Development of ELISA for Quantifying Hemocyanin in Hemolymph of Banana Shrimp (Fenneropenaeus merguiensis)

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Abstract

Hemocyanin (HC) is a copper-binding protein that represents as a main protein in hemolymph of mollusks and arthropods. HC is found to be a precursor of antifungal peptide and converted to be a phenoloxidase-like enzyme by sodium dodecyl sulphate (SDS) treatment. Phenoloxidase (PO) plays important roles in immune response of crustaceans, suggesting that HC may also be involved in immune system. In this study, HC was purified from hemolymph of Fenneropenaeus merguiensis by ultracentrifugation and subsequently by preparative polyacrylamide gel electrophoresis (PAGE). Purified HC showed single protein band in non-denaturing PAGE and arranged in a doublet of 75 and 79.4 kDa in SDS-PAGE. Anti-HC antibody raised against purified HC was highly specific to HC in the hemolymph and was used to develop an enzyme linked immunosorbent assay (ELISA) for quantifying HC. By ELISA, HC content was 85.7% of total protein in the shrimp hemolymph. After bacterial injection, the expression of HC levels in the hemolymph was increased and reached the highest at 12 h post-injection. These results indicated that HC is inducible and may be involved in shrimp immune response to pathogenic bacteria.

Keywords: Hemocyanin Phenoloxidase Fenneropenaeus merguiensis Shrimp immunity

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Introduction

The aquaculture of penaeid shrimp is worldwide economically important for intertropical developing countries. Banana shrimp, Fenneropenaues merguiensis, is one of economically aquatic shrimp species in Thailand. The intensification of shrimp farming has been accompanied by the development of diseases from microbial infection (Destoumieux-Garzón et al., 2001). Vibriosis is one of the most dangerous pathogen in shrimp diseases caused by bacteria belonging to the genus Vibrio. Vibrio harvevi has been particularly devastated in the marine culture of F. merguiensis (Tansutapanit and Ruangpan, 1987). Thus, study towards a shrimp immune response should help in the design for diseases control. Invertebrates lack adaptive immunity and rely completely on their innate immune system (Hoffmann et al., 1999). Shrimps have developed effective systems for detecting and killing microbial invasions that are mainly based on the activity of blood cells, hemolymph coagulation and participation in the engulfment of invading microorganisms (Destoumieux et al., 1997).

In shrimp hemolymph, hemocyanin (HC) represents as a main protein up to 95% of the total protein (Van Holde and Miller, 1995). HC is a copper-containing protein that found in two phyla, mollusks and arthropods. It has multiple functions including oxygen carrier, storage protein (Paul and Pirow, 1998), and precursor of an antibacterial and antifungal peptide (Destoumieux-Garzón et al., 2001; Destoumieux et al., 1997). Hepatopancreas has been shown to be the site of HC synthesis (Spindler et al., 1992). Arthropod HCs are composed of hexamers or multiples of hexamer of subunits with molecular mass of around 75 kDa (Adachi et al., 2005). Each polypeptide subunit contains 600 - 660 amino acid residues depending on species (Markl, 1986) and consists of two copper-binding sites which are Cu (A) and Cu (B), each of them are coordinated by three histidine residues (Van Holde and Miller, 1995). In addition, HC was converted to be a phenoloxidase-like enzyme similar to phenoloxidase (PO) isolated from hemocytes (Adachi et al., 2001). A close relationship between PO and HC was deduced based on their similar sequences, physicochemical properties and functions (Lee et al., 2004; Pless et al., 2003). PO is also a copper-containing enzyme

that found in hemocytes of mollusks, arthropods and insects. It plays important roles in the cuticle sclerotization and immune response (Söderhäll and Cerenius, 1998). Therefore, HC may also be involved in shrimp immune response like PO. However, little is known about the structure and immune function of crustacean HC. Therefore, this study aimed to purify and characterize HC from hemolymph of F. merguiensis to produce anti-HC antibody and to develop a sensitive ELISA for quantifying HC content in the hemolymph of shrimps injected with pathogenic bacteria.

Materials and Methods

1. Purification of HC

F. merguiensis was collected from Nakhon Si Thammarat province and cultured temporarily in laboratory tanks. Hemolymph was withdrawn and mixed with anticoagulant solution. After removal of hemocytes, the plasma was subjected to ultracentrifugation (UC) (Adachi et al., 2001). Fractions containing high concentrations of HC were further purified by preparative PAGE. Its purity was analyzed by non-denaturing PAGE and SDS-PAGE.

2. Preparation and purification of anti-HC antibody

Purified HC was used to raise antibody in an albino rabbit. Anti-HC antibody was partially purified by ammonium sulfate precipitation and separated by DEAE-Sephacel column (Auttarat et al.,2006). The specificity of anti-HC antibody was analyzed by Western blotting.

3. Enzyme-linked immunosorbent assay (ELISA)

To develop a sensitive ELISA for determining HC levels in hemolymph, assay conditions were optimized including concentrations of anti-HC antibody (1^{o}Ab) or of anti-rabbit IgG horseradish peroxidase conjugated (2^{o}Ab), pH of coating buffer and hemolymph contents. A standard curve of HC was obtained with purified HC according to the method of Auttarat et al. (2006).

4. Expression of HC levels in the hemolymph of V. harveyi injected shrimps

In the immune challenge experiments, shrimps were injected with V. harveyi ($5x10^{7}$ cells/shrimp). At 0, 6, 12, 18 and 24 h post-injection, hemolymph was withdrawn

and left to clot at 4 °C overnight. The supernatant was cleared by centrifugation and immediately used or stored as aliquots at -20 °C before ELISA analysis. Shrimps injected with saline solution were maintained as a control group.

Results and Discussion

1. Purification of HC and anti-HC antibody production

HC was isolated from shrimp hemolymph by UC and subsequently by preparative PAGE. After purification, HC showed only a single protein band in non-denaturing PAGE (Fig. 1A, lane 4), indicating that this HC preparation was pure. In SDS-PAGE, purified HC revealed two protein bands with M_r of 75 and 79.4 kDa (Fig. 1B, lane 4), similar to that of Penaeus japonicus (67 and 77 kDa) (Adachi et al., 2001) and Bathynomus giganteus (70 and 72 kDa) (Pless et al., 2003). Anti-HC antibody was successfully raised against purified HC in an albino rabbit. Specificity of the antibody was demonstrated by Western blotting (Figure1C). It showed strong cross reactivity with HC in the hemolymph and pure form; none was found for other proteins.

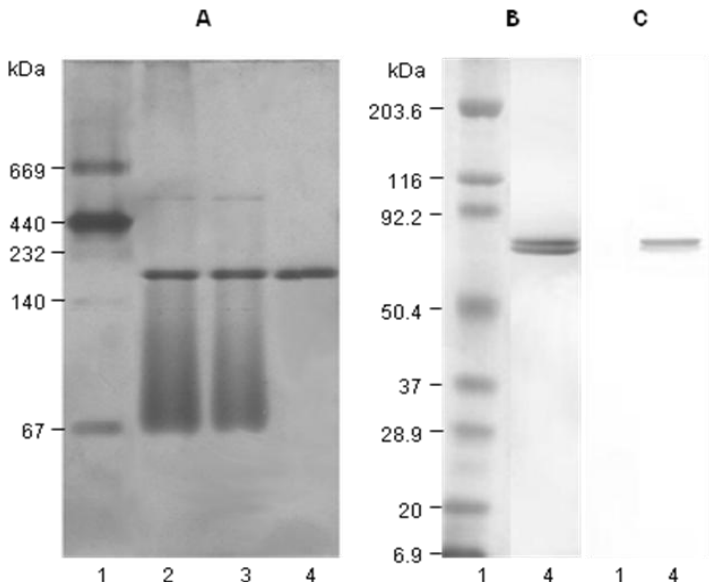

Figure1 HC protein patterns in non-denaturing PAGE (A), SDS-PAGE (B) and Western blot analysis (C). Lane 1, molecular weight markers; lane 2, plasma; lane 3, fraction 13 from UC; lane 4, purified HC from preparative PAGE.

2. Development of ELISA

In order to get a very sensitive assay, ELISA was developed. Figure 2 shows the optimum conditions of ELISA to quantify HC in the shrimp hemolymph by using 1^{o}Ab at 1:2,000 dilution, 2^{o}Ab at 1:25,000 dilution, the coating buffer at pH 10.5 and hemolymph at 1:200 dilution. The standard curve of purified HC is a linear over the range of 10 - 69 µg with a correlation coefficient R^2 = 0.995 (Figure 3).

3. Determination of hemolymph HC by ELISA

Hemolymph from 6 normal shrimps was diluted to 1:200 and analyzed for HC content by ELISA. The results revealed that the concentration of HC in hemolymph was 111.07 ± 3.58 mg/ml while total protein was 129.65 ± 4.63 mg/ml, implying that HC content was 85.7% of total protein in hemolymph of normal banana shrimp. Thus, HC is a main protein in F. merguiensis hemolymph similar to the case of Litopenaeus vannamei (Cariolou and Flytzanis, 1993).

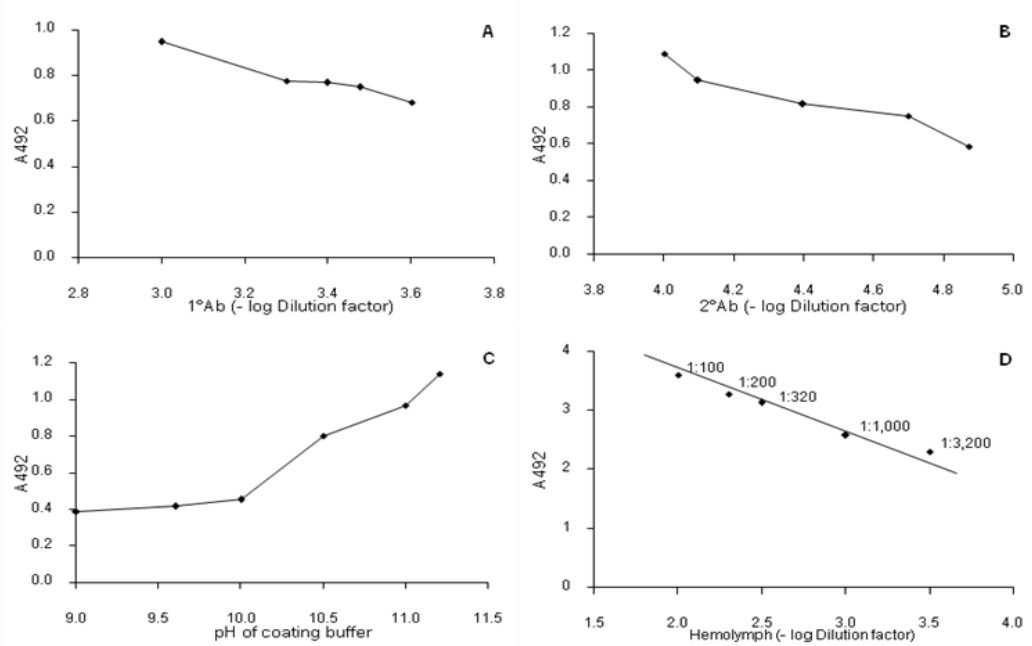

Figure2 Optimization of ELISA conditions; concentrations of 1^{o}Ab (A) and 2^{o}Ab (B), pH of coating buffer (C) and dilution factor of hemolymph (D).

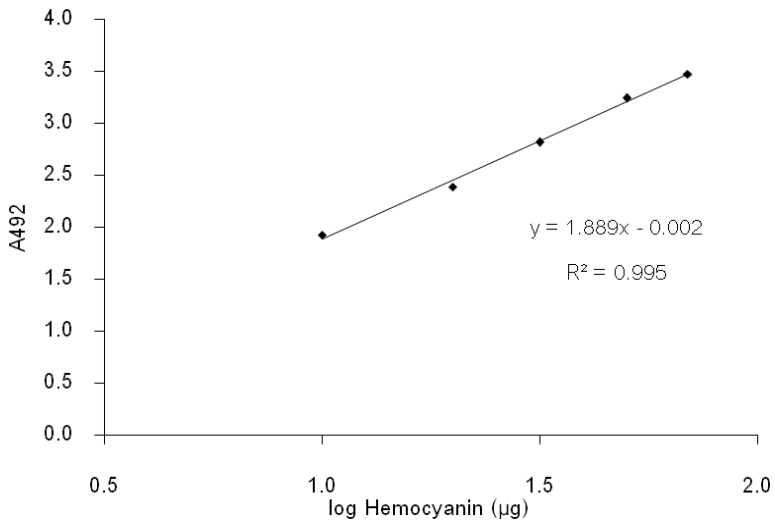

Figure3 Standard curve of purified HC for ELISA. Each well on microtiter plate was coated with various amounts of purified HC (10 - 69 µg). ELISA was performed at the optimum condition by using 1^{o}Ab (1:2,000 dilution), 2^{o}Ab (1:25,000 dilution), 0.4 mg/ml OPD and 0.01% H_2O_2 and the reaction was incubated for 30 min. The absorbance was measured at 492 nm by an ELISA microplate reader.

4. Expression of HC levels in the hemolymph of V. harveyi injected shrimps

To investigate the responses of HC in the shrimp immune defense, banana shrimps were injected with V. harveyi and hemolymph was collected at different time post-injection. By ELISA analysis using the specific anti-HC antibody, HC levels in the hemolymph of the control shrimps were not significantly different at 0 - 24 h after saline injection. In contrast, HC concentrations in the hemolymph of V. harveyi injected shrimps were increased at 6 h and reached the highest at 12 h post-injection and fell back to normal level at 24 h (data not shown here). This result suggested that HC was inducible in response to pathogenic bacterial challenge.

Conclusion

HC was purified from hemolymph of F. merguiensis by UC and preparative PAGE. It showed a single protein band in non-denaturing PAGE and two bands (75 and 79.4 kDa) in SDS-PAGE. The antibody raised against purified HC was used in ELISA. In this study, very sensitive ELISA was successfully developed to specifically quantify HC. HC content was found to be 85.7% of total protein in hemolymph of normal banana shrimp. HC levels in the hemolymph of bacterial injected shrimps was increased at 6 h and reached the maximum at 12 h post-injection. These results indicated that HC may be involved in shrimp innate immune response to pathogenic bacteria challenge.

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