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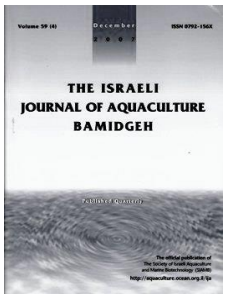

Improved Sampling of Hemolymph and Screening of Anti-Coagulants of Hemocytes in the Snail *Babylonia areolata*

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Keywords: anti-coagulant; *Babylonia areolata*; hemocyte

Abstract

Our aim was to improve sampling of hemolymph from the snail *Babylonia areolata* in order to evaluate the physiological and immune capacities of hemocytes in aquaculture. We also identified appropriate types of hemolymph anti-coagulants for *B. areolata.* Hemolymph samples were collected using an improved foot plantaris puncture method. We screened five types of anti-coagulants from Penaeid shrimp (A), marine decapods (B), abalone (C), and oyster (D), as well as a home-made anti-coagulant (E), to act against *Babylonia areolata* hemocytes on the basis of cell death rate and hemocyte aggregation. We improved the former foot plantaris puncture method and identified the home-made anti-coagulant as the best anti-coagulant from the five which we tested.

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Introduction

Babylonia areolata is a commercially important aquaculture species cultured along the southeast coast of mainland China. Due to its rich nutrition, rapid growth, and pleasing palatability, *B. areolata* has been identified one of the most important marine gastropods for human consumption (Fu et al., 2017; Lü et al., 2016). In China, especially in Hainan Province *B. areolata* was cultured in an area of over approximately 450 000 square metres in 2013 and generated approximately RMB 250 million yuan (Fu et al., 2017; Lü et al., 2016). However, *B. areolata* production has decreased drastically in recent years because of the increased incidence of bacterial diseases such as vibriosis, proboscis intumescence disease, and shell cast disease (Feng et al., 2008). Thus, production of disease-resistant animals is crucial and depends on increasing understanding of marine invertebrate immunology. However, limited information is available about the cyto-immunity of marine gastropods compared with that of bivalve mollusks.

With less effective humoral factors in invertebrates, cellular immunity plays a more important role in invertebrates than in vertebrates. Hemocytes play a pivotal role in shellfish immunity (Soares-da-Silva et al., 2002). Observation of the function of hemocytes may help us understand the defensive reaction in immune systems of gastropods. However, mollusk hemocytes *in vitro* are easily aggregated by pseudopod interconnections after they leave their body (Auffret and Oubella, 2013; Zhou et al., 2017). In mollusks, hemocyte aggregation can induce clump formation which in turn affects hemostasis and wound healing. Hemocyte aggregation is difficult to reverse, making observation and research difficult (Li, 2001), often compromising experimental designs. Another important consideration is the clotting progress. Hemocyte aggregation and clotting make it difficult to evaluate hemocyte immune capacities. Thus, hemocyte experiments use anti-coagulants to maintain the normal form of hemocytes *in vitro* and prevent hemocyte aggregation for a reasonable time. The anti-coagulant should not only prevent hemocyte aggregation but should also maintain cell integrity and hemocyte function and should also keep them as close as possible to their original physiological condition *in vitro*. However, the type and effective concentration of blood anti-coagulant that is the most suitable for gastropod hemolymph is still unknown. *B. areolata* (as in all snails) has an open circulation system, and its hemolymph contains hemocyanin. Appropriate anti-coagulants should be used in research on hemocyte functions of *B. areolata*. Almost all current hemocyte research in snails involves the use of buffer solution as an anti-coagulant (Sminia et al., 1979; Zhou et al., 1988) and the use of anti-coagulants from shrimp and shellfish as references in experiments on other gastropod hemocytes. Until now, an anti-coagulant specific for gastropod hemocytes has been unavailable. Based on the few reports regarding gastropod hemolymph, the aim of this study was to improve sampling of hemolymph from the snail *B. areolata*, to select an anti-coagulant for the hemolymph, to investigate the effects of the five types of anti-coagulants (one of them was home-made), and identify the appropriate type of hemolymph anti-coagulant for *B. areolata*.

Materials and Methods

Samples.

B. areolata adults (3-4 cm) were purchased from a farm in Dongshan, Fujian Province. The snails were maintained in a cement pond (2 m× 3 m× 3) with flowing water (24-28°C, salinity 26-29, pH 7.8-8.5). A layer of fine calcareous sand was added to the pond to allow burrowing. The snails were fed daily with oysters and chopped fresh fish.

Sampling of hemolymph.

B. areolata adults (3-4 cm shell length) were used for hemolymph sampling. Water adhering to the snail's surface and foot was removed and the snails were cleaned with absorbent paper. Hemolymph samples were obtained with an improved foot plantaris puncture method. Hemolymph was extruded using a 1.5 mL blue pipettes with the tip cut-off. This was inserted into the snail's foot while the snail's head and foot were forced to retract into the shell. Approximately 100 μL of hemolymph was collected from each snail and the hemolymph was immediately transferred into 1.5 mL Eppendorf tubes containing the same quantity of anticoagulant to avoid hemocyte aggregation. The mixture was gently shaken to avoid clumping of hemocytes and then stored at 4°C.

Screening of anti-coagulants.

Anti-coagulant A was prepared in accordance with the Penaeid shrimp anti-coagulant formulation of Vargas-Albores et al. (1993). Anti-coagulant B was prepared in accordance with the *Carpinus manas* formulation of Söderhäll and Smith (1983). Anti-coagulant C was prepared in accordance with the abalone anti-coagulant formulation of Lebel et al. (1996). Anti-coagulant D was prepared in accordance with the oyster anti-coagulant formulation of Xue et al. (2001). Anti-coagulant E was prepared in-house. The home-made anti-coagulant solution (E) consisted of 2.05 g glucose, 0.8 g sodium citrate ($2H_2O$), 0.42 g NaCl, and 10 Mm HEPES in 100 mL distilled water. pH was adjusted to 6.1 with 10% citric acid. The solution was sterilized at 121°C.

Hemolymph from nine adult snails (3-4 cm shell length) was pooled. Snail hemolymph and one type of anti-coagulant were mixed in equal amounts and stored at 4°C in an Eppendorf tube. Ten microliters of hemolymph mixture and an equal amount of 2% trypan blue solution were mixed on glass slides at different time intervals (2, 4, 6, 9, 12, 24, 36, and 48 h). The mixture was observed through an oil immersion lens after 5 min to determine the number of dead cells per 100 cells at each time interval. Cell count was repeated three times. Hemocyte mortality rates at each time interval were calculated for each anti-coagulant. The state of hemocyte aggregation in each anti-coagulant was observed and recorded, including the numbers of cells in 10 fields of view for every sample and the average number of cells in each hemocyte cluster.

Results

Cell survival or death was determined by trypan blue staining. Live cells were white, whereas dead cells were blue (Fig. 1). After 2 h, anti-coagulant E had the lowest hemocyte mortality rate of only 2.1%. The hemocyte mortality rates in anti-coagulants D, B, and A were 17.8%, 19.3%, and 22.5%, respectively. Anti-coagulant C had the highest hemocyte mortality rate of 48.7%. Hemocyte mortality rates increased in each anti-coagulant at time intervals up to 6 h. After 9 h, the hemocyte death rate for anti-coagulant E was 9.3%. It was the lowest among all five anti-coagulants for that time interval. For anti-coagulant B the hemocyte death rate was 42.2%. The hemocyte mortality rates for anti-coagulants D and C reached 62% and 83.6%, respectively; it was the highest at 84.8% for anti-coagulant A and remained the highest up to the 24 h time interval for anti-coagulant A (Fig. 2). The hemocyte mortality rate for anti-coagulant E somewhat increased but was the still lowest among all five anti-coagulants.

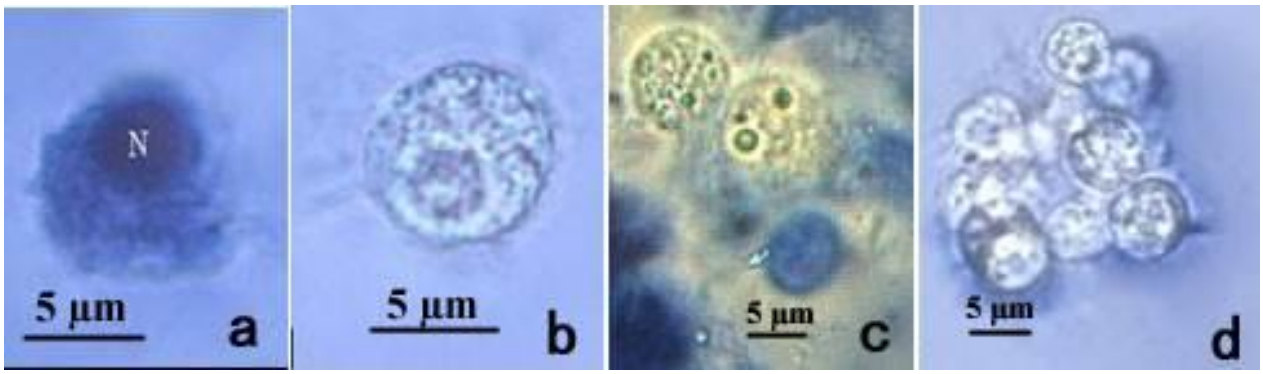

Fig. 1. Hemocytes in trypan blue reagent. (a), (b), and (c) show hemocytes with different responses to hemocyte staining with trypan blue reagent. Dead cells appear blue, of small volume, and with a large nuclear-cytoplasmic ratio, whereas live cells are transparent and colorless, of large volume, and with a small nuclear-cytoplasmic ratio. (a) shows a dead cell, while (b) shows a live cell. In (c), the colorless cells are alive, whereas the blue cells are dead. (d) shows a hemocyte cluster. N stands for the cell nucleus.

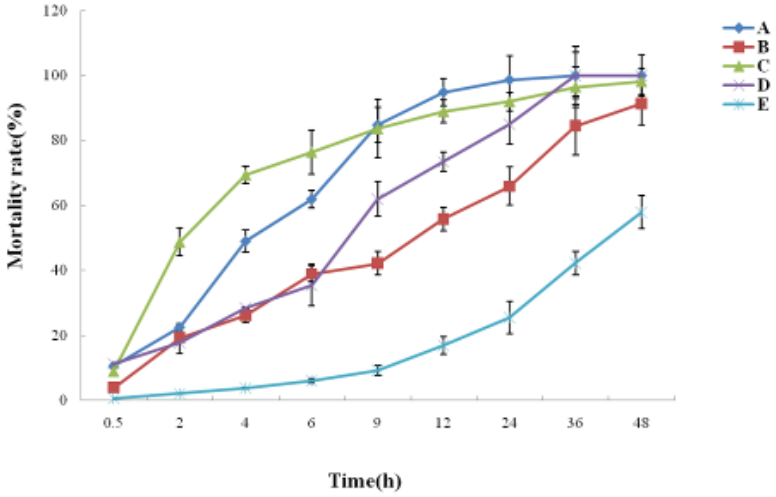

Fig. 2. Death rate of *Babylonia areolata* hemocytes in five anti-coagulants.
A = Penaeid shrimp anti-coagulant;
B = marine decapod anti-coagulant;
C = abalone anti-coagulant;
D = oyster anti-coagulant; and
E = home-made anti-coagulant

No obvious trend was observed in the number of hemocyte clusters and the number of cells in each hemocyte cluster for the five types of anti-coagulants. The number of hemocyte clusters for anti-coagulant C and the average number of cells in each hemocyte cluster were lowest at the 48 h time interval. The average number of cells in each hemocyte cluster for anti-coagulant E was moderate among all five types of anti-coagulant. The other three types of anti-coagulant showed irregular changes (Figs 3, 4).

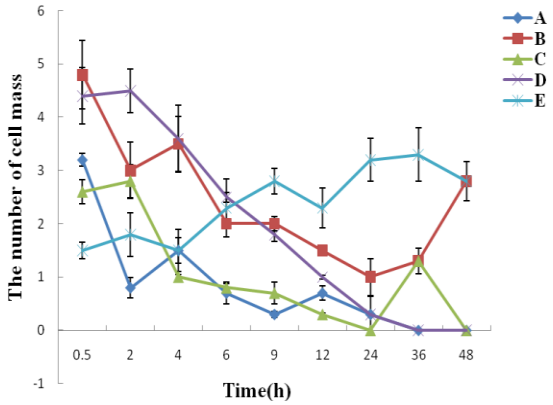

Fig. 3. Aggregated hemocyte cluster of *Babylonia areolata* hemocytes in five anti-coagulants (Names of anti-coagulants as in Fig. 2.)

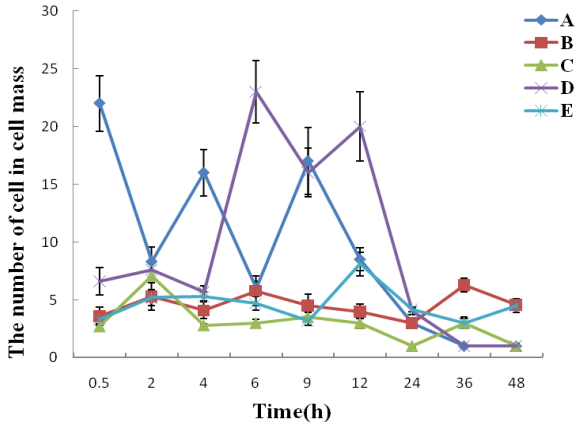

Fig. 4. Number of cells in the hemocyte cluster of *Babylonia areolata* hemocytes in five anti-coagulants (Names of anti-coagulants as in Fig. 2.)

In summary, hemocyte mortality rate was lowest for anti-coagulant E. Anti-coagulant E exhibited the most even hemocyte distribution and most effective anti-coagulant action. Anti-coagulant C also had effective anti-coagulant action, while its hemocyte mortality rate was high.

Discussion

Hemolymph collection method.

Snail hemolymph collection methods include foot plantaris puncture (Sminia et al., 1979), capsular discission (Jeong and Heyneman, 1976), heart puncture (Cheng and Auld, 1977), head and foot flesh suction (Wang et al., 1994), and head and foot flesh extrusion (Tan et al., 2001). Each method has its particular advantages and disadvantages. While the foot plantaris puncture method is convenient and simple, hemolymph is sometimes wasted as the glutinous hemolymph easily sticks to the wall of the collecting tube and may block the tube nozzle due to the small dimensions of the pipette. The capsular discission method is an incision similar to the heart puncture method. It is possible to use both methods to collect high-quality hemolymph, however the accurate location of the heart must be determined before drilling a hole in the snail shell. The head and foot flesh suction method requires lengthy stimulation time, and only a very small quantity of hemolymph fluid is collected. The head and foot flesh extrusion method is applicable only for blood smears and is not recommended for hemolymph sampling.

In our *B. areolata* hemolymph collection experiment we used an improved foot plantaris puncture method. The principle of this method is similar to the head and foot flesh extrusion method. The sampled fluid, which is blue due to its ion content, is the same as the hemolymph in the lung's pulmonary vein in terms of unit volume and cell density, and is recognized as hemolymph fluid (Sminia, 1975). Instead of using a slender pipette as in the foot plantaris puncture method, we used pipette tips with cut-off heads which are convenient to use and prevent the hemolymph fluid from blocking the pipette nozzle.

Some of our experiences with hemocyte fluid collection are summarized below: The colorless, seawater-like fluid squeezed out from the gap between the shell and the foot when stimulating the foot plantaris with the tip of the liquid-transferring pipette could not be taken for hemolymph collection, however the slightly blue dense fluid subsequently extruded was hemolymph. In general, 90 μL of hemolymph could be sampled from a snail of approximately 3.05 cm shell length and 6.12 g weight. To avoid waste and shortage of the hemolymph, the foot plantaris was not strongly stimulated during sampling, and the hemolymph was extracted slowly. The hemolymph would also merge with other fluids if the foot plantaris was stimulated too strongly or if the hemolymph was squeezed out too quickly. This phenomenon could affect the result of the experiment. Thus, we found that the foot plantaris should be stimulated gently with the tip of the liquid-transferring apparatus, and no squeezing should be used. These were our modifications and improvements on the former foot plantaris puncture method.

Anticoagulant screening.

Anti-coagulants are extremely important in hemocyte research. In hemocyte research failure is often due to lack of an appropriate anti-coagulant, resulting in hemocyte death or aggregation. In this study, we selected four anti-coagulants previously identified, and in addition we prepared an anti-coagulant (E). All five of the anti-coagulants used in this study were screened for cell mortality rate and aggregation with anti-coagulants recorded over different time intervals. The mortality rates of snail hemocytes were very high in the prawn (Vargas-Albores et al., 1993), abalone (Lebel et al., 1996), and oyster (Xue et al., 2001) anti-coagulants, and aggregation was extensive. However, the home-made anti-coagulant E resulted in longer viability for snail hemocytes and greatly reduced aggregation. Consequently, we considered it to be the best anti-coagulant for hemocyte research of *B. areolata*. Based on these results we recommend the use of anti-coagulant E in hemocyte immunology research of *B. areolata*. We do not recommend the use of

anti-coagulants A, B, and D because of their high mortality rates and poor anti-coagulant effects.

Results from this experiment supplied valuable experience for gastropod research and will benefit the development of snail hemocyte research, contribute to the knowledge of gastropod immunology, gain greater insight into the cytology-based mechanisms of immune defense and disease resistance of hemocytes in the future, and also provide reference data for *B. areolata* aquaculture. In addition, anti-coagulant E also performed best in other hemocyte experiments on the abalone *Haliotis diversicolor* and *Scylla serrata* (unpublished data). Anti-coagulant E has the potential to become an ideal anti-coagulant for marine gastropods and crustaceans.

Conclusion

We modified and improved the foot plantaris puncture method for hemolymph sampling. And succeeded in demonstrating that anti-coagulant E prepared in our laboratory was the most appropriate hemolymph anti-coagulant for *Babylonia areolata*.

Acknowledgements

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