Identification and agglutination properties of hemocyanin from the mud crab (Scylla serrata)

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Infectious diseases have significantly delayed the growth of crab aquaculture. Identification of the immune molecules and characterization of the defense mechanisms will be pivotal to the reduction of these diseases. Hemocyanin is an important non-specific immune protein present in the hemolymph of both mollusks and arthropods. However, little is known about the hemocyanin from the mud crab Scylla serrata. In this study, we identified the S. serrata hemocyanin using affinity proteomics and investigated its agglutinative properties. The results showed that S. serrata hemocyanin consists of five subunits with molecular weights of 70, 72, 75, 76 and 80 kDa, respectively. It demonstrated agglutination activities against seven bacterial species at concentrations ranging from 7.5 to 30 μg/ml. Agglutination was inhibited by 50–200 mM of N-acetylneuraminic acid, α-D-glucose, α-galactose and α-xylose. The 76 kDa subunit was identified as the protein that primarily binds bacterial cells and we speculate that it functions as the agglutinating subunit. We showed that outer membrane proteins (Omp) of bacteria could completely inhibit agglutination and that the agglutination activities of hemocyanin against Escherichia coli ΔOmpA and ΔOmpX mutants were significantly decreased, suggesting that these two Omps may be important ligands of hemocyanin. Together, the data collectively suggests that the 76 kDa subunit of S. serrata hemocyanin mediates agglutination through recognition of OmpA and OmpX proteins in bacteria.

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1. Introduction

Scylla serrata, a euryhaline species, generally inhabits intertidal zones and estuaries throughout the Indo-Pacific region including the coast of southeastern China [1]. It is an important commercial crustacean species in China. However, the intensification of crab farming has been accompanied by the development of many infectious diseases, especially from viruses (e.g., reovirus virus and white spot syndrome virus), bacteria (Vibrio parahaemolyticus) and parasites (Hematodinium sp.) [2,3], which cause mass mortalities during disease outbreaks resulting in vast economic losses. It is well-known that crustaceans (including crabs) lack the true adaptive immune response found in vertebrates and they rely exclusively on innate-immune mechanisms that include both cellular and humoral responses. Therefore, studies on the innate immune system are needed to provide new insights into the control of infectious diseases and the development of a sustainable crab farming.

Over the past several years, several non-specific immune proteins in crab species have been identified including antimicrobial peptide [4–6], prophenoloxidase [7], anti-lipopolysaccharide factor (ALF) [8–10], antioxidant enzyme [11], superoxide dismutase [12] and α2-macroglobulin [13]. Recently, hemocyanin, the main protein component of hemolymph from mollusks and arthropods, was reported as a novel and important defense molecule of the non-specific innate immune system [14,15]. Besides its primary function as a respiratory protein for many arthropods, it has been suggested that hemocyanin can be functionally converted into a phenoloxidase-like enzyme by different substances [16–18] and act as an antiviral agent against a variety of viruses, or generate reactive oxygen species as an antimicrobial strategy [19,20]. In addition, it has been reported that isolated antibacterial and antifungal peptides from shrimp are similar to the C-terminus of hemocyanin [21,22]. Furthermore, our previous reports suggested that the hemocyanin from the shrimp Litopenaeus vannamei can react with
anti-human Ig as an antigen, bind to bacteria as an agglutinin, and bind to vertebrate erythrocytes as a hemolysin [23–25]. However, little is known about S. serrata hemocyanin. Thus its compositions and immune features need further examination.

In this study, our results suggest that mud crab (S. serrata) hemocyanin consists of five subunits that show agglutination activities against pathogenic bacteria and that the 76 kDa subunit of hemocyanin was enough to mediate agglutination. Furthermore, we confirmed that some outer membrane proteins (including OmpX and OmpA) were recognized as ligands by hemocyanin, which represents hemocyanin as a novel pattern recognition receptor (PRR).

2. Materials and methods

2.1. Animal and preparation of mud crab S. serrata sera

S. serrata were purchased from a local farm in Shantou, Guangdong province, P.R. China. Haemolymph samples were taken with a sterile syringe from an arthrodial membrane at the base of a walking leg, and were allowed to clot overnight at 4 °C. The sera were collected after centrifuging at 6000 g for 20 min and stored at −20 °C until analyzed.

2.2. Isolation, purification and identification of S. serrata hemocyanin

Protein purification using affinity chromatography has been previously described [25]. Briefly, rabbit anti-hemocyanin IgG (5 mg, prepared by immunization in the laboratory, purified by ammonium sulfate precipitation and ion-exchange chromatography), was covalently coupled to CNBr-activated sepharose 4B (0.5 ml) in carbonate buffer (0.5 M NaCl, 0.1 M NaHCO3, pH 8.3), and packed into a 2.0 ml syringe. A 200 μl aliquot of S. serrata sera was loaded onto the affinity column and the column was washed with phosphate buffered saline (PBS, 0.01 M, pH 7.4) until absorbance at 280 nm reached baseline. Bound proteins were eluted with 3 ml of glycine–HCl buffer (0.1 M, pH 2.4) and the eluates were neutralized with 300 μl of Tris–HCl buffer (1 M, pH 8.0). Eluted proteins were concentrated by ultrafiltration–centrifugation.

Identification of the proteins eluted from the column was carried out using polyacrylamide gel electrophoresis (PAGE), SDS-PAGE, immunoblotting and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry analysis as described previously [23]. In brief, PAGE and SDS-PAGE were performed using a 3% stacking gel (pH 8.9) in Tris-Glycine buffer (pH 8.3). Following SDS-PAGE, separated proteins were transferred to a PVDF membrane for 2 h at 0.8 mA/cm² in transfer buffer (25 mM Tris, 0.1 M Glycine and 20% Methanol). The membrane was blocked for 60 min with 5% skim milk in TBS (20 mM Tris, 150 mM NaCl, pH 7.4) at 37 °C. After rinsing 3 times with TBS for 5 min each, the PVDF membrane was incubated with primary antibody (rabbit anti-hemocyanin IgG, 1:1500) and second antibody (goat anti-rabbit IgG-HRP antibody, 1:3000, SINO-AMERICAN, China) for 1 h and 45 min respectively, at 37 °C. The membrane was then washed and developed with DAB (3'-diminobenzidine).

For MALDI-TOF mass spectrometry analysis, the protein bands were excised from SDS-PAGE gel and digested with trypsin and 0.5 μl of the peptide mixture was mixed with the matrix a-cyano-4-hydroxy-cinnamic 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 submitted to Mascot (http://www.matrixscience.com/cgi/search_form.pl? FORMVER = 2&SEARCH = PMF) for NCBI nr databases search.

2.3. Agglutination assays

Seven bacterial species including V. parahaemolyticus, Vibrio alginolyticus, Vibrio harveyi, Vibrio fluvialis, Aeromonas hydrophila, Staphylococcus aureus and E. coli K12 were selected for this analysis. These bacteria were cultured separately in routine broth medium or LB medium for overnight at 28 °C or 37 °C. Cells were harvested, washed and diluted to 10⁶ CFU/ml in TBS–Ca²⁺ (0.05 M Tris, 0.75% NaCl, 0.05 M CaCl₂). The agglutination of the seven bacteria by S. serrata hemocyanin was performed at 37 °C for 30 min. Hemocyanin (0.6 mg/ml, initial concentration) was 2-fold diluted in TBS–Ca²⁺ and 20 μl of each bacterial suspension was added. Agglutination was determined by light microscopy and was scored as positive (+) or negative (−) compared to an untreated control suspension of bacteria in the TBS–Ca²⁺ buffer. Agglutinative titer was defined as the highest dilution of the test samples.

2.4. Agglutination inhibition assays by saccharides

The agglutination of bacteria by S. serrata hemocyanin was further confirmed by inhibition tests using nine reagents: α-D-glucose, α-D-galactose, α-D-xylose, α-D-sorbitol, sucrose, maltose, α-D-lactose, mannitol and N-acetylenuraminic acid. For this purpose, hemocyanin was diluted to 20-fold (30 μg/ml) with TBS–Ca²⁺ and mixed separately with an equal volume of each of the nine test compounds (200 mM) that were serially diluted with TBS–Ca²⁺ on a clean glass slide for 10 min at room temperature. Suspensions of the seven bacteria (10⁶ CFU/ml) were added to each sample and incubated for 30 min at 37 °C. The minimal concentration of each compound that completely inhibited the agglutination activity was recorded.

2.5. Analysis of the agglutinating subunit of hemocyanin

To investigate the active subunit of hemocyanin in agglutination, SDS-PAGE and immunoblotting were performed. A suspension of E. coli K12 was incubated with 0.015 mg/ml hemocyanin (400 μl)
at 37 °C for 2 h, the pellets were collected by centrifugation at 12 000 rpm for 1 min and washed twice with 0.01 M PBS buffer. The resulting pellets were solubilized in 2× protein loading buffer and heated for 3 min before being applied to a 10% (w/v) polyacrylamide separating gel with a 3% (w/v) polyacrylamide stacking gel. A suspension of _E. coli_ K12 incubated with PBS was used as a control. Immunoblotting was carried as described above.

### 2.6. Identification of ligands for receptor hemocyanin in the agglutinative reactivity

Agglutination inhibition assays by lipopolysaccharides (LPS) and outer membrane proteins (Omps) of _E. coli_ K12 were used to investigate the agglutinative target recognized by _S. serrata_ hemocyanin. LPS (2.4 mg/ml) and Omps (1.2 mg/ml) serially diluted with TBS—Ca²⁺ were used in agglutination assays as described above. Agglutination assays using _E. coli_ K12 Omp-deletion mutants were also conducted. Six mutants (ΔOmpA, ΔOmpX, ΔOmpW, ΔOmpT, ΔOmpC and ΔOmpFdL) were selected for this analysis (and were provided by State Key Laboratory of Biocontrol, College of Life Sciences, Zhongshan University, Guangzhou 510275, People’s Republic of China). They were separately cultured, harvested, washed and diluted to 10⁸ CFU/ml in TBS—Ca²⁺. The agglutinative activity assay was conducted as described above.

### 3. Results

#### 3.1. Mud crab _S. serrata_ hemocyanin consisted of five subunits

Mud crab _S. serrata_ hemocyanin from hemolymph was isolated with the use of affinity chromatography. Following separation by PAGE, only one band was obtained (Fig. 1A). Following an SDS-PAGE separation and immunoblotting analysis with rabbit anti-hemocyanin IgG (Fig. 1B), five subunits with approximate masses of 70, 72, 75, 76 and 80 kDa were strongly stained by Coomassie Brilliant Blue R-250 and three of the bands (the 75, 76 and 80 kDa proteins)

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**Table 1**

PMF search results of the five subunits of mud crab _S. serrata_ hemocyanin using Mascot search with NCBI nr databases.

<table>
<thead>
<tr>
<th>Band</th>
<th>Protein name</th>
<th>Accession No.</th>
<th>Protein MW</th>
<th>Protein PI</th>
<th>Pep. count</th>
<th>Protein score</th>
<th>Protein score C.I.%</th>
<th>Best ion Score</th>
<th>Best ion C.I.%</th>
</tr>
</thead>
<tbody>
<tr>
<td>P70</td>
<td>hemocyanin subunit 2 (CaeSS2)</td>
<td>gi:56748935</td>
<td>74 987.6</td>
<td>5.4</td>
<td>6</td>
<td>137</td>
<td>100</td>
<td>98</td>
<td>100</td>
</tr>
<tr>
<td>P72</td>
<td>hemocyanin subunit 2 (CaeSS2)</td>
<td>gi:56748935</td>
<td>74 987.6</td>
<td>5.4</td>
<td>9</td>
<td>164</td>
<td>100</td>
<td>67</td>
<td>99.994</td>
</tr>
<tr>
<td>P72</td>
<td>hemocyanin subunit [Callinectes sapidus]</td>
<td>gi:7582388</td>
<td>77 054.6</td>
<td>5.26</td>
<td>12</td>
<td>115</td>
<td>99.998</td>
<td>46</td>
<td>99.175</td>
</tr>
<tr>
<td>P75</td>
<td>hemocyanin subunit [Callinectes sapidus]</td>
<td>gi:7582388</td>
<td>77 054.6</td>
<td>5.26</td>
<td>19</td>
<td>334</td>
<td>100</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>P75</td>
<td>hemocyanin subunit 2 (CaeSS2)</td>
<td>gi:56748935</td>
<td>74 987.6</td>
<td>5.4</td>
<td>8</td>
<td>111</td>
<td>99.995</td>
<td>64</td>
<td>99.988</td>
</tr>
<tr>
<td>P76</td>
<td>hemocyanin subunit [Callinectes sapidus]</td>
<td>gi:7582388</td>
<td>77 054.6</td>
<td>5.26</td>
<td>14</td>
<td>274</td>
<td>100</td>
<td>88</td>
<td>100</td>
</tr>
<tr>
<td>P76</td>
<td>hemocyanin subunit 2 (CaeSS2)</td>
<td>gi:56748935</td>
<td>74 987.6</td>
<td>5.4</td>
<td>9</td>
<td>88</td>
<td>99.055</td>
<td>35</td>
<td>89.678</td>
</tr>
<tr>
<td>P80</td>
<td>hemocyanin subunit 3 [Cancer magister]</td>
<td>gi:57901143</td>
<td>77 370.6</td>
<td>5.2</td>
<td>7</td>
<td>175</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>P80</td>
<td>hemocyanin subunit 2 (CaeSS2)</td>
<td>gi:56748935</td>
<td>74 987.6</td>
<td>5.4</td>
<td>7</td>
<td>175</td>
<td>100</td>
<td>98</td>
<td>100</td>
</tr>
</tbody>
</table>

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**Fig. 2.** MALDI-TOF MS peptide mass fingerprint (PMF) spectra of 75 kDa protein.
reacted with the IgG. The five bands were subjected to MALDI-TOF mass spectrometry and subsequent database (NCBI nr) search. The peptide mass fingerprint (PMF) spectra of 75 kDa protein is shown in Fig. 2. All five proteins showed high homology with hemocyanin subunits from other crab species including Carcinus aestuarii, Callinectes sapidus and Cancer magister (Table 1). Together, these results suggested that S. serrata hemocyanin was purified to homogeneity and was composed of five subunits.

3.2. Hemocyanin showed agglutinative activities

To characterize agglutination of bacterial species by S. serrata hemocyanin, V. parahaemolyticus, V. alginolyticus, V. harveyi, V. fluvialis, A. hydrophila, S. aureus and E. coli K12 were selected. We found that hemocyanin could agglutinate all of these bacteria, although their agglutination titres varied with the bacterial species and ranged from 7.5 to 30 μg/ml (Fig. 3 and Table 2). Inhibition tests were also performed using α-D-glucose, D-galactose, D-xylene, D-sorbitol, sucrose, maltose, α-D-lactose, mannitol and N-acetylneuraminic acid. Of the nine compounds, α-D-glucose (200 mM), D-galactose (200 mM) and D-xylose (200 mM) inhibited the agglutination activities of hemocyanin with V. alginolyticus and S. aureus, A. hydrophila, E. coli K12 respectively, whereas N-acetylneuraminic acid (50–100 mM) inhibited agglutination of all seven bacterial species (Table 3). These results suggested that S. serrata hemocyanin possessed agglutination properties.

3.3. The 76 kDa subunit of hemocyanin was responsible for its agglutinative activities

To further investigate hemocyanin subunits contributing to agglutination, an E. coli K12 suspension was treated with the purified hemocyanin and proteins bound to the bacterial cells were examined by SDS-PAGE and immunoblotting analysis. As shown in Fig. 5, an obvious band (with a mass of about 76 kDa) was found in the protein samples treated with hemocyanin but not in those treated with 0.01 M PBS. This result suggests that mud crab S. serrata hemocyanin mediates agglutination via its 76 kDa subunit.

3.4. Outer membrane proteins were ligands for receptor hemocyanin

To characterize the bacterial pathogen-associated molecular pattern (PAMP) recognized by hemocyanin, an agglutination inhibition strategy was applied to detect hemocyanin ligands by using LPS and Omps of E. coli K12. The results indicated that E. coli K12 Omps could completely inhibit agglutination activities against E. coli K12, V. parahaemolyticus, V. alginolyticus, V. fluvialis, V. harveyi, A. hydrophila and S. aureus. However, LPS had no effect on any of the agglutination properties (Table 2 and Fig. 3). To corroborate this initial determination, the agglutination activities of hemocyanin against six E. coli Omp-deletion mutants was assessed. Our results showed that deletion of OmpX and OmpA resulted in a 75% decrease in agglutination activity whereas the absence of OmpFdL

Table 2

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Agglutinative titer</th>
<th>Agglutinative activity [μg/ml]</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. parahaemolyticus</td>
<td>80</td>
<td>7.5</td>
<td>0.133</td>
</tr>
<tr>
<td>V. alginolyticus</td>
<td>80</td>
<td>7.5</td>
<td>0.133</td>
</tr>
<tr>
<td>V. harveyi</td>
<td>20</td>
<td>30</td>
<td>0.033</td>
</tr>
<tr>
<td>V. fluvialis</td>
<td>40</td>
<td>15</td>
<td>0.067</td>
</tr>
<tr>
<td>A. hydrophila</td>
<td>40</td>
<td>15</td>
<td>0.067</td>
</tr>
<tr>
<td>S. aureus</td>
<td>40</td>
<td>15</td>
<td>0.067</td>
</tr>
<tr>
<td>E. coli K12</td>
<td>80</td>
<td>7.5</td>
<td>0.133</td>
</tr>
</tbody>
</table>

a The highest dilution of the testing samples in the presence of different bacteria.

b Protein concentration/Agglutinative titer.

c Agglutinative titer/Protein concentration.
contributed to an increase in hemocyanin-specific agglutination activity (Figs. 4 and 6). These results suggest that some outer membrane proteins (at least OmpX and OmpA), are ligands for hemocyanin.

4. Discussion

The multi-functional hemocyanin superfamily consists of a large group of proteins which display huge molecular variances [14,26]. So far, about 50 different hemocyanins from crustaceans, insects, chelicerates, myriapods and onychophorans have been characterized [27]. However, hemocyanin from the mud crab (S. serrata) has not previously been investigated. In this study, the S. serrata hemocyanin was isolated by affinity chromatography and found to consist of five subunits. This is in agreement with the structure of hemocyanin from the mediterranean crab Carcinus aestuarii and the semi-terrestrial crab Ocypode quadrata [28,29].

Previously it was reported that the 80 and 52 kDa subunits from Litopenaeus setiferus serum lectin showed a 23% and 22% homology to hemocyanin subunits from L. vannamei [30]. We subsequently found that hemocyanin from shrimp hemocyanin could bind to eight bacterial pathogens and four animal erythrocytes [23,24]. This suggested that S. serrata hemocyanin may have agglutinative properties. In the present study, we report for the first time that S. serrata hemocyanin can recognize and agglutinate seven bacterial species. Furthermore, the agglutination could be stopped by 50–100 mM of N-acetylneuraminic acid, which was in agreement with the report that shrimp hemocyanin, similar to the sugar-binding lectins, contained a calcium-dependent protein domain specific for binding to N-acetylated sugar moieties [23,30–33]. Nonetheless, the agglutination of hemocyanin was 1–2 orders of magnitude lower than that of serum lectin from L. setiferus [30]. It has been suggested that hemocyanin originated more than 550 million year ago from an oxygen-consuming phenoloxidase [34,35] and that the coagulation cascade in crustaceans is linked to prophenoloxidase activation, with the oxygen carrier hemocyanin functioning as a substitute for prophenoloxidase [36]. As this present study showed that hemocyanin had agglutinative activities, it raises the possibility that hemocyanins, phenoloxidases and blood clotting proteins may have evolved from a common ancestral protease cascade. The multi-functional hemocyanin also retains immunological activity.

It has been documented that immune function of hemocyanins is conformation- or oligomer-dependent [16,25,37]. Other evidence indicates that the phenoloxidase activity of hemocyanin from the tarantula Eurypelma californicum is limited to just two of its seven subunits [16]. Two subunits of hemocyanin from shrimp Penaeus japonicus existed different activities in antiviral defense [37].

<table>
<thead>
<tr>
<th>Saccharides or Omps</th>
<th>Minimum inhibitory concentration by saccharides (mM) or Omps (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V. parahaemolyticus</td>
</tr>
<tr>
<td>α-D-glucose</td>
<td>N</td>
</tr>
<tr>
<td>α-D-galactose</td>
<td>N</td>
</tr>
<tr>
<td>α-D-xylose</td>
<td>N</td>
</tr>
<tr>
<td>α-D-sorbitol</td>
<td>N</td>
</tr>
<tr>
<td>Sucrose</td>
<td>N</td>
</tr>
<tr>
<td>Maltoolose</td>
<td>N</td>
</tr>
<tr>
<td>α-D-lactose</td>
<td>N</td>
</tr>
<tr>
<td>Mannitol</td>
<td>N</td>
</tr>
<tr>
<td>N-acetylneuraminic acid</td>
<td>100</td>
</tr>
<tr>
<td>Omps of E. coli K12</td>
<td>1.2</td>
</tr>
</tbody>
</table>

* Not inhibited.

Fig. 4. Inhibition of agglutinative activities of mud crab S. serrata hemocyanin (30 μg/ml). A, Inhibited by saccharides (4400×): 1, negative control using V. alginolyticus; 2, with V. alginolyticus by 200 mM α-D-glucose; 3, negative control using V. harveyi; 4, with V. harveyi by 200 mM N-acetylneuraminic acid. B, Inhibited by E. coli K12 Omps (2120×): 1, negative control using E. coli K12; 2, with E. coli K12 by 1.2 mg/ml E. coli K12 Omps; 3, negative control using V. parahaemolyticus; 4, with V. parahaemolyticus by 1.2 mg/ml E. coli K12 Omps.
were investigated. Our results showed that the 76 kDa subunit of S. serrata hemocyanin tightly bound the bacterial cell, suggesting that the agglutinative activities of S. serrata hemocyanin may be attributable to this subunit.

It is thought that the innate immune system relies on its ability to rapidly detect pathogen-associated molecular patterns (PAMPs) displayed by invading pathogenic microbes. With respect to S. serrata hemocyanin, our results show that the E. coli outer membrane proteins OmpA and OmpX are ligands. Omps represent important virulence factors and play essential roles in bacterial pathogenesis by enhancing the adaptability of bacterial pathogens to various environments [38]. OmpA is involved in bacterial virulence and growth and is acted as a PAMP-associated molecule to elicit cytotoxic T lymphocytes [39]. Neutrophil elastase kills E. coli by degrading OmpA [40] and recognition of OmpA by macrophages may be an initiating event in the antibacterial host response [41]. OmpX from E. coli is responsible for promoting bacterial adhesion to and entry into host cells, and these proteins have a role in the resistance against attack by the host complement system [42]. The N-proximal loop of OmpX forms an IgG-accessible epitope at the cell surface [43]. Previous findings in our laboratory indicated that shrimp hemocyanin have a conserved Ig-like domain binding site [25] and that OmpX is the pathogen agglutination target recognized by this hemocyanin [24]. These details and our experimental results support the contention that OmpA and OmpX are ligands for S. serrata hemocyanin. Notably, hemocyanin was still able to agglutinate E. coli cells carrying deletions in the ompA and ompX genes, suggesting that hemocyanin-dependent agglutination may depend upon multiple targets. Further investigations are required to determine other targets for hemocyanin binding.

In conclusion, the present study shows that mud crab S. serrata hemocyanin is composed of five subunits of ranging in size from 70 to 80 kDa. This hemocyanin can bind outer membrane proteins including E. coli OmpA and OmpX and agglutinate several species of pathogenic bacteria. Agglutination seems particularly dependent upon the 76 kDa subunit.

Acknowledgments

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