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Identification and characterization of glycosylation sites on *Litopenaeus vannamei* hemocyanin

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The respiratory glycoprotein hemocyanin has been implicated in immune-related functions. Using lectin blotting, we show that the binding of shrimp (*Litopenaeus vannamei*) hemocyanin to concanavalin A decreases markedly with O-glycosidase treatment but not with PNGase F. Twelve O-glycosylation sites, three on the large hemocyanin subunit and nine on the small hemocyanin subunit (HMCs), were identified by LC-MS/MS. Importantly, when the glycosylation sites at Thr-537, Ser-539, and Thr-542 on the C terminus of HMCs were replaced with alanine, the resultant mutant hemocyanin had reduced carbohydrate content, coupled with a fourfold reduction in bacterial agglutination and 0.2-fold reduction in antibacterial activities toward *Vibrio parahaemolyticus* and *Staphylococcus aureus*. These results suggest that the glycosylation sites on shrimp hemocyanin are closely related to its immunological functions.

Keywords: agglutination; antibacterial; glycosylation; hemocyanin; *Litopenaeus vannamei*

Hemocyanin is a copper-containing glycoprotein, which is primarily responsible for oxygen transport in both arthropods and mollusks [1,2]. There are, however, differences in the structure and glycosylation of molluscan and arthropodan hemocyanins [3,4]. It has been shown that arthropodan hemocyanin occurs as hexamers or multiples of hexamers while molluscan hemocyanin exists as decamers or di-decamers [5,6] and contains one, two, or three structural subunits in the native molecule [7] In addition, the carbohydrate content and monosaccharide composition are different between arthropodan and molluscan hemocyanins [8,9].

Recent studies have shown that hemocyanin is multifunctional, playing important roles in several physiological, pathophysiological, defense, metabolism, and immune-related functions [10–12]. For instance, Laino [13] initially reported that hemocyanin of the spider *Polybetes pythagoricus* could functionally be converted into a phenoloxidase-like enzyme. Following this, hemocyanin from *Limulus polyphemus* and *Cancer pagurus* as well as other species was found to also possess phenoloxidase activity upon SDS treatment [14,15]. Apart from this, molluscan hemocyanin from *Rapana venosa* and *Helix lucorum* has been shown to possess antiviral activities against Herpes Simplex Virus 1 (HSV-1) in a dose-dependent way [16], while arthropodan hemocyanin from shrimp *Penaeus monodon* was shown to possess antiviral activities against both DNA and RNA viruses [17]. Similarly, Dolashka-Angelova [18] reported that the structural subunit of hemocyanin from the mollusks *Helix aspersa* had a high antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli,* while recombinant hemocyanin of *Litopenaeus vannamei* was shown to possess antibacterial activity against

Abbreviations
CCH, *Concholepas concholepas* hemocyanin; FLH, *Fissurella latimarginata* hemocyanin; FU, functional unit; GalNAc, *N*-Acetylgalactosamine; LB, Luria-Bertani; NC, nitrocellulose; PVDF, polyvinylidene fluoride.

Vibrio parahaemolyticus [19]. Aside its antimicrobial function, recent accumulating evidences indicate that hemocyanin also has antitumor activity. It has been reported that *Concholepas concholepas* hemocyanin (CCH) from the Loco or Chilean abalone and *Fissurella latimarginata* hemocyanin (FLH) from the Lapa negra possess immunostimulatory properties such as antitumor activity [11], while Stenzl [20] showed that the functional unit (FU) RvH1-c of hemocyanin from *R. venosa* possessed strong anticancer activities against bladder cancer permanent cells. Furthermore, our previous studies revealed that hemocyanin of *L. vannamei* attenuated the proliferation of HeLa cells by modulating the mitochondrial pathway apoptosis as well as exerted a significant antitumor effect on Sarcoma-180 (S180) tumor-bearing mice by enhancing the immunity and antioxidative potential of the mice [12,21]. Besides this, our earlier studies suggested that *L. vannamei* hemocyanin could react with human IgM, IgG, or IgA as an antigen [22–24], bind to bacteria and animal erythrocytes as an agglutinin and hemolysin [23,25] as well as act as an immune-enhancing protein [26].

A number of studies have indicated that the functional diversity of hemocyanin was related to its glycosylation. Dolashka-Angelova *et al.* found that the hemocyanin of *R. venosa*, *H. lucorum*, and *Megathura crenulata* were glycosylated, with the glycosylated *R. venosa* hemocyanin FU, RvH2-c, exhibiting antiviral activity while the non-glycosylated unit, RvH2-b, showed no antiviral activity [3,16]. Similarly, Arancibia found that the arrangement of the *N*-Acetylgalactosamine (GalNAc) residues on keyhole limpet hemocyanin (KLH) and FLH was one of the factors accounting for their different antitumor activity [27]. In addition, our previous studies revealed that the diversity in the glycosylation of *L. vannamei* hemocyanin was related to its agglutinative activity against bacteria, as hemocyanin with five different glycans had different agglutinative activity against *E. coli*, *S. aureus*, and erythrocytes [28]. Moreover, the agglutinative activity of hemocyanin was significantly reduced after a reduction in the glycan level [28,29]. However, prior to this study, little was known about the glycosylation types and sites on *L. vannamei* hemocyanin as well as how this relates to its immune-related activities.

In this study, we showed that the small subunit hemocyanin of *L. vannamei* had O-glycans on its C terminus at residues Thr537, Ser539, and Thr542. Moreover, these modifications were found to be closely associated with hemocyanin's bacterial agglutination and antibacterial activities.

Materials and methods

Experimental animals and preparation of shrimp sera

Penaeid shrimps (*L. vannamei*), with average length 10–14 cm and weight 15–20 g, irrespective of sex, were purchased from a local supplier, Shantou Huaxun Aquatic Product Corporation (Shantou, China). Shrimps were then transferred to laboratory tanks with aerated seawater at room temperature for at least 2 days acclimatization before experiments. Hemolymph was drawn directly from the pericardial sinus using a sterile needle with syringe, and then allowed to clot overnight at 4 °C. The sera were separated as previously described [30] and stored at −20 °C until use. All animal experiments were carried out in accordance with the guidelines and approval of the Animal Research and Ethics Committees of Shantou University, China.

Purification and identification of hemocyanin

Purification of hemocyanin was performed by affinity chromatography as previously described with some modifications [29]. Briefly, *L. vannamei* sera (2 mL) were loaded onto an affinity chromatography column with a ligand of rabbit anti-shrimp small subunit hemocyanin antibody, followed by washing the column with PBS (0.01 M, pH 7.4) until the absorbance at 280 nm reached baseline. Bound proteins were eluted with glycine-HCl buffer (0.1 M, pH 2.4) and neutralized immediately with Tris-HCl buffer (1 M, pH 8.0).

The purified hemocyanin was identified by gel electrophoresis and immunoblotting. SDS/PAGE was carried out under reducing conditions on a 10% separating gel (pH 8.8) with a 5% stacking gel (pH 6.8). Following the SDS/PAGE, gels were either stained with Coomassie Brilliant Blue R-250 (RuJi, GuangZhou, China) or transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Milford, MA, USA) with a semidry transfer apparatus (DYCP-40C; LiuYi, Beijing, China). The PVDF membranes were then blocked for 2 h with 5% skimmed milk (WeiJia, Guangzhou, China) in TBST (20 mM Tris, 0.15 M NaCl, pH 7.4, 0.05% Tween-20) at room temperature. After being washed with TBST three times, the membranes were incubated with rabbit anti-shrimp hemocyanin antiserum diluted (1 : 2000) in 5% skimmed milk at room temperature for 45 min [28]. After being washed three times with TBST, the membranes were incubated with goat anti-rabbit IgG-HRP antibodies (Sigma-Aldrich, Burlington, MA, USA) diluted (1 : 20 000) in 5% skimmed milk at room temperature for 30 min. Membranes were finally washed and developed with ECL chemiluminescence western blotting detection kit (Millipore).

Deglycosylation assays

The deglycosylation of hemocyanin was carried out using Peptide-*N*-Glycosidase F (PNGase F) or O-glycosidase

(New England Biolabs, Hitchin, UK) according to the manufacturer's instructions. Briefly, purified hemocyanin was treated as follows: 5 μL 10 X G7 Buffer, 5 μL NP-40, 5 μL Neuraminidase (only for O-glycosidase), and 40 μL (300 $\mu g \cdot mL^{-1}$) hemocyanin were mixed with 3 μL PNGase F or O-glycosidase and incubated at 37 °C for 4 h as previously described [29].

Carbohydrate detection

Dot-lectin blotting was used to compare the difference in glycosylation between hemocyanin and deglycosylated hemocyanin as previously described with modification [24]. Briefly, a nitrocellulose (NC) membrane (Beyotime, Beijing, China) was cut into the desired size, soaked in TBS (20 mM Tris, 150 mM NaCl, pH 7.4) for 5 min, and allowed to dry on a filter paper. Then, 1.5 μL of the hemocyanin or deglycosylated hemocyanin sample (0.3 $mg \cdot mL^{-1}$) was spotted onto the NC membrane using a pipette. After allowing to dry, the NC membranes were blocked with 5% BSA (MP Biomedicals, Santa Ana, CA, USA) in TBST at room temperature for 2 h, followed by incubation at room temperature with a 1 : 2000 dilution of Biotin-ConA (Sangon Biotech, Shanghai, China) and 1 : 20 000 dilution of Avidin-Peroxidase (Sangon Biotech) for 45 min and 30 min, respectively. Finally, the membranes were washed and developed with ECL Chemiluminescence western blotting detection kit as described above. The gray values of the dots on membranes were determined using ADOBE PHOTOSHOP (Version 13.0.1). The relative gray value was calculated as follows: Relative Gray Value = (Gray value of treated group − Gray value of background)/(Gray value of untreated group − Gray value of background).

Mass spectrometry analysis

The purified hemocyanin samples were sent to Applied Protein Technology (Shanghai, China) for the identification of the glycosylation sites using LC-MS/MS. Briefly, the hemocyanin samples were alkylated with 100 μL 0.05 M iodoacetamide, 8 M urea in 0.1 M Tris/HCl pH 8.5. After 20 min of incubation in the dark, three washes with 8 M urea and three additional washes with 40 mM ammonium bicarbonate (NH_4HCO_3) were performed. Finally, the enzymatic digestion was performed with 4 μg trypsin in 40 μL of 40 mM NH_4HCO_3 overnight at 37 °C [31]. Following this, the peptides were desalinized using Cartridges (66872-U; Sigma, St. Louis, MO, USA) and dissolved in 40 mM NH_4HCO_3. The peptides were then mixed with 0.1% formic acid and purified using Easy Nlc, a Nano-HPLC liquid phase system (Thermo, Bremen, Germany) as previously described [32]. The purified peptides were then analyzed by Q-Exactive mass spectrometer (Thermo Finnigan, San Jose, CA, USA). The original mass spectrometry data were analyzed by BYONIC software (PMI-Byonic-Com: v2.0-3), after which the predicted protein sequence data were analyzed using the online website (http://www.functionalglycomics.org/) so as to identify the glycan composition.

RNA extraction and cDNA synthesis

Total RNA was extracted from *L. vannamei* hepatopancreas using the RNAiso Plus kit (Takara, Dalian, China) according to the manufacturer's instructions. The extracted RNA was treated with RNase-Free DNase (Takara) to remove contaminating DNA, and cDNA synthesized using the M-MLV RTase cDNA Synthesis Kit (Takara) following the manufacturer's instructions.

Gene cloning of small subunit hemocyanin

A pair of hemocyanin gene-specific primers HMCs-F (5′-TACGTAATGAGGGTC TTAGTGGTTCT-3′) and HMCs-R (5′-GCGGCCGCATTGAAGACCTTAACTT GGAT-3′) were designed based on the cDNA sequence of small subunit hemocyanin (GenBank accession number: X82502.1) with the SnaB I and Not I restriction sites at the 5′ and 3′ ends of the primers for PCR amplification. The PCR cycling conditions were as follows: 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min, and a final extension 72 °C for 10 min. The PCR products were extracted and purified as previously explained [33], ligated into the pMD-19T vector (Takara) followed by transformation into *E. coli* DH5a (Promega, Madison, WI, USA). Recombinant clones grown on MacConkey agar (Sigma) were identified using blue-white screening, while positive clones were picked and confirmed by sequencing.

Site-directed mutation of small subunit hemocyanin

To construct the hemocyanin mutants, the primer pho-HMC (5′-ATTGAACGCAAGTCCGCGGAAGCTTCAG TAGCTGTACCGGACGTGC') was designed using PRIMER PREMIER 5 (Version 5.00) (Palo Alto, CA, USA) based on the cDNA sequence of small subunit hemocyanin (GenBank accession number: X82502.1), followed by synthesis of the primer by Beijing Genomics Institute (BGI, Beijing, China) with a phosphate on the 5′ terminal. The site-directed mutagenesis reactions were performed with the StarMut Multi Site-directed Mutagenesis Kit (GeneStar, Beijing, China) according to the manufacturer's instructions. PCR cycling conditions were 95 °C for 2 min, 30 cycles of 95 °C for 1 min, 55 °C for 30 s, and 65 °C for 4 min. The PCR products were digested with Dpn I and then transformed into *E. coli* DH5a (Promega). The recombinant plasmid was purified and ligated into the pPIC9K vector (Invitrogen, Waltham, MA, USA) followed by transformation into *Pichia pastoris* GS115 (Fig. S2). Recombinant clones grown

on MacConkey agar (Sigma) were picked and confirmed by sequencing [34]. The nucleotide and deduced amino acid sequences of hemocyanin and the hemocyanin mutants were analyzed using the online BLAST tool at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) and the BIOEDIT SEQUENCE ALIGNMENT EDITOR (Version 7.0.4.1) (Carlsbad, CA, USA).

Eukaryotic expression, purification, and identification of recombinants

Recombinant clones of hemocyanin and hemocyanin mutants were cultured at 28 °C overnight in Buffered Glycerol-complex Medium, BMGY (1% Yeast extract, 2% Peptone, 100 mmol·L^{-1} PBS (pH 6.0), 1.34% YNB, 4×10^{-5}% Biotin, 1% Glycerol), followed by centrifugation at 339 ***g*** for 10 min at 4 °C to collect the yeast. Next, the yeast was cultured in Buffered Methanol-Complex Medium, BMMY (1% Yeast extract, 2% Peptone, 100 mmol·L^{-1} PBS (pH 6.0), 1.34% YNB, 4×10^{-5}% Biotin, 1% methanol), and then, methanol was added for the induction of hemocyanin expression. Supernatants were collected and filtered through an Amicon Ultra-15 centrifugal filter (Millpore, Tullagreen, Ireland) to concentrate the recombinant hemocyanin proteins. The purified recombinant proteins, wild-type hemocyanin (wt-rHMCs) and mutant hemocyanin (mut-rHMCs), were identified by western blot analysis using anti-hemocyanin antibody as described above. The samples were also analyzed by ConA lectin blotting. For this, samples were first separated by SDS/PAGE, and then transferred onto PVDF membranes as described in the identification of hemocyanin above. Following this, membranes were blocked with 5% BSA in TBST at room temperature for 2 h, and then incubated with Biotin-Con A and Avidin-Peroxidase as described for Dot-lectin blotting. Finally, membranes were developed with an ECL Chemiluminescence western blotting detection kit.

Agglutinating activity assays

The bacteria, *V. parahaemolyticus* and *S. aureus*, were used for the agglutinating activity analysis as previously described [28]. Briefly, bacteria were cultured in Luria-Bertani (LB) media (Tryptone 10 g·L^{-1}, Yeast extract 5 g·L^{-1}, NaCl 10 g·L^{-1}) overnight at 37 °C, then harvested by centrifugation, washed three times with normal saline, and diluted to 10^8 CFU·mL^{-1} in TBS-Ca^{2+} (50 mmol·L^{-1} Tris-HCl, 0.75% NaCl, 50 mmol·L^{-1} $CaCl_2$, pH 7.2). Native HMC, mut-rHMCs, and wt-rHMCs (150 μg·mL^{-1}) were diluted twofold in TBS-Ca^{2+}, then incubated with the same volume (10 μL) of each bacteria suspension at 37 °C for 30 min, respectively. The bacteria agglutination was observed using a light microscopy (BX51; OLYMPUS, Tokyo, Japan), and the agglutinative efficiency determined as the reciprocal of the highest dilution of the tested samples.

Antibacterial activity assays

The antibacterial activity analysis was carried out as previously described [24]. The antibacterial activities of the filtered (0.22 μm) hemocyanin samples including native HMC, mut-rHMCs, and wt-rHMCs were assessed by the number of bacterial colonies growing or present on LB Petri dish. Bacteria (*V. parahaemolyticus* and *S. aureus*) were grown in LB media overnight at 37 °C and collected by centrifugation, washed three times with 0.85% NaCl, resuspended, and then diluted with 50 mM Tris-HCl (pH 7.2) to 10^3 CFU·mL^{-1}. Meanwhile, each hemocyanin sample (100 μg·mL^{-1}) was mixed with half volume of the same bacteria suspension. After the mixture was incubated at room temperature for 30 min, 50 μL of incomplete nutrient media (0.04% bacto-tryptone, 0.02% yeast extract, 0.75% NaCl, 50 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.2) was added and then incubated for an extra 1.5 h. The mixture of bacteria suspension and hemocyanin sample were, respectively, spread on LB Petri dishes, then cultured for 12 h at 37 °C and the number of bacteria determined by counting the colony-forming units (CFU). As control, the buffer used for diluting the bacteria was mixed with the bacteria and cultured. The inhibition rate was calculated as follows: Inhibition rate = (clones in negative control − clones in the experimental group)/clones in negative control × 100%. All samples were prepared and analyzed in triplicates, with each experiment repeated three times. Data are expressed as means ± standard error (SD).

Results

Analysis of purified hemocyanin and identification of the glycosylation types

The affinity chromatography purified hemocyanin was analyzed by SDS/PAGE and immunoblotting. As shown in Fig. 1A, two bands with molecular weights around 77 and 75 kDa were observed and could specifically react with anti-shrimp hemocyanin antibodies, suggesting that a good separation had been achieved. Next, to characterize the glycosylation types on hemocyanin, dot-lectin blotting was used, by first treating the purified hemocyanin samples with PNGase F (cleaves N-linked glycoproteins and glycopeptides) or O-glycosidase (cleaves O-linked glycoproteins and glycopeptides) before analysis. As shown in Fig. 1B, the binding of lectin with hemocyanin decreased by about 60% with O-glycosidase treatment, while treatment with PNGase F showed no significant reduction in binding to lectin. Moreover, hemocyanin diluted 25-fold could still bind ConA, whereas O-glycosidase treated hemocyanin at the same dilution showed no binding to ConA.

Location of O-glycosylation sites on *L. vannamei* hemocyanin subunits

For a global assessment of the glycosylation diversity in hemocyanin, samples were digested with trypsin and analyzed by LC-MS/MS. As the presence of N-glycans on shrimp hemocyanin could not be detected, we went about to explore the O-glycan structures of hemocyanin so as to identify putative O-glycosylation sites. The LC-MS/MS data revealed 12 glycosylation sites on hemocyanin (Fig. S1 and Table S1), including three sites on large subunit hemocyanin (HMCl, shaded gray in Fig. 2A) and nine sites on small subunit hemocyanin (HMCs, shaded gray in Fig. 2B). Details of the predicted O-glycans composition of hemocyanin are presented in Table S2 and Table S3. These results suggest that hemocyanin possessed many O-glycosylation sites, which are mainly located on the Ig-like domain in the C terminus.

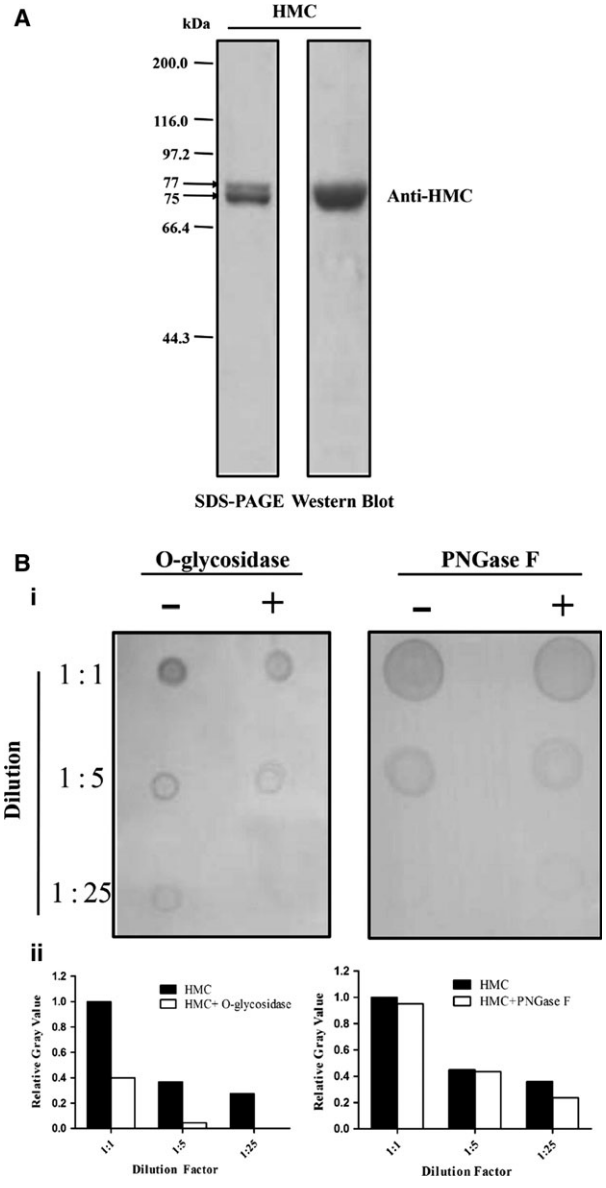

Fig. 1. Analysis of purified hemocyanin and identification of the glycosylation types. (A) SDS/PAGE and western blot analysis of affinity chromatography purified HMC. (B) Dot-lectin blotting analysis of hemocyanin treated with O-glycosidase or PNGaseF (i) dot blots, (ii) quantified gray values of dot-lectin blotting.

Mutation of potential immune-related glycosylation sites on hemocyanin

Our previous studies showed that the Ig-like domain of *Penaeus vannamei* hemocyanin could react with anti-human Ig [23,24] and was also significantly upregulated during Taura syndrome virus infection [35]. All these suggest that the Ig-like domain of hemocyanin possessed immunology function. Further evidence indicated that the Ig-like domain of hemocyanin could interact with bacteria and had agglutination activity toward *E. coli* K12, compared with the other domains of hemocyanin [24]. Aside from this, we also previously showed that an 18.4 kDa peptide on the C terminus of small subunit hemocyanin (double underlined in Fig. 2B) had strong agglutination and antibacterial activities against pathogenic bacteria [36]. Based on these, we selected three potential immune-related glycosylation sites on this C-terminal peptide to generate a mutant of small subunit hemocyanin, mut-rHMCs, by replacing threonine (Thr) and serine (Ser) residues at positions 537, 542, and 539 with alanine (Ala) (Fig. 3A). The mutant was cloned into the pPIC9K vector and expressed in yeast *P. pastoris* (Fig. S2). Lectin blotting analysis revealed that wild-type recombinant small subunit hemocyanin, wt-rHMCs, had higher binding ability to Con A than mut-rHMCs, with the binding activity of mut-rHMCs being almost 90% lower (Fig. 3B). The results show that hemocyanin could be mutated at these three sites, with the resultant mutations impacting on the glycosylation of hemocyanin, therefore suggesting their crucial role in hemocyanin.

Glycosylation sites on the C terminus of small subunit hemocyanin are closely related to bacterial agglutination activity and antibacterial activity

To further investigate whether mutation of the glycosylation sites on the C terminus of small subunit hemocyanin had any impact on its antimicrobial activity, *V. parahaemolyticus* and *S. aureus* were used for agglutination activity analysis. The results revealed that the agglutination activity of mut-rHMCs was

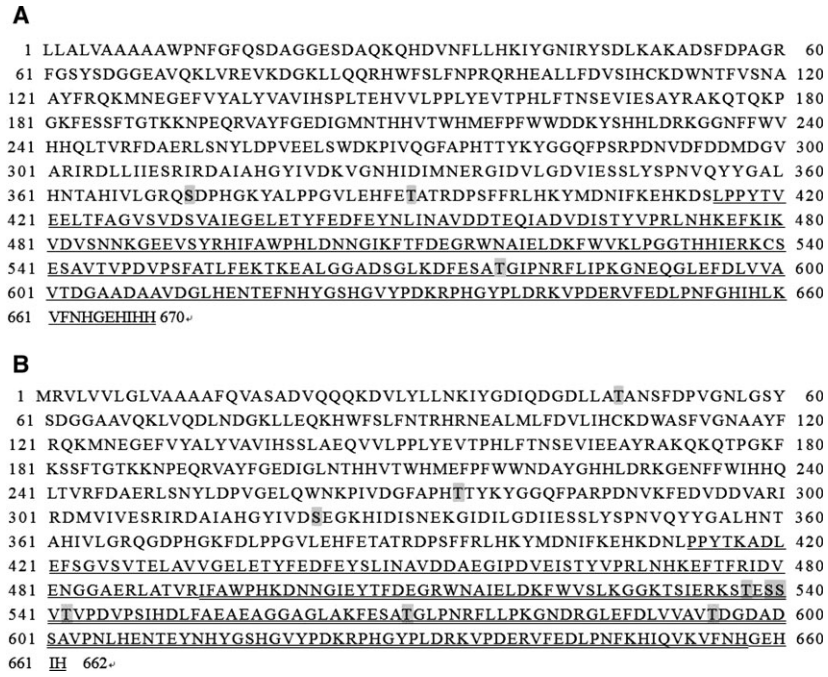

Fig. 2. Location of O-glycosylation sites on *Litopenaeus vannamei* HMC subunits. (A) Large subunit hemocyanin (HMCl), (B) Small subunit HMCs. Glycosylation sites are shaded gray. The Ig-like domain of hemocyanin is underlined while the 18.4 kDa peptide on the C terminus of small subunit hemocyanin is double underlined.

weaker than that of wt-rHMCs, with about fourfold reduction (Fig. 4A, Table 1). Similarly, although both wt-rHMCs and mut-rHMCs showed strong antibacterial activities at a concentration of 100 $\mu g \cdot mL^{-1}$ toward *V. parahaemolyticus* and *S. aureus* compared with PBS treatment, the antibacterial activity of mut-rHMCs was relatively lower (20%) than wt-rHMCs (Fig. 4B). This further indicates that mutation of these three glycosylation sites reduced the agglutination and antibacterial activities of hemocyanin.

Discussion

Glycosylation is a common and highly diverse type of posttranslational protein modification. Incidentally, most cell surface and secreted proteins are glycosylated, which is crucial in cell signaling and cell proliferation as well as other biological processes [37]. It has been suggested that protein glycosylation generally involves the attachment of glycans to serine (Ser), threonine (Thr), or asparagine (Asn) residues, with the most abundant type of O-glycosylation being the attachment of GalNAc to serine (Ser) or threonine (Thr) residues [38]. Hart indicated that protein glycosylation with O-GlcNAc could modulate cardiac mitochondrial function, signaling, as well as affect protein expression, degradation, and crosstalk [39]. In addition, O-GlcNAc glycosylation plays an important role in diseases such as the etiology of diabetes and neurodegeneration [39]. Similar to most proteins, hemocyanins are glycoproteins, with large differences in their carbohydrate content and monosaccharide composition, which regulate immunostimulatory properties [3]. Becker reported that the *Concholepas* hemocyanin (CCH) subunit CCHA, which has both N- and O-linked moieties, showed better antitumor effects in the bladder carcinoma cell line MBT-2 than subunit CCHB, in which O-linked glycans were nearly absent [40]. When the glycosylation sites on the gastropod *Helix pomatia* β-hemocyanin were studied, it revealed that the FU *Hp*e was glycosylated at Asn-111 and Asn-387 [41]. However, the glycosylation sites of arthropod hemocyanins have not been reported. Here, we report for the first time the glycosylation sites of *L. vannamei* hemocyanin and showed that it was only deglycosylated by O-glycosidase (Fig. 1B). Furthermore, our previous study showed that the agglutinative activity of hemocyanin toward *Vibrio fluvialis* and *Vibrio alginolyticus* was reduced by about fourfold and eightfold, respectively, after deglycosylation, suggesting that O-glycosylation of *L. vannamei* hemocyanin closely relate to its immune function [29].

Seven glycosylation sites were identified on the secretory component of secretory immunoglobulin A (sIgA) and a single site on the J-chain, while five sites were found on the IgA heavy (H) chain [42]. Due to the importance of glycosylation in biological processes, researchers became interested in exploring the glycosylation sites on hemocyanin. Gielens reported that there

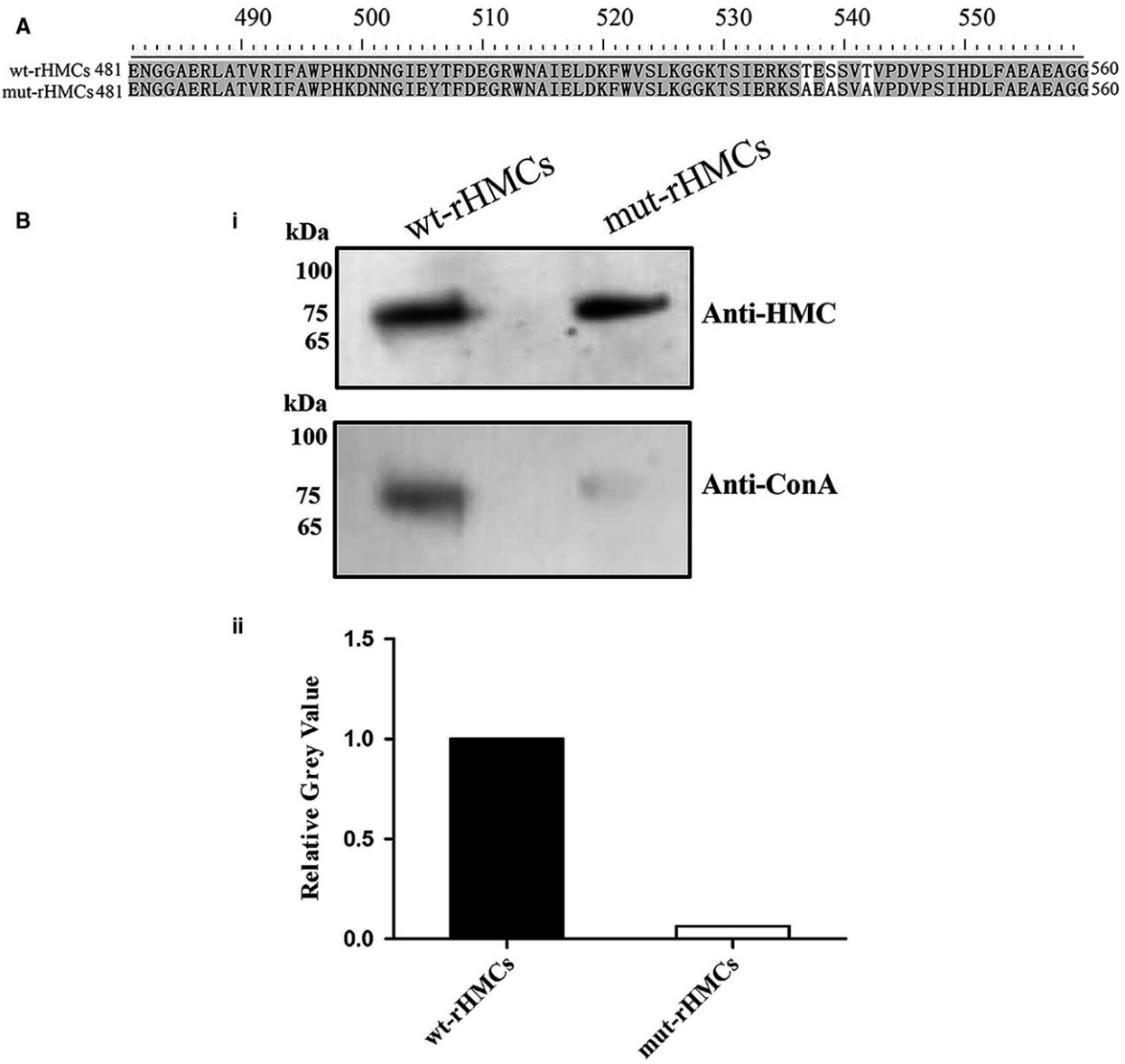

Fig. 3. Mutation of potential immune-related glycosylation sites on small subunit hemocyanin. (A) Partial protein sequence alignment of wild-type recombinant small subunit hemocyanin (wt-rHMCs) and mutant small subunit hemocyanin (mut-rHMCs) in which residues Thr-537, Ser-539, and Thr-542 were replaced with Ala (mut-rHMCs). (B) Analysis of carbohydrate content of wt-rHMCs and mut-rHMCs. (i) Dot-lectin blotting, (ii) Relative gray values of dot-lectin blotting.

were six glycosylation sites on the FUs of *H. pomatia* hemocyanins with glycosylation on Asn-387 being the common one [43]. Employing mass spectrometry, we were able to detect 12 glycosylation sites on *L. vannamei* hemocyanin, with three sites found on the large subunit while nine sites were on the small subunit (Fig. 2). Till now, little was known about the existence of glycosylation sites on *L. vannamei* hemocyanin and the functional role of this glycosylation. The present study was, therefore, an attempt to explore the glycosylation sites on *L. vannamei* hemocyanin as well as determine the impact on immunological functions in shrimp.

We recently found that shrimp seem to have different immune defensive strategies against different virulent pathogens, with the N and C termini of small subunit hemocyanin having different functions, such as agglutinative activity toward *E. coli* K12 and chicken erythrocytes [19,24]. Of particular note is the fact that, a 18.4 kDa peptide in the C terminus of small subunit

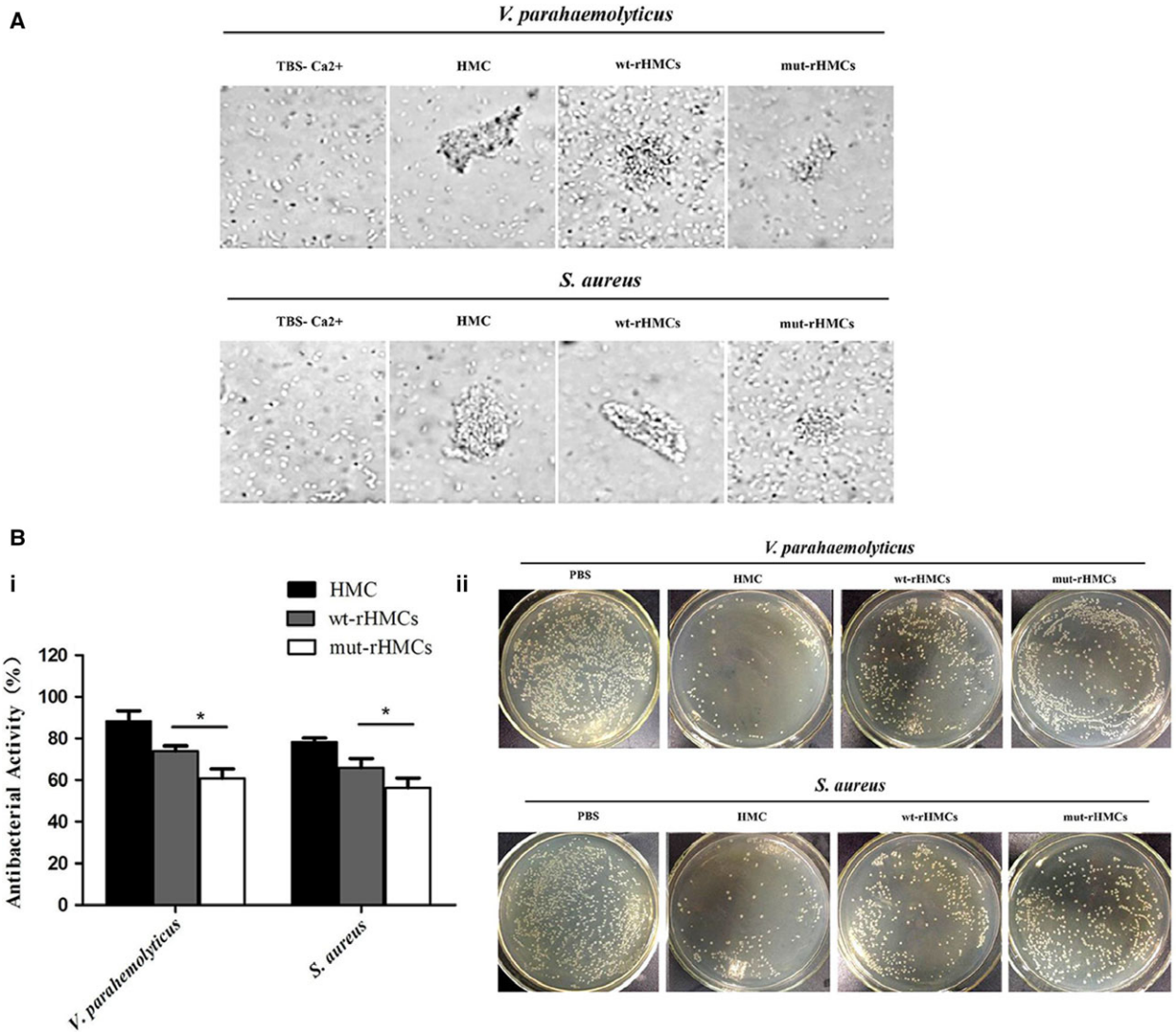

Fig. 4. Mutation of glycosylation sites on C terminus of small subunit hemocyanin affects its bacterial agglutinative and antibacterial activities. (A) Agglutinative activity analysis of native HMC, mutant hemocyanin in which residues Thr-537, Ser-539, and Thr-542 were replaced with Ala (mut-rHMCs), and recombinant hemocyanin (wt-rHMCs) expressed in yeast *Pichia pastoris* toward *Vibrio parahaemolyticus* and *Staphylococcus aureus*. Proteins were used at a concentration of 25 $\mu g \cdot mL^{-1}$. (B) Antimicrobial activity analysis of native HMC, mut-rHMCs, and wt-HMC toward *V. parahaemolyticus* and *S. aureus*. Proteins were used at a concentration of 100 $\mu g \cdot mL^{-1}$. The antibacterial activity of the native hemocyanin was set to 100% to which the other samples were normalized. Significant difference relative to wt-rHMCs were determined by one-way ANOVA and indicated by asterisks ($*p < 0.05$).

Table 1. Comparison of agglutinative activities between wt-rHMCs and mut-rHMCs at concentration of 150 $\mu g \cdot mL^{-1}$.

Bacterium	*Vibrio parahaemolyticus*	*Staphylococcus aureus*
Agglutinative titer [a]/Agglutinative activity ($\mu g \cdot mL^{-1}$)[b]		
HMC	512/0.19	1024/0.09
mut-rHMCs	128/0.78	128/0.78
wt-rHMCs	512/0.19	512/0.19

[a]The highest dilution of the test samples in the presence of different bacteria. [b]Protein concentration/agglutinative titer.

hemocyanin, which we previously found to have strong agglutination and antibacterial activities against pathogenic bacteria, was found in this study to contain three of the glycosylation sites (Thr-537, Ser-539, and Thr-542) [36]. Even though arthropods can produce mucin-type O-glycans, there was a Hex (for glucose, mannose, galactose) type glycan on site Thr-537, which might be a new type glycan in arthropods. Moreover, the agglutinative and antibacterial activity of small subunit hemocyanin significantly decreased upon

mutation (T537A, S539A, and T542A). Given that we have previously showed that native shrimp hemocyanin had strong bacterial agglutinative and antimicrobial activities [24], we went on to determine how the agglutinative and antimicrobial activity of the mut-rHMCs compared with native hemocyanin or wt-rHMCs. While mut-rHMCs still had some bacterial agglutinative and antibacterial activities against *V. parahaemolyticus* and *S. aureus*, this was significantly reduced compared with native hemocyanin and wt-rHMCs, that is, about fourfold reduction in agglutinative activity and 0.2-fold reduction in antibacterial activity (Fig. 4). Thus, mutation of the three glycosylation sites impacted more on the agglutinative activity than the antibacterial activity of hemocyanin, signifying their importance in agglutination and antibacterial activity. While the mutant seems to have a significant reduction in its agglutinative and antibacterial activity, this was not completely lost, suggesting that there might still exist other unidentified glycosylation sites, for which reason further future research is needed. Taken together, our results have revealed that glycosylation of the three sites found on the C terminus of the small subunit of hemocyanin was closely associated with the immune activity of hemocyanin, especially its agglutinative and antibacterial activities.

In conclusion, there exist great diversification in the glycosylation level and sites between the small and large subunit of *L. vannamei* hemocyanin. More importantly, three glycosylation sites on the C terminus of the small subunit of hemocyanin seem to be associated with its immune function, as mutation of these sites resulted in significant reduction in the agglutinative and antibacterial activity. It will therefore be of interest to further explore in future studies which of these three glycosylation sites is of crucial importance in the immunological function of hemocyanin as well as how these glycosylation sites regulate the immune activity of hemocyanin *in vivo*.

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Author contributions

YZ conceived and coordinated the study. ZZ and RL designed, performed and analyzed the experimental data. JJA, FW and MZ supervised the work and provided technical assistance. YZ, ZZ, RL and JJA wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Mass spectrum of peptide from hemocyanin purified by affinity chromatography after typsin digested.

Fig. S2. Construction of pPIC9k-mut-*hmc* plasmid.

Table S1. Summary of glycosylation sites on A-HMC.

Table S2. Analysis of glycosylation sites and carbohydrate structure prediction on large subunit of HMC (AJ250830.1).

Table S3. Analysis of glycosylation sites and carbohydrate structure prediction on small subunit of HMC (X82502.1).