

## Immunological Study of Phagocytosis and Serum Lectin of *Scapharca subcrenata*

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### ABSTRACT

Lectin-like activity was demonstrated in the serum of the ark shell *Scapharca subcrenata*. Agglutinating activities of the serum were found against erythrocytes of human blood groups A B and O, and chicken, black bone chicken and mouse. The agglutinating titers were 256, 256, 8, 16, 64, 64 respectively. The experiment showed that the lectin-like activity was stable at a wide temperature and pH range. The activity could be inhibited by L-rhamnose, L-arabinose, maltose, D-galactose and sucrose. The three types of hemocytes of *S. subcrenata* phagocytosed *Candida albicans* cells *in vitro*. The optimum temperature of phagocytosis was 30°C, and the percentage of phagocytosing hemocytes attained to 62 %. When the experimental bivalves were challenged with *Vibrio* cells, the phagocytic activity of the hemocytes was much higher than controls injected with physiological saline. Subsequently, the phagocytic activity decreased, and returned to close to the level of controls at 72 h after injection.

### INTRODUCTION

The ark shell *Scapharca subcrenata* is a bivalve mollusc distributed along the coast of China, Korea and Japan. It is a common clam species and is commercially important in these countries.

Outbreaks of epidemic diseases cause mortalities in aquaculture farms all over the world making it important to study immune defense systems and to find an effective approach to combat disease. Bivalves do not possess an acquired immunity equivalent to that of vertebrates. Innate immune reactions, including phagocytic, cytotoxic or inflammatory responses by hemocytes, plus lysosomal enzymes and lytic and cytotoxic molecules in the serum all play an important role in the defense mechanism of the bivalve (Roch, 1999).

To investigate the immune defense system of the ark shell, we studied the lectin-like properties of the serum and the phagocytic reaction of the hemocytes.

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## MATERIALS AND METHODS

### Animals

*Scapharca subcrenata*, about 40mm in height, were collected from the coast of Qingdao and kept undisturbed in laboratory tanks for one week. Healthy ones were selected for experiments. During the culture, they were fed with microalgae *Isochrysis galbana* and *Platymonas subcordiformis*.

### Hemolymph collection

Hemolymph (1-1.5 ml/ark shell) was collected directly from adductor of the ark shell by using a thin dropper after careful cutting the adductor muscle. One part of the hemolymph was kept at 4°C for 12 h, then was centrifuged at 4 ° for 5min at 3000 x g. The supernatant was collected and kept below -20 ° for the measurement of serum lectin activity. The other part was mixed with the same volume of Alsever's solution to give a cell suspension for the phagocytosis assay.

### Agglutinating activity against erythrocytes

Human blood group A, B, O, chicken, black bone chicken (*Gallus domesticus*) and mouse erythrocytes were collected with Alsever's solution. The erythrocytes were harvested after centrifugation and washed with physiological saline twice, then were suspended in the same solution. Agglutination of erythrocytes was tested in a 96-well Titertek U-plate, in which samples were prepared by serial 2-fold dilution with physiological saline. The results were scored in terms of titer value according to the method of Shiomi *et al.* (1980).

### Stability study on serum lectin

In the heat-stability test, the serum was pretreated by waterbath at 20°C, 37°C, 50°C, 65°C, 80°C for 10 min and 30 min respectively, then agglutinating activity on black bone chicken erythrocytes was tested.

To study the influence of pH value on agglutinating activity, the serum of ark shell was prepared by serial 2-fold dilution with buffer solutions with different pH values ranging from 5 to 12. Agglutinating activity on black bone chicken erythrocytes was then tested.

### Agglutination inhibition test

Each of 12 kinds of monosaccharides and oligosaccharides (300 mmol/L, 30 mmol/L, 3 mmol/L), 4 fold dilution serum of ark shell, and the same volume of black bone chicken erythrocyte suspension were mixed in 96-well Titertek U-plate, and the agglutinating reaction was observed.

### Phagocytosis assay

*Candida albicans*, supplied by Applied Microbiology Lab, Ocean University of China, was used for the phagocytosis assay. After culture at 28 ° for 48 h on slant medium, the cells were washed with physiological saline and harvested in a centrifuge tube. After further centrifugation and resuspension in physiological saline, the yeast was diluted to a final concentration of 10<sup>8</sup> cells/ml and stored at 4 ° until use.

Reaction solution was designed as a mixture of 500 (1 hemocyte suspension and 40 (1 yeast suspension, and was treated in waterbath at 4 °C, 10 °C, 20 °C, 30 °C, 37 °C respectively. After incubation for 30 min, the reaction was stopped immediately by ice bath. The reaction solution was settled on glass slide, which was then fixed and stained by Wright's (Merck) solution. Observation was made under a NIKON E-800 optical microscope, and the phagocytic index, i.e. percentage of phagocytosing hemocytes was evaluated.

### **Effect of immune stimulation on the phagocytic activity**

The ark shell were divided into three groups. One group of 30 ark shell were injected with 20 µl of sterilized physiological saline suspension of *Vibrio anguillarum* (10<sup>8</sup> cells/ml) at the pelecypodium. The second group of 30 ark shell were treated with 20 µl physiological saline as control. Hemolymph was sampled at 4 h, 12 h, 72 h respectively after injection. The third group of 10 ark shell was left untampered. The hemolymph was sampled and a percentage of phagocytosing hemocytes was investigated.

## **RESULTS**

### **Properties of the serum lectin**

Serum from the abductor muscle of *Scapharca subcrenata* can agglutinate erythrocytes of human blood groups A, B, O, and of chicken, black bone chicken and mouse *in vitro* (Table 1).

**Table 1.** Hemagglutinating titer of the serum of *S. subcrenata*.

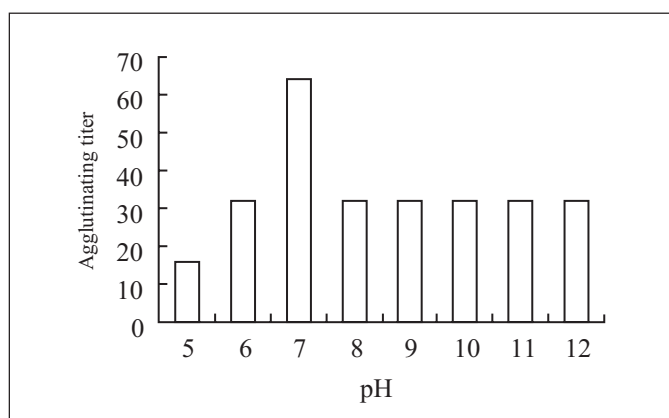
Erythrocyte	Human blood group A	Human blood group B	Human blood group O	Chicken	Mouse	Black bone chicken
Agglutinating titer	256	256	8	16	64	64

**Table 2.** Heat-stability of hemagglutination of the serum from *S. subcrenata*.

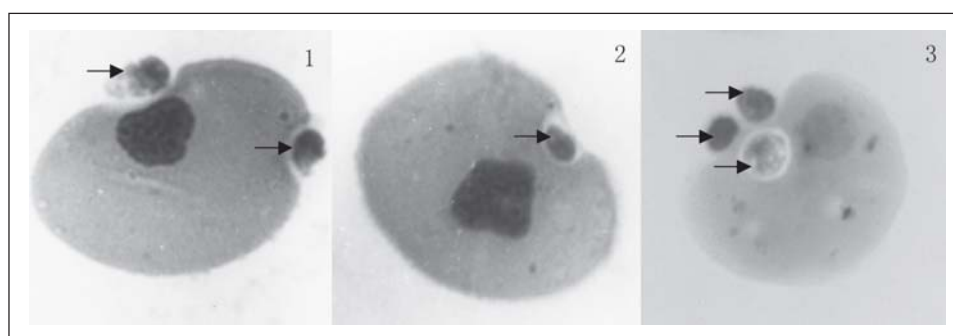
Treatment	20°C	37°C	50°C	65°C	80°C
10min	64	64	8	4	0
30min	64	32	8	0	0

After the serum was pretreated at different temperatures, the agglutinating titers using black bone chicken erythrocytes are shown in Table 2. The serum lectin-like activity kept its stability in a narrow temperature range. When the temperature reached 50°C, the agglutinating activity fell sharply.

Hemagglutination of black bone chicken erythrocytes occurred over a wide pH range, and the maximum activity appeared at pH 7.0 (Fig.1).



**Figure 1.** Agglutinating activities of serum at different pH value. Serum of ark shell was prepared by serial 2-fold dilution by buffer solution with different pH value ranging from 5 to 12. Agglutinating activity was tested here on black bone chicken erythrocytes.



**Figure 2.** Phagocytosis of *Candida albicans* by an erythrocyte of *Scapharca subcrenata*. Stained by Wright's (Merck) solution. Arrows indicate *Candida albicans* cells.

**Table 3.** Agglutination inhibition of various saccharides on serum

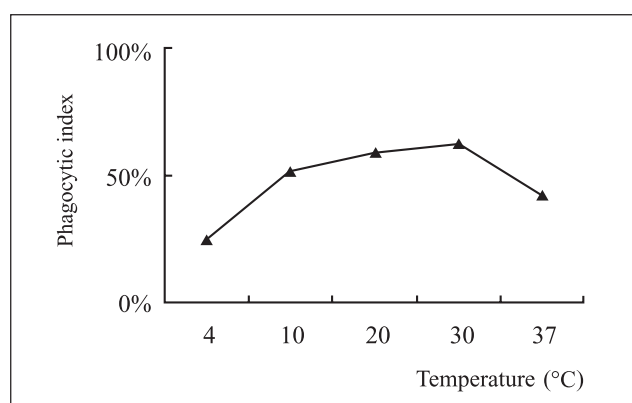
Concentration	D-fructose	L-rhamnose	L-arabinose	D-glucose	D-mannose	D-xylose
100mM	-	+	+	+	-	+
10mM	-	+	+	-	-	-
1mM	-	-	-	-	-	-

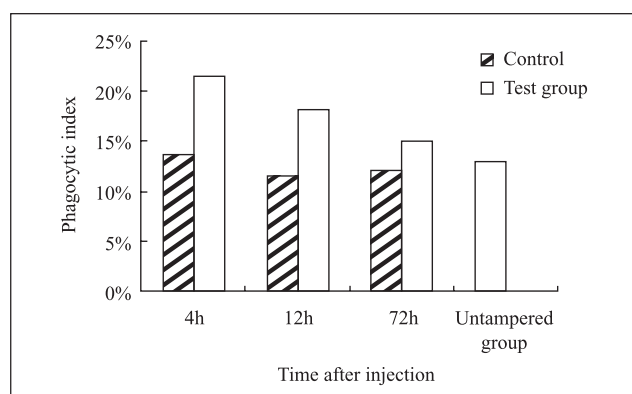
Concentration	Maltose	Lactose	D-galactose	Sorbose	Trehalose	Sucrose	Control
100mM	+	(+)	+	(+)	(+)	+	-
10mM	+	(+)	+	-	-	+	-
1mM	-	-	-	-	-	-	-

\* “+” strong inhibition; “(+)” weak inhibition; “-” no inhibition.

Among the 12 kinds of monosaccharides or oligosaccharides tested, L-rhamnose, L-arabinose, maltose, D-galactose and sucrose showed strong agglutinating inhibition activity using black bone chicken erythrocytes. D-glucose, D-xylose, and lactose showed weak agglutinating inhibition reaction. Other saccharides showed no effect (Table 3).



**Figure 3.** Effect of temperature on phagocytosis of *Scapharca subcrenata* hemocyte *in vitro*.



**Figure 4.** Effect of *Vibrio* cells on phagocytosis of *Scapharca subcrenata* hemocytes *in vitro*.

### The phagocytosis by the hemocytes of *Scapharca subcrenata*

Three main types of hemocytes were recognized according to their morphological characteristics, all of them phagocytosed *Candida albicans* cells *in vitro*, which illustrates the important role hemocytes play in the immune activity of the bivalve. At first, foreign yeast cells adhered to the surface of the hemocyte, then by an extension of the hemocyte cell membrane, the yeast cell was incorporated within the hemocyte as a phagosome (Fig. 2).

### Effect of temperature on the phagocytic index of the *Scapharca subcrenata*

*In vitro* phagocytic index of hemocytes was related to the environmental temperature (Fig. 3). In our study, the optimum temperature for phagocytosis *in vitro* was 30°C, and with a phagocytic index of 62%.

### Effects of immune stimulation on phagocytosis by hemocyte

After *Scapharca subcrenata* was challenged by *Vibrio* cells for 4 h, the phagocytic index increased markedly. Then the phagocytic index began to decrease gradually and was close to that of control group after 72 h of stimulation (Fig. 4). This increase didn't occur in *Scapharca subcrenata* injected with saline solution.

### DISCUSSION

It is reported that the hemolymph from the Pacific oyster, *Crassostrea gigas* (Vasta *et al.*, 1982), house mussel, *Modiolus modiolus* (Tunkijjanukij *et al.*, 1997), and sea mussel, *Crenomytilus grayanus* (Belogortseva *et al.*, 1998) have the ability to agglutinate vertebrate erythrocytes *in vitro*. Hemagglutinins were confirmed to exist in the hemolymph of these animals, and most of them are lectin-like, which have specific receptors for carbohydrate determinants (Lackie, 1980). In our experiment, lectin-like factors was found in the serum from the adductor muscle of *Scapharca subcrenata*, and behaved differently in agglutinating various kinds of vertebrate erythrocytes.

In the present study, the agglutinating activity of the serum lectin in *Scapharca subcrenata* was thermolabile and active over a wide pH reaction range, which is similar to that from other kinds of shellfish, such as *Saxidomus giganteus* (Johnsen, 1964), *Crassostrea virginica* (Tamplin *et al.*, 1989), *Patinopecten yessoensis* (Mori *et al.*, 1980a) and *Crassostrea gigas* (Mori *et al.*, 1980b).

Lectins interact with vertebrate erythrocytes or microorganisms by binding to the saccharides receptors on the surface of the cells (Sharon *et al.*, 1972). The interaction can be inhibited with the addition of specific saccharides which combine with the active sites of lectins. Thus specific saccharides act as regulators in the interaction between specific lectins and foreign cells. According to Parish's (1977) hypothesis, self/non-self discrimination in invertebrates is mainly based on the recognition of carbohydrate determinants by soluble or cell-bound oligomers of glycosyl-transferases. Lectins played an important role in immune regulation and signaling (Tunkijjanukij *et al.*, 1998). Lectins may also increase phagocytosis of bacterial pathogens by acting as opsonins (Olafsen *et al.*, 1992).

Erythrocytes, hyalinocytes, and granulocytes performing phagocytosis were observed on the blood film under an optical microscope, showing that these three types of hemocytes could act as phagocytic executant in cellular immune system of *Scapharca subcrenata*. It was reported that erythrocytes in ark shells possess the same function as in mammals i.e. transportation of oxygen (Chen *et al.*, 1996). Evidence was found in the present experiment that erythrocytes also played a role in the defense system of the ark shell.

Both granulocytes and hyalinocytes of *Tapes philippinarum* were able to phagocytose yeast and plasma (Cima *et al.*, 2000). In the presence of yeast cells, hydrolytic enzymes and oxidative enzymes were released to counter the pathogen. Our results with *S. subcrenata* are in agreement with the observation on *Tapes philippinarum*, whereas, in some other shellfish, only one cell type was reported to be involved in phagocytosis (Montes *et al.*, 1995; Moore *et al.*, 1977; Nakayama *et al.*, 1997). We also found that immune stimulation by virulent *Vibrio* cells could increase the cellular immune function of *S. subcrenata*. It was reported previously that the stimulation of short-term, low-concentration pollution was

beneficial to the improvement of the phagocytic activity of bivalves (Pipe *et al.*, 1995). After the snail *Planorbarius corneus* was injected with bacterial cells for 2 h, the bacterial elimination rate reached a peak value of 76%. It was found that the invading cells were almost removed after 192 h (Ottaviani *et al.*, 1986).

The conclusion drawn from the present study is that the phagocytosis by *S. subcrenata* hemocytes can be stimulated by foreign matter, such as bacterial cells. This reaction contributes to the immune defensive mechanisms of the mollusc. More work on the immune mechanisms of agglutination and phagocytosis and their interaction will be carried on in our laboratory.

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