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Identification and characterization of the related immune-enhancing proteins in crab *Scylla paramamosain* stimulated with rhubarb polysaccharides

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ABSTRACT

Recently, considerable interest has been focused on immunostimulants to reduce diseases in crab aquaculture. However, information regarding to the related immune-enhancing proteins in crabs is not available yet. In this study, rhubarb polysaccharides were tested for enhancement of the immune activity in crab *Scylla paramamosain*. Compared with those in the control group, values of, phenoloxidase (PO), alkaline phosphatase (AKP) and alkaline phosphatasein (ACP) activity in the, experimental group were improved significantly 4 d after the treatment. Furthermore, 15 and 17 altered proteins from haemocytes and hepatopancreas, respectively, were found in rhubarb polysaccharide-treated crabs using 2-DE approach. Of these, hemocyanin, chymotrypsin, cryptocyanin, C-type lectin receptor, and ferritin protein were identified by mass spectrometry. In addition, RT-PCR, analysis showed that the mRNA levels of hemocyanin and chymotrypsin increased about 2.4- and 1.4-fold in the experiment group. Moreover, the hemocyanin gene in *S. paramamosain* (SpHMC) was, cloned and characterized. SpHMC contains one open reading frame of 2022 bp and encodes a polypeptide of 673 amino acids. It is clustered into one branch along with crab hemocyanin in a phylogenetic tree. The mRNA transcripts of SpHMC were detected mainly in the tissues of, hepatopancreas, hemocyte and intestines, and its levels were up-regulated significantly in hemocytes, of *S. paramamosain* treated with *Vibrio parahemolyticus*, *Beta streptococcus* or poly I:C for 6–48 h. Taken together, these studies found 5 related immune-enhancing proteins and a novel heomcyanin homologue with potential pathogen-resistant activities in crab.

1. Introduction

Mud crab (*Scylla paramamosain*) is widely cultured in the coast of southeast China. However, within the past ten years, the crab industry has suffered from a great economic loss due to viral, bacterial and parasitic diseases, such as the infection of mud crab dicistrovirus (Zhang et al., 2011), *Vibrio cholerae* and *Vibrio vulnificus* (Wang, 2011), and an unnamed parasite (Kvingedal et al., 2006). Since the invertebrates lack a true adaptive immune system (Du, 2001), researchers suggested that the understanding of crab immunology will be helpful to establish strategies for crab's disease control (Huang et al., 2006; Ko et al., 2007; Vaseeharan et al., 2007; Lin et al., 2008).

Recently, a large variety of immunostimulants have been applied to control crab diseases. For example, Sui et al. found that poly-β-hydroxybutyrate could protect Chinese mitten crab *Eriocheir sinensis* larvae from *Vibrio anguillarum* resulting in higher survival and better growth (Sui et al., 2012). Sun et al. illustrated that CpG oligodeoxynucleotides (ODNs) enhanced the immunoprotection efficiency and growth rate of *E. sinensis* (Sun et al., 2013). Zhou et al. demonstrated that β-glucan from *Saccharomyces cerevisiae* also could improve the growth, immune responses as well as the resistance to Vibrio in *E. sinensis* (Zhou et al., 2008). However, the related immune-enhancing proteins and underlying mechanism of various immunostimulants to activate or boost the innate immune system in crab has not been investigated.

In this study, 5 related immune-enhancing proteins were identified in the mud crab *S. paramamosain* by proteomic and RT-PCR strategies. Of these, the SpHMC gene was further cloned and

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characterized, which was associated with the resistance of crab to pathogens. The results will be important for the investigation of crab immune-enhancing mechanism.

2. Materials and methods

2.1. Experimental animals

Live healthy green mud crabs (*S. paramamosain*) (average weight: 150 g) were obtained from Shantou Huaxun Aquatic Product Corporation (Shantou, China). They were acclimatized at room temperature for one week before the experiments were carried out.

2.2. Preparation and injection of rhubarb polysaccharides

Rhubarb was purchased from a local pharmacy of Shantou city, Guangdong, China. Rhubarb polysaccharides were prepared as described by Bendjeddou et al. (2003). Briefly, after drying at 50 °C for 12 h, the rhubarb was soaked in distilled water at a ratio of 100 g/500 ml for 12 h, and then boiled for 30 min. The herbs were further filtered through gauze, and boiled until the solution was concentrated to 100 ml. The solution was then mixed with 3 times volume of ethanol. After standing at room temperature for 4 h, the mixture was centrifuged at 8000 rpm for 15 min. The precipitate was dissolved and extracted by alcohol for three times before vacuum freeze-drying for 24 h, then dissolved with ddH_2O and centrifuged at 8000 rpm for 15 min, and the supernatant was harvested. After determining the polysaccharide concentration by the method of GB/T 9695.31-91, the rhubarb polysaccharides were stored at 4 °C until analysis.

For the immunostimulation test, an experimental group and a control group were set up with each group having five crabs. Each crab was injected with 100 μl rhubarb polysaccharides (5.6 g/ml) or equal volume of 0.85% sodium chloride.

2.3. Preparation of the sera, hemocyte and hepatopancreas proteins

The *S. paramamosain* sera were prepared as our previous description (Yan et al., 2011a,b,c). Hemolymph 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.

The hemocyte protein was prepared from five rhubarb polysaccharide-treated crab's pool hemolymph as Lee et al. reported (Lee et al., 2000) with some modifications. In brief, the *S. paramamosain* hemolymph was extracted with a 1 ml syringe and mixed with two times volume of anticoagulant (0.48% citric acid, 1.32% sodium citrate, 1.47% glucose). After centrifugation at 1000 rpm for 5 min (4 °C), the precipitate was resuspended in 1 ml sodium eacodylate buffer (CAC, 0.21% sodium eacodylate, 2.64% NaCl, 0.11% $CaC1_2$, 0.53% $MgC1_2$), centrifuged at 1000 rpm for 5 min (4 °C). After resuspending with 0.5 ml CAC buffer, the suspension was ultrasoned in ice-water for 3 min, and then centrifuged at 12,000 rpm for 10 min (4 °C). After determining the protein concentration by the Bradford method (Bradford, 1976), the supernatant was used as hemocyte proteins and kept at −20 °C with phenylmethanesulfonyl fluoride (PMSF, final concentration was 1 mM) until analysis.

The extraction of hepatopancreas proteins was performed according to Hu et al. (2010) with some modifications. Briefly, hepatopancreas (200 mg) was removed from five crabs 4 d after the immunostimulant treatment and pooled together. The sample was soaked in 1 ml CAC buffer and ultrasoned in ice-water for 3 min, then centrifuged at 12,000 rpm for 10 min (4 °C). The supernatant was used as hepatopancreas proteins, and kept at −20 °C with a final concentration of 1 mM PMSF for further assays.

2.4. Immune parameter assays

The phenoloxidase (PO) activity was assayed spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA), which was modified from the method reported previously by Hernández-López et al. (Hernández-López et al., 1996). 10 μl of *S. paramamosain* sera (50 mg/ml) were added to 200 μl of phosphate buffer solution (PBS, 0.01 mM, pH 6.4). 10 μl of L-DOPA (0.01 mM) were then added as the substrate. The mixture was incubated at 25 °C for 2 min. The optical density at 490 nm was measured every 3 min using a microplate reader. An increment of 0.001 absorbance every min under this condition was defined as one unit of PO activity. The assay was conducted in triplicate.

The alkaline phosphatase (AKP), acid phosphatase (ACP), lysozgme (Lys) and superoxide dismutase (SOD) activities were measured with a corresponding kit (Jiancheng Bioengineering Institute, Nanjing, China). 50, 200 and 30 μl of *S. paramamosain* sera was used to determine the AKP (or ACP), Lys and SOD activity, respectively. An AKP (or ACP) enzyme unit was defined as 100 ml sera generated 1 mg phenol from p-nitrophenyl phosphate (pNPP) substrate at 37 °C for 15 or 30 min (15 min for AKP, and 30 min for ACP). The concentration of Lys in sera was calculated according to the formula provided by the kit after reacted at 37 °C for 15 min with 0 °C for 3 min. A SOD enzyme unit was defined as the value when 50% SOD in 1 ml sera was inhibited compared with control group after treated at 37 °C for 40 min with 25 °C for 10 min. Those enzyme activities were expressed as units/ml for sera. These assays were carried out in triplicate.

2.5. Two-dimensional gel electrophoresis (2-DE)

2-DE of the hemocyte or hepatopancreas proteins was performed as previously described (Qiao et al., 2011). In brief, a total of 40 μg of hemocyte or hepatopancreas proteins in rehydration buffer (containing 7 M urea, 2 M thiourea, 4% CHAPS, 0.2% DTT and 3.4 ml of IPG buffer, pH 3–10) was used to rehydrate the IPG strip (7 cm, pH 3–10 for hemocyte proteins and pH 4–7 for hepatopancreas proteins, Bio-Rad, Hercules, CA) for 16 h. The isoelectric focusing (IEF) was performed at a constant temperature of 20 °C using a continuous increase in voltage (up to 4000 V) until reaching 32,000 (for hemocyte proteins) or 22,000 Vh (for hepatopancreas proteins). Prior to the second dimension, the focused IPG was incubated for 15 min in an equilibration buffer containing 20% w/v glycerol, 2% SDS, 0.375 M Tris–HCl (pH 8.8), 2% DTT, then further equilibrated for 15 min in a similar buffer in which 2% DTT was replaced with 2.5% of iodoacetamide. The strip was placed onto top of a 12% SDS-PAGE gel. Low-melting point agarose was used to cover the IPG strip and filter paper. The gel was run in a Mini Protean Tetra cell (Bio-Rad, Hercules, CA) at 40 V for 30 min and subsequently at 100 V for 2.0 h. After SDS-PAGE, the gel was stained with Coomassie G-250.

2.6. Imaging analysis

The 2-DE gel images were analyzed with PDQuest software version 8.0 (Bio-Rad, Hercules, CA). Comparative analysis of protein spots was performed by matching corresponding spots across different gels. Each of the matched protein spots was rechecked manually. After normalization with total intensity volume of all spots present in each gel, intensity volumes of individual spots were subjected to statistical analysis to compare the values in the control group to that of the experimental group. Only differentially