubes were allowed to stand for 1 h at room temperature.

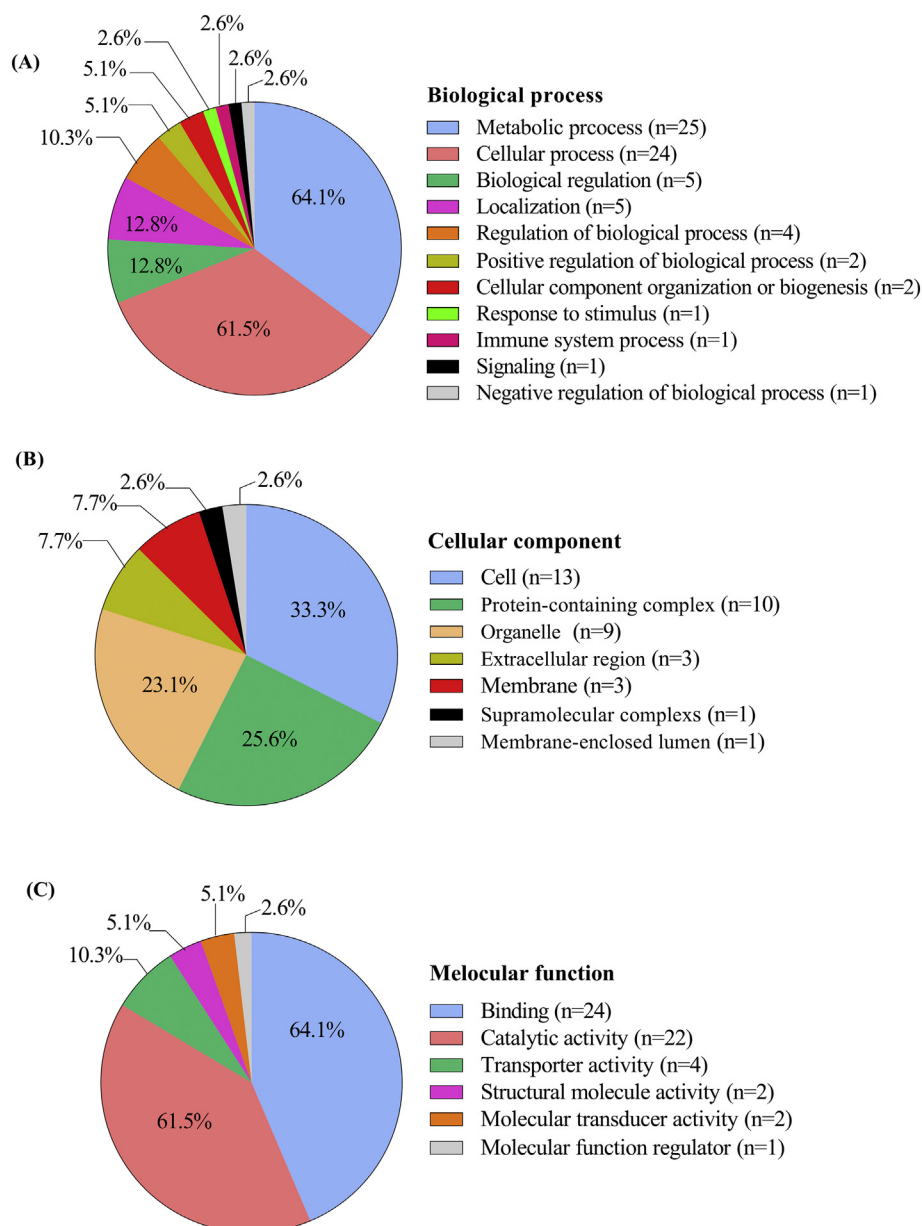


**Fig. 2.** GO analysis of the identified hemocyanin interacting proteins. The GO items of the identified proteins were downloaded from the Uniprot database and classified by WEGO program according to biological process (A), cellular component (B), and molecular function (C).

**Table 2**

Summary of *L. vannamei* annotated proteins identified to interact with hemocyanin.

| Protein ID <sup>a</sup>    | Description                                                       | Protein score | Number of unique Peptide | Coverage (%) | Protein mass (Da) |
|----------------------------|-------------------------------------------------------------------|---------------|--------------------------|--------------|-------------------|
| gi 29838466 gb AAO92933.1  | 1,3-beta-D-glucan-binding protein [ <i>Litopenaeus vannamei</i> ] | 3475.66       | 41                       | 0.360385144  | 164196.59         |
| gi 115345804 gb ABI95361.1 | Hemolymph clottable protein [ <i>Litopenaeus vannamei</i> ]       | 548.22        | 8                        | 0.06302521   | 189370.8          |
| gi 115492980 gb ABI98020.1 | Arginine kinase [ <i>Litopenaeus vannamei</i> ]                   | 483.99        | 1                        | 0.047752809  | 40419.59          |
| gi 124518460 gb ABN13875.1 | Hemocyte transglutaminase [ <i>Litopenaeus vannamei</i> ]         | 374.02        | 5                        | 0.087186262  | 85603.8           |
| gi 124665660 gb ABK60045.2 | Alpha-2-macroglobulin [ <i>Litopenaeus vannamei</i> ]             | 346.78        | 8                        | 0.069565217  | 168586.41         |
| gi 164685130 gb ABY66598.1 | Pyruvate kinase 3 [ <i>Litopenaeus vannamei</i> ]                 | 277.45        | 5                        | 0.118443316  | 64424.92          |
| gi 147724160 gb ABQ45957.1 | Prophenoloxidase 2 [ <i>Litopenaeus vannamei</i> ]                | 127.37        | 2                        | 0.030390738  | 78984.64          |
| gi 288816877 gb ADC55251.1 | ATP synthase subunit alpha [ <i>Litopenaeus vannamei</i> ]        | 61.93         | 1                        | 0.023636364  | 59416.42          |
| gi 309099430 gb ADO51769.1 | Elongation factor-2 [ <i>Litopenaeus vannamei</i> ]               | 54.58         | 1                        | 0.017730496  | 95049.08          |

<sup>a</sup> Proteins involved in hemolymph clotting were highlighted with gray shading.

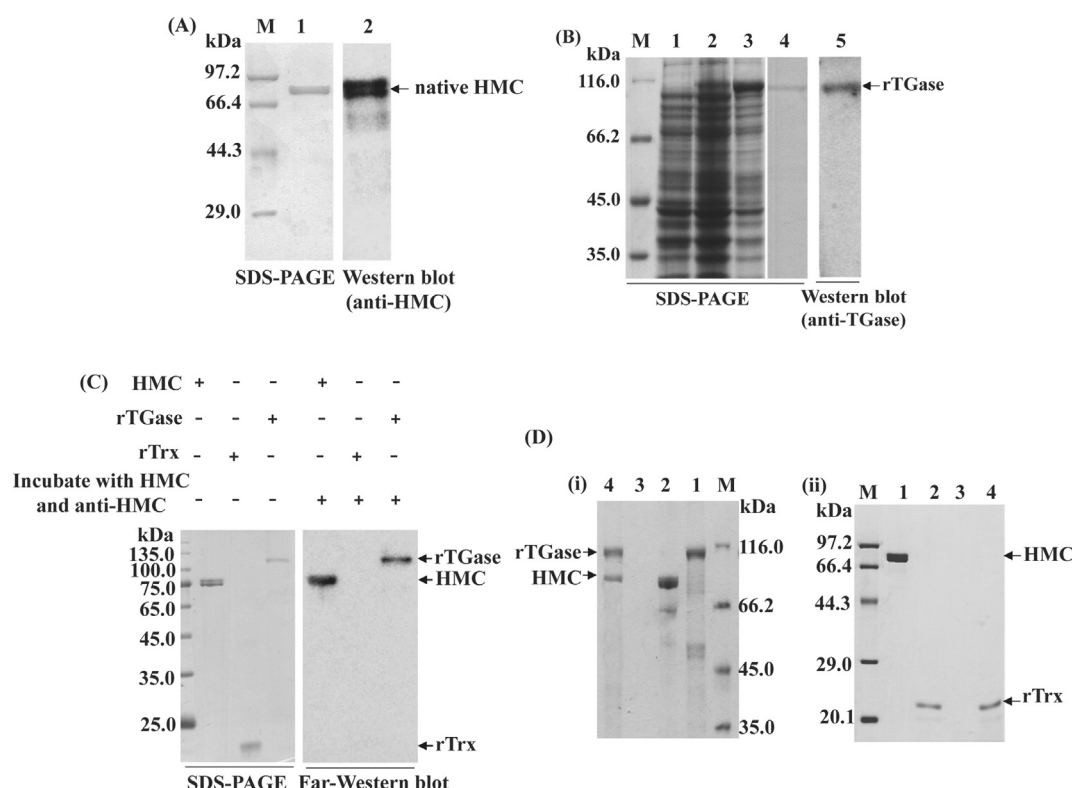


**Fig. 3.** *L. vannamei* hemocyanin directly interacts with TGase. (A) SDS-PAGE (lane 1) and Western blot (lane 2) analysis of the purified native hemocyanin (HMC). (B) Expression and purification of *L. vannamei* recombinant TGase (rTGase). Lanes 1–3, SDS-PAGE analysis of IPTG-uninduced (lane 1) and IPTG-induced bacteria lysis (lane 2), and the inclusion bodies of IPTG-induced bacteria lysis (lane 3); Lanes 4–5, SDS-PAGE (lane 4) and Western blot (lane 5) analysis the purified rTGase. (C) Far-Western blot analysis of the interaction between hemocyanin and TGase. The purified HMC, rTrx and rTGase were separated by SDS-PAGE (Left panel) and then blotted to PVDF membrane. The blotted membrane was further incubated with the purified HMC, followed by Western blot analysis with anti-HMC antibody (Right panel). (D) His-pull down analysis of the interaction between HMC and TGase. The purified rTGase and rTrx proteins were separately bound to the NTA-Ni agarose beads, followed by incubation with the purified HMC. The wash and elution fractions were analyzed by SDS-PAGE analysis. Lanes 1–2, Input samples of rTGase (i-lane 1) and HMC (i-lane 2), and HMC (ii-lane 1) and rTrx (ii-lane 2). Lanes 3–4, Wash (lane 3) and elution (lane 4) fractions from the beads. M, Protein marker.

### 3. Results

#### 3.1. Identification of the potential interacting proteins of *L. vannamei* hemocyanin

Hemocyanin is the main component of plasma proteins and has critical roles in shrimp immunity. In this study, to better understand the interaction networks of hemocyanin, Co-IP assay was performed to identify the putative interactors of hemocyanin. As shown in Fig. 1, compared to the control rabbit IgG, two differential bands marked B1 (about 75–180 kDa) and B2 (about 55–75 kDa) were observed in the Co-IP sample using the anti-HMC antibody, indicating that proteins in these two bands might interact with hemocyanin. Next, these two gel bands were excised and subjected to LC-MS/MS analysis. The MS/MS spectra were searched against Crustacea Protein Database using the Mascot software, which led to the identification of a total of 39 possible hemocyanin interacting proteins (Supplementary Table S1).

To determine the molecular characterization of the hemocyanin-interacting proteins, GO analysis was carried out according to the biological process, cellular component and molecular function. In biological process, the interacting proteins were mainly involved in metabolic process (64.1%) and cellular process (61.5%) (Fig. 2A). For cellular component, the results showed that most of the proteins were localized in cell (33.3%), protein-containing complex (25.6%) and organelle (23.1%) (Fig. 2B). In terms of molecular function, the majority of the proteins were related to binding (64.1%) and catalytic activity (61.5%) (Fig. 2C). In addition, of the 39 identified proteins, *L. vannamei* annotated proteins were teased out for further analysis. Nine proteins

were identified including TGase and clottable protein (CP), which had high protein scores (Table 2). Given that TGase and CP were two key mediators of hemolymph clotting system [31], we reasoned that hemocyanin might be involved in the control of hemolymph clotting in shrimp.

#### 3.2. Hemocyanin directly interacts with TGase

To confirm the Co-IP and LC-MS/MS results, *L. vannamei* TGase was selected for further studies. The interaction between hemocyanin and TGase was verified using Far-Western blot and His-pull down assays. First, native hemocyanin was purified from shrimp plasma using gel filtration chromatography (Fig. 3A), while rTGase was expressed in *E. coli* cells and then purified by nickel column chromatography (Fig. 3B). The purified hemocyanin and rTGase were further confirmed by Western blot analysis with anti-HMC and anti-TGase antibodies, respectively (Fig. 3A and B). For Far-Western blot assay, purified hemocyanin, rTGase and rTrx (negative control) were resolved by SDS-PAGE and then blotted onto PVDF membrane. After extensive washes, the membrane was incubated with purified hemocyanin, followed by Western blot analysis with anti-HMC antibody. The results showed that hemocyanin could bind rTGase, but not rTrx (Fig. 3C). In the His-pull down analysis, purified rTGase and rTrx were first incubated with Ni-NTA agarose beads, respectively. Subsequently, the beads were extensively washed and further incubated with purified hemocyanin. The proteins that bound to the beads were eluted and separated by SDS-PAGE. It was shown that hemocyanin could physically interact with rTGase (Fig. 3D-i), but not rTrx (Fig. 3D-ii). Therefore, the results from these assays

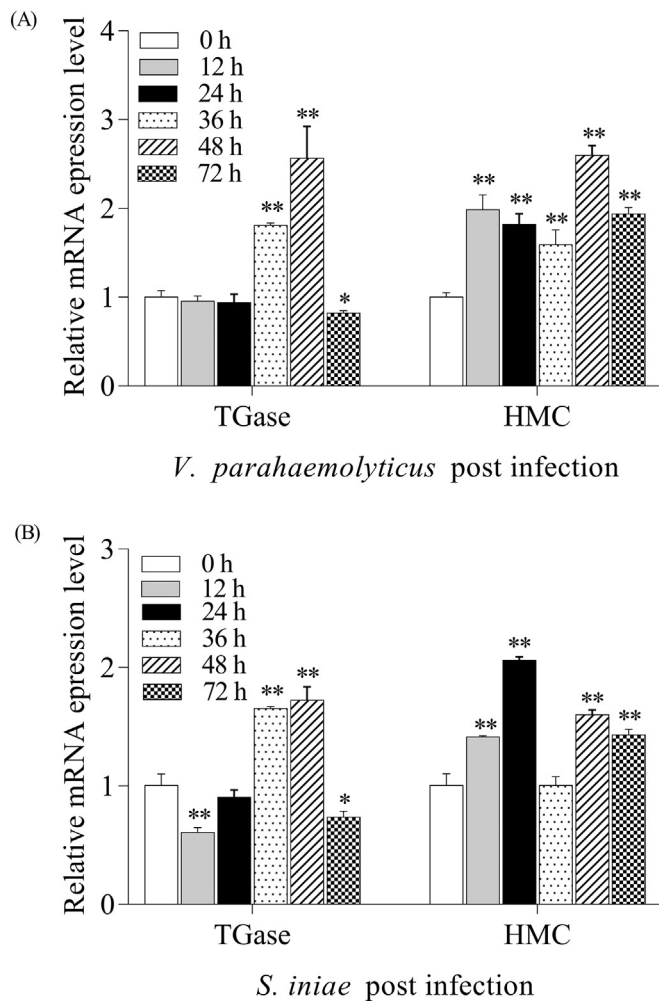


**Fig. 4.** *L. vannamei* hemocyanin and TGase show similar expression patterns upon pathogen infection. Shrimps were injected with *V. parahaemolyticus* and *S. iniae*, respectively. At different timepoints post infection, the expression levels of hemocyanin (HMC) and TGase in *V. parahaemolyticus* (A) and *S. iniae* (B) injected groups were measured using qPCR analysis. Triplicate qPCRs were performed for per sample and the data were shown as Mean  $\pm$  SD. Significant differences were calculated using the student's *t*-test relative to the expression at 0 h (\* indicates  $p < .05$  and \*\* indicates  $p < .01$ ).

demonstrate that *L. vannamei* hemocyanin directly interacts with TGase.

### 3.3. Hemocyanin and TGase show similar expression patterns during infection

The interaction of hemocyanin with TGase indicated that they might have a close relation in shrimp immune response. To explore this, the expression profiles of hemocyanin and TGase were determined using qPCR analysis at various time points post pathogen challenge. As shown in Fig. 4A, hemocyanin and TGase showed similar expression patterns post *V. parahaemolyticus* infection. The expression of these two genes were generally elevated between 0 h and 48 h, both reaching a peak of approximately 2.5-fold at 48 h post infection, compared to baseline expression at 0 h. For *S. iniae* infection, the expression profiles of hemocyanin and TGase also followed a similar trend. The expression of hemocyanin was induced from 0 h to 24 h, and then down-regulated from 36 h to 72 h, while the expression of TGase increased from 36 h to 48 h, followed by a down-regulation at 72 h (Fig. 4B). The maximum expression of hemocyanin and TGase post *S. iniae* challenge were observed at 24 h (2.0-fold) and 48 h (1.7-fold), respectively compared to

0 h. The similarity in expression patterns shown by hemocyanin and TGase suggest that these two genes might play synergistic roles in the immune response to pathogens in *L. vannamei*.

### 3.4. Hemocyanin modulates TGase expression and hemolymph clotting

Given that hemocyanin and TGase interacted with each other and followed a similar expression pattern during pathogen infection, we went about to further determine the regulatory roles of hemocyanin on TGase using RNAi technique. First, the gene knockdown effect of siHMC was analyzed in shrimp. The result showed that compared to the siNon-injected control, the expression of hemocyanin significantly decreased at 72 h post siHMC injection, indicating a successful *in vivo* knockdown of hemocyanin (Fig. 5A-i). Subsequently, the consequence of hemocyanin depletion on TGase expression was analyzed by qPCR. The results showed that the expression of TGase significantly reduced by about 4-fold after hemocyanin depletion as compared to control (Fig. 5A-ii). Meanwhile, in order to further determine whether hemocyanin affects clotting, hemolymph clotting analysis was performed. It was found that hemolymph clotting was observed in the siNon-injected sample (Fig. 5B-i), while little clotting occurred in the siHMC-injected sample (Fig. 5B-ii) or in the negative control (i.e., hemolymph from siNon-injected shrimp incubated with anti-coagulant) (Fig. 5B-iii). These results suggest that *L. vannamei* hemocyanin could modulate the expression of TGase and regulate hemolymph clotting.

## 4. Discussion

Invertebrates primarily depend on their innate immune system to respond to pathogens infection. In shrimp, a variety of immune molecules and immune signaling pathways have been identified, with their interactions reported to play pivotal roles in innate immunity [32–34]. Several recent studies have shown that hemocyanin is a multifunctional glycoprotein with various immunological properties and involved in the modulation of immune functions [22,23,35]. However, little is known about the immune-related interaction networks of hemocyanin in shrimp *L. vannamei*. In this study, we employed Co-IP and LC-MS/MS analysis to identify the interacting partners of *L. vannamei* hemocyanin in shrimp plasma. A total of 39 candidate proteins were found to associate with hemocyanin protein (Supplementary Table S1). GO analysis revealed that most of the identified proteins were localized in cell and involved in metabolic process and binding in terms of biological process, cellular component and molecular function (Fig. 2). Of note, two clotting-related proteins TGase and CP were identified, suggesting the involvement of hemocyanin in the hemolymph clotting system.

Synonymous with blood clotting in vertebrates, the hemolymph clotting system is an integral part of the innate immune system in invertebrates [36], which prevents the loss of hemolymph and the intrusion of pathogens during injury or infection. In crustaceans, hemolymph clotting is mainly mediated by TGase and CP [24,31]. Upon tissue damage or infection, TGase is released from hemocytes or other tissues to catalyze the cross-linking and aggregation of the plasma protein CP in a calcium-dependent manner [31]. Although both TGase and CP have been cloned and characterized in some shrimp species [24], the molecular mechanism by which these proteins regulate the formation of hemolymph clots has not been reported in shrimp. Our current and previous Co-IP data suggest that hemocyanin possibly interacts with TGase [25]. This observation is further substantiated in this study, where we demonstrated that *L. vannamei* hemocyanin indeed directly interacts with TGase (Fig. 3), suggesting a role of hemocyanin in shrimp hemolymph clotting by binding TGase. Interestingly, it has been demonstrated that hemocyanin is associated with the fibrils of the *Limulus* hemolymph clot [37]. In addition, a recent study has reported that spider hemocyanin is incorporated into the hemolymph clots and represented more than half of the protein content of clots [38]. From the foregoing report, couple with the fact that shrimp hemocyanin

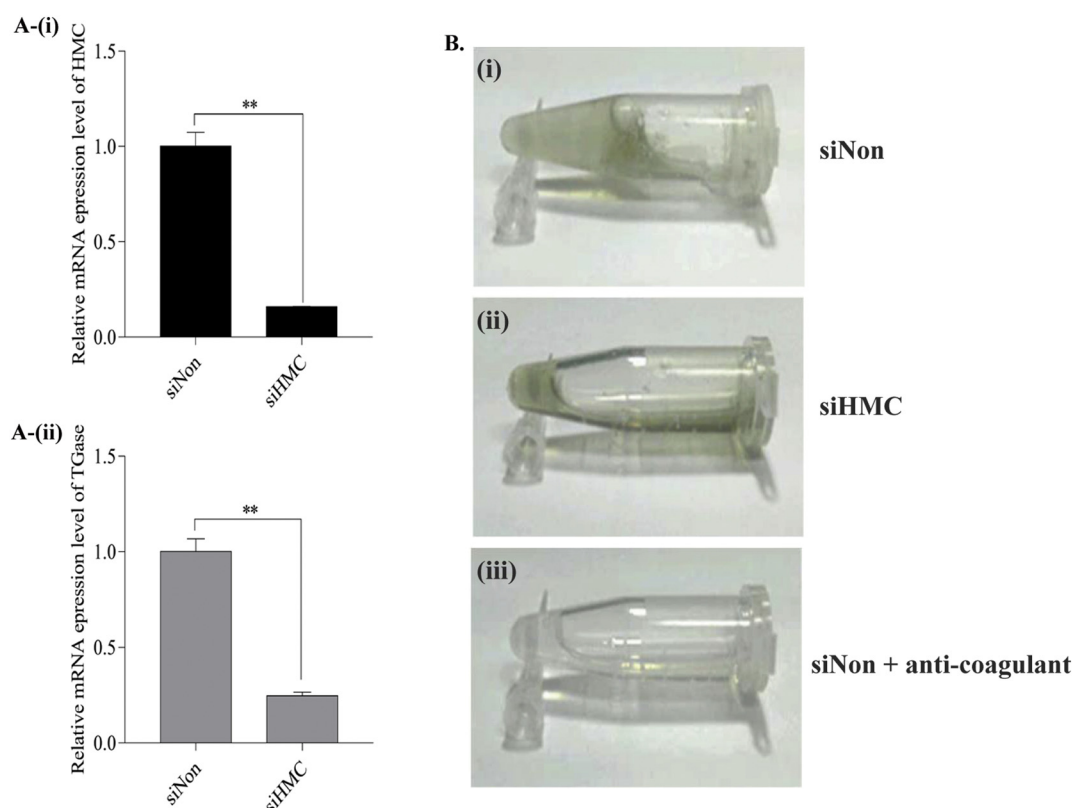


**Fig. 5.** *L. vannamei* hemocyanin modulates TGase expression and hemolymph clotting. Expression of TGase (A) and hemolymph clotting analysis (B) after hemocyanin (HMC) knockdown. Shrimps were injected with 5.0  $\mu$ g siHMC or siNon (negative control). At 72 h post siRNA injection, the expression level of HMC (A-i) and TGase (A-ii) was measured using qPCR. Meanwhile, hemolymph of each group was collected with or without the use of anticoagulant, and then immediately transferred to a centrifuge tube. Clotting was visualized and captured after tubes were standing for 1 h at room temperature. B-i and ii, hemolymph was directly withdrawn from siNon- and siHMC-injected shrimps, respectively; B-iii, hemolymph was collected from siNon-injected shrimps and half-diluted with anti-coagulant.

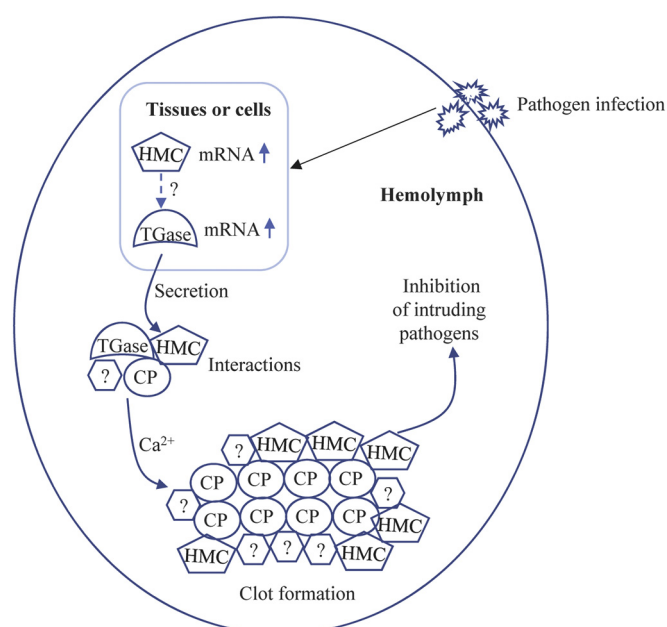


**Fig. 6.** Proposed model illustrating the roles of hemocyanin in hemolymph clotting. In shrimp, hemocyanin (HMC) is up-regulated upon pathogen challenge and modulates the expression of TGase. Thereafter, hemocyanin and TGase are released into hemolymph where hemocyanin interacts with the clotting-related proteins (TGase, CP, etc.) and thereby affect the formation of hemolymph clots.

constitutes > 90% of hemolymph plasma proteins [26], it is plausible to state that hemocyanin plays a role in hemolymph clotting by interacting with clotting-related proteins such as TGase.

Two types of TGase (TG I and TG II) have been identified to be involved in the hemolymph clotting of *L. vannamei* [39–41]. The TGase identified in the current study, which interacted with hemocyanin was TG I. It has previously been shown that although TG I was widely distributed in various shrimp tissues, the greatest TGase activity was detected in the hepatopancreas [39]. Given that hemocyanin is also believed to be synthesized in the hepatopancreas in crustacean [42], it is reasonable to speculate that probably there is a close relationship between hemocyanin and TGase in the hepatopancreas. In fact, it was observed in the current study that the expression of hemocyanin and TGase in the hepatopancreas followed a similar pattern upon challenge with *V. parahaemolyticus* and *S. iniae* (Fig. 4). Moreover, the expression of TGase was significantly down-regulated after hemocyanin was depleted in hepatopancreas (Fig. 5A). These results suggest that the expression of TGase is modulated by hemocyanin, and that these two proteins might function synergistically in the immune response against pathogenic bacteria.

In order to provide direct evidence that hemocyanin plays a role in the hemolymph clotting system of shrimp, hemolymph clotting was determined after *in vivo* knockdown of hemocyanin. The results revealed that compared to control, there was a significant inhibition in hemolymph clotting following hemocyanin depletion in shrimp (Fig. 5B). Considering all that has been stated above, we propose that hemocyanin can be up-regulated upon pathogen infection, which then modulates the expression of TGase via an unknown mechanism. The synthesized hemocyanin and TGase are then released into the hemolymph where hemocyanin interacts with clotting-related proteins such

as TGase, CP and other proteins. These interactions might promote clot formation in shrimp hemolymph, therefore inhibiting the intrusion of pathogens (Fig. 6).

## 5. Conclusion

In this study, we identified 39 putative hemocyanin interacting partners from *L. vannamei* plasma by Co-IP and LC-MS/MS analysis. Furthermore, these results are the first to reveal that hemocyanin might positively regulate hemolymph clotting in shrimp by interacting with TGase and modulating its expression. These findings provide more insight into the functions of hemocyanin in immune response and will be helpful for shrimp disease control and prevention.

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## Conflict of interest

The authors declare that there is no conflict of interest.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jpro.2019.04.013>.

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