MATERIALS AND METHODS

Experiment Part I : Partial Purification and Characterization of Lectin from Hemolymph of Banana Prawn

1. Animals

Adult banana prawns, *P. merguiensis* (body length 11-14 cm) of both sex were collected from Koh Si Chang, Chon buri, Thailand. The prawns were maintained in an aquarium with natural sea water (salinity 28-30 ppt, temperature 27-28 °C). Only the hemolymph from intermolt, apparently healthy prawns were used.

2. Hemolymph Preparation

Hemolymph samples from intermoult prawns were collected using a sterile plastic syring by inserting a 25-gauge needle which contained AC-1 anticoagulant (0.45 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid and 10 mM EDTA, pH 7.0, modified from Söderhäll and Smith, 1983) into the pericardial sinus. The samples were transferred to polystyrene tubes held on ice, pooled and centrifuged at 7,700xg for 15 min at 4°C. The cell pellet was discarded, and the hemolymph was stored at -20°C until use for all analyses (modified from Maheswari *et al*., 1997).

3. 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 supernatant (serum) was used for hemagglutinating activity and antibacterial activity.

4. Hemagglutination Assay

The hemagglutination (HA) assays were performed in U-bottomed microtiter plate by two-fold serial dilution of a 50 µl serum sample with an equal volume of 0.15 M NaCl or 0.05 M Tris-HCl, 0.15 M NaCl, 10 mM $CaCl_2$ pH 7.6 (TBS/Ca). After dilution, 50 µl of 2% human A, B, O and AB erythrocytes from Burapha hospital and animal erythrocytes from Mahidol University, Thailand was added to each well and incubated for 1 h at 25 °C. The hemagglutination titers were recorded as the reciprocal of the highest dilution of the sample causing complete agglutination of erythrocytes. Control for all assays consisted of the substitution of the sample by buffer. Each experiment was performed in triplicate (Goto-nance *et al*., 1996). The trypsinisation human erythrocytes used Shiomi's method (Shiomi *et al*., 1980). Trypsinized human 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% erythocyte suspension was prepared in TBS. The results were expressed by the titer value, which is the maximum dilution for positive agglutination.

5. Agglutination of Bacteria

As representative of test bacteria, nine species were used in this study which six isolates of *Vibrio angillarum, V. cholerae, V. fluvialis, V. harveyi*, *V. parahemolyticus* and *V. mimicus* were obtained from Microbiology Laboratory, Institute of Marine Science, Burapha University, Chonburi, Thailand. Other bacterial isolates as *Staphylococcus aureus* TISTR 517, *Micrococcus luteus* TISTR 884, *Escherichia coli* TISTR 887 and *Pseudomonas aeruginosa* TISTR 1467 were obtained from Microbiological Resources Center,Thailand Institute of Scientific and Technological Research.

Bacteria 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, 0.5 ml of culture was transferred to 5 ml TSB or TSB/NaCl and incubated at 30°C, 1-5 h. Bacteria were harvested in the log phase by

centrifugation at 1000xg for 15 min, washed three times with TBS pH 7.6 and suspended in 5 ml TBS before heat-killing at 65 °C. Agglutination of bacteria was carried out by adding 80 µl banana prawn serum with 20 µl of bacterial suspension or 200 µl affinity purified lectin (1.28 µg protein) with 20 µl of bacterial suspension. After incubation for 1 h at 25 °C, the results were read under a microscope and compared to suspensions of bacteria without test samples.

6. Temperature and pH stability

Heat stability was examined by incubating the serum at 4, 25, 35, 40, 60, 70, 80 or 100 °C for 30 min. After cooling in ice box, the remaining agglutinating activity against 4% human erythrocyte suspension was examined as previously described. The effect of pH on the hemagglutinating activity was tested by dialyse the serum (300µl) against the 0.05 M acetate buffer (pH 4 to 6) and 0.05 M Tris-HCl buffer (pH 7.6-10) for 24 h 4 °C. After dialysis, all the samples were re-equilibrated by dialysis against 0.05 M Tris-HCl, 0.15 M NaCl pH 7.6. The dialysate were centrifuged and the resulting supernatant was tested for hemagglutinating activity using human A erythrocytes.

7. Divalent Cation Dependency

To examine the divalent cation requirement of *P. merguiensis* lectin, the serum was dialysed against 0.01 M EDTA in 0.1 M Tris-HCl buffer pH 7.6 for 48 h. The serum was then dialysed against 0.15 M NaCl for 24 h to remove EDTA. The serum was tested for hemagglutinating activity against human A erythrocytes in the presence of 10 mM $CaCl_2$, $MgCl_2$ or $MnCl_2$ in 0.05 M Tris-HCl, 0.15 M NaCl pH 7.6 (Goto *et al*., 1992).

8. Inhibition of Hemagglutination by Sugars

Inhibition tests by sugars and glycoproteins were conducted using the dialytic serum against TBS showing a titer of 1:8. The samples (50 µl) were allowed

to react with various concentration of inhibitors (50 µl) for 1 h. Fifty µl of 4% human A erythrocytes suspension was then added to the mixture and the agglutination was measured after standing for 1 h at room temperature. The results were expressed by the minimum concentration of the inhibitors that completely inhibit the hemagglutination. The following sugars (200 mM) and glycoprotein (0.25%) were used: D-fructose, D-ribose, D-xylose, L-arabinose, D-galactose, D-glucose, D-mannose, D-rhamnose, L-fucose, D-sorbital, D-galactosamine, D-glucosamine, N-acetyl-D- glucosamine, N-acetyl-D-galactosamine, N-acetylneuraminic acid, α-methyl-D-mannopyranoside, cellobiose, lactose, maltose, melibiose, sucrose, raffinose, mucin Type II from porcine stomach (PSM) and fetuin from fetal calf serum.

9. Protein determination

Protein concentrations were determined according to Bradford (Bradford, 1976) with bovine serum albumin as a standard.

10. Lectin Purification and Characterization

Affinity Chromatography

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 AB, 1999). The coupling procedure for use with CNBr-activated Sepharose 4B (15 g) was followed the affinity chromatography principles and the methods handbook (Amersham Phamacia Biotech AB, 1999). After swelling and washing the gel, 3.0 g mucin in 75 ml 0.1 M $NaHCO_3$ buffer, pH 8.3 containing 0.5 M NaCl was added, and the suspension was incubated at 25°C for 18 h with gentle shaking. The unbound arm was blocked with 1.0 M ethanolamine in 0.1 M Tris-HCl buffer, pH 8.0. Then, the excess adsorbed protein was washed at least three cycles with coupling buffer followed by 0.1 M acetate buffer containing 0.5 M NaCl, pH 4.0 and 0.1 M Tris-HCl buffer containing 0.5 M

NaCl, pH 8.0. The mucin coupled to CNBr-activated Sepharose 4B was packed into a 1.5×15 cm column. The sorbent was equilibrated with TBS-Ca and used for the affinity purification of *P. merguiensis* lectin (Watanachote *et al*., 2006).

The affinity sorbent was prepared by immobilization of mucin type II from porcine stomach on CNBr-activated Sepharose 4B column which was previously equilibrated with TBS/Ca-2 was used for the affinity purification of *P. merguiensis* lectin. Hemolymph 20 ml was dialyzed against 0.05 M Tris-HCl buffer [containing 0.15 M NaCl, pH 7.6 (TBS) and 10 mM $CaCl_2$ (TBS-Ca)] then was applied directly to affinity chromatography column. The agglutinin bound to the affinity matrix was eluted with TBS/Ca-2 containing 25 mM EDTA (TBS/Ca-2/EDTA) and the eluted fractions were assayed for their agglutinating activity, using human A erythrocytes. The fractions (1 ml) which exhibiting hemagglutination activity were collected and dialyzed against 0.01 M TBS for 48 h and lyophilized using LYOALFA-6 (Telstar Industial, Spain) before storage at -20°C and further purified by gel filtration chromatography.

Gel Filtration Chromatography

Affinity purified *P. merguiensis* lectin was applied to a column of Sephacryl S-200 HR (Phamacia Biotech, Sweden), 1.5×100 cm. The column was previously equilibrated with TBS-Ca. Fractionation was carried out at a flow rate of 18 ml/h, and fractions of 1 ml collected. The hemagglutination titer of each fraction was measured. The fractions containing hemagglutinating activity were pooled, dialysed against TBS/Ca-2, and stored at -20 °C until used. To estimate the approximate molecular weight of hemagglutinins, five reference proteins, ribonucleaseA (MW 13,700), chymotrypsinogen (MW 25,000), ovalbumin (MW 43,000) and albumin (MW 67,000) were passed through the same column. All the chromatographic procedures were performed at the steady flow rate of 18 ml/h at 4 °C, and the effluents (1 ml fractions) collected during adsorption, washing, re-

equilibration and elution were tested for hemagglutinating activity using trypsinised human A erythrocytes. (modified from Bulgakov *et al.*, 2004)

SDS-PAGE

The purity and approximate molecular weight 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 and Hoefer SE 600 Series for 13-cm (Amersham pharmacia biotech, USA). Visualization of protein bands was performed by colloidal Coomassie brilliant blue G-250 (Fermentas, USA). The protein molecular weight markers range of 10-200 kDa (Fermentas, USA) were used as standards to calculate molecular weight by using ImageQuant TL V2005 (Amersham Bioscience,USA).

2-D gel electrophoresis

The lysis buffer (30 mM Tris, 7 M urea, 2 M Thiourea, 4% CHAPS, pH 8.5 stored at –15°C to -30°C) 1-2 ml was added directly to affinity purified lectin and vortex for an additional 30 sec, then centrifuged 13,000×g at 4°C for 15 min. Then, the supernatant was removed and transfered to a clean tube and discard and insoluble pellet. Samples were cleaned by Ettan^{TM}Sample Preparation kits and Reagents 2D Clean-up kit (Amersham Biosciences, USA). Protein was determined by Bio-Rad protein assay kit with BSA as the standard. Unused extract was stored in aliquots at -80°C.

The protein extracts were separated by 2-Dimensional Gel Electrophoresis. Isoelectric focusing (IEF) was perfomed in the Immobiline^{TM} DryStrip pH 3-10, 13 cm, IPG strips in a disposable cassette. Hemolymph volumes were adjusted in order to analyze the same amount of protein, 200 μg for each set of control or injected samples. Hemolymph was loaded directly onto the strips and rehydrated for 12 h at ambient temperature.

Running Conditions for pH 3-10: Temperature 20ºC; Current 50 µA per strip.

Step Voltage Mode	Voltage(V)	kVh
1.Step and Hold	500	0.5
2.Gradient	1000	0.8
3.Gradient	8000	11.3
4.Step and Hold	8000	4.4

The second dimension was performed in 12.5% SDS-Polyacrylamide gels using the Hoefer SE 600 Series (Amersham pharmacia biotech). Proteins in the analytical gels were stained with colloidal Coomassie brilliant blue G-250 staining. Then, a gel was scanned by Image Scanner II (Amersham Biosciences) using LabScan5 software. The evalution of the gel was analyzed by ImageMaster 2D Platinum software 5 (Amersham Biosciences, USA).

11. Protein identification by LC-MS/MS

Spots of interest were excised from the gels within 18 h after colloidal Coomassie blue staining protein and digesting using trypsin. Peptide was analyzed by using LC-MS/MS (liquid chromatography mass spectrometry) model Finnigen LTQ Linear Ion Trap Mass Spectrometer (Thermo Electron Corporation) at Bioservice Unit, National Center for Genetic Engineering and Biotechnology. The MS/MS spectra of the most intense peaks were obtained following each full-scan mass-spectrum. Protein identification relied on the comparison of the measured mass of the tryptic peptides with the predicted masses from nrFASTA databases.

Experiment Part II : Affinity Purified Lectin from Hemolymph of *Penaeus merguiensis* After *Vibrio harveyi* Infection and Bacterial Clearance Activity

1. Hemolymph Preparation

Hemolymph was obtained from each animal by inserting a 25-gauge needle, 1 ml which contained 0.2 ml anticoagulant into the pericardial sinus. The hemolymph was centrifuged at 7,700xg for 15 min at 4°C. The supernatant, hemolymph was dialyzed against TBS-Ca. The hemolymph was applied directly to affinity chromatography.

2. Hemagglutination Assay

The hemagglutination was assayed by using human ABO, sheep, rabbit, guinea pig, rat, hamster and enzyme treated human A erythrocytes was carried out using a 2% erythrocytes suspension as previously mention.

3. Affinity Chromatography Purification of Penaeus merguiensis Lectin

The affinity sorbent was prepared by immobilization of mucin type II from porcine stomach on CNBr-activated Sepharose 4B column as previously mention (Watanachote *et al*., 2006).

4. Test bacterial species

As representative of test bacteria, nine species were used in this study which six isolates of *V. angillarum, V. cholerae, V. fluvialis, V. harveyi*, *V. parahemolyticus* and *V. mimicus*. Other bacterial isolates, *S. aureus* TISTR 517, *M. luteus* TISTR 884, *E. coli* TISTR 887 and *P. aeruginosa* TISTR 1467 were used for antibacterial assay. Bacteria were grown in 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, 0.5 ml of culture was 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.

5. Antibacterial activity

Antibacterial assays of hemolymph of both affinity purified and gel filtration purified lectin in 0.01 M TBS-Ca were elucidated with *Vibrio* spp., *P. aeruginosa*, *S. aureus*, *M. luteus* and *E. coli*. Twenty microliters of bacterial suspension were added to each mixture of 80 µl of sterile hemolymph affinity and gel filtration purified lectin (~ 1.28 µg of protein) samples and incubated for 1 h at 25°C (modified from Tunkijjanukij and Olafsen, 1998). Antibacterial activity was assayed as reductions of colony forming units (CFU), after ten-fold serial dilutions, the mixture of the tested samples and bacterial suspension (control) were spreaded on tryptic soy agar (TSA) containing an additional 1.5% NaCl (TSA/NaCl) for *Vibrio* spp., and incubated overnight at 35°C. This assay was performed in triplicate and antibacterial activity was determined by calculating differences between numbers of CFU in the presence of test samples and in the controls according to the formula:

$$\% \text{ inhibition} = \frac{\text{No. of colonies in control} - \text{No. of colonies in test}}{\text{No. of colonies in control}} \times 100$$

6. Bacterial clearance

A single colony of *V. harveyi* was transferred to TSB/NaCl and incubated overnight at 200 rpm at 30°C. Then, 0.5 ml of this culture was added to 49.5 ml of TSB/NaCl and incubated for 5 h. Ten ml of the culture were centrifuged and the pellet resuspended in 1 ml TBS to give approximately 10^9 cells/ml.

Determination of bacterial clearance rate was performed on six sets of prawn, twelve prawns of each set were injected intramuscularly at the 6th abdominal segment with 10 µl of the bacterial suspension. The twelve prawns from each feed-treatment group were removed immediately after injection at 1, 15, 30, 45, 60 and 120 min post-injection to determine the number of bacterial cells/ml in hemolymph. Hemolymph (50 µl) was collected from the pericardial sinus into a syring containing 100 µl of anticoagulant solution and added immediately to a tube containing 350 µl of TSB. Hemolymph mixture was ten-fold diluted in normal saline then 100 µl of diluted mixture were spread onto plates of thiosulphate citrate bile sucrose agar (TCBS) to obtain the reduction of colony forming unit (CFU). After incubation at 35°C for 10 h, total counts were calculated according to dilution and recorded as the mean CFU count ± standard deviation for the triplicate counts (modified from Sritunyalucksana *et al*.,1999).

Hemolymph mixtures were centrifuged at 7,700×g for 15 min at 4°C. The supernatant was used for comparison of protein pattern at each time after post injection with *V. harveyi* by SDS-PAGE using 10% running gel, 13 cm. The evaluation of the protein band was analyzed by ImageQuant TL V2005 (Amersham Bioscience, USA). The hemagglutination titer of supernatant at each time was also measured. Hemagglutination specific activity was calculated according to the formula:

$$\text{Hemagglutination specific activity} = \frac{\text{hemagglutinatinating activity (titer/ml)}}{\text{protein (mg/ml)}}$$

Experiment Part III : Antibacterial Protein in Serum Hemolymph and Hemocyte Lysate Supernatant of *Penaeus merguiensis*

1. Animals

Adult banana prawns, *P. merguiensis* were maintained in an aquarium with sea water. Only the hemolymph from intermolt, apparently healthy prawns were used.

2. 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.

3. Hemolymph Preparation

Hemolymph was obtained from each animal as mention in the experiment part I.

4. 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 resuspened in 1.0 ml TBS. The cell suspension was then homogenized with a sonicator (Ultras Homogenizor-VP30S, Taitec,Japan) equipped with a microtip (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 before hemagglutinating and antibacterial activity assay.

5. 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 hemacytometer under light microscope. Determination of the viable cells were incubated 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.

6. Hemagglutination Assay

The hemagglutination was assayed by using human ABO, sheep, rabbit, guinea pig, rat, hamster and enzyme treated human A erythrocytes was carried out using a 2% erythrocytes suspension as mention in the experiment part I.

7. Antibacterial Activity

Antibacterial activities of serum, HLS and purified proteins were performed using *V. angillarum, V. fluvialis, V. harveyi*, *V. mimicus*, *V. parahemolyticus, S. aureus* and *E. Coli.* Twenty microliters 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. Antibacterial activity was determined by calculating differences among numbers of CFU in the presence of test samples and in the controls as previously mention.