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Characterization of the circulating hemocytes in mud crab (*Scylla olivacea*) revealed phenoloxidase activity

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ABSTRACT

This study focused on an isolation and characterization of the circulating hemocytes in mud crab, *Scylla olivacea*. Isolation of specific cell types of hemocytes from crab hemolymph was accomplished by using 60% Percoll density gradient centrifugation. Four separated bands of the hemocytes were successfully obtained. Characterization of these isolated hemocytes by light microscope using trypan blue-rose bengal staining, rose bengal–hematoxilin staining, and phase contrast revealed four distinct types of hemocyte cells. Using their specific morphology and granularity, they were identified as hyaline cell (HC), small granular cell (SGC), large granular cell (LGC) and mixed granular cell (MGC). Transmission electron microscopy (TEM) revealed more details on specific cell size, size of cytoplasmic granule, and nuclear to cytoplasmic ratio, and confirmed the classification. Relative abundance of these cells types in the hemolymph of an adult crab were 15.50 ± 8.22% for HC, 55.50 ± 7.15% for SGC, 13.50 ± 5.28% for LGC, and 15.50 ± 3.50% for MGC. Proteomic analysis of protein expression for each specific cell types by two-dimensional electrophoresis identified two highly abundant proteins, prophenoloxidase (ProPO) and peroxinectin in LGC. Determination of phenoloxidase (PO) activity in each isolated cell types using *in vitro* and *in situ* chemical assays confirmed the presence of PO activity only in LGC. Based on an increased PO activity of crab hemolymph during the course of White Spot Syndrome Virus (WSSV) infection, these results suggest that prophenoloxidase pathway was employed for host defense mechanism against WSSV and it may link to the role of large granular hemocyte.

1. Introduction

Crustacean hemocytes play an important role in host innate immune responses. They are involved in cellular immune responses, such as phagocytosis, encapsulation, mediation of cell–cell communication and also in humoral immune responses, such as proteolytic cascades (Johansson et al., 2000). General classification of hemocyte cells in crayfish and penaeid shrimps is based on a presence or absence of cytoplasmic granules (Giulianini et al., 2007; Smith and Soderhall, 1983; Soderhall and Smith, 1983; Sritunyalucksana et al., 2005). Their typical types of hemocyte cells are identified as hyaline cell (without evident granule), semi-granular cell (with some small granules), and granular cell (with high numbers of large granule). These hemocytes demonstrate not only their different morphological features but they also show distinct role in immunity. For instant, the hyaline cell of *Carcinus maenas*, acts as phagocytic cells in the present of yeast particle (Thörnqvist et al., 1994). Semi-granular cell of crayfish, *Pacifastacus leniusculus*, demonstrated a function for cell encapsulation (Kobayashi et al., 1990). Moreover, the granular cell the freshwater crayfish *Astacus leptodactylus* can participate in prophenoloxidase (ProPO) system (Giulianini et al., 2007). In other two species of crayfishes, *Pacifastacus leniusculus* (Johansson and Söderhäll, 1985) and *Procambarus clarki* (Lanz et al., 1993), ProPO system induced in both semigranular and granular cells. Moreover in crabs, *Carcinusmaenas*, ProPO system was demonstrated only in granular cells (Soderhall and Smith, 1983).

Circulating hemocytes were reported as the targets for viral infection in crayfish, penaeid shrimp and crabs (Jiravanichpaisal et al., 2001; Kanchanaphum et al., 1998; Wongprasert et al., 2003). During the course of infection, the number of total hemocytescan be dramatically varied (Lorenzon et al., 1999; Smith et al., 1984). White Spot Syndrome Virus (WSSV) infection has a significant effect on the proportion of different hemocyte cell types

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in *P. leniusculus* (Jiravanichpaisal et al., 2001). The number of granular hemocytes in virus-infected crayfish was observed significantly higher than in mock-injected and non-injected crayfish. Moreover semi-granular hemocytes in crayfish were reported to be more susceptible to WSSV infection than hyaline and large granular cells. This led to a dramatic reduction of semi-granular cells during the course time of WSSV infection (Jiravanichpaisal et al., 2006).

Mud crabs (*Scylla* species) can be infected by various species of pathogens including bacteria, fungi and viruses. Crabs carrying pathogens can lead to a high mortality of other crustacean species, for example, White Spot Syndrome Virus, a deadly pathogens for marine shrimp (Rajendran et al., 1999). Nonetheless, some species of mud crabs can be tolerant to viral infection at a relatively high dose without showing gross signs of disease or mortality (Kanchanaphum et al., 1998; Supamattaya et al., 1998). This phenomenon suggests a persistent mechanism to WSSV and differences in innate immune system between mud crabs and other crustaceans.

Since the mechanism of immune responses in crustaceans depends on cellular and humoral immune system, presenting by specific hemocytes, the purpose of this study was to characterize *Scylla olivacea*'s hemocyte cells and determine their specific roles in adaptive ability to WSSV infection. The experiment involved an isolation and identification of crab hemocytes, based on cell morphology and granularity. Differential protein expressions among the identified cell types were then investigated by proteomic two-dimensional gel electrophoresis. The results revealed two dominated proteins, peroxinectin and prophenoloxidase in the large granular cell (LGC). The enzymatic activity of phenoloxidase was then examined and confirmed by biochemical assays. The finding suggests that LGC can play an active role in innate immune response, and involves in defensive prophenoloxidase mechanism of mud crab.

2. Materials and methods

2.1. Preparation of mud crab, S. olivacea

Male mud crabs (*S. olivacea*) with approximated 350-g body weight were obtained from local market in Salaya, Nakhonpathom, Thailand. The crabs species were identified using their morphology and confirmed by the nucleotide sequence of mitochondria 16s rRNA gene as previously described (Somboonna et al., 2010). The crabs were acclimatized in artificial seawater of 30 ppt salinity for at least three days before an experiment. They were fed daily with sea bass meat. The water was regularly changed every day.

2.2. WSSV inoculum and challenging

WSSV stock inoculum was obtained from Centex Shrimp, Thailand. The original inoculum was prepared from the hemolymph serum of moribund WSSV-infected black tiger shrimp. Hemocytes were removed by centrifugation at 800*g* for 10 min. WSSV in the serum was quantified by real time PCR as described by Srisala et al. (2008). WSSV of 10^5 copies/g of crab was inoculated into the soft layer between carapace and swimming legs. During four-day post infection, viral infectivity, total hemocyte count, differential hemocyte count, and phenoloxidase activity were quantified and reported.

2.3. Detection of WSSV infection by semi-quantitative PCR

DNA was extracted from total hemocytes of control and infected crabs during 4 dpi. DNA extraction method was modified from Lo et al. (1997). In brief, total hemocytes were harvested by centrifugation at 800*g*, 5 min, at 4 °C from the hemolymph-anticoagulant (0.45 M NaCl, 10 mM glucose, 10 mM EDTA, 26 mM sodium citrate, 30 mM citric acid, pH 4.6) mixture. The pellets were washed twice with anticoagulant with the same centrifuged condition. The hemocytes pellets were vigorously mixed with DNA extraction buffer (100 mM, sodium chloride, 10 mM Tris–Cl pH, 8.0, 25 mM EDTA, pH 8.0, 0.5% N-lauroyl sarcosine). The reactions were incubated with 0.5 mg/ml RNase A for 30 min at 37 °C. Then, the reactions were incubated with 0.5 mg/ml proteinase K for 1 h at 56 °C. The reactions were vigorously mixed with 1% cetyltrimethyl ammonium bromide (CTAB) and incubated at 65 °C for 10 min. Phenol: chloroform extraction was performed twice. The aqueous phase was transferred to the new tube. DNA was then obtained by isopropanol precipitation.

The PCR 25 μl reactions included 100 ng DNA template, 1× PCR buffer, 3 mM $MgCl_2$, 0.2 mM dNTP, 0.3 μM of internal control primers (16sar_L 5′-CGC CTG TTT ATC AAA AAC AT-3′, 16sar_R 5′-GGT CTG AAC TCA GAT CAC GT-3′), 0.5 μM WSSV primer (WSSV_F 5′-ATG AGA ATG AAC TCC AAC TTT AA-3′, WSSV_R 5′- CAG AGC CTA GTC TAT CAA TCA T-3′), and 0.125 U of *Taq* DNA polymerase. The PCR cycles were 95 °C for 5 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, and 72 °C for 5 min. The expected PCR products were 556 bp of internal control, mitochondria 16s rRNA gene (accession number AF109321) and 446 bp of wssv419-like protein gene (accession number AY850066).

2.4. Isolation of mud crab hemocytes

An isolation of mud crab hemocytes was performed by Percoll™ (GE healthcare) density gradient centrifugation (Imjongjirak et al., 2009). Crab hemolymph was withdrawn by 21G needle containing 5 ml of anticoagulant in asyringe. The collected hemolymph was gently mixed with anticoagulant (0.45 M NaCl, 10 mM glucose, 10 mM EDTA, 26 mM sodium citrate, 30 mM citric acid, pH 4.6) in 1:1 ratio and immediately placed on ice to avoid clotting. The hemocytes were loaded and separated in 60% Percoll™ gradient (60% Percoll, 0.45 M NaCl) with centrifugation speed at 2900*g*, 4 °C for 30 min. Each separated band of hemocytes was slowly aspirated into a new tube. The cells were then collected by low speed centrifugation at 1900*g*, 4 °C for 2 min. The cells were rinsed several times with anticoagulant and centrifuged.

2.5. Total and differential hemocytes count

Total hemocyte count (THC) was performed to determine the total number of hemocyte per milliliter of crab hemolymph. The 50-μl of hemolymph–anticoagulant mixture was combined with 50 μl trypan blue dye (0.4% trypan blue in PBS). A 10 μl of mixture was filled into hemocytometer and examined on light microscope as previously described (Sritunyalucksana et al., 2005).

Differential cell count (DHC) was performed to yield the relative abundance of each specific hemocyte cells per milliliter of crab hemolymph. The 50-μl crab hemolymph–anticoagulant mixture was incubated in 50 μl trypan blue dye and 5 μl rose bengal dye (1.2% rose bengal dye, 50% ethanol). The cell mixture was wet mounted on a clean glass slide and cover slip. At least 100 cells were counted and related to THC of individual crabs.

2.6. Characterization of mud crab hemocytes

Cell morphology and granularity of the isolated or mixed hemocytes were examined by light microscopy using wet mount staining, permanent staining and phase contrast. For wet mount staining, the isolated cells were stained similar to those in DHC. For permanent staining, the isolated hemocytes were stained using rose bengal and hematoxylin dyes (Sritunyalucksana et al., 2005).