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Morphology and phagocytic ability of hemocytes from *Cristaria plicata*

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ABSTRACT

Cristaria plicata is one of the most important freshwater mussels for pearl production in China, whose hemocytes were characterized using light, phase contrast and electron microscopy. In accordance with the presence or the absence of granules in the cytoplasm, two main types of hemocytes were recognized, granulocytes and agranulocytes. The granulocytes possessed cytoplasmic granules, while agranulocytes had none or a few cytoplasmic granules. In addition, in line with the diameter of granulocytes and cytoplasmic granules, granulocytes were further divided into large and small granulocytes. According to the diameter of agranulocytes and the different N/C ratios, agranulocytes could also be divided into hyalinocytes and lymphoid hemocytes. The mean concentration of the hemocytes of *C. plicata* was about $(2.37 \pm 0.51) \times 10^6$ cell mL^{-1}. The two main cell types were small granulocytes (56.60% ± 1.41) and hyalinocytes (36.80% ± 2.11), while large granulocytes (4.20% ± 0.23) and lymphoid hemocytes (2.40% ± 0.12) were of the minority. With the rise of water temperature, total hemocyte counts showed a significant increase. The hemocytes presented different tinctorial properties for Wright's staining in smears. Small granulocytes and hyalinocytes exhibited acidophils and basophils, while large granulocytes and lymphoid hemocytes showed only acidophils and basophils respectively. Three types of hemocytes, large granulocytes, small granulocytes and hyalinocytes, could phagocytize yeast and *Bacillus subtilis* cells. Small granulocytes appeared to be more efficient in phagocytosis than large granulocytes and hyalinocytes, and large granulocytes showed to be more efficient than hyalinocytes. Water temperature had a great effect on the phagocytic ability of hemocytes.

1. Introduction

Hemocytes of bivalve were involved in healing wound, transport of calcium and protein in regeneration of the shell, intracellular digestion (Cheng, 1981). However, their more important role resided in internal defense, such as recognition and phagocytosis or encapsulation nonself materials (Foley and Cheng, 1974).

Classification of bivalve hemocytes existed many debates by variable hemocyte morphology and identified method. Cheng (1981) and Hine (1999) considered that the hemocytes of the whole Mollusca phylum were distinguished into two fundamental types, granulocytes and hyalinocytes (or agranulocytes). granulocytes and agranulocytes were also split into several subpopulations (Cima et al., 2000; Wootton and Pipe, 2003; Zhang et al., 2006). Besides granulocytes and agranulocytes in bivalve molluscs, fibrocytes, morula-like cells and vesicular hemocytes were observed (Foley and Cheng, 1974; Cheng and Foley, 1975; Nakayama et al., 1997; Auffret, 1989).

Hemocytes were recognized to be the main cellular mediators of the defense system in mollusc (Cheng and Manzi, 1996). Their mechanisms involve phagocytosis of pathogens and their degradation by lytic enzymes and/or the production of reactive oxygen metabolites (Pipe et al., 1995; Winston et al., 1996) or their encapsulation if they are too large to be phagocytosed (Cheng, 1981). Phagocytosis focused on characterization and morphological description of phagocytes (Nakayama et al., 1997; Zhang et al., 2006), or the analysis of the phenomenon at biochemical and molecular levels (Cima et al., 2000; Azumi et al., 2002).

Hemocytes of marine bivalves have become the focus of studies (Renault et al., 2001; Dyrynda et al., 1997; Cima et al., 2000). However, relatively little is known in relation to morphology and phagocytosis of hemocytes from freshwater mussel. *Cristaria plicata* is one of economical importance in freshwater mussel and is known as "pearl bivalves" in aquaculture industry in China. The present study was designed to implement fundamental studies in hemocyte characterization and phagocytosis of the bivalve.

2. Materials and methods

2.1. Experimental animals

C. plicata was collected from Poyang Lake in China. Shell length was 100–130 mm. Before experiments were undertaken, the bivalves

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were maintained in aquariums for at least 5 days. The room temperature was 20 °C ± 2.

2.2. Hemocytes collection

Approximately 0.5 mL of hemolymph was extracted from the posterior adductor muscle sinus of each bivalve by a 2.5 mL syringe and 5-gauge needle into 1 mL Alsever's solute (20.8 g/L glucose, 8.0 g/L sodium citrate, 22.5 g/L sodium chloride, pH7.2). The hemolymph samples were prepared for the following experiments.

2.3. Light microscopy

2.3.1. Phase contrast microscope

One drop of hemolymph samples was added onto the glass slide, and settled for 20 min at room temperature. Finally, observations were done using a phase contrast microscope (Olympus IX51).

2.3.2. Cytochemical staining

Wright's staining techniques were employed to differentiate the subpopulations. Smears were prepared. One drop of hemolymph sample placed on glass slides, and settled for 30 min in moist chamber at room temperature. After the hemocytes were adhered on the glass surface, the supernatants were moved by the absorbent paper. The smears were dried in the air, then stained for 10 min with Wright's solution. Smears were rinsed in distilled water, dried again, and sealed by neutral tree gum, finally, observed by optical microscope (Olympus CX41).

2.3.3. Hemocyte count and size measurement

Total hemocyte counts (THC) and differential hemocyte counts (DHC) were counted by a blood cell counting chamber from 30 bivalves, and relative percentages were calculated by living cells. Cell and nucleus diameter of hemocytes were measured in 100 cells by a Filar eye-piece graticule. Nuclers/cytoplasm (N/C) ratio was calculated. The SPSS software was exploited for analysis a significant difference among each group data. Values of $P<0.05$ were considered significant.

2.3.4. Water temperature effect on hemocyte counts

Water temperature was controlled at 10, 15, 20, 25 and 30 °C respectively. The bivalves were placed into aquariums at different water temperature for 4–5 days. Hemolymph was extracted from the posterior adductor muscle sinus. Hemocytes were counted to utilize 30 bivalves at each temperature group.

2.4. Transmission electron microscopy

Hemolymph samples were centrifuged 3000 rpm for 10 min. The supernatants were discarded. The pellets were washed twice in Pipes buffer, then fixed in a fixative solution (2.5% glutaraldehyde for 2 h under pH7.2) for 2 h at 4 °C, and post-fixed in 1% osmium tetroxide for 1 h at 4 °C. After being washed in Pipes buffer, the hemocytes were embedded in 1.5% agar at 40 °C and quickly centrifuged 10,000 rpm for 5 min. The pellets were dehydrated and embedded in Epon. Ultrathin. Sections (50–70 nm) were stained with uranyl acetate and lead citrate, and examined in a transmission electron microscope JEOL H600 with 75 kV.

2.5. Phagocytosis experiment

The phagocytosis assay was adapted from the method of Zhang et al. (2006). Two species of bacteria, yeasts and *Bacillus subtilis,* were used. Prior to the phagocytosis assay, both bacteria types of cells were suspended in distilled water (about 10^8) and activated 1 h at room temperature (20–23 °C).

Fresh hemolymph (1 mL) was immediately mixed with an equal volume of bacteria suspension in culture tubes. The mixtures were incubated for 1 h at 20 °C. Then the mixtures chilled on ice water to terminate phagocytosis for 30 min (Nakayama et al., 1997). Finally, smears were prepared and stained with Wright's solution. The surplus mixed liquor of the hemolymph and bacteria cells were centrifuged 3000 rpm for 10 min. The pellets were used to make ultrathin sections, which observed by a transmission electron microscope.

The effect of water temperatures on the phagocytic ability of hemocytes was designed. The mixture of hemolymph and bacteria cells were incubated for 1 h at different temperature, 10 °C, 20 °C, 30 °C, and 37 °C, respectively, then smears were prepared as described above. The phagocytic ratio was expressed as the percentage of phagocytized hemocytes in total hemocytes, which estimated in 20 randomly selected microscope fields at ×400. The phagocytic index (i.e. the number of yeast cells per phagocytic hemocyte) was evaluated after counting 20 fields in each glass side with at least 200 cells (Zhang et al., 2006).

3. Results

3.1. Hemocyte identification and characterization

3.1.1. Phase contrast microscope

According to the presence or the absence of cytoplasmic granules, hemocytes of *C. plicata* were identified into granulocytes and agranulocytes under phase contrast microscope. Granulocytes had abundant refringent cytoplasmic granules and rough cell appearance, while agranulocytes had none or a few refringent cytoplasmic granules and laevis cell appearance (Fig. 1). Granulocytes had two differential subpopulations. One possessed abundant large clear and high refringent cytoplasmic granules. These subpopulation hemocytes were called large granulocytes. They could produce few pseudopodia, occasionally, extend a few small projections. Therefore, they always showed spheroid. Occasionally, large granulocytes were easy to rupture, whose large cytoplasmic granules were released (Fig. 1A). The other possessed many small vague and low refringent cytoplasmic granules. These hemocytes were called small granulocytes. They could extend many radial or root-like pseudopodia, and be easy to adhere to the surface of glass slides (Fig. 1B). The sizes of small granulocytes were obviously smaller than those of large granulocytes.

There were also two different morphologic types of agranulocytes under phase contrast microscope. One was amoeboid cells, always extended veil-like or root-like pseudopodia, with an ovoid, or round, eccentric nucleus, and copious cytoplasm often containing a variety of organelles. These type hemocytes were called hyalinocytes (Fig. 1C). The other was spherical cells, displayed a few short radiate pseudopodia, with a central spherical nucleus surrounded by a rim of scant cytoplasm lacking organelles. These type hemocytes were called lymphoid hemocytes (Fig. 1D). The sizes of hyalinocytes were obviously larger than those of lymphoid hemocytes.

3.1.2. Cytochemical staining

Large granulocytes were round or ovoid cells, and were acidophilic in Wright's stain smears. The nuclei were round or kidney-shape and eccentric. Cytoplasmic granules were deep red (Fig. 1a). Small granulocytes were ovoid or fan-shaped cells in smears. Some showed basophilic, cytoplasmic granules stained blue. Others showed acidophilic, and the granules were stained deep red or pink (Fig. 1b1, b2). The nuclei were round or horseshoe shape, and commonly eccentric. Hyalinocytes were polymorphic cells in smears, and showed two tinctorial properties. One was basophilic, and cytoplasm was stained blue. The other was acidophilic, and cytoplasm was stained plum (Fig. 1c1, 1c2). The nuclei were round or ovoid, eccentric. Lymphoid hemocytes showed basophilic in smears (Fig. 1d), and were round in