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Separation of Shrimp Haemocytes (*Penaeus monodon*) and the Opsonic Effect of Serum on Phagocytic Activity

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Abstract

Two cell types of shrimp haemocytes (*Penaeus monodon*) were separated by continuous gradient centrifugation. Granular haemocytes and hyaline cells were demonstrated in vitro for their phagocytic activity using glutaraldehyde-fixed sheep red blood cells (SRBC[f]). The phagocytic activity of granular haemocytes was two times greater than that of hyaline cells. Haemocytes opsonized by shrimp serum increased phagocytic activity compared to the normal one, suggesting that opsonin in serum is one of the factors that play a role in the enhancement of shrimp phagocytosis.

Introduction

Black tiger shrimp, *Penaeus monodon* culture has become the most important aquaculture active in Thailand. However with rapid expansion, more disease problems have occurred. One approach to disease problems in shrimp culture is to increase the shrimp internal defense response. Immunity in invertebrates is defined as non-specific internal defense response that included both cellular and humoral components. The shrimp's circulating blood cells or haemocytes play an essential role in the cellular immune response, mediating vital reactions such as phagocytosis, encapsulation, nodule formation and killing of foreign cells (Ratcliffe et. al., 1985). Phagocytosis is one of the major defense mechanisms when the foreign particles or microorganisms are introduced

into the shrimp. Smith and Soderhall (1983) reported that phagocytic activity of the crayfish *Astacus astacus* and *Pacifastacus leniusculus* was evident only for hyaline cells and semigranular cells. Soderhall et. al., (1986) also reproted phagocytic activity for hyaline cells of the crab *Carcinus maenas*. Goldenberg et. al., (1984) demonstrated that phagocytosis of sheep erythrocytes (SRBC) by haemocytes of the American lobster, *Homarus americanus*, was greatly enhanced by SRBC opsonization with lobster haemolymph serum. Similar results were obtained with the kuruma prawn (*Penaeus japonicus*), showing that opsonic factor in haemolymph serum increased the phagocytic activity (Kondo et. al., 1992). Previous studies have reported that numerous substances, such as B-1,3 glucans, lipopolysaccharides, peptidoglycan and endotoxin or dead bacteria, can enhance the immune response of crustaceans (McKay and Jenkin, 1970; Paterson et. al., 1974; Smith and Soderhall, 1983; Soderhall et. al., 1985; Itami et. al., 1989; Boonyaratpalin et. al., 1995).

The purpose of this study was to describe a method for separating the haemocyte populations of the black tiger shrimp, *Penaeus monodon*, through density gradient centrifugation and to determine the precise phagocytic activity of those various cell types with and without opsonization.

Materials and Methods

Shrimp samples

Black tiger shrimp (*P. monodon*) were obtained from culture farms where there were no reports of viral diseases. They were held in chlorinated sea water with aeration at 28 ± 1 °C and salinity between 28 ± 30 ppt. They were fed four times daily with commercial shrimp feed for one to two weeks before the start of experiments.

Preparation of gradients

Haemolymp was separated through continuous gradient according to the method of Soderhall and Smith (1983) and Kondo et. al., (1992) with some modifications. Briefly, 7 ml of 60% Percoll (Pharmacia, Uppsala Sweden) in 2.8% NaCl gradients were formed in round bottom polycarbonate tubes by centrifugation in an angle-head rotor (KOKUSAN-H200R, Japan) at 11,000 Xg for 30 min at 4 °C.

Bleeding and haemocyte separation

Haemolymph was withdrawn from the ventral sinus, using a 3-ml plastic syringe containing 0.5 mg cysteine/ml modified k-199 medium (Table 1) as an anticoagulant. The ratio of haemolymph to anticoagulant was 1:3 (v/v). One millilitre of the diluted haemolymph was then added immediately to the top of preformed Percoll gradients and centrifuged in a swing out rotor (KOKUSAN-H200R, Japan) at 1,700 xg for 10 min at 4 °C. Either granular or hyaline

cells were collected from the separation bands. Cell viability was measured by trypan blue exclusion. The number haemocytes was adjusted to $2x10^5$ cells/ml with modified k-199 medium.

Preparation of fixed SRBC

SRBC were prepared by washing these three times using phosphate buffer saline (PBS) before suspension in the same buffer as 2% (v/v). The SRBC were then fixed with 0.25% glutaraldehyde solution in 50 mM Tris-HCI, pH 7.6 containing 500 mM NaCl(TBS) and washed five times with TBS. The fixed $SRBC^f$ were suspended in the same buffer, giving a final concentration of 10% suspension (v/v).

Preparation of serum

Haemolymph was bled from the shrimp using plastic syringes and stored at 4°C for 24 hr. The clot was then removed by centrifugation at 12,000 xg for 30 min at 4°C and the serum was stored at -20°C until use.

Preparation of serum-treated SRBC

$SRBC^f$ were washed with the modified k-199 medium, adjusted to $5x10^8$ cells and incubated in shrimp serum at 20°C for 1 hr. The serum treated $SRBC^f$ were washed three times with modified k-199 medium and suspended in the same medium at a concentration of $1x10^7$ cells/ml.

Phagocytosis assay

From each gradient purification, 200-ml of hyaline cells and granulocyte suspension ($2x10^5$ cells/ml) was added to a 3.5 cm Petri dish containing a 22x22-mn precleaned glass slide and separately incubated at room temperature until monolayers were formed. Two milliliters of $SRBC^f$ and serum treated $SRBC^f$ containing $1x10^7$ cells/ml were inoculated separately onto these monolayers. After incubation in a moisture chamber for 45 min at room temperature, the haemocyte monolayers were then fixed for 5 min using a 0.125% glutaraldehyde solution in modified k-199 medium and stained with Giemsa. In order

Table 1. Components of modified k-199 medium.

Component	g/liter
M-199 (2x)	500 ml/liter
Hepes	2.38
$MgCl_2.6H_2O$	3.3
$MgSO_4 .7H_2O$	3.0
NaH_2PO_4	0.05
NaCl	11
$CaCl_2 .2H_2O$	0.9
L-glutamine	0.15

* Finally adjusted to pH 7.6 with 1 N NaOH.

to determine the percentage of phagocytosis, the total cells and number of phagocytosing cells were counted, using a phase-contrast microscope (Olympus-BH-2, Japan). Four hundred cells per slide were counted using two slides for each treatment.

Statistical tests

Analysis of variance and Duncan's new multiple range test were used to compare the means. Results with $p<0.05$ were considered significant.

Results

Separation of black tiger shrimp haemocytes

Two layers of black tiger shrimp haemocytes resulted in Percoll continuous density gradient centrifugation as shown in Figure 1. The haemocytes of each layer were observed under a phase-contrast microscope. The upper layer comprised of large numbers of hyaline cells with few or no granules, while the lower layer comprised of granular cells.

Opsonic effect of serum and phagocytosis of haemocytes on black tiger shrimp

Both hyaline cells and granular cells had phagocytic activities against $SRBC^f$. The phagocytic activity of the granular cells was two times higher than that of the hyaline cells. $SRBC^f$ treated with haemolymph serum gave a significant increase in phagocytic activity for both haemocyte types (Table 2).

Discussion

The results presented in this paper demonstrated that morphologically distinct haemocyte populations from *Penaeus monodon* could be isolated through density gradient centrifugation on Percoll, and that the separation probably also represented a functional difference of haemocyte types.

In this experiment, both hyaline cells and granular cells of black tiger shrimp had the capacity to phagocytose $SRBC^f$. Similar results were obtained in a study by Kondo et. al., (1992) where all three types of kuruma prawn haemocytes were ingested $SRBC^f$ and the phagocytic activities of semigranular cells and granular cells were significantly higher than that of hyaline cells. Smith and Ratcliffe (1978) demonstrated that phagocytosis is mainly achieved by phagocytic cells which have a variable number of small (less than 1.0 mm) intracellular granules. By contrast in our experiments, hyaline cells with few or no granules also showed phagocytic activity. It is not known whether the crustacean haemocyte types themselves showed a species-related difference in phagocytic activity.

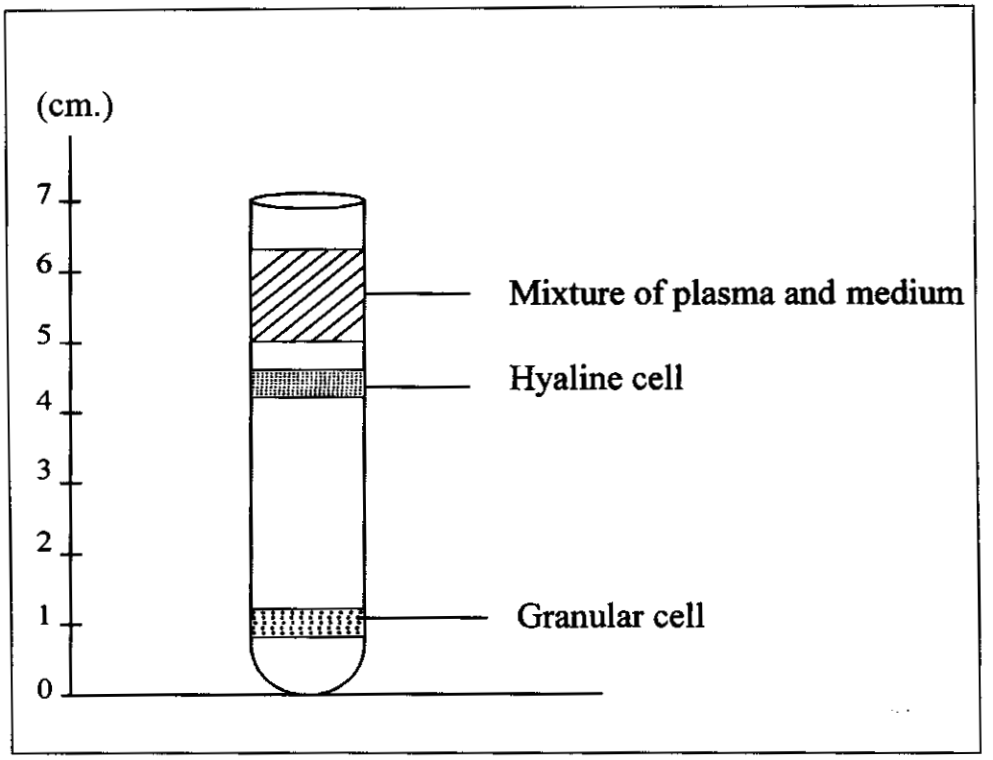

Fig. 1. Separation of black tiger shrimp haemocytes by Percoll continuous density gradient centrifugation.

Table. 2 Opsonic effect of black tiger shrimp serum on the phagocytosis of glutaraldehyde-fixed sheep red blood cells by hyaline cells and granular cells.

Treatment	% phagocytosis	
	Hyaline cells	Granular cells
$SRBC^f$	8.42 + 0.901[a]	16.56 + 2.349[a*]
$SRBC^f$ + serum	24.90 + 4.204[b]	38.302 + 5.392[b*]

*[1]Means with different letters are significantly different ($p<0.05$) when compared within cell types. Asterisks indicate significant differences between treatments.

Opsonization of $SRBC^f$ with black tiger shrimp serum in our experiments significantly increased phagocytic activity. Tyson and Jenkin (1973) reported significantly enhanced in vitro phagocytosis following pre-incubation of foreign particles in crayfish serum. The serum is known to contain agglutinins against vertebrate erythrocytes and their adsorption from the serum was found to abolish its opsonic properties (Mckay and Jenkin, 1970). Ratanapo and Chulavatnatol (1990) reported the presence of a sialic acid-specific lectin called monodin in the serum of *P. monodon*. Kondo et. al., (1992) also reported that the opsonic activity of kuruma prawn serum for phagocytosis was dependent on lectins in the serum. Therefore, the opsonic activity of lectins in shrimp serum should be investigated further.

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