Journal of Science, Technology, and Humanities : January - June 2008, Vol. 6, No. 1, pp. 5-17

ANTIBACTERIAL PROTEINS IN THE SERUM HEMOLYMPH AND HEMOCYTE LYSATE SUPERNATANT OF THE BANANA PRAWN, *PENAEUS MERGUIENSIS*.

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ABSTRACT

As invertebrates do not synthesize specific antibodies, a number of antibacterial proteins and lectins have been isolated from their serum hemolymph and hemocytes. Antibacterial activity in serum hemolymph and hemocyte lysate supernatant (HLS) of the banana prawn, *Penaeus merguiensis* was investigated *in vitro*. The marine bacterium, *Vibrio* spp., *Escherichia coli* and *Staphylococcus aureus* were used as the test organisms. Hemocytes from *P. merguiensis* are classified into three main populations: hyaline, small-granular and large-granular under light microscope like other crustaceans. Hyaline cell refers to hemocytes that their cells contain no cytoplasmic granules, whereas granulocytes (small-granular and large-granular) contain abundant granules. Granulocytes and hyaline cells are present at 91 and 5 % of the circulating hemocytes, respectively. Proteins in serum hemolymph and HLS possess high antibacterial activity against Gram-negative bacteria. Antibacterial proteins are mainly located in serum and hemolymph. The serum hemolymph and HLS of banana prawn agglutinate erythrocytes of different vertebrate species. Lectin from hemolymph of banana prawn was isolated by affinity chromatography on mucin-CNBr-activated Sepharose 4B and gel filtration chromatography on Sephacryl S-200. The corresponding protein consisted of two subunits: 30.09 (PML1) and 28.01 kDa (PML2). The purified lectin strongly reduced the viable count of the test bacteria. These results show that one of the antibacterial proteins in the serum hemolymph and HLS from *P. merguiensis* is a lectin and involves in shrimp immune responses.

Keywords: Lectin, *Penaeus merguiensis*, antibacterial activity, *Vibrio* species, serum, hemolymph, hemocyte.

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INTRODUCTION

One of the fastest growing aquaculture productions is that of the penaeid shrimp. The banana prawns, *Penaeus merguiensis*, are found in Indo -West pacific: from the Persian Gulf to Thailand, Hong Kong, the Philippines, Indonesia and Australia. From 1980 to 2004, the production of captured shrimp increased more than two-fold, while aquaculture of shrimp increased more than four-fold. However, most shrimp cultures in the Asia-Pacific region have similar disease problems caused by pathogenic bacteria and virus. *Vibrio* species, the major of bacterial diseases, are normal part of the bacterial flora in aquatic environments (Lightner and Redman, 1988; Ruangpan and Kitao, 1991). The isolation of Vibrio species from water or diseased shrimp are *V. parahemolyticus, V. harveyi, V. alginolyticus, V. mimicus and V. damsela* (Lui et al., 2004; Vandenberghe et al., 1999).

Because marine invertebrates are naturally exposed to microorganisms, the defense systems against microorganisms are dependent on an innate immune system by a cellular and humoral factors. The cellular defense system includes phagocytosis, encapsulation, nodule formation, melanization and coagulation (Franc and White, 2000; Johansson et al., 2000; Montano-Perez et al., 1999). The other system is a humoral immunity, which is a relatively short-term protective memory to include lectin, lysin, antimicrobial peptides and other killer substances to eliminate or inactivate the pathogenic attack (Armstrong et al., 1996; Cooper and Lemmi, 1981).

For crustaceans, hemocytes are divided into three types, hyaline, semi-granular and granular cells (Bachere et al., 1995). The hyaline cells are involved in the initiation of the hemolymph coagulation. (Hose et al., 1990). The semi-granular cells, which contain small granules, display some phagocytic capacities and specialize as small invader encapsulation (Persson et al., 1987). The semi-granular cells recognize lipopolysaccharides and β-1,3-glucans, while the granular cells do not respond to these polysaccharides (Söderhalländ Cerenius, 1992). Agglutinin or lectins are proteins or glycoproteins from hemolymph of crustaceans as potential molecules involved in immune recognition in phagocytosis through opsonization (Marques and Barracco, 2000). In addition, lectins from invertebrates play a role as defense molecules having antibacterial activity or bacterial agglutination activity (Arason, 1996; Tunkijjanukij and Olafsen, 1998). Bactericidal activities against Gram-negative bacteria have been clarified in the hemolymph of *P. monodon* using the colony-forming units inhibition assay (Adam, 1991). Antibacterial activities in marine crustacean decapods are reported in *Pandalus borealis* (northern shrimp), *Pagurus bernhardus* (hermit crab), *Hyas araneus* (spider crab) and *Paralithodes camtschatica* (king crab) (Haug et al., 2002). The penaeidins are the first antimicrobial peptides found in penaeid shrimps. Penaeidins are isolated from the hemocytes of the Pacific white shrimp *(Litopenaeus vannamei)*. Their structures are characterized along with their antimicrobial activities (Destoumieux et al., 1997). The antimicrobial lectin, named scyllin, is isolated from the edible crab *(Scylla serrata)* (Chattopadhyay et al., 1996). Therefore, the production of antimicrobial peptides and proteins is important for host defense. The larger antimicrobial proteins, containing more than 100 amino acids, are often lytic enzymes or contain domains that target specific microbial macromolecules. Almost all antimicrobial peptides are cationic and amphipathic. Antimicrobial peptides have been found in the epithelial layers, phagocytic cells and body fluids of multicellular animals (Ganz, 2003).

This study was aimed to compare antibacterial proteins in serum hemolymph and hemocyte lysate supernatant of *P. merguiensis* which showed inhibitory effect on *Vibrio* spp. collected from natural habitat.

MATERIALS AND METHODS

Animals

Adult banana prawns, *Penaeus merguiensis* (body length 11-14 cm) of both sexes were collected from Koh Si Chang, Chonburi, Thailand. The prawns

were maintained in an aquarium containing sea water (salinity 28-30 ppt, temperature 27-28° C). Only the hemolymph from intermolt, apparently were healthy prawns was used.

Serum preparation

Serum samples from the intermolt prawns were collected by using a 25-gauge needle into the pericardial sinus. The samples were transferred to microtube, held on ice and allowed to clot at 4° C for 1 h. Then, the samples were centrifuged at 5000xg for 15 min at 4° C. The serum (supernatant) was used for hemagglutinating activity and antibacterial activity.

Hemolymph preparation

Hemolymph was obtained from each animal by inserting a 25-gauge needle into the pericardial sinus. The hemolymph 1 ml which contained 0.2 ml anticoagulant (0.45 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid and 10 mM EDTA, pH 7.0) was centrifuged at 7,700xg for 15 min at 4° C. The supernatant, hemolymph was dialyzed against 0.05 M Tris-HCl buffer [containing 0.15 M NaCl, pH 7.6 (TBS) and 10 mM CaCl2 (TBS-Ca)]. The hemolymph was later used to apply directly to affinity chromatography.

Hemocyte lysate supernatant preparation

The 0.5 ml hemolymph was drawn from each shrimp by using a 25-gauge needle, which contained 1.0 ml anticoagulant. Hemocytes were collected by centrifuging these extracts at 3000xg for 5 min at 4° C. Then, the hemocytes were washed twice with TBS. Supernatant was eliminated and hemocyte pellets from four prawns were pooled and resuspended in 1.0 ml TBS. The cell suspension was then homogenized with a sonicator (Ultras Homogenizor-VP30S, Taitec, Japan) equipped with a micro-tip (out put 5, duty cycle 50%) and centrifuged at 8000xg for 15 min at 4° C. The hemocyte lysate supernatant (HLS) was kept at -20° C until use for hemagglutinating and antibacterial activity assay.

Total hemocyte count

For hemocyte counts, 250 µl of fresh hemolymph were diluted with 500 µl of anticoagulant. The total hemocyte counts (THC) and differential cell counts were determined in a hemocytometer under a light microscope. Determination of the viable cells were incubated using 90 µl diluted fresh hemolymph with 10 µl 0.15% trypan blue in TBS for 5 min. Viable cells did not take up the trypan blue stain, while dead hemocytes appeared blue.

Hemagglutination assay

The hemagglutination was assayed by using erythrocyte from human ABO, sheep, rabbit, guinea pig, rat, hamster and enzyme treated human group A erythrocytes was carried out using a 2% erythrocytes suspension. Trypsinized human group A erythocytes were prepared by incubating 4% erythocytes suspension with 0.2% trypsin in 0.05 M TBS pH 7.6 at 37° C for 1 h. After washing four times, a 2% erythrocyte suspension was prepared in TBS. Fifty micro-liter of serial two-fold dilution of the test samples in TBS was added to the same volume of 2% erythrocytes suspension in 96 well microtiter U-plates. After incubation for 1 h at room temperature, the extent of agglutination was examined visually. The hemagglutinating activity was expressed as the titer that was the reciprocal of the highest dilution giving detectable agglutination.

Protein determination

Protein concentrations of the serum hemolymph and HLS were determined by the Bio-Rad Protein Assay according to Bradford method (Bradford, 1976) with bovine serum albumin (BSA) as a standard.

Affinity chromatography purification of *Penaeus merguiensis* lectin

The affinity sorbent was prepared by immobilization of mucin type II from porcine stomach (PSM) (Sigma Chemical Co., USA) on CNBr-activated Sepharose 4B (Amersham Phamacia Biotech,1999). The sorbent was equilibrated with TBS-Ca and used for the affinity purification of *Penaeus merguiensis*

lectin. The dialyzed hemolymph (20 ml) was applied to the mucin- CNBr-activated Sepharose 4B column at a flow rate of 0.2 ml/min. The column was washed sequentially with TBS-Ca until the absorbance at 280 nm of the effluent was stable at zero, and the lectin which was eluted with 25 mM EDTA-TBS. Lectin binding was monitored by the hemagglutination assay against trypsinized human A erythrocytes (Watanachote et al., 2006). The fractions (1 ml) exhibiting hemagglutination activity were collected, dialyzed against 0.01 M TBS for 48 h and lyophilized using LYOALFA-6 (Telstar Industrial, Spain) before storage at -20°C and use in antibacterial activity assay.

SDS-PAGE

The purity and approximate molecular mass of the purified lectin were estimated by SDS-PAGE under reduced condition with a 12.5% running gel and a 4% stacking gel in buffer system of Laemmli (1970) using Hoefer miniVE vertical electrophoresis system for 7-cm. Visualization of protein bands was performed by colloidal Coomassie brilliant blue G-250 (Fermentas, USA) and silver stain. The protein molecular mass markers ranged from 10-200 kDa (Fermentas, USA) were used as standards to calculate molecular mass.

Bacterial preparation

Five isolates of *Vibrio angillarum, V. fluvialis, V. harveyi, V. mimicus and V. parahemolyticus* were collected from a shrimp farm and sea water at Rayong and Chonburi provinces. Then, the bacteria were isolated at Microbiology Laboratory, Institute of Marine Science, Burapha University, Chonburi, Thailand. Other bacterial isolates, *Escherichia coli* TISTR 887 and *Staphylococcus aureus* TISTR 517 were obtained from Microbiological Resources Center, Institute of Scientific and Technological Research, Thailand. All bacteria except *Vibrio* were grown in tryptic soy broth (TSB). *Vibrio* spp. cultures were grown in 5 ml TSB supplemented with 1.5% NaCl (TSB/NaCl) at 30° C, 16-18 h with continuous shaking. Then, the cultures 0.5 ml were transferred to 5 ml TSB or TSB/NaCl and incubated at 30° C, 1-5 h. The bacterial suspension with 10^6-10^7 cells/ ml (~ 0.3 AU at 600 nm) was used in antibacterial assay.

Antibacterial activity

Antibacterial activities of serum, HLS and purified proteins were performed using *Vibrio* spp., *S. aureus*, and *E. coli*. Twenty microliter of bacterial suspension in TSB with 10^6-10^7 cells/ml were added to 80 µl of each sterile serum, affinity and gel filtration purified lectin (~1.28 µg of protein) or HLS (~2 µg of protein) and incubated for 1 h at 25° C (modified from Tunkijjanukij and Olafsen, 1998). After that ten-fold serial dilutions of the mixture of the tested samples and bacterial suspension (control) were spread on tryptic soy agar containing an additional 1.5% NaCl (TSA/NaCl for *Vibrio* spp.) and overnight incubation at 35° C. The assays were performed in triplicate. Antibacterial activity was shown as reductions of colony forming units (CFU) and determined by calculating differences among numbers of CFU in the presence of test samples and in the controls according to the formula:

$$\% \text{ inhibition} = \frac{\text{No. of colonies in control} - \text{No. of colonies in test}}{\text{No. of colonies in control}}$$

RESULTS

1. *Penaeus merguiensis* hemocytes

In *Penaeus merguiensis*, there are three types of circulating cells under a light microscope. The cell type one, hyaline observed in shrimp hemolymph was considered as a typical oval or elongated shape, agranule and measured approximately 2.8 µm. Cell type two, small-granular, has rounded shape, 2.5 µm and small intracellular granules. The third type of the cells was detected with 2 µm in size and contain s numerous dark large granules hemocyte (LGH), as shown in Figure 1. According to the type of hemocyte, the THC and different cell counts were measured by light microscope. Mean THC was $4.74 \pm 1.32 \times 10^6$ cells/ml. The viable cell populations withdrawn from

the banana prawn consisted of 56±15% viable cells. The percentage of differential hemocyte counts is shown in Figure 2. Small granule hemocyte was the most abundant population, 83.3±9.4% and the LGH was 8.2±4%, while hyaline the least abundant was 5±2% on average.

The SDS-polyacrylamide gel electrophoresis of hemolymph serum and hemocyte lysate supernatant (HLS) proteins performed by using silver stain showed that the protein from hemolymph and serum could be divided into fifteen clear bands, while the protein from HLS with three or four bands which three bands were markedly dominants of Mr 69.5, 57.0 and 43.5, as shown in Figure 3. The band of Mr 57.0 kDa was detected in hemolymph serum and HLS.

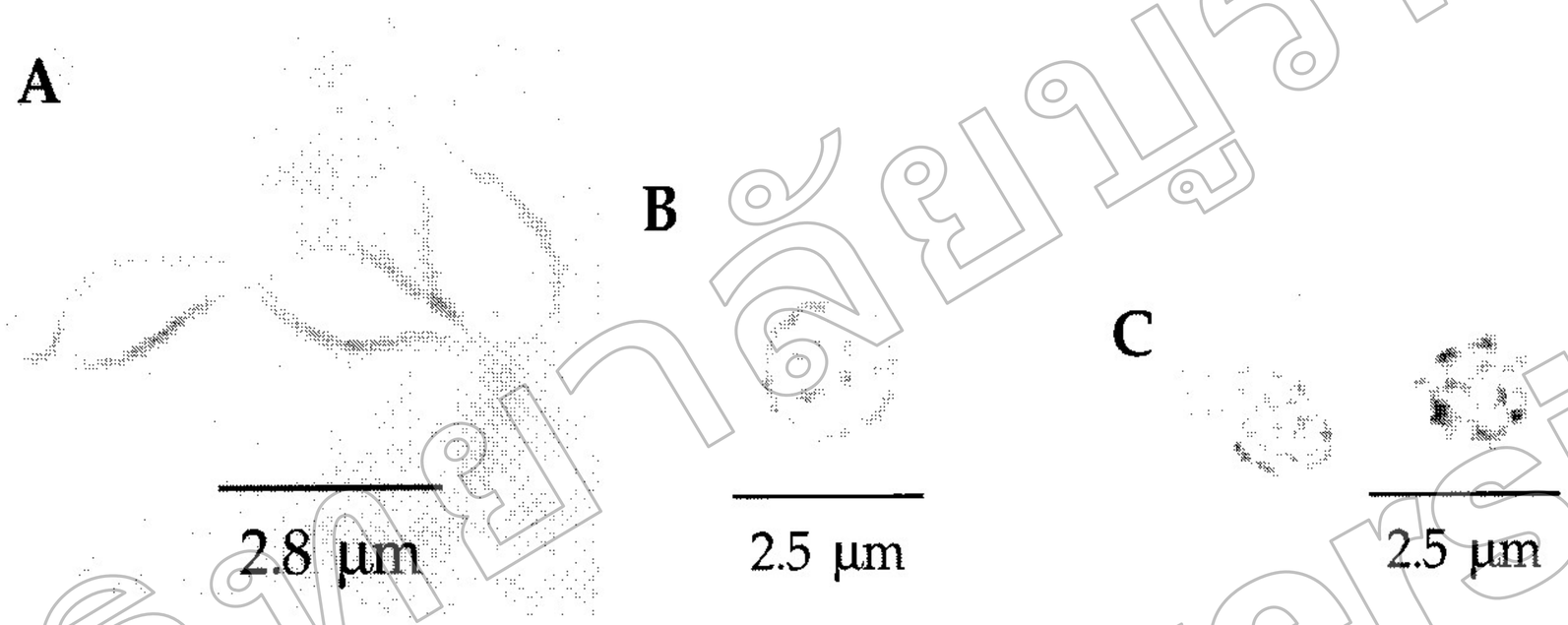

Figure 1. Light micrographs of shrimp hemocytes, *Penaeus merguiensis*. (A) hyaline hemocytes; (B) a small granule hemocyte (SGH); (C) large granule hemocytes (LGH).

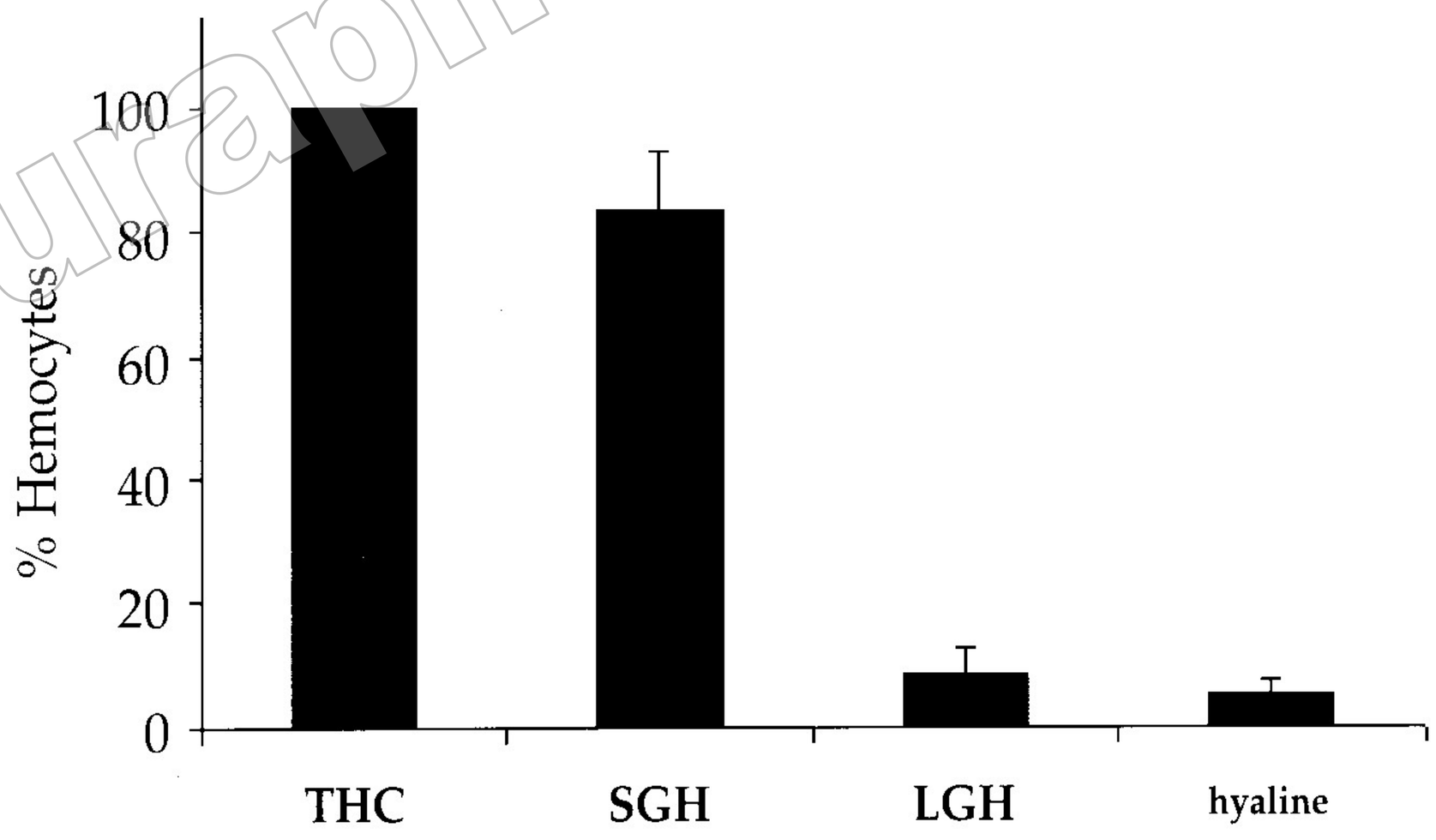

Figure 2. Total and differential count of hemocytes in *Penaeus merguiensis* hemolymph (values are averge ±SD, N=30). Total hemocyte count (THC), small granule hemocyte (SGH), large granule hemocyte (LGH).

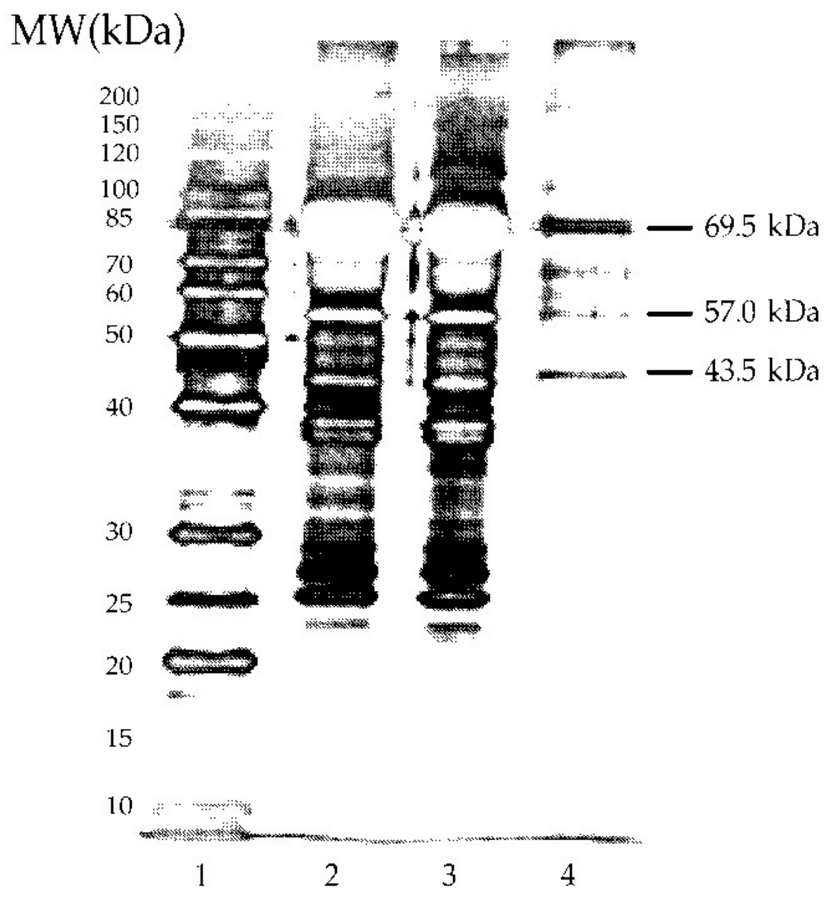

Figure 3. 12.5% SDS-PAGE of hemolymph (lane 2), serum (lane 3), and hemocyte lysate supernatant (lane 4) proteins in reducing condition. Lane 1: molecular weight marker proteins. The proteins in lanes 2 and 3 were 2 μg proteins, while lane 4 was 5 μg protein. The proteins in s were treated with dithiothreitol (DTT). Protein bands were visualized with Silver staining.

2. Hemagglutinin activity

The agglutination assay of the serum hemolymph and HLS of *Penaeus merguiensis* using human and animal erythrocytes as agglutinogens can be seen in Table 1. All test samples caused agglutinating activity with at least one type of erythrocyte. Agglutination of rabbit erythrocyte was higher than that of the other mammalian species tested, and their agglutination titers increased when trypsinized human A cells were used. The maximum titers were produced by serum and hemolymph against trypsin treated human A erythrocytes, while HLS agglutinated rabbit erythrocytes. Hence, the serum hemolymph and HLS contained a lectin.

Table 1. Hemagglutinating activity (HA) of serum, hemolymph and hemocyte lysate supernatant from *P. merguiensis* against different types of mammalian erythrocytes.

Erythrocytes	**HA (titer)**		
	Serum	**Hemolymph**	**Hemocyte lysate supernatant**
Human A	64	16	0
Trypsin treated human A	256	256	16
Human B	32	16	0
Human O	32	16	0
Sheep	128	0	0
Rabbit	128	64	256
Guinea pig	64	0	0
Rat	64	0	0
Hamster	0	0	0
Protein (mg/ml)	143.0	30.50	0.1411 (n=9)

3. Affinity purified lectin from hemolymph of *Penaeus merguiensis.*

Based on the hemagglutinating inhibition results demonstrating an apparent specificity of serum agglutinin of *Penaeus merguiensis* for mucin (PSM) as previously described (Watanachote et al., 2006), the banana prawn hemolymph was affinity purified on PSM-CNBr activated Sepharose 4B. The hemolymph which had passed through the affinity matrix and the effluent collected after subsequent washing of this matrix with 50 mM Tris-HCl buffer saline/ 0.01M $CaCl_2$ (TBS/Ca) showed one lectin peak. The lectin peak (PML) was eluted from the column with 50 mM Tris-HCl buffer saline/ 25 mM EDTA (TBS /EDTA), and tested for hemagglutinin with human A, as shown in Figure 4. Active fractions were combined and concentrated by lyophylization for antibacterial assay.

The 0.5 ml of purified and concentrated lectin from affinity chromatography was applied on a Sephacryl S-200 column. The elution profile from Sephacryl S-2 column gave one lectin peak (PML). After that, the purity of lectin was subjected to SDS-PAGE. The electrophoretic profile (SDS-PAGE) of the lectin purification is shown in Figure 5. The affinity purified lectin gave five bands, while after the two step purification, affinity and gel filtration chromatography gave two bands of Mr 30.09 and 28.01 kDa.

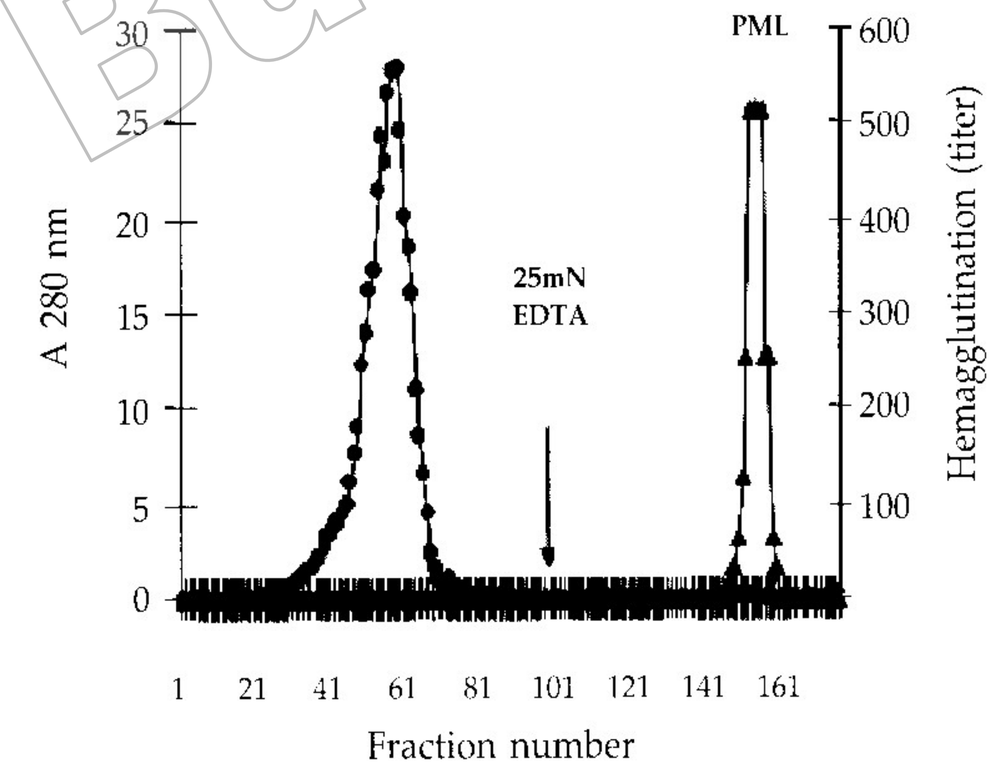

Figure 4. Affinity chromatography of the *P. merguiensis* lectin on mucin CNBr-activated sepharose 4-B. ● — ● = absorbance at 280 nm, ◆ — ◆ = hemagglutinin titer against trypsin treated human A erythrocytes.

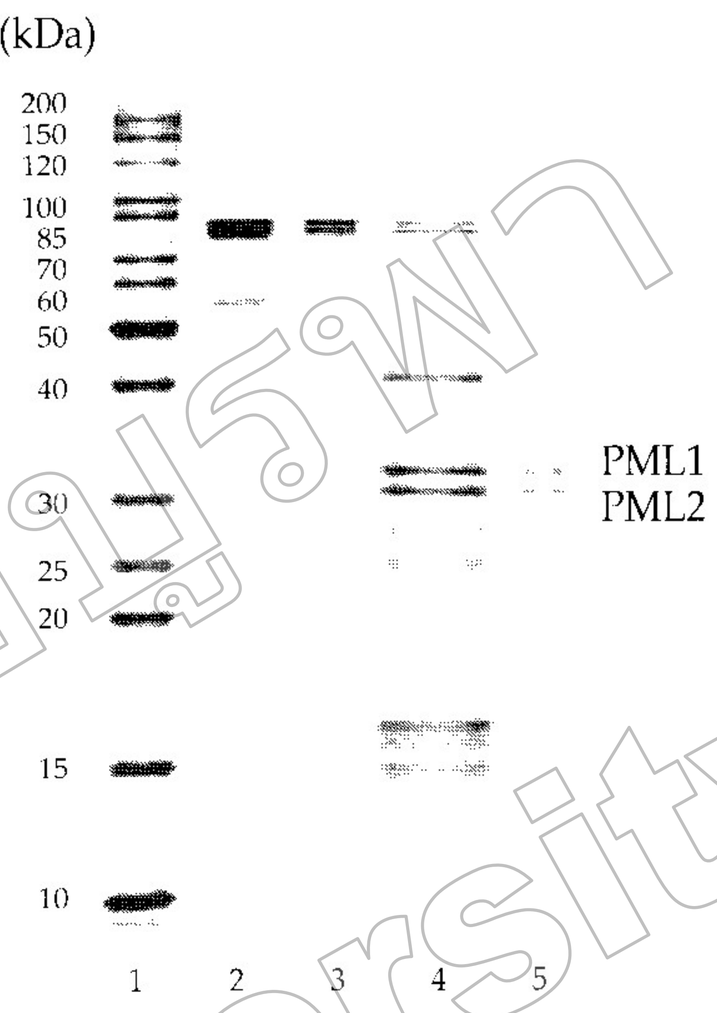

Figure 5. 12.5% SDS-PAGE of serum (lane 2), hemolymph (lane 3), affinity purified lectin (lane 4) and affinity and gel filtration purified lectin (lane 5) proteins in reducing condition were treated with 2-mercaptoethanol. Lane 1: molecular weight marker proteins. The proteins in lanes 2-5 were 0.5 µg proteins. Visualization of protein bands was performed by colloidal Coomassie brilliant blue G-250.

4. Antibacterial activity

The antibacterial activity of serum, affinity purified lectin, gel filtration purified lectin (~ 1.28 µg proteins) and HLS (~ µ2 g proteins) from hemolymph of banana prawn is presented in Figure 6. The serum, HLS and affinity purified lectin exhibited approximately 20-70% antibacterial effect against all test strains of *Vibrio* species. The affinity purified lectin had strong inhibitory effect on *V. angillarum, V. cholerae* and *V. fluvialis* with 68.75, 62.05 and 61.16%, respectively. Serum of the banana prawn demonstrated antibacterial activity against *Vibrio* species ranging from 17.64-

45.32%, but no inhibitory effect with *V. harveyi*. Serum of the banana prawn showed the highest antibacterial activity against *V. alginolyticus*, approximately 45%. HLS extracted from the hemocytes of banana prawn contained antibacterial activity. HLS showed slight inhibitory against all test strain bacteria which affected *Vibrio* species, ranging from 24.94-57.83%, and gave the highest inhibitory against *V. parahemolyticus*. Using 2 µg proteins showed a ntibacterial activity against *E. coli* and *S. aureus* with 56.85 and 29.98%, respectively. In liquid growth inhibition of each assay, HLS, affinity purified lectin and gel filtration purified lectin showed highly inhibitory marked activity against both Gram-negative and Gram-positive bacteria. Nevertheless, HLS, affinity purified lectin and gel filtration purified lectin exhibited more antibacterial activity on Gram-negative than Gram-positive bacteria, *S. aureus*. Among the Gram-negative bacteria, *V. alginolyticus*, *V. cholerae* and *V. harveyi* affinity purified lectin was two times more active than HLS at 1.28 and 2 µg proteins. Apparently, gel filtration purified lectin gave higher activity than affinity purified lectin. However, no activity was found for serum against *V. harveyi* and *S. aureus*.

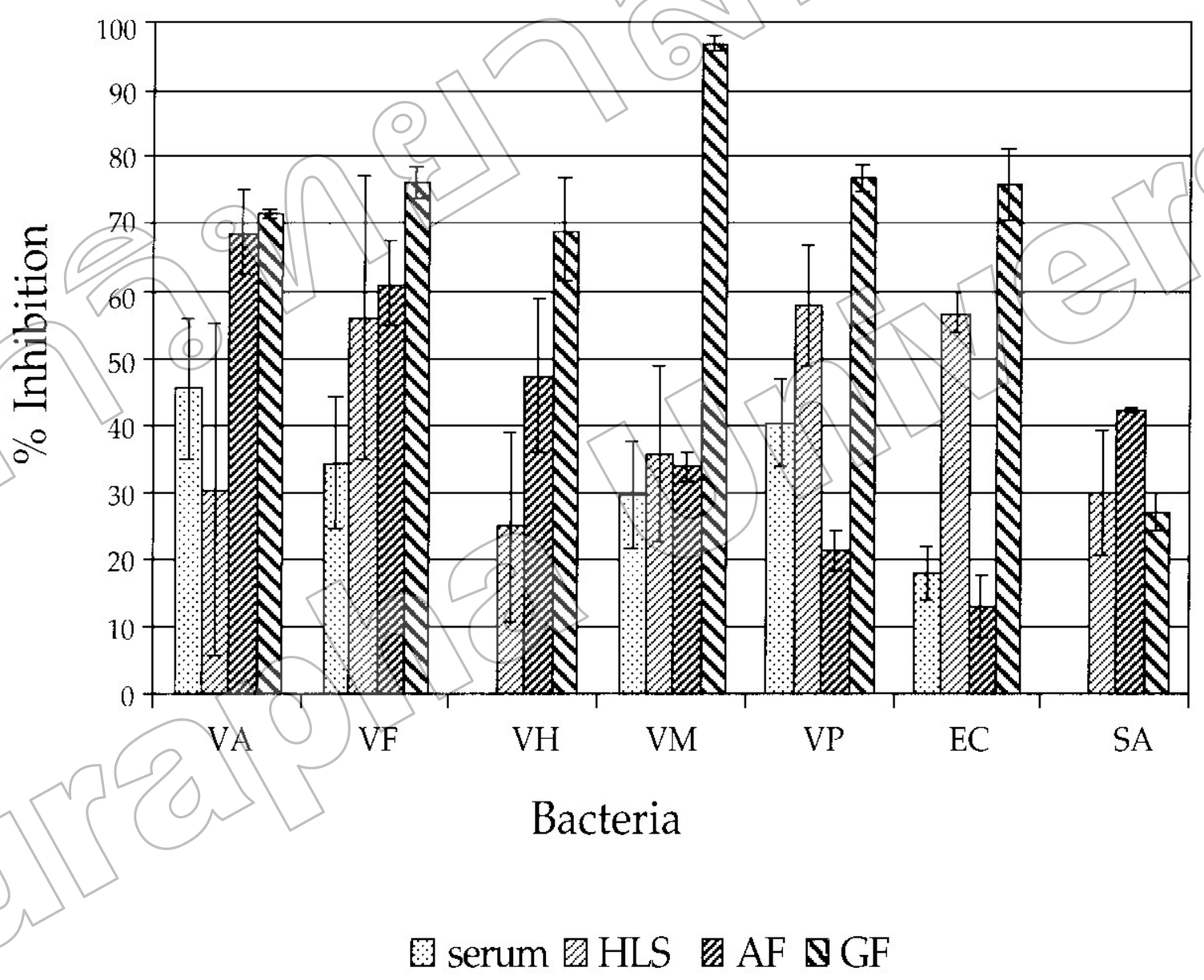

Figure 6. Antibacterial activities of serum, affinity purified lectin (AF), gel filtration purified lectin (GF) (~ 1.28 µg proteins) and HLS (~ 2 µg proteins) from hemolymph and hemocyte of *Penaeus merguiensis*.

(VA = *Vibrio alginolyticus*, VF = *V. fluvialis*, VH = *V. harveyi*, VM = *V. mimicus*, VP = *V. parahemolyticus*, EC = *Escherichia coli* and SA = *Staphylococcus aureus*)

DISCUSSION

Characterization of hemocyte of *Penaeus merguiensis*

The circulating hemocytes of invertebrates are essential for self defense against infections in immunology. In general, crustacean hemocytes were classified as hyaline, semi-granular and granular cells (Söderhäll and Smith, 1983). Hemocyte classification was based on size and number of intracellular granules. A hemocyte classification system was developed, which relates cellular morphology at the light and electron microscope levels, cytochemistry, and three essential functions: clotting, phagocytosis and encapsulation. However, this classification is confusing and contains a very high number of name and description of different cell types (Hose et al., 1990; Azumi et al., 1996). According to Martin and Graves (1985), the penaeid shrimp hemocytes are often classified into agranular cell (hyaline cell), small-granular (semi-granular) and large-granular (granular). In the present study, *P. merguiensis* hemocytes were classified into three types under light microscope as hemocyte from *P. californiensis* and *P. zonangulus* (Martin and Graves, 1985; Cardenas et al., 2000). Like other crustaceans, *Farfantepenaeus californiensis*, *Litopenaeus vannamei* and *L. stylirostris* have three main populations differing in presence and size of cytoplasmic granules. These results are different from the previously report of *P. monodon* that the hemocytes were classified in five types which the cells were stained with Grunwald-Giemsa and detected under light microscope (van de Braak, 2002).

On the surface of all eukaryotic cell are plasma membrane proteins attached to carbohydrate. This carbohydrate-rich cell coat may have an affinity for lectins. Thus, lectins are powerful tools for the study of carbohydrate and their derivatives, both in solution and cell surfaces (Lis and Sharon, 1986). In two decapods, the ridgeback prawn *Sicyonia ingentis* and the American lobster *Homarus americanus* hemocytes were classified by using wheat-germ agglutinin (WGA), which specifically binds N-acetyl-D-glucosamine. Fluorescence and electron microscopy were used to show that WGA stains the cytoplasmic granules in the granulocytes but not the hyaline cells (Martin et al., 2003). Our results showed that the extract from hemocyte of banana prawn contained an agglutinin, which strongly agglutinated rabbit erythrocyte. Therefore, the lectin from HLS may be different from serum and hemolymph.

Total hemocyte count

THC of banana prawn was $4.74\pm1.32\times10^6$ cell/ml and consisted of 56±15% viable cells. The numbers of circulating hemocytes were different between penaeid shrimps. *Penaeus californiensis*, *P. ingentis*, *P. monodon*, *F. californiensis*, *L. vannamei and L. stylirostris* exhibited a wide range of THC, from 11×10^3-50.9×10^6 cells/ml, by using hemocytometer (Martin and Graves, 1985; van de Braak, 2002; Vargas-Albores, 2005). The total hemocyte counts of the penaeid shrimps of *F. californiensis*, *L. vannamei*, *L. stylirostris and P. merguiensis* (in this study) were 4.04 ± 1.63, 9.35 ± 2.10, $14.16\pm5.30\times10^6$ cells/ml (Vargas-Albores et al., 2005) and $4.74\pm1.32\times10^6$ cells/ml respectively. Sequeira et al. (1995) reported that the *P. japonicus* hemocyte cell population was related to sex and molt cycle. Several studies suggest that a number of invertebrate hemocyte cells depends on environmental factors, such as temperature, pH and salinity, as well as chemical need, to maintain the hemocyte during in *vitro* study (Paterson and Stewart, 1974; Oliver and Fisher, 1995). In addition to traditional methods such as microscopy and protein chemistry, flow cytometry has been used to investigate the circulating hemocytes for evaluation of invertebrate hemocyte responses to immunological stimuli (Cardenas et al., 2000). Giulianini et al. (2007) analyzed automatically the ultrastructural features of freshwater crayfish, *Astacus leptodactylus*, by means of image-analysis software in combination with traditional THC by using light-/electron microscopy. The invertebrates *Tachypleus tridentatus* (Japanese horseshoe crab), *F. californiensis*, *L. vannamei* and *L. stylirostris* contain granular hemocyte (small-granular and large-granular) which comprise 99, 72, 75 and 66% of the circulating hemocyte, respectively (Iwanaga, 2002; Vargas-Albores et al., 2005), whereas *P. merguiensis* contain 88% of THC.

Granular hemocytes are involved in the defense mechanism against foreign materials by phagocytosis and encapsulation, instead of hyaline cells which are involved in the initiation of hemolymph coagulation (Hose et al., 1990).

Hemagglutinin and antibacterial activity

It is a well-known fact that invertebrates appear to be lack of important substance of the immune system as defined in vertebrate, including immunoglobulins. Thus, the humoral and cell-associated lectins from banana prawn were preliminary studied to understand their role on antibacterial activity. For some characterization and biological properties of hemolymph purification lectin, sugar specificity were also observed in our previous study (Watanachote et al., 2006; Watanachote et al., 2007). Serum hemolymph and HLS from the prawn contain agglutinin for human and animal erythrocytes. The HLS demonstrated the most agglutinating activity against rabbit erythrocyte and gave three major bands, Mr 69.5, 57.0 and 43.5 kDa by SDS-PAGE which were different from the protein bands of serum and hemolymph. Hemolymph of the banana prawn gave two lectin subunits, PML1 (Mr 30.09 kDa) and PML2 (Mr 28.01 kDa). This means that agglutinin from HLS may be different from serum and hemolymph agglutinin. The previous results showed that five types of lectin, named tachylectin (TL)-1 to 5, and several bacterial agglutinin in the horseshoe crab have been identified in circulating hemocytes and hemolymph plasma (Iwanaga, 2002).

Our results show that serum HLS and affinity purified lectin of *Penaeus merguiensis* have antimicrobial activity *in vitro*. Our earlier study of purified lectin from *P. merguiensis* hemolymph showed strong inhibitory action against *Vibrio* spp. (Watanachote et al., 2007). Here, we have been attempted with respect to location of antibacterial activity in hemolymph of the prawn. HLS protein concentration of approximately 2 µg/100 µl was reduced in colony counts of *Vibrio* spp., *E. coli* and *S. aureus*, ranging from 24.94-57.83%, while serum affected all the tested strains, ranging from 17.64-45.32%, when using approximately 1 µg protein in 100 µl of reaction mixture. These results indicated that antibacterial substance was probably circulated more in hemolymph than in hemocyte, or antibacterial substance was related to hemocyanin (Watanachote et al., 2007; Alpuche-Osorno et al., 2005). Nagai et al. (2001) showed that the clotting enzyme of the Japanese horseshoe crab functionally converted hemocyanin to phenoloxidase. HLS antibacterial activity was higher against Gram-negative bacteria than Gram-positive bacteria, *S. aureus*. The HLS antimicrobial properties of shore crab, *Carcinus maenas*, exhibited heat stable and independent of divalent cation. Also, HLS are not due to phenoloxidase activity (Chisholm and Smith, 1992). Antibacterial activity resides exclusively in the granular cells and non-lytic in character and operates mainly against Gram-negative organisms (Chisholm and Smith, 1992). Like HLS from *C. maenas*, the hemocytes from *Galathea strigosa*, *Nephrops norvegicus*, *Crangon crangon* and *Glyptonotus antracticus* contain antibacterial factors, and antibacterial potencies vary from species to species (Chisholm and Smith, 1995). However, phenoloxidase activity is weak in *G. strigosa* and absent in *G. antracticus* (Smith and Söderhäll, 1991). The resistance of live *Micrococcus luteus* suggested that the blue crab *Callinectes sapidus* antibacterial activity was not a lysozyme (Noga et al., 1996). Therefore, the agglutinin in the hemocyte of *P. merguiensis* may be a lectin, recognizable molecule that plays a role in the antibacterial activity.

Therefore, further studies should be investigated focusing on the purified agglutinins in different hemocyte cells and their sugar specificities that are useful for hemocyte classification and the role of hemolymph or hemocyte lectin on phagocytosis and opsonization.

ACKNOWLEDGEMENT

This research was supported by the Center for Agricultural Biotechnology through the fund from Subproject Graduate Study, Kasetsart University, Thailand.

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