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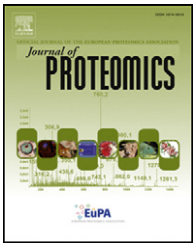

Comparative proteomic identification of the hemocyte response to cold stress in white shrimp, *Litopenaeus vannamei*

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ABSTRACT

To understand molecular responses of crustacean hemocytes to cold stress, flow cytometer analysis and two-dimensional electrophoresis proteomic approach were used to investigate altered proteins in hemocytes of *Litopenaeus vannamei* during cold stress treatment. Through flow cytometer analysis, 13 °C for 24 h post-cold stress was selected as the suitable temperature and condition for cold stress treatment. MALDI-TOF/TOF MS analysis revealed that 6 forms of 6 proteins were significantly up-regulated, including three enzymes (cystathionase, glyceraldehyde 3-phosphate dehydrogenase and glyoxalase 1) and one immune-related protein (oncoprotein nm23), whereas 24 forms of 3 proteins were significantly down-regulated in the treated shrimp (hemocyanin, hemocyte transglutaminase and transketolase). There were 20 spots identified as hemocyanin meaning that it may play important roles in environmental regulation in shrimp. Real-time fluorescence quantitative PCR confirmed that the levels of transcription of the hemocyanin, partial mRNA for hemocyanin, cystathionase, glyoxalase 1 and oncoprotein nm23 genes were found to relate well with that of their translation products after cold stress treated, while only the levels of hemocyte transglutaminase transcripts were not corresponded with that of their translation products. Further investigation of these data may lead to better understanding of the molecular responses of crustacean hemocytes to cold stress.

1. Introduction

The white shrimp *Litopenaeus vannamei* (*L. vannamei*), has high commercial value and excellent property of breeding. In recent years, *L. vannamei* has become the main aquatic animal cultured in coastal regions of China. However, environmental changes have become an important reason for increased prevalence of shrimp disease, leading to the significant reduction of shrimp production. Temperature is one of the most important physical factors controlling growth of marine shrimp. In shrimp farms the seasonal range of water temperatures may vary from 15 to 32 °C, but the best survival temperature for *L. vannamei* juveniles is between 20 and 30 °C [1], and for large shrimp (>5 g), the temperature optimum is about 27 °C [2]. Low water temperature affects shrimp health by suppressing the immune system and disturbing physiological processes [3].

Shrimp innate immunity consists of cellular and humoral responses. Hemocytes in the hemolymph are the major components of cellular immune responses in crustaceans. Hemocytes play a central role in the immune response of shrimp, which rely mainly on recognition of nonself [4], phagocytosis [5], melanization and coagulation which are mediated by the release of hemocytic effectors such as the prophenoloxidase (proPO)-activating system [6,7] and transglutaminase [8], or antimicrobial peptides [9]. However, decrease of circulating hemocytes would depress the immune ability, increase the susceptibility against

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pathogens, and even endanger the survival of shrimp [10]. It has been reported that temperature stress can change the total hemocyte count (THC) [3,11]. Therefore THC may be a stress indicator of immune system activity. Reactive oxygen species (ROS), including hydroxyl radicals (HO^-), superoxide anions (O_2^-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and peroxyl (ROO^-) are constantly generated in organisms. The balance between the production of ROS and the antioxidant defense mechanisms can be disturbed by environmental stress. Overproduction of ROS may lead to oxidative damage to tissue macromolecules including DNA, proteins and lipids [12], and subsequently induce cell apoptosis [13]. In the normal cell, cytoplasmic free-Ca^{2+} (cf-Ca^{2+}) which can modulate many physiological or pathological processes is strictly controlled. Cf-Ca^{2+} overloads or disturbances in local intracellular distribution determine toxicity or cell death (necrosis or apoptosis) [14].

While hemocytes physiological responses of the shrimp immune system to environmental stress have been extensively studied [1,3,15,16], little is known about its molecular elucidation of shrimp immune responses to cold stress. Recently, a few reports have indicated that proteomic based techniques are a useful method in the identification of shrimp immune-related proteins [17–20]. In these studies, the pathogenicity of viral infections has been the main focus of such studies. However, alterations in protein expression and function in cold stress shrimp remain unknown.

Here, flow cytometry which analyzes the individual cell in a short time, could provide an objective, reproducible and sensitive characterization of a large number of hemocytes. In this study, we used flow cytometry to detect the respiratory burst (RB), cf-Ca^{2+} concentration and apoptotic cell for determining the suitable temperature for cold stress of hemocytes. THC was also used as an indicator of the cold stress. A proteomic approach was applied to investigate altered proteins in hemocytes of *L. vannamei* during cold stress. The results provide important information on shrimp immune responses against cold stress.

2. Materials and methods

2.1. Animals

White shrimp *L. vannamei* with average weight 7.50±0.33 g were obtained from a shrimp farm in Panyu (Guangdong, China). They were transported into the lab and acclimated in 250 L air-pumped circulating diluted seawater tanks at least 1 week prior to experiments. During the acclimation stage, the water salinity and temperature in tanks were consistent with the conditions of the culture ponds (salinity 5‰ and temperature 28±1 °C). Commercial shrimp diet was given two times per day. Only shrimp in the intermolt stage were used. Healthy shrimp were randomly selected and divided into control and cold stress groups.

2.2. Cold stress

One hundred and twenty shrimp individuals were randomly divided into 6 tanks (60 cm×40 cm×35 cm), with 20 individuals per plastic aquarium exposure chamber and air pump for aeration. Three replicate exposure chambers were employed per treatment or control group. An artificial climate incubator (temperature range 5 °C–50 °C) was used for cooling. Water temperature was decreased from 28 °C to 13 °C within 3 days at a rate of 2.5 °C/8 h.

2.3. Sample collection

A total of hemolymph of six shrimps from each tank was collected at the cold stress procedure when the water temperature was maintained at 28 °C, 23 °C, 18 °C, 13 °C, 13 °C for 24 h and 48 h, with each tank one shrimp for one pool. 300 μL of hemolymph was extracted from each shrimp by a 25-gauge needle and 1 mL syringe containing an equal volume of ice-cold anticoagulant solution ((27 mM trisodium citrate, 385 mM sodium chloride, 115 mM glucose, pH 7.5). The hemolymph from each shrimp was transferred into a separate microcentrifuge tube held on ice. Each pool (×3) was composed of hemolymph from six individuals. A drop of diluted hemolymph sample was removed to a hemocytometer to measure THC with lightmicroscope. 200-μL hemolymph were diluted for analysis of RB, cf-Ca^{2+} and apoptotic cell ratio. The remaining hemolymph was divided equally and transferred into centrifugal tubes on ice and centrifuged at 3000 rpm for 10 min at 4 °C. The separated hemocytes were preserved at −80 °C after liquid nitrogen flash freezer. Half was for the proteomic analysis, the other half was for the real-time fluorescence quantitative PCR (RT-PCR).

2.4. Flow cytometer analysis

2.4.1. RB

To monitor the level of RB, the cell-permeant probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) was used as described by Xian et al. [21]. After it permeates through the cell membrane, DCFH-DA is deacetylated by cytosolic enzymes to dichlorofluorescein (DCFH), which is polar and trapped with the cells. Initially, DCFH was considered as a specific indicator for H_2O_2. Later it was found to be also oxidized by nitric oxide (NO), peroxynitrite, hydroxyl (HO^-) and peroxyl (ROO^-). These ROS oxidizes the nonfluorescent DCFH to highly fluorescent 2′, 7′-dichlorofluorescein (DCF, $\lambda_{excitation}$=498, $\lambda_{emission}$=522). A volume of 200-μL dilute hemolymph was diluted with anticoagulant solution to obtain a final concentration of 1×10^6 cells mL^{-1}, and then incubated with 5 μM DCFH-DA (Sigma) for 30 min in the dark, totally three pools. Then the fluorescence of the cell suspensions was recorded with the flow cytometer. RB activity, represented as the oxidation of DCFH to DCF by ROS described above, was expressed as mean fluorescence of DCF.

2.4.2. Cf-Ca^{2+} concentration in hemocytes

To detect the level of calcium concentration, the cell-permeant probe Fluo-3/acetoxymethyl ester (fluo-3/AM, Sigma) was used as described by Aton et al. [22]. After it permeates through the cell membrane, fluo-3/AM is de-esterified in the cytoplasm by esterase. Then this de-esterified form binds cytoplasmic free calcium and emits a green fluorescence ($\lambda_{excitation}$=506, $\lambda_{emission}$=526) detected using fluorescence channel 1 (FL1). A volume of 200-μL diluted hemolymph from each of the three pools was diluted with anticoagulant solution to obtain a final