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HEMOCYANIN-DERIVED PHENOLOXIDASE ACTIVITY IS DEPENDENT ON DODECAMERIC STRUCTURE IN SHRIMP *LITOPENAEUS VANNAMEI*

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Abstract – Hemocyanin (Hc) is a multifunctional protein in both mollusks and arthropods. Phenoloxidase (PO) activities are the most important physiological functions for Hcs after conversion. In shrimp, Hc occurs as two oligomer forms, dodecamers and hexamers. Differences in the transport oxygen capacity and agglutination activity between the two oligomers of shrimp Hc have been found. In the present study, we investigated the differences in the Hc-derived PO activity between the dodecameric and hexameric Hc forms of the shrimp *Litopenaeus vannamei*. The two oligomers were separated by non-denaturing polyacrylamide gel electrophoresis, converted by trypsin cleavage and their PO activities were determined by oxidation of L-DOPA. The dodecamers exhibited PO activity after enzymatic conversion while the hexamers did not exhibit PO activity. This result provides new insight into the structural/functional relationships of Hcs.

*Key words***:** hemocyanin; dodecamer; phenoloxidase; *Litopenaeus vannamei*

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INTRODUCTION

Hemocyanins (Hcs) are respiratory proteins in mollusks and arthropods that have a variety of physiological functions, such as maintenance of osmotic pressure (Paul et al., 1998), regulation of molting and circadian rhythms (Jaenicke at al., 1999); cuticle composition (Paul et al., 1994), and regulation of the agglutination of red blood cells and bacteria (Pan et al., 2008). Weak phenoloxidase (PO) activity has also been observed in Hcs from both arthropods and mollusks after proteolytic cleavage of the N-terminal portion of HCs (Decker et al., 2004; Jiang et al., 2007; Siddiqui et al., 2006) or after incubation with sodium dodecyl sulfate (SDS) (Decker et al., 2004; Nagai et al., 2000; Pless et al., 2003). Phenoloxidase, which is ubiquitously present in animals, oxidizes phenols to quinones to form melanin (Jiang et al., 2007). The melanization pathway through the action of PO is a major component of the innate immune response of arthropods. Therefore, the Hcs are currently accepted as important immune molecules.

Hcs are heteromeric proteins containing 70-80 kDa subunits. In arthropods, Hcs occur as integral numbers of hexamers (1×6, 2×6, 4×6, 6×6 and 8×6), depending on the species (Cong et al., 2009; Perbandt et al., 2003). In shrimp, Hcs commonly exist as hexamers (1×6) and dodecamers (2×6) (García-Carreño et al., 2008; Hagner-Holler et al., 2005). These two oligomers possess different oxygen carrying capacities and agglutination activities. Beltramini et al. (2005)

found that the oxygen affinity of Hc dodecamers of *Penaeus monodon* is stronger than that of hexamers. In our previous work, we observed that only the dodecameric form of *L. vannamei* exhibits the activity of agglutination of red blood cells and bacteria while the hexameric form does not exhibit this activity (Pan et al., 2008). Thus, clarification of the functional importance of the two oligomer types of Hc may be critical in understanding the role of this multifunctional protein. However, little is known about the difference in PO activity between the two oligomers.

In the present study, we tested if the two Hc oligomers of Pacific white shrimp (*L. vannamei*) had different PO activities. The two Hc oligomers from hemolymph were separated by non-denaturing gel electrophoresis (native-PAGE). Both oligomers were proteolyzed by trypsin and L-DOPA was used as a substrate to determine potential differences in Hc-derived PO activities.

MATERIALS AND METHODS

Collection of hemolymph

Shrimps (*L. vannamei*), weighing 10-15 g from a natural source were purchased from a local market (Baiyang) in Hangzhou. Hemolymph was extracted from the pericardial sinus of each individual shrimp using a 1 mL pyrogen-free disposable syringe and was then allowed to clot overnight at 4°C. The hemolymph was centrifuged at 3 000 xg for 10 min at 4°C and stored at -80°C until analysis.

Protein concentration determination and purity assessment

Protein concentrations were determined by the Bradford method. The purity of hemocyanin in the hemolymph was checked by SDS-PAGE and the ratio of optical density (OD) values at 340 and 280 nm (OD_{340}/OD_{280}) (Adachi et al., 2001).

Detection of agglutination activity

The Hc agglutination activity was detected using a direct agglutination assay and pooled human red blood cells (RBCs) as previously described (Pan et al., 2008). Blood was collected from several volunteers and diluted to a 0.5% suspension in TBS-Ca^{2+} (50 mM Tris-HCl, 150 mM NaCl and 10 mM $CaCl_2$, pH 7.4). Twenty μL serial two-fold dilutions of shrimp hemolymph in TBS-Ca^{2+} were added to glass slides, followed by the addition of an equal volume of 0.5% RBCs. After incubation for 10 min at 37°C, agglutination was observed under a light microscope (Nikon TE2000-U).

Polyacrylamide gel electrophoresis (PAGE)

Sodium dodecyl sulfate (SDS)-PAGE was performed under reducing conditions according to the standard procedure on 7.5% slab polyacrylamide gels. Native-PAGE was carried out using 5% slab polyacrylamide gels at 4°C without SDS in the gels and electrophoretic buffer, and without SDS and β-mercaptoethanol in the sample loading buffer. SDS and native gels were stained with Coomassie Brilliant Blue R-250.

In-gel protein digestion and nano-LC-MS analysis

The protein bands corresponding to hexameric and dodecameric hemocyanin in native-PAGE gels were excised and the gel strips cut into slices of equal size (about 1 mm). The gel slices were destained and dried. In-gel trypsin digestion was performed overnight at 37°C. The resulting peptides from the digestion were extracted and dried. The peptide samples were then analyzed by nano-LC (Dionex Ultimate 3000) using a C_{18} reverse phase column coupled to an ESI mass spectrometer (Bruker Daltonics maXis). Mass spectra were compared to the NCBInr database with the taxonomy of *L. vannamei* using the Mascot search engine 2.0.

Phenoloxidase activity assay

Freshly extracted Hcs with aggregating activity were separated by native-PAGE. Bands corresponding to dodecamers and hexamers (containing about 30 μg of protein) were excised, cut into pieces and placed

into tubes. A blank gel strip without any protein was also excised and used as a negative control. To each tube, 1 mL of bovine trypsin (1 mg/mL in 100 mM potassium phosphate buffer, pH 6.8) was added, and the tubes were incubated for 1 h at room temperature (25°C). Phenoloxidase activity was determined as described by Ashida et al., (1971). Briefly, 1 mL of 10 mM L-DOPA (100 mM potassium phosphate buffer, pH 6.8) was added to the tubes and incubated for 30 min. The OD values of the solutions were determined by spectrophotometry at 490 nm. The assays were carried out twice in triplicate.

RESULTS AND DISCUSSION

The hemolymph was diluted and submitted to SDS-PAGE (Fig. 1A). Only two protein bands were observed and identified as two Hc subunits of *L. vannamei* with molecular masses of 73.6 and 75.2 kDa by mass spectrometry. The purity of the Hc in the hemolymph was monitored by the OD_{340}/OD_{280} ratio, which was 0.198. This was in agreement with the value reported for Hc of *Penaeus vannamei* (García-Carreño et al., 2008). The present study suggested that Hc was the main protein component of hemolymph, representing up to 95% of the total amount of proteins, which was in agreement with Hagner-Holler et al. (2005).

We have previously demonstrated that only the dodecameric form and not the hexameric form possesses agglutination activity and that dodecamers spontaneously disaggregate into hexamers *in vitro* (Pan et al., 2008). To determine if the dodecamers were still intact, we assayed the agglutination activity of the hemolymph before examination of its PO activity. Fig. 1B shows representative agglutination images of human RBCs, with agglutinative titers up to 32. This result indicated that Hc existed mainly in the dodecameric form in the hemolymph.

In our previous work, we found that dodecameric Hc is the predominant oligomer form in the hemolymph of *L. vannamei* and that Hc hexamers and dodecamers reciprocally interchange *in vitro* (Pan et al., 2008). Therefore, pure oligomers cannot be obtained using common protein purification methods, such as ion-exchange chromatography and gel-filtration chromatography. To separate the two oligomeric forms we used native-PAGE. Two protein bands were observed in native gels (Fig. 1C). The two bands were excised and identified by LC-MS analysis; both bands were determined to be mixtures of the two Hc subunits (Table 1), the upper band corresponding to the dodecameric and the lower band to the hexameric form. This is in agreement with previously results that dodecamer and hexamer are the main oligomeric forms in *Litopenaeus* Hc (García-Carreño et al., 2008; Pan et al., 2008).

Table 1. Two protein bands corresponding to hexameric and dodecameric Hc in native-PAGE gels were identified by nano-LC-MS analysis.

Gel bands	Access No.	Protein	Score	Matches
Top band	gi|7414468	Hc	460	18(15)
	gi|854403	Hc	441	15(11)
Bottom band	gi|854403	Hc	2054	60(57)
	gi|7414468	Hc	812	28(26)

The PO activity of the two oligomers was compared after conversion of the dodecamer to hexamer by trypsin. After addition of L-DOPA as substrate, the dodecamer solution color gradually changed to chrome yellow (Fig. 1D) and the mean OD value of the solution at 490 nm increased 3.5 fold after 30 min when compared with the control (Fig. 1E) that did not change color. No color change was observed in the hexamer tubes. This result suggests that dodecameric Hc possessed PO activity that the hexameric Hc did not.

Arthropod Hcs belong to a protein family that exists as different oligomeric forms *in vivo*. Most studies of multimeric proteins focus on the link between their structural and functional properties, such as variations in oxygen-binding properties (Beltramini et al., 2005) and agglutination activity (Pan et al., 2008). Shrimp Hcs depend on two different forms of Hcs for oligomeric organization. In the present study, we demonstrated that the proPO moiety is activated to the PO moiety after proteolytic

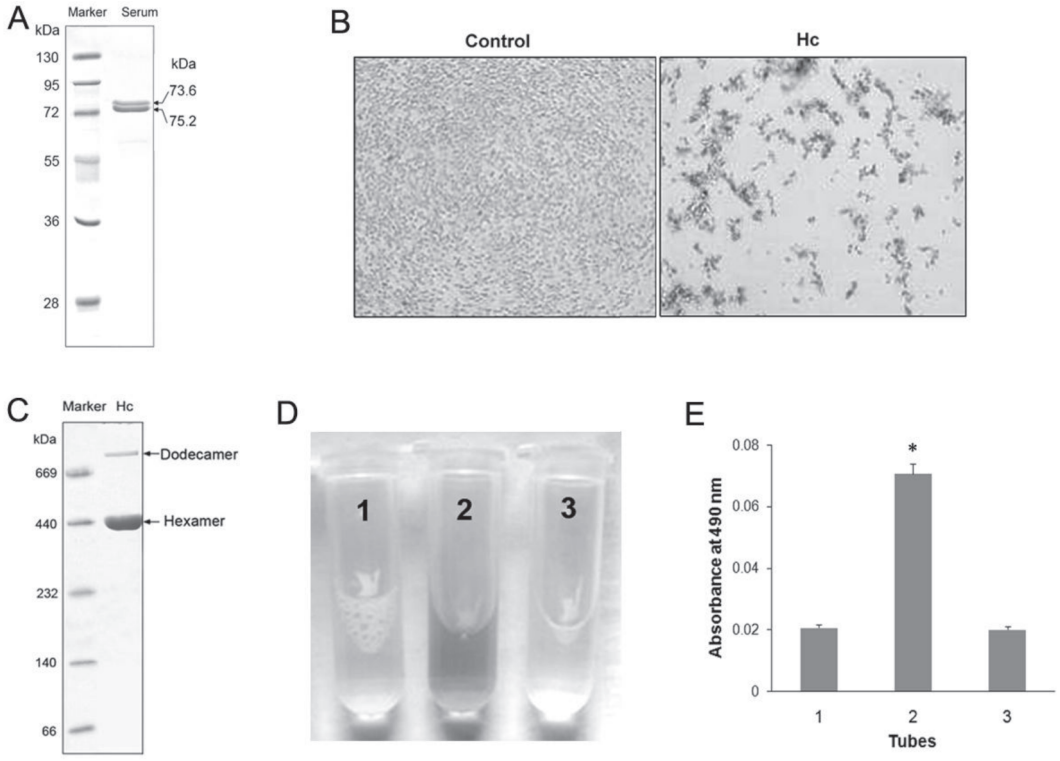

Fig. 1. Dodecameric HC exhibited PO activity after enzymatic conversion. A – SDS-PAGE analysis of *L. vannamei* hemolymph. B – *L. vannamei* Hc agglutination with human RBCs. Agglutination was examined by light microscopy. C– *L. vannamei* Hc separated by Native-PAGE. D and E – PO activity assay of two Hc oligomers; D – Two oligomers from native gel strips were incubated with 1 mg/mL trypsin at room temperature for 1 h; 10 mM L-DOPA was added to assay PO activity; tube 1 – blank gel strip without protein used as control; tube 2 – dodecamer-containing; tube 3 – hexamer-containing. D – Absorbance of the three tubes at 490 nm. Student's *t*-test was used for evaluation of results of OD_{490}; *P* values of <0.05 were considered statistically significant.

conversion of the two Hc. Our results show that only dodecameric Hcs exhibit PO activity, observed as the oxidization of L-DOPA after conversion by trypsin. No oxidation reaction occurred when hexameric Hcs were incubated with L-DOPA after proteolytic conversion, indicating that hexameric Hcs do not exhibit PO activity. The detailed mechanism of higher-order oligomer-dependent proPO function is still unclear and needs to be investigated. One explanation of the functional differences is based on the unique amino acid residues involved in the formation of Hc homodimers from *L. vannamei* that differ from other known crustacean Hcs (Beltramini et al., 2005). It is possible that the dodecamers adapt a unique structural pattern that provides the active sites for binding to substrates. In hexamers, the active sites are most likely restricted.

Elucidating oligomer-dependent immune function may be of importance in understanding Hcs. Pulmonary surfactant protein-D (SP-D) is a C-type lectin synthesized in respiratory epithelial cells in the lung. The assembly of SP-D trimers into dodecamers is required for proper regulation of surfactant phospholipid homeostasis (Zhang et al., 2001). Mutations of human mannose-binding lectin, which compromises an assembly of higher-order oligomers, results in reduced ligand-binding capacity, thereby reducing the capability to activate a complementary system (Larsen et al., 2004).

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Authors' contribution

The first two authors contributed equally to this work.

REFERENCES

Adachi, K., Hirata, T., Nagai, K. and *M. Sakaguchi* (2001). Hemocyanin a most likely inducer of black spots in kuruma prawn *Penaeus japonicus* during storage. *J. Food Sci.* **66**, 1130-1136.

Ashida, M. (1971). Purification and characterization of pre-phenoloxidase from hemolymph of the silkworm *Bombyx mori*. *Arch. Biochem. Biophys.* **144**, 749-762.

Beltramini M., Colangelo N., Giomi F., Bubacco L., Di Muro P., Hellmann N., Jaenicke E. and *H. Decker* (2005). Quaternary structure and functional properties of *Penaeus monodon* hemocyanin. *FEBS J.* **272**, 2060-2075.

Cong Y., Zhang Q., Woolford D., Schweikardt T., Khant H., Dougherty M., Ludtke S. J., Chiu W. and *H. Decker* (2009). Structural mechanism of SDS-induced enzyme activity of scorpion hemocyanin revealed by electron cryomicroscopy. *Structure* 17, 749-58.

Decker H., Ryan M., Jaenicke E. and *N. Terwilliger* (2001). SDS-induced phenoloxidase activity of hemocyanins from *Limulus polyphemus*, *Eurypelma californicum*, and *Cancer magister*. *J. Biol. Chem.* **276**, 17796-17799.

García-Carreño F.L., Cota K. and *M. A. Navarrete Del Toro* (2008). Phenoloxidase activity of hemocyanin in whiteleg shrimp *Penaeus vannamei*, conversion, characterization of catalytic properties, and role in postmortem melanosis. *J. Agric. Food Chem.* **56**, 6454-6549.

Hagner-Holler S., Kusche K., Hembach A. and *T. Burmester* (2005). Biochemical and molecular characterisation of hemocyanin from the amphipod *Gammarus roeseli*, complex pattern of hemocyanin subunit evolution in Crustacea. *J. Comp. Physiol. B* **175**, 445-452.

Jaenicke E., Foll R. and *H. Decker* (1999). Spider hemocyanin binds ecdysone and 20-OH-ecdysone. *J. Biol. Chem.* 274, 34267-34271.

Jiang N., Tan N.S., Ho B. and *J.L. Ding* (2007). Respiratory protein-generated reactive oxygen species as an antimicrobial strategy. *Nat. Immunol.* **8**, 1114-122.

Larsen F., Madsen H.O., Sim R.B., Koch C. and *P. Garred* (2004). Disease-associated mutations in human mannose-binding lectin compromise oligomerization and activity of the final protein. *J. Biol. Chem.* **279**, 21302-21311.

Nagai, T. and *S. Kawabata* (2000). A link between blood coagulation and prophenoloxidase activation in arthropod host defense. *J. Biol. Chem.* **275**, 29264-29267.

Pan J.Y., Zhang Y.L., Wang S.Y. and *X. X. Peng* (2008). Dodecamer is required for agglutination of Litopenaeus vannamei hemocyanin with bacterial cells and red blood cells. *Mar. Biotechnol.* **10**, 645-652.

Paul R., Bergner B., Pfeffer-Seidl A., Decker H., Efinger R. and *H. Storz* (1994). Gas transport in the hemolymph of arachnids I. Oxygen transport and the physiological role of hemocyanin. *J. Exp. Biol.* **188**, 25-46.

Paul, R. and *R. Pirow* (1998). The physiological significance of respiratory proteins in invertebrates. *Zoology* **100**, 319-327.

Perbandt, M., Guthoehrlein, E. W. and *W. Rypniewski* (2003). The Structure of a functional unit from the wall of a gastropod hemocyanin offers a possible mechanism for cooperativity. *Biochemistry* **42**, 6341-6346.

Pless D.D., Aguilar M.B., Falcon A., Lozano-Alvarez E. and *E.P. Heimer de la Cotera* (2003). Latent phenoloxidase activity and N-terminal amino acid sequence of hemocyanin from *Bathynomus giganteus*, a primitive crustacean. *Arch. Biochem. Biophys.* **409**, 402-410.

Siddiqui N.I., Akosung R.F. and *C. Gielens* (2006). Location of intrinsic and inducible phenoloxidase activity in molluscan hemocyanin. *Biochem. Biophys. Res. Commun.* **348**, 1138-1144.

Zhang L., Ikegami M., Crouch E.C., Korfhagen T.R. and *J. A. Whitsett* (2001). Activity of pulmonary surfactant protein-D (SP-D) *in vivo* is dependent on oligomeric structure. *J. Biol. Chem.* **276**, 19214-19219.