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Hemocyanin from shrimp *Litopenaeus vannamei* shows hemolytic activity

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ABSTRACT

Hemocyanin is an extracellular copper-containing protein present in the hemolymph of both mollusks and arthropods. The traditionally recognized function of hemocyanin is for oxygen transport. Lately, it was demonstrated that hemocyanin is a multifunctional protein, especially participating in multiple roles of immune defense. For better understanding its actions in immune defense, the hemolytic activity of hemocyanin from shrimp *Litopenaeus vannamei* and the mechanism were investigated in this study. The results showed that shrimp hemocyanin exhibited hemolytic activity against vertebrate erythrocytes. The hemolysis displayed dependencies on hemocyanin concentration, pH, temperature and divalent cations. The highest activity occurred at a concentration of 0.125 mg ml^{-1}, and pH 6.0, 40 °C in the presence of calcium. Moreover, from the incubation products of erythrocytes with hemocyanin, besides two subunits of hemocyanin, two molecules around 150 and 230 kD were isolated and speculated as oligomers of hemocyanin. Further evidence revealed that the hemolysis could be inhibited to different degrees by osmoprotectants with high molecular masses, suggesting that it follows a colloid-osmotic mechanism. These results indicate that *L. vannamei* hemocyanin has a novel function with hemolytic activity, partly related to a colloid-osmotic mechanism mediated by its oligomers.

1. Introduction

Hemocyanin is a large, copper-containing protein present in the hemolymph of both mollusks and arthropods. It is colorless in the deoxy form and blue in the oxygenated form. The traditional role of hemocyanin is the transport and storage of molecular oxygen. Subsequently, it was demonstrated that hemocyanin is a multifunctional protein involved in several physiological processes such as protein storage, osmoregulation, molt cycle and exoskeleton formation [1–3]. More recent reports reveal that hemocyanin may be a novel important type of non-specific innate immune defense molecule [4–14]. One of its main immunological functions is the phenoloxidase activity, which could be activated by sodium dodecyl sulfate (SDS) [5], trypsin [6], clotting enzyme [7] and antimicrobial peptides [8], etc. Besides, hemocyanin and its degenerated fragments possess antimicrobial properties. Hemocyanin from horseshoe crab *Carcinoscorpius rotundicauda* could be activated by microbial proteases to produce reactive oxygen species (ROS), resulting in formation of a strong antimicrobial defense [9]. C-terminal fragments with broad antifungal activities were produced from hemocyanin within the first hours of an infection in penaeid shrimps, *Penaeus vannamei* and *Penaeus stylirostris* [10]. Crustacean hemocyanin could be processed by a cysteine-like proteinase to generate an antimicrobial peptide under acidic conditions, and this production could be further enhanced by injection of lipopolysaccharide (LPS) and glucan into the animal [11]. Moreover, hemocyanin also displays antiviral activity. Hemocyanin isolated from shrimp *Penaeus monodon* acted as an antiviral agent against a variety of viruses including DNA and RNA viruses [12]. Two subunits of hemocyanin from shrimp *Penaeus japonicus* showed differences in antiviral defense [13]. Further, our previous evidence indicated that *Litopenaeus vannamei* hemocyanin could aggregate with eight species of bacteria pathogenic for shrimp and four types of animal erythrocytes directly [14]. These results detailed hemocyanin's roles in many immune activities.

It has been demonstrated that hemolysin is a major part of the immediately effective, non-specific and natural defenses of most living invertebrates against invading pathogens, and it plays an important role as a humoral factor in the invertebrate innate immune system [15,16]. However, so far little is known about the contact–hemolytic activity of hemocyanin.

In the present study, the hemolytic activity of hemocyanin from shrimp *L. vannamei* was investigated via affinity chromatography

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and hemolytic activity assay. Furthermore, the dependencies on pH, temperature, divalent cation and hemocyanin concentration were characterized. More importantly, the possible structure of hemocyanin molecules in hemolysis and its action mechanism were deduced. The present results reveal a novel immune function of hemocyanin and its mechanism, which will assist in the investigation of multifunctional hemocyanin.

2. Materials and methods

2.1. Animal and preparation of shrimp sera

Penaeid shrimps (*L. vannamei*), weighing 15–20 g, irrespective of sex, were purchased from a local supplier and kept in aerated seawater. Haemolymph was drawn directly from the pericardial sinus using a sterile tube, and then allowed to clot overnight at 4 °C. The sera were separated after centrifuging at 3000 rpm for 20 min and stored at −20 °C until analysis [14].

2.2. Purification and identification of hemocyanin

Hemocyanin purification was performed by affinity chromatography as we described previously [14] with modification. Briefly, an affinity chromatography column with a ligand of rabbit anti-shrimp hemocyanin subunit around 75 kDa antibody was installed according to the conventional method. *L. vannamei* sera (200 μl) were loaded onto the affinity column, and then the column was washed with PBS (0.01 M, pH 7.4) until absorbance at 280 nm reached baseline. Bound protein (potential hemocyanin) was eluted with glycine–HCl buffer (0.1 M, pH 2.4), and neutralized immediately with Tris–HCl buffer (1 M, pH 8.0). After determining by the method of Bradford [17], about 0.3 mg protein was acquired. Then the purified potential hemocyanin was identified by gel electrophoresis and immunoblotting assays. Native-PAGE was performed in an 8% polyacrylamide separating gel with a 3% polyacrylamide stacking gel, with the absence of SDS in gels and the absence of both SDS and β-mercaptoethanol in sample loading buffer. SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out under reducing conditions on a 10% polyacrylamide separating gel with a 5% polyacrylamide stacking gel. SDS and native gels were stained with Coomassie Brilliant Blue R-250. Following native-PAGE and SDS-PAGE, immunoblotting assays were further performed, respectively. The proteins were transferred to a PVDF membrane with a semi-dry transfer apparatus according to the manufacturer's instructions, respectively. The membrane was blocked for 1 h with 5% skim milk in TBS (20 mM Tris, 0.15 M NaCl, pH 7.4) at room temperature, then incubated with rabbit anti-shrimp hemocyanin antisera (1:1500 dilution) and goat anti-rabbit IgG-HRP (1:8000 dilution) antibodies at room temperature for 1 h, respectively. Finally, the membrane was washed and developed with substrate (3′3-diminobenzidine, DAB) until optimum color developed.

2.3. Preparation of erythrocyte suspension

Blood from healthy sheep, mouse, herring and chicken were centrifuged at 2000 rpm for 10 min and erythrocytes were obtained. The cells were washed three times with 0.01 M PBS (pH 7.4) and centrifuged at 500 rpm for 5 min each time, then the erythrocytes were diluted to 0.5% suspension in 0.01 M PBS (pH 6.0) containing 0.15 M NaCl and 10 mM $CaCl_2$.

2.4. Determination of hemolytic activity

Hemolytic activity of hemocyanin was determined as previously described [15]. In brief, hemocyanin (0.9 ml) was mixed with 0.5% (v/v) erythrocyte suspension (0.3 ml). After 1 h incubation at 37 °C, unbroken cells and cell debris were removed by centrifugation at 3500 rpm for 10 min, and hemolysis was determined by the absorbance at 540 nm in supernatants. The 0.5% (v/v) erythrocyte suspension treated with double distilled water and PBS-Ca^{2+} (0.01 M, pH 6.0) was used as a 100% and 0% hemolysis control, respectively. All samples were prepared in triplicate.

To further investigate the process of hemocyanin-dependent hemolysis, dynamic analysis of hemolysis of chicken erythrocytes was performed. 0.5% (v/v) chicken erythrocyte suspension (10 μl) was mixed with 0.125 mg ml^{-1} hemocyanin (10 μl) on a slide. After incubation at 37 °C for 15, 30, 45 and 60 min, respectively, digital photo-micrographs were taken with Olympus BH-2 microscope.

2.5. Determination of the optimum parameters of hemolytic activity

To obtain the optimum parameters of the hemolytic activity of hemocyanin, different conditions were performed. (i) *pH*. The following buffers containing 0.15 M NaCl and 10 mM $CaCl_2$ were used for preparation of chicken erythrocyte suspension: pH 2.0, 0.01 M HCl; pH 4.0, 10 mM sodium acetate buffer; pH 6.0 and 7.4, 10 mM sodium phosphate buffer; pH 8.0, 10 mM Tris–HCl buffer; pH 10.0, sodium borate buffer. (ii) *Temperature.* 0.2 mg ml^{-1} hemocyanin was mixed with 0.3 ml of 0.5% (v/v) chicken erythrocyte suspension after incubation at 4, 20, 37, 40 or 60 °C for 1 h (iii) *Divalent cation*. Hemocyanin (0.125 mg ml^{-1}) was added to chicken erythrocyte suspension supplemented with 10 mM $CaCl_2$, $MgCl_2$, $BaCl_2$ or $ZnCl_2$. (iv) *Hemocyanin concentration*. 0.025, 0.05, 0.075, 0.1, 0.125, 0.15, 0.2, 0.25, 0.3 or 0.4 mg ml^{-1} of hemocyanin was incubated with chicken erythrocyte suspension. All of the other procedures were carried out in the same conditions as described above for determination of hemolytic activity of hemocyanin.

2.6. Analysis of the structure of hemocyanin molecules in hemolysis

The structure of hemocyanin molecules in hemolysis was investigated by SDS-PAGE and immunoblotting as previously described [18]. Briefly, 0.5% (v/v) erythrocyte suspension (0.3 ml) was incubated with 0.2 mg ml^{-1} hemocyanin (0.9 ml) at 37 °C for 1 h, the erythrocyte membranes were collected by centrifugation at 3500 rpm for 10 min and washed twice with 0.01 M PBS buffer. The erythrocyte membrane pellets were solubilized in 2×protein loading buffer at room temperature rather than boiling for 5 min as the procedure of the conventional SDS-PAGE in order to preserve any potential oligomeric forms of the membrane-bound hemocyanin molecules before being applied to an 8% (w/v) polyacrylamide separating gel with a 3% (w/v) polyacrylamide stacking gel. Protein samples separated by the first SDS-PAGE were blotted onto PVDF membranes, and immunoblotting was carried out under the same conditions as described above. Following the first SDS-PAGE, the bands of potential oligomers of hemocyanin around 150 and 230 kDa were further purified as Kang described [19]. In brief, the first SDS-PAGE gel was stained by Coomassie Brilliant Blue fast staining reagent (45% ethanol, 10% acetic acid, 1 mg ml^{-1} Coomassie Brilliant Blue R-250) for 20 min and then destained by 250 mM KCl. The two bands were excised and transferred into a dialysis bag, respectively. After electrophoresis in the SDS-PAGE electrode buffer overnight at 4 °C, the eluate in the bag was concentrated by pre-cooling acetone at −20 °C for 30 min, then solubilized in protein loading buffer and heated for 5 min. The following procedures including the second SDS-PAGE and immunoblotting were the same as previously described.