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Bacterial Agglutination by Sialic Acid-Specific Lectin in the Hemolymph of the Banana Shrimp, *Penaeus* (*Fenneropenaeus*) *merguiensis*

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ABSTRACT: A lectin from the hemolymph of the banana shrimp *Penaeus* (*Fenneropenaeus*) *merguiensis* expressed higher agglutination activity against rabbit erythrocytes than those from human, and its activity was Ca^{2+}-dependent. The hemagglutinating activity of the hemolymph lectin was stable up to 55 °C and optimal at pH 7.5-8.0. N-acetylated sugars, ManNAc, GlcNAc, GalNAc, and NeuNAc, were effective inhibitors of the lectin induced hemagglutinating activity, with NeuNAc being the most powerful. Porcine stomach mucin and fetuin showed high inhibitory activity to the hemolymph lectin-induced hemagglutination. In addition, the hemolymph lectin selectively agglutinated *Vibrio harveyi* and *V. parahemolyticus*, which are pathogenic to *P. merguiensis*, and to a lesser extent, *V. vulnificus*, but had no effect on the non-pathogenic *V. cholerae*, *Samonella typhi* and *Escherichia coli*. This observation suggests that lectin present in the hemolymph of *P. merguiensis* may contribute to the defense mechanism of this species against bacterial infections.

KEYWORDS: lectin, crustacean, *Penaeus* (*Fenneropenaeus*) *merguiensis*, banana shrimp, hemolymph.

INTRODUCTION

Lectins are naturally occurring proteins or glycoproteins which bind selectively and non-covalently to carbohydrate residues. The main characteristic of this class of protein is their ability to interact specifically with carbohydrates and combine with glyco-components of the cell surface.[1] They have been considered to serve as recognition molecules in cell-cell or cell-matrix interactions.[2] Lectins with diverse physiological roles are distributed in a wide range of organisms from bacteria to animals and plants. Invertebrates lack an adaptive immune system and they depend on an innate immune system for defense against invading microorganisms, which has been a subject of interest.[3] For example, stimulation of hemolymph lectin activity by pathogenic bacteria has been demonstrated in Pacific oyster, *Crassostrea gigas* and silk moth, *Antheraea pernyi*.[4,5] A survey of the serum obtained from marine invertebrates revealed the presence of sialic acid-specific lectins. The relevance of these lectins in the host defense system relies on the observation that sialic acid, an important constituent of many glycoconjugates, is present on different cell surfaces.[6,7] In crustaceans, sialic acid-specific lectin of Indian horseshoe crab, *Carcinoscorpus rotunda cauda*, could agglutinate many types of bacteria and a lectin identified from the hemolymph of the blue crab *Callinectes sapidus* has also been found to possess agglutinating activity against several serotypes of *Vibrio* spp.[8,9] Bacterial agglutination has been demonstrated with lectins from the black tiger shrimp *Penaeus monodon*, brown shrimp *Penaeus californiensis*, edible crab *Scylla serrata*, freshwater prawn *Macrobrachium rosenbergii*, and *Liocarcinus depurator* as well, suggesting their possible roles as anti-bacterial agents.[10-14]

To the best of our knowledge, no lectins have yet been reported in the hemolymph of the banana shrimp *Penaeus* (*Fenneropenaeus*) *merguiensis*. This shrimp is one of the most economically important species in the tropical countries. We hereby describe the anti-bacterial activity of the lectin in the hemolymph of *P. merguiensis* against certain pathogenic bacteria of this species.

MATERIALS AND METHODS

Animals and Hemolymph Preparation

Adult *P. merguiensis* (25-40 g BW) were collected from Nakhon Si Thammarat, Thailand and transferred to the Aquatic Health Research Center, Prince of Songkla University (PSU). They were maintained in 30-35 ppt seawater with adequate aeration and fed with pellets 4 times a day. Hemolymph was withdrawn from the

pericardial sinus with a hypodermic syringe provided with a 26 gauge needle. Clotting was allowed at 4 °C overnight, and the hemolymph was then centrifugated at 2,500× g at 4 °C for 20 min. The cell-free hemolymph was immediately used or stored as aliquots at -20 °C for further analysis. The protein content of the hemolymph was measured according to the method of Lowry *et al*.[15] using bovine serun albumin (BSA) as standard.

Hemagglutination Assays

Whole blood was obtained from a healthy rabbit kept in the Animal Facilities at the Faculty of Science, PSU and immediately mixed with heparin. Erythrocytes were washed several times in TBS (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl) by centrifugation at 2,500 × g for 10 min at 4 °C and resuspended in the same buffer as 2% cell suspension. Hemagglutinating activity (HA) of the *P. merguiensis* hemolymph lectin was assayed in a 96-well microtiter U plate (NUNC, Denmark) according to a two-fold serial dilution procedure. The samples were diluted with TBS and then 25 µl each was mixed with 25 µl of a 2% suspension of rabbit erythrocytes. The mixture was left at room temperature for 1 h, and HA was observed. Positive hemagglutination was obtained when the erythrocytes did not sediment to the bottom of the well forming a red button. The titer was recorded as the highest dilution that still caused agglutination. The HA was defined as the reciprocal of the titer and specific HA was expressed as the activity per mg protein.

Specificity of the lectin to human erythrocyes was performed in a similar manner. Human red blood cells types A, B, AB, and O were bled from healthy donors.

Sugar Specificity of Hemagglutinating Activity

Sugar specificity of the hemolymph lectin was investigated by competitive inhibition using the following sugars and glycoproteins: D-galactose, D-glucose, D-mannose, N-acetyl galactosamine (GalNAc), N-acetyl glucosamine (GlcNAc), N-acetyl mannosamine (ManNAc), N-acetyl neuraminic acid (NeuNAc), porcine stomach mucin, fetal calf serum fetuin, fetal calf serum asialofetuin and bovine submaxillary mucin. Stock solutions of the sugars and glycoproteins were prepared in TBS, pH 7.5, and stored at -20 °C until use. For the inhibition test, 25 µl of serial two-fold dilutions of the various sugars or glycoproteins was first added to each well of a 96-well microtiter plate. Thereafter, an equal volume of hemolymph lectin (titer 1:64) was added to each well, mixed by gentle shaking, and incubated for 1 h at room temperature. Finally, 50 microliter of a 2% rabbit erythrocyte suspension was added. The minimum concentration of the inhibitors required to completely block agglutination was observed after 1 h of incubation at room temperature.

Effects of Temperature, pH and Divalent Cations on Hemagglutinating Activity

The effect of temperature on the HA of the hemolymph lectin was investigated after incubation of the hemolymph in TBS for 20 min at various temperatures. After cooling at 4 °C, the residual HA was measured against a 2% rabbit erythrocyte suspension, as described previously.

The effect of pH on the HA of the hemolymph lectin was examined by allowing hemagglutination to occur in various buffers containing 0.15 M NaCl for 1 h at room temperature as described previously. The following buffers were used: 50 mM sodium acetate, pH 4-6; 50 mM Tris-HCl, pH 6-9 and 50 mM glycine-NaOH, pH 9-11.

To determine the effect of EGTA on HA, hemolymph lectin was dialyzed overnight against deionized water. The HA activity was then tested in TBS, pH 7.5 in the presence of various concentrations of EGTA. To test the dependency of HA on divalent cations, the hemolymph lectin was diluted with TBS containing 0.075 mM EGTA to a titer of 1:64 and then 25 microliter of the solution was mixed with the same volume of TBS containing various concentrations of Ca^{2+} or Mg^{2+}. After standing at room temperature for 45 min, HA was determined by addition of 50 microliter of a 2% rabbit erythrocyte suspension and read after 1 h of incubation at room temperature.

Preparation of Bacteria

Three pathogenic bacteria of *P. merguiensis*: *Vibrio vulnificus*, *V. parahemolyticus* and *V. harveyi*, were obtained from the Aquatic Health Research Center, PSU. Each isolate was grown overnight with a tryptic soy agar slant containing 2% NaCl at 37 °C. Other bacterial isolates, *Escherichia coli*, *Salmonella typhi* and *V. cholerae*, were obtained from the Fish and Fisheries Product Quality Control and Research Center, Songkhla, Thailand. Each was grown with a Luria- Bertani medium slant at 37 °C overnight. Growth of bacteria in liquid medium was initiated by using a single colony of bacteria in 5 ml of tryptic soy broth containing 2% NaCl. The overnight bacterial culture was then inoculated into tryptic soy broth containing 2% NaCl to give a final 0.2% inoculum and incubated at 37 °C with continuous rotary shaking at 200 rpm. An aliquot of the bacterial culture was removed at 1 h intervals during the growth period of 15 h. The bacterial growth was followed by measuring the absorbance of the collected aliquots at 600 nm. The bacterial cells in each suspension were harvested, washed three times and resuspended in TBS. The turbid suspensions were adjusted to approximately 10^9 cells/ml.

Bacterial Agglutination Test

Hemolymph lectin was two-fold serially diluted with TBS. Fifty microliters of the diluted lectin were mixed in each tube with 50 µl of the bacteria suspension ($5x10^7$ cells) and left at room temperature for 1 h. Afterwards, the suspension was shaken twice at a full speed on a Vortex-Genie 2 for 20 sec each. One drop of the mixture was then placed on a slide and covered with a cover-slip. The agglutination was observed under a light microscope with dark-field illumination. The bacterial agglutination titer (BAU) was expressed as the highest dilution of the lectin giving a complete agglutination of the bacterial cells. As a control, TBS was used in the same manner instead of the lectin solution.

Results and Discussion

Hemagglutination Properties of the Hemolymph Lectin

Lectin in the hemolymph of *P. merguiensis* could agglutinate erythrocytes from both rabbit and human, with higher titer against rabbit erythrocytes at a minimum lectin concentration of 0.55 mg/ml. The reaction against human erythrocytes showed no specificity as to blood groups (Table 1). Its agglutination

Table 1. Hemagglutinating activity of *Penaeus merguiensis* lectin in hemolymph.

Species	Hemagglutination (unit/mg protein)	Minimum concentration required (mg/ml)
Human blood A erythrocyte	35.9	1.10
Human blood B erythrocyte	35.9	1.10
Human blood O erythrocyte	35.9	1.10
Human blood AB erythrocyte	35.9	1.10
Rabbit erythrocyte	71.8	0.55

activity was similar to that of lectins found in *Litopenaeus schmitti*, *Penaeus indicus* and *Litopenaeus setiferus* which did not show specificity to human blood groups.[16-18]

Treatment of hemolymph lectin with EGTA abolished the HA completely at 0.075 mM (Fig. 1). The activity was completely restored with 0.075 mM $CaCl_2$. At concentrations up to 200 mM, $MgCl_2$ showed no capacity to rescue the HA of the EGTA-treated lectin. The finding suggests that the HA of hemolymph lectin of *P. merguiensis* is calcium-dependent, similar to the HA of most arthropod lectins. A variety of crustacean lectins have been demonstrated to be C-type[19], a lectin family characterized by calcium-dependent binding activity,

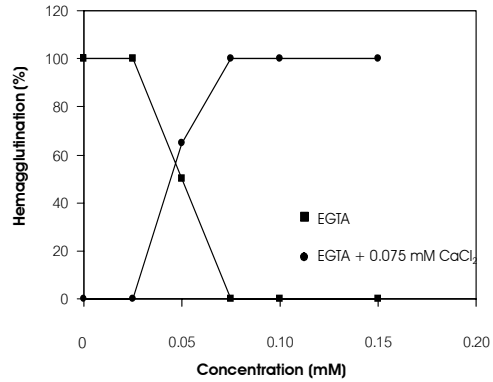

Fig 1. Effect of EGTA and $CaCl_2$ on the hemagglutinating activity of hemolymph lectin of *Penaeus merguiensis*. The hemagglutinating activity of dialyzed hemolymph lectin was assayed in the presence of different concentrations of EGTA (■). Hemolymph lectin diluted in TBS containing 0.075 mM EGTA, was assayed for hemagglutinating activity in the presence of various concentrations of $CaCl_2$ (●).

including those of *P. monodon*, *P. stylirostris*, *P. californiensis* and *Parapenaeus longirostris*.[11,20-22] By contrast, the lectins of *L. schmitti*, *P. indicus* and *P. japonicus* does not require calcium for their activity.[16,23,24]

Hemolymph lectin retained full HA over the temperatures range of 0-50 °C, but the activity decreased markedly between 55 and 70 °C, and the activity was completely abolished at 75 °C (Fig. 2). The presence of 50 mM $CaCl_2$ caused a slight delay in the heat-inactivation. The optimum pH of hemolymph lectin

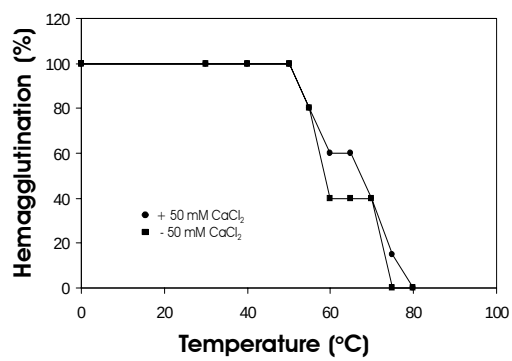

Fig 2. Thermal stability of hemolymph lectin of *Penaeus merguiensis*. Hemolymph lectin was incubated in TBS for 20 min at various temperatures. After cooling at 4 °C, the remaining hemagglutinating activity was determined against a 2% rabbit erythrocyte suspension at room temperature. The residual activity is shown as the percentage of hemagglutinating activity at each temperature in relation to that of the native protein. The same experiments were performed in parallel in the presence of 50 mM $CaCl_2$.

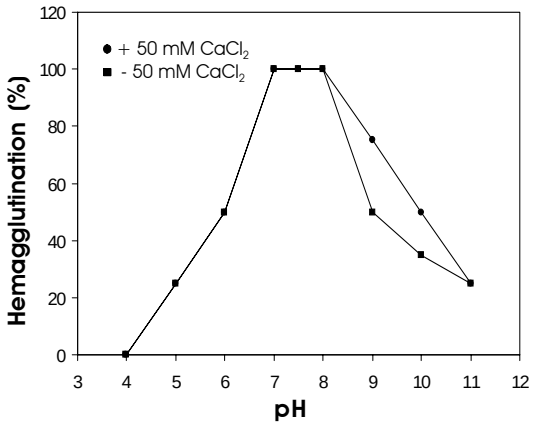

Fig 3. Effect of pH on the hemagglutinating activity of hemolymph lectin of *Penaeus merguiensis*. The hemolymph was incubated in various buffers containing 0.15 M NaCl for 45 min at room temperature. The following buffers were used: 50 mM sodium acetate, pH 4-6, 50 mM Tris-HCl, pH 6-9 and 50 mM glycine-NaOH, pH 9-11. The residual activity was determined against a 2% rabbit erythrocyte suspension at room temperature and shown as the percentage of hemagglutinating activity at each pH in relation to that at pH 7.5. The same experiments were carried out in parallel in the presence of 50 mM $CaCl_2$.

to agglutinate rabbit erythrocytes was 7.0-8.0; the activity was reduced below pH 7.0 and above pH 9.0, and completely abolished at pH 4.0 (Fig. 3). Again, the HA increased in the presence of 50 mM $CaCl_2$.

Sugar Specificity of the Hemolymph Lectin

The sugar specificity of the hemolymph lectin was examined by competitive inhibition of various sugars and glycoproteins on the HA of hemolymph lectin against rabbit erythrocytes. Many N-acetylated sugars were inhibitory with NeuNAc (or sialic acid) being the most effective (Table 2). A 1.0 mM concentration of NeuNAc, at 1:64 titer, inhibited the hemolymph lectin, while 4-fold higher concentrations of GalNAc and GlcNAc and a 16-fold higher concentration of ManNAc, were required to inhibit the same activity of the lectin. D-galactose, D-glucose, or D-mannose, at 200 mM, had no inhibitory activity. The same preferential affinity to NeuNAc has been reported in lectins from *P. monodon*, *P. indicus*, *P. paulinensis*, *L. setiferus* and *L. schmitti*.[16,18,20,21,23] In contrast, lectins from *P. californiensis*, *M. rosenbergii* and *Diogenes affinis* do not apparently discriminate among different N-acetylated aminosugars.[11,25,26] Lectins of the crayfish *Pacifastacus leniusculus* and the crab *S. serrata* have a binding affinity only to sialoglycoconjugates, but not to free NeuNAc.[27,28]

Table 2. Inhibition of hemagglutinating activity of hemolymph lectin of *Penaeus merguiensis* by sugars and glycoproteins.

Inhibitors	Minimum concentration for inhibition[a]
N-Acetyl neuraminic acid	1.0 mM
N-Acetyl glucosamine	4.0 mM
N-Acetyl galactosamine	4.0 mM
N-Acetyl mannosamine	16.0 mM
Porcine stomach mucin	0.67 mg/ml
Fetal calf serum fetuin	1.0 mg/ml
Fetal calf serum asialofetuin	NI[b]
Bovine submaxillary mucin	NI[b]
D-Glucose	NI[b]
D-Galactose	NI[b]
D-Mannose	NI[b]

[a] Minimum concentration to completely inhibit the hemolymph lectin at 1:64 titer in the presence of 2% rabbit erythrocyte suspension.
[b] NI: no inhibition of agglutination at 200 mM sugars or at 5 mg/ml glycoproteins.

Lectins from other invertebrates, such as the oyster *Creassostrea virginica* and the mussel *Mytilus edulis* show specificity for GlcNAc.[29,30] Those of the snail *Achatinina fulica* and the mollusc *Helix pomatia* are specific for O-acetylated and N-acetylated sugar residues, respectively, suggesting that specificity for N-acetylated sugars may be preserved in invertebrates.[31,32]

From the glycoproteins tested, porcine stomach mucin and fetal calf serum fetuin inhibited of the hemolymph lectin of *P. merguiensis* to a similar extent. Fetal calf serum asialofetuin and bovine submaxillary mucin (BSM) at concentrations up to 5 mg/ml had no effect on the HA (Table 2). Unlike that of *P. merguiensis*, lectins from hemolymph of *L. schmitti*, *P. monodon*, and *L. setiferus*, were inhibited by BSM.[16,18,20] Lectins reported from other decapod crustaceans, such as from the giant freshwater prawn *M. rosenbergii*, *Cancer antennarius*, and *Liocarcinus depurator*, also showed specificity for sialic residues.[13,14,33] These observations suggest that sialic or acetylated moieties may be required for interaction with the lectin.

Bacterial Agglutination of the Hemolymph Lectin

Among three pathogenic *Vibrio* species of penaeid shrimp, *V. harveyi* is the predominant one. To elucidate the possible role of hemolymph lectin in the bacteria-infected shrimp, lectin-induced agglutination of the three types of bacteria: *V. vulnificus*, *V. parahemolyticus* and *V. harveyi*, was investigated and they were found to be agglutinated strongly by the lectin. In contrast, the lectin did not agglutinate *V. cholerae*, *E. coli* and *S. typhi*. Figure 4 shows the agglutination of *V. harveyi* by the hemolymph lectin, the pattern of which is similar to that of *V. vulnificus* and *V. parahemolyticus* (data not shown). Agglutination assays revealed that a lectin

Table 3. Agglutinating activity of hemolymph lectin of *Penaeus merguiensis* against various pathogenic and non-pathogenic bacteria.

Bacterial strain	Agglutination (BAU/mg protein)[b]	Minimum concentration required (mg/ml)[c]
Vibrio harveyi[a]	4.48	8.9
Vibrio parahemolyticus[a]	4.48	8.9
Vibrio vulnificus[a]	2.24	17.8
Vibrio cholerae	0	No agglutination
Escherichia coli	0	No agglutination
Salmonella typhi	0	No agglutination

[a] Pathogenic bacteria.
[b] Agglutinating activity units tested in the presence of each species of bacteria.
[c] Minimum concentration required for a complete agglutination of the bacteria by the hemolymph lectin.

concentration of 8.9 mg/ml was sufficient to agglutinate 10^9 cells/ml of *V. harveyi* and *V. parahemolyticus* (Table 3). Agglutinating activity against *V. vulnificus* was also observed, but was lower by 2 fold. Under the same condition, the hemolymph lectin did not cause any agglutination of *V. cholerae*, *E. coli* and *S. typhi* (Table 3).

Concerning the recognition of microorganisms, many studies have examined the specificity of crustacean lectins towards different bacteria or components of their cell walls.[10,14,23,25,33] In this study, the hemolymph lectin strongly agglutinated major infectious bacteria, *V. harveyi*, *V. parahemolyticus,* and to a lesser degree, *V. vulnificus*, but not the non-pathogenic *E. coli, S. typhi* and *V. cholerae*. This selective agglutination towards bacterial cell wall has been noticed previously.[11,22,27,33] A preference for recognition of *Vibrio* species was also observed in the agglutination of bacteria by hemolymph of the eastern oyster *C. virginica* and the blue crab *C. sapidus*.[9,34] Our results of the selective binding of hemolymph lectin to some of the shrimp pathogenic bacteria suggest that the lectin plays a role in the defense against these pathogenic bacteria.

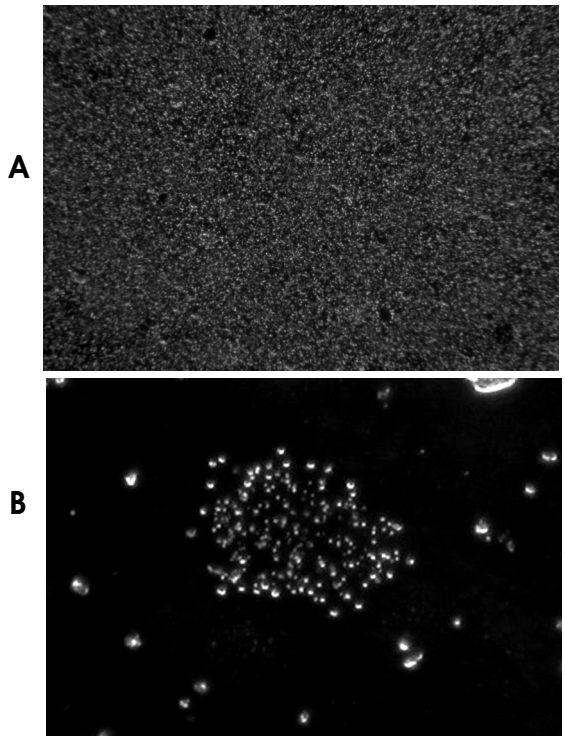

Fig 4. Agglutination of hemolymph lectin of *Penaeus merguiensis* against *Vibrio harveyi*. The bacterial sample ($5x10^7$ cells) was mixed with TBS (A) or the hemolymph lectin (B). After incubation at room temperature for 1 h, one drop of the mixture was placed on a slide and covered with a cover-slip. The agglutination was observed under light microscopy with dark-field illumination..

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