Chapter 2

Materials and methods

Materials

1. Animals

1.1 Banana shrimp *Peneaus merguiensis*

Live mature female and male *P. merguiensis* were collected from Trang Coastal Aquaculture Station and the Sichon district in Nakhon Si Thammarat province. They were 10-14 cm long and weighed between 30-40 g.

Ovarian development in female shrimps was classified into 4 stages on the basis of the colour and size of the ovary observed through the external carapace according to Quinitio *et al*. (1989) and Primavera (1988). Shrimps in stage 1 have ovaries that are thin, transparent and not visible through the dorsal exoskeleton. Ovaries in stage 1 (undeveloped or immature) and stage 5 (spent) often cannot be distinguished from each other. Stage 2 is referred to as the early maturing stage. The ovaries are flaccid and white to olive green in colour and discernible as a linear band through the exoskeleton. The developing ova have yolk granules. In stage 3 (late maturing) the ovarian tissues have a glaucous appearance with the anterior portion thick and expanded. They are clearly visible through the exoskeleton. Stage 4 begins when the ovaries are classified as ripe (mature) and have grown and expanded through the exoskeleton of the first abdominal segment. The ovaries appear dark olive green and consist mostly of yolky oocytes.

1.2 White shrimp *Penaeus vannamei*

The hemolymph of adult male *P. vannamei* was collected from a shrimp farm in Songkhla province.

1.3 Black tiger shrimp *Penaeus monodon*

P. monodon was obtained from a shrimp farm in Trang province.

1.4 Giant freshwater prawn *Macrobrachium rosenbergii*

The hemolymph of *M. rosenbergii* was collected from Chumphon province.

1.5 Rabbits

To produce antiserum against purified lectin and to draw the blood for hemagglutination test, two albino rabbits weighing 1.5 kg and aged 3 months were used. The rabbits were fed with normal food at the Southern Laboratory Animal Facility, Prince of Songkla University (PSU).

2. Chemicals

All chemicals used were of analytical grade and purchased from the following companies.

Chemical	Company
Acetic acid	Merck
Acrylamide	Fluka
Agar	BD Biosciences
Agarose	Sigma
Ammonium persulphate	Merck
Ammonium sulphate	Fluka
Anisole	Fluka
Beta-mercaptoethanol	Fluka
Bicinchoninic acid assay reagent kit	Pierce
Bisacrylamide (N, N' methylenediacrylamide)	Fluka
Boric acid	Sigma
Bovine serum albumin	Sigma
Bovine submaxillary mucin	Sigma
Broad range molecular weight marker	Promega
Bromophenol blue	Merck
Calcium chloride	Ajex
Carboxymethyl-Cellulose	Sigma

Chemical	Company
Citric acid	Ajex
Coomassie Brilliant Blue R-250	Sigma
Coomassie plus protein assay reagent kit	Pierce
Copper sulfate	Fluka
DEAE-Sephacel	Sigma
3,3'-Diaminobenzidine	Sigma
Ethanol	BDH Laboratory
Fetuin-agarose	Sigma
Folin-Ciocalteu's phenol reagent	Merck
Freund's adjuvant complete	Sigma
Freund's adjuvant incomplete	Sigma
Glycerol	Sigma
Glycine	Fluka
Glycoprotein staining kit	Molecular Probes
Heparin	Difco Laboratories
Hydrochloric acid	Merck
Hydrogen peroxide	Carlo Erba
Low molecular weight standard	Amersham Pharmacia Biotech
Magnesium chloride (six hydrate)	Sigma
Methanol	Merck
N-Acetyl galactosamine	Sigma
N-Acetyl glucosamine	Sigma
N-Acetyl mannosamine	Sigma
N-Acetyl neuraminic acid	Sigma
N,N,N',N'Tetramethylethylenediamine	Fluka
Octyl-β-D-glucopyranoside	Sigma
o-Phenylenediamine tablets dihydrochloride	Sigma

Chemical	Company
Peptide-N-glycosidase F	Sigma
Peroxidase conjugated goat anti-rabbit IgG (whole molecule)	Sigma
Porcine fetal calf asialofetuin	Sigma
Porcine fetal calf fetuin	Sigma
Porcine stomach mucin	Sigma
Phosphoric acid	BDH Laboratory
Plate count agar	Difco Laboratories
Potassium dihydrogen phosphate	Fluka
Potassium phosphate, dibasic	Baker Analyzed
Potassium sodium tartate	Ajex
Pro-Q Emerald 300 glycoprotein staining kit	Molecular Probes
Pyridine	Sigma
Silver staining kit	Bio-Rad
Sodium carbonate	Fisher Scientific
Sodium chloride	Sigma
Sodium dodecyl sulphate	Riedel de-Haen
Sodium hydroxide	Labscan Asia
Standard bovine serum albumin	Pierce
Superose-12 HR 10/30 column	Pharmacia
Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar	HiMedia
3,3′, 5, 5′-Tetramethylbenzidine (TMB) substrate kit for peroxidase	Vector Laboratories
Trichloroacetic acid	Carlo Erba
Trifluoroacetate	Fluka
Trifluoromethanesulfonic acid	Sigma
Tris (hydroxymethyl) aminomethane	Sigma
Triton X-100	Merck
Tryptic soy broth	BD Bioscience
Tween-20	Labchem

3. Instruments

Instrument	Model	Company
Amino acid analyzer	Pico-Tag	Waters
BioImaging Systems	BioDoc-IT System	UVP
Centrifuge	5415C, 5804R	Eppendorf
ELISA plate reader	Elx808	Bio-Tek
Fast Protein Liquid Chromatography	-	Pharmacia
High performance liquid chromatography	-	Hewlett Packard
High pressure system sterilizer	ES-315	TOMY
Incubator	1510E	Shel lab
Micropipette	-	Eppendorf, Gilson
Microscope	Leica DML	Leica
Microtube pump MP-3	MP-3N	Eyela
Mini IEF cell	-	BioRad
Orbital Incubator Refrigerated	-	Gallenkamp
pH meter	Accument15	Fisher
Power supply	1000/500	Bio-Rad
Refrigerated super speed centrifuge	J2-21	Beckman
Standard lab oven	-	Binder
Ultracentrifuge	L8-70M	Beckman
UV transilluminator	M-20	UVP
UV-VIS spectrophotometer	160A	Shimadzu
Vortex	G-560E	Scientific Industries
Water bath (unstirred digital bath)	JW20	Julabo

Methods

1. Preparation of hemolymph samples

Hemolymph was collected from pericardial sinus with a 26G gauge needle attached to a syringe and transferred immediately to tubes. Serum was obtained by allowing the hemolymph to clot at 4°C overnight. Serum was cleared by centrifugation at 16,000xg for 30 min at 4°C and the supernatant was immediately used or stored as aliquots at -20°C for futher use.

2. Hemagglutination assays

Rabbit erythrocytes were obtained from a healthy animal. Erythrocytes were washed several times with TBS (50 mM Tris-HCl, pH 7.5-0.15 M NaCl) by centrifugation at 2,500xg for 10 min each at 4°C and then suspended in the same buffer as 2% erythrocytes. The hemagglutinating activity (HA) of lectin was assayed in a microtiter U plate (NUNC) according to a 2-fold serial dilution procedure. The samples were diluted with 25 μl of TBS on plates and mixed with 25 μl of 2% rabbit erythrocyte suspension. The mixture was allowed to stand at room temperature for 1 h, and then the HA was observed. The activity was defined as the titer value of maximum dilution with positive agglutination of 2% rabbit erythrocytes. Positive hemagglutination was obtained when the erythrocytes did not sediment to the bottom of the well forming a red button. Specific hemagglutinating activity was defined as HA unit/mg protein.

3. Determination of protein

3.1 Bradford method (Bradford, 1976)

Twenty μl of each sample was mixed with 1 ml of Coomassie plus protein assay reagent kit or Bradford reagent (0.01% Coomassie brilliant blue G-250 - 4.7% ethanol - 8.5% phosphoric acid) for 2-3 min. The absorbance of each samples were measured at 595 nm between 2-60 min after addition of the reagent. The calibration graph was prepared from 1, 3, 5, 7 and 9 μg of the standard protein (BSA, bovine serum albumin) assayed using the same procedure.

3.2 Bicinchoninic acid (BCA) method (Smith *et al*., 1985)

Twenty-five μl of each standard or samples was added into the appropriate microtiter plate wells. The diluents were mixed with 200 μl of BCA assay reagent kit for 30 second. The plate was covered and incubated at 37°C for 30 min. The absorbance was measured

at or near 562 nm on an ELISA plate reader after cool the plate to room temperature. The standard curve was prepared from 0-250 μg/ml of BSA.

3.3 Lowry method (Lowry *et al*., 1951)

A hundred μl of shrimp hemolymph diluted with TBS was mixed with 3 ml of alkaline-copper reagent (2% Na_2CO_3 in 0.1 N NaOH: 1% potassium sodium tartrate: 0.5% $CuSO_4$ in the ratio of 100:1:1) at room temperature for 10 min. The mixture was then added and immediately mixed with 0.3 ml of diluted Folin-Ciocalteu reagent (Folin-phenol : distilled water in the ratio of 1:1) for 30 min. The optical density at 620 nm (O.D. 620) was determined by using the UV-Vis spectrophotometer 160A. The calibration curve was prepared using BSA as standard.

4. Polyacrylamide gel electrophoresis (PAGE)

4.1 Nondenaturing PAGE

A (10 x 10 x 0.1 cm) slab gel composed of stacking gel (3 cm) and separating gel (6 cm) was used. Nondenaturing PAGE 4-10% gradient PAGE was performed according to the method of Davis (1964). Gel compositions were as below.

Composition	Stacking gel	Separating gel	
	3% (5 ml)	4% (3 ml)	10% (3 ml)
30% Acrylamide-0.8% bisacrylamide	0.50 ml	0.40 ml	1.00 ml
0.5 M Tris-HCl, pH 6.8	0.63 ml	-	-
1.5 M Tris-HCl, pH 8.8	-	1.50 ml	1.50 ml
10% Ammonium persulphate	50 μl	30 μl	30 μl
TEMED	5 μl	3 μl	3 μl
Distilled water	3.82 ml	1.07 ml	0.47 ml

The purity of lectin during the purification steps was carried out by nondenaturing PAGE using the Davis buffer system (Davis, 1964). Samples were mixed with a sample buffer (0.2 M Tris-HCl, pH 6.8, 8 mM EDTA, 40% glycerol and 0.4% bromophenol blue) in a ratio of 3:1. A separating gel was run with a constant current of 15 mA for 2 h at room temperature.

4.2 SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli (1970) using a slab gel (10 x 10 x 0.1 cm), composed of stacking gel (3 cm) and separating gel (6 cm) with a linear gradient of 6-18% gel in the presence or absence of 1% β-mercaptoethanol. Compositions of the gels are shown as below.

Composition	Stacking gel	Separating gel	
	3% (5 ml)	6% (3 ml)	18% (3 ml)
30% Acrylamide-0.8% bisacrylamide	0.5 ml	0.6 ml	1.8 ml
0.5 M Tris-HCl, pH 6.8	1.25 ml	-	-
1.5 M Tris-HCl, pH 8.8	-	0.75 ml	0.75 ml
0.2 M EDTA	50 µl	30 µl	30 µl
10% SDS	50 µl	30 µl	30 µl
10% Ammonium persulphate	50 µl	30 µl	30 µl
TEMED	5 µl	3 µl	3 µl
Distilled water	3.10 ml	1.56 ml	0.36 ml

Samples were mixed with a sample buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 20% glycerol in the presence or absence of 1% β-mercaptoethanol and 0.4% bromophenol blue as the tracking dye) in a ratio of 3:1. They were then boiled for 30 min. Electrophoresis was carried out in the electrode buffer (25 mM Tris-0.192 M glycine, 0.1% SDS, pH 8.3). The gel was run at a constant voltage of 250 V for 2 h. The molecular masses of the subunits of purified lectin were estimated by measuring their relative mobility in SDS-PAGE as compared to those of low molecular weight standards (Amersham Pharmacia Biotech): phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa).

4.3 Protein staining

4.3.1 Coomassie Brilliant Blue R-250 staining

After electrophoresis, the gel was immersed in Coomassie Blue (0.08% Coomassie Brilliant Blue R-250-50% methanol-7.5% acetic acid) for 2 h and fixed for 30 min in

a fixing solution (50% methanol-7.5% acetic acid). The gel background was destained using destaining solution (5% methanol-7.5% acetic acid).

4.3.2 Silver staining

After electrophoresis, proteins were fixed in the gel with 40% methanol-10% acetic acid for 15 min and subsequently washed twice with 5% ethanol- 10% acetic acid for 15 min with gentle shaking. The gel was then transferred to an oxidizing solution, shaken gently for 3 min then washed with deionized water several times until its background became clear. The gel was and then transferred into a silver stain kit for 20 min and washed with deionized water for 15 sec. It was then immersed in a developing solution while shaking gently and the solution was changed several times until protein bands appeared in the gel. This reaction was then stopped by 3% acetic acid.

4.3.3 Gel code blue staining

The gel was washed 2-3 times with deionized water for 10 min and then immersed in gel code blue stain reagent for 30 min. After washing the gel with deionized water, the blue band was visualized over a faint blue background.

4.4 Glycoprotein staining

The carbohydrates of purified lectin were stained using Pro-Q Emerald 300 glycoprotein gel stain kit. The staining was performed using the protocol suggested by the manufacturer.

SDS-PAGE of purified lectin samples and glycoprotein standards (2 μg) were run on a 10% slab gel. After electrophoresis, the gel was fixed in 50% methanol-5% acetic acid for 45 min or overnight. The gel was then washed with 3% acetic acid for 10-20 min twice. It was then transferred into oxidizing solution containing periodic acid for 30 min and washed 3 times with 3% acetic acid. The gel was stained in Pro-Q Emerald 300 staining solution in the dark for 90-120 min and then washed with 3% acetic acid for 12-20 min. Stained gel was visualized using a 300 nm UV transilluminator.

5. Purification of lectin from serum

5.1 By Fetuin-agarose column

Lectin from *P. merguiensis* serum was purified by affinity chromatography using a Fetuin–agarose column. Five ml of clear serum were applied onto a column previously equilibrated with TB-NaCa (50 mM Tris-HCl, pH 7.5-0.3 M NaCl-0.1 M $CaCl_2$). After being retained in the column at 4°C for 12 h to allow for complete binding, unbound material was eluted from the column with an equilibrating buffer at a 15ml/h flow rate. Fractions of 1 ml were collected and the optical density was measured at 280 nm (O.D.280) until absorbance of the eluent at 280 nm was below 0.01. The lectin was desorbed with 100 mM GlcNAc (N-acetyl glucosamine) in TB-NaCa. . The eluted fractions were dialyzed overnight against TBS at 4°C. The samples were assayed for their hemagglutinating activity (HA) by the 2-fold dilution procedure in the presence of a 2% solution of rabbit erythrocytes. The active fractions with a high HA were pooled, concentrated and dialyzed overnight against 50 mM Tris-HCl, pH 7.5 at 4°C. Further purification of lectin was performed by gel filtration chromatography.

5.2 By Superose 12 HR 10/30 culumn

The active samples from the Fetuin-agarose column with high HA were applied to a fast protein liquid chromatography (FPLC) system on a Superose 12 HR 10/30 column equilibrated earlier with TB-NaCa. The protein was then eluted by the same buffer with a flow rate of 0.5 ml/min. Fractions (0.5 ml each) were collected, O.D.280 and HA was determined. The fractions with a high HA were pooled, concentrated and dialyzed overnight against 50 mM Tris-HCl, pH 7.5 at 4°C. The purity of lectin was tested by nondenaturing PAGE or kept at -20°C for further analysis.

5.3 Preaparative PAGE

Preparative PAGE was performed in the same manner as nondenaturing PAGE using a slab gel (10x10x0.1 cm) with a 10% polyacrylamide. The putative lectin fraction (150 μg protein) was subjected to electrophoresis at a constant current of 15 mA for 2 h. A small gel portion was cut vertically from each side and stained with 2% Coomassie Blue for 10 min. These portions were then replaced next to the rest of the gel that had not been stained; the gel at the same level corresponding to the stained lectin band was cut horizontally and placed in a dialysis bag. The protein was eluted from the gel by submerged electrophoresis in 0.25 M Tris-0.192 M

glycine, pH 8.3 at a constant current of 15 mA for 18 h. The eluate was concentrated by centrifugation at 1,500xg for 10 min at 4°C in a Nanosep 30 K Omega centrifuge tube (Pall Life Sciences). The purity of the lectin during the purification steps was verified by means of nondenaturing PAGE.

6. Carbohydrate inhibition assays

The carbohydrate specificity of the lectin was investigated by competitive inhibition using the following sugars and glycoproteins: D-(+)-galactose, D-(+)-glucose, D-(+)-mannose, N-acetyl-galactosamine (GalNAc), N-acetylglucosamine (GlcNAc), N-acetyl-mannosamine (ManNAc), N-acetyl neuraminic acid (Neu5Ac), porcine stomach mucin (PSM), fetal calf serum fetuin, fetal calf serum asialofetuin and bovine submaxillary mucin (BSM). Stock solutions of sugars or glycoproteins were prepared in TBS and stored at -20°C until use. For the inhibition test, serial 2-fold dilutions of 25 µl of the various sugars and glycoproteins were first added to each well of the 96 well microtitre plates. Next, 25 µl of purified lectin (agglutination titer at 1:64) was added to each well, mixed by gentle shaking, and incubated for 1 h at room temperature. Finally, 50 µl of 2% rabbit erythrocyte suspension was added and the minimum concentration of inhibitors required to block agglutination was determined after 1 h incubation at room temperature.

7. Two-dimensional electrophoresis (2-D PAGE)

Purified lectin was analyzed by 2-D PAGE to confirm purification to homogeneity and its isoelectric pH (pI) value determined. The first dimension used isoelectric focusing (IEF). The purified homogenous lectin was subjected to agarose gel isoelectric focusing in a Mini IEF Cell using 2% ampholytes in a continuous gradient of pH ranging from 3-10 (a mixture of ampholytes pH 3-10 : pH 4-5 in a ratio of 1:5). Thirty-seven micrograms of purified lectin were applied to the gel plate and focused sequentially at a constant voltage of 100 V for 15 min, 200 V for 15 min and 450 V for 60 min, respectively. Thereafter, the gel was subjected to the second dimension that was 12% SDS-PAGE. Afterwards, protein bands were visualized by staining with Coomassie Blue. The pI value of purified lectin was estimated using standard

protein markers (carbonic anhydrase, pI 7.0; ovalbumin, pI 5.1; glucose oxidase, pI 4.2; amyloglucosidase, pI 3.8).

8. Analysis of amino acid composition

The amino acid composition of purified lectin was determined following acid hydrolysis with 6 M HCl at 110^{o}C for 24 h in glass tubes sealed under N_2. After hydrolysis, HCl was removed by evaporation and the samples were analyzed according to the manufacturer's recommendations with an amino acid analyzer by derivatization of free amino acids with a phenylisothiocyanate (PITC) pre-column before further separation by a reversed phase–high performance liquid chromatography (RP-HPLC) Pico-Tag column.

9. Analysis of carbohydrate composition

9.1 Composition analysis of neutral amino sugars by HPAEC-PAD

Purified lectin (25 μg) was hydrolyzed with 2 M trifluoroacetate (TFA) at 100^{o}C for 4 h. After drying the hydrolysate, 1% of the sample was dissolved in water and determined by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC/PAD) using a CarboPac PA-1 column eluted with a gradient of 0.05M- 1M of sodium hydroxide. System performance (HPAEC/PAD) was monitored by the performance of sugar standards. The printout of the results shows the profile and individual monosaccharide content expressed in nanomoles present in the volume injected. The aforementioned monosaccharides were identified by elution position. Blanks were required for optimum quantitative results.

9.2 Sialic acid determination by RP-HPLC with fluorescence detection

Seventy-five grams of purified lectin were dissolved in a final concentration of 2 M acetic acid and heated to 80^{o}C for 3 h. The released sialic acids were collected by ultra-filtration through a 3,000 NMWCO filter and derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB). The fluorescent sialic acid derivatives were analyzed by RP-HPLC with an on-line fluorescence detection. Analysis was performed in Glycotechnology Core Resource, University of California, San Diego.

The method allows detection of as less as 250 femtomole of individual species, and separates most of the known members of the sialic acid family. Identification is based on

known standards run in parallel, and quantitation is done in reference to known amounts of sialic acids derivatized and injected in parallel.

10. Deglycosylation

10.1 Enzymatic N-deglycosylation by peptide N-glycosidase F (PNGaseF) digestion

Purified lectin (33 μg) was dissolved with 90 μl of ultrapure water in micro centrifuge tube and 5 μl of denaturant solution containing 2% octyl-β-D-glucopyranoside with 100 mM β-mercaptoethanol supplied by Sigma deglycosylation kit was added. The reaction mixture was boiled in a hot water bath for 10 min. After cooling, 5 μl of 1x reaction buffer was added and 50 μl of solution was transferred to two separate tubes as a control and a test sample. Five μl (500 units/ml) of PNGaseF enzyme was added to the test sample while 5 μl of water was added to the control sample. The reaction mixtures were then mixed and spinned briefly and incubated at 37°C for 1 h. The reaction was stopped by heating at 100°C for 10 min and analyzed by SDS-PAGE.

10.2 Chemical deglycosylation by trifluoromethanesulfonic acid (TFMS)

Lyophilized purified lectin (50 μg) was treated with 250 μl of 9:1 TFMS: anisole at 4°C for 6 h. The reaction mixture was cooled to below -20°C by placing in dry ice-ethanol contained in a small beaker, and then neutralized by addition of 60% pyridine in distilled water cooled to below -20°C. The neutralized reaction mixture was dialyzed at 4°C against 50 mM Tris-HCl buffer, pH7.5 with several changes of buffer, then freeze-dried before dissolving with distilled water and run on SDS-PAGE with the untreated lectin as reference.

11. Preparation of anti-lectin antibody

Anti-lectin antibody was raised against purified lectin in a rabbit. An albino rabbit was immunized by intracutaneous injection with 20 μg of purified lectin mixed with 1 ml Freund's complete adjuvant. Another booster injection was given a week later in the same manner. Two weeks following the second boost, the rabbit was administered with 20 μg of purified lectin mixed with 0.8 ml Freund's incomplete adjuvant. Blood was drawn from the rabbit two weeks after the last injection and allowed to clot overnight at 4°C. After centrifugation at 2,500xg for 20 min, the serum was precipitated by adjusting to 50% ammonium sulfate saturation

and left at 4°C for 10 h. The precipitate obtained after centrifugation at 22,000xg for 30 min was resuspended and dialyzed against 10 mM sodium phosphate, pH 7.4 for 16 h. The dialysate was applied to a 2.6 cm x 10 cm DEAE-Sephacel column equilibrated with the same buffer. Unabsorbed anti-lectin antibody was eluted as the first peak (Wallace, 1965) using the equilibrating buffer with a flow rate of 30 ml/h. Fractions (3 ml each) containing anti-lectin antibody were pooled, concentrated and used for dot blotting and ELISA.

12. Dot blot analysis

Serum and tissue extracts were spotted onto a nitrocellulose membrane and left to aired-dry for 1 h. After rinsing the membrane with TBS, protein-binding sites on the membrane were blocked in TBS containing 3% BSA for 4 h at room temperature or for 10 h at 4°C. The membrane was again rinsed three times for 10 min each with TBST (TBS containing 0.05% Tween 20) and then incubated with anti-lectin antibody (1°Ab) (1:2,000 dilution in TBST containing 1% BSA) for 2 h at room temperature. After washing three times with TBST, the membrane was transferred in the same buffer containing horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (2°Ab) (1:20,000 dilution). It was held at room temperature for 1 h and then rinsed with TBST three times and following with TBS twice. Any immunoreaction was visualized by incubating the membrane in a substrate solution of 0.05 mg/ml 3, 3'-diaminobenzidine/ml (DAB) and 0.005% H_2O_2 in 50 mM Tris-HCl buffer, pH 7.5. The colour reaction was stopped by transferring the membrane into water. Purified lectin was used as positive control while BSA was negative control. Both of them were carried out in the same manner.

13. Affinity blot analysis

To detect glycosylation and to determine sugar residues of purified lectin, the lectin was run on 10% SDS-PAGE. It was then blotted onto nitrocellulose membrane according to the method of Towbin *et al.* (1979). After blocking with 5% nonfat milk, the membrane was incubated with 30 μg/ml biotinylated lectins (shown in Table 6). As a control, a 200 mM concentration of a competing sugar of each lectins was included. After washing with TBST, the membrane was incubated with 0.5% streptavidin-peroxidase in TBST for 2 h. Reactive bands

were visualized with 3,3′, 5, 5′-Tetramethylbenzidine (TMB)/H_2O_2 substrate kit for peroxidase by transferring the membrane to a staining dish containing TMB substrate solution. The membrane was incubated for 10-30 minutes at room temperature. The colour reaction was stopped by transferring the membrane into water.

Table 6 Biotinylated lectins used in the affinity blotting

Lectin source	Common name	Abbreviation	Sugar specificity
Arachis hypogaea	Peanut	PNA	Galβ1,3 GalNAc > α and βGal
Artocarpus	Jackfruit	Jacalin	Galβ1,3 GalNAc
Abrus precatorius	Jequirity bean	APA	Gal β1,3 GalNAc > βGal
Buahinia purpurea	Camel's foot tree	BPA	N-acetyl-D-galactosamine
Bandeiraea simplicifolia		BS-I	α-Gal
Caragana arborescens	Pea tree	CAA	GalNAc
Erythrina cristagalli	Coral tree	ECA	LacNAc >Lac>GalNAc> Gal
Galanthus nivalis	Snowdrop bulb	GNA	Manα1,3Man> Manα1,6Man> Manα1,2Man
Glycine max	Soy bean	SBA	α and βGalNAc > α and βGal
Lycopersicon esculentum	Tomato	LEA	GlcNAcβ1,4GlcNAc oligomers
Lotus tetragonolobus	Asparagus pea	Lotus	α-L-fucose
Maclura pomifera	Osage orange	MPA	αGal, [β1,3GalNAc]
Maackia amurensis	Maackia	MAA	NANA (α2,3) Gal
Phaseolus vulgaris	Red kidney bean	PHA-L	αMan > αGlc = αGlcNAc
Pisum sativum	Garden pea	PEA, PSA	α Man > β1,4Gal=β1,2GlcNAc
Psophocarpus tetragonolobus	Winged bean	PTA	αGal, αGalNAc
Triticum vulgaris	Wheat germ	WGA	GlcNAc (β1,4GlcNAc) $_{1\text{-}2}$> βGlcNAc> NeuAc
Ulex europaeus	Gorse	UEA-I	αFuc
Vicia faba	Fava bean, broad bean	VFA	αMan > Glc = GlcNAc
Wistaria floribunda	Japanese wisteria	WFA	GalNAcα1,6Gal >αGalNAc> βGalNAc

14. Western blot analysis

Proteins separated with nondenaturing 4-10% gradient PAGE were transferred onto a nitrocellulose membrane in 25 mM Tris, 192 mM glycine (pH 8.3) buffer containing 10% methanol at a constant current of 500 mA for 2 h. After rinsing the membrane with TBS, protein-binding sites on the membrane were blocked with TBS containing 3% BSA for 4 h at room temperature or for 10 h at 4°C. The membrane was rinsed three times for 10 min each with TBST and then incubated with anti-lectin antibody (1°Ab) (1:1,000 dilution in TBST containg 1% BSA) for 2 h at room temperature. After washing three times with TBST, the membrane was transferred in the same buffer containing 2°Ab (1:20,000 dilution). The membrane was held at room temperature for 1 h and then rinsed with TBST three times and following with TBS twice. Visualization of any immunoreaction was carried out by incubating the membrane in a substrate solution of 0.05 mg/ml DAB and 0.005% H_2O_2 in 50 mM Tris-HCl buffer, pH 7.5. The colour reaction was stopped by transferring the membrane into water.

15. Development of enzyme linked immunosorbent assay (ELISA)

To develop ELISA for quantifying lectin levels in the hemolymph, optimal conditions of the assay were performed.

15.1 The optimal concentration of antibodies

In the experiment, 2 antibodies, anti-lectin antibody (1°Ab) and HRP conjugated goat anti-rabbit IgG (2°Ab) were used. The optimal concentration of each antibody was determined by using 350 ng of purified lectin and 1°Ab was diluted with an assay buffer (50 mM phosphate buffer, pH 7.4 containing 0.15 M NaCl and 0.05% Tween 20, PBST) in the range of 1:1,000 to 1:5,000 dilutions or 2°Ab was diluted in the range of 1:10,000, 1:25,000, 1:50,000 and 1:75,000 dilutions with the same buffer. Purified lectin (150 μl) was coated to each well of microtiter polystyrene plate at 4°C overnight. The plate was washed three times with PBST. Nonspecific binding sites were blocked by incubating the plate at room temperature for 2 h with 250 μl of a super blocking buffer and washed three times with PBST. Thereafter, 150 μl of 1°Ab was added and incubated for 2 h at room temperature. The plate was then washed three times with

PBST followed by addition of 150 µl of 2^{o}Ab per well and incubation for 2 h at room temperature. After washing again as described above, then 150 µl of a substrate solution (0.4 mg/ml *o*-phenylenediamine (OPD) in 50 mM citrate buffer, pH 5 containing 0.012% H_2O_2) was added to each well. The enzyme reaction was allowed to proceed for 30 min and then stopped by addition of 50 µl of 2 M H_2SO_4. Absorbance was immediately read at 492 nm (O.D.492) using an ELISA microplate reader.

15.2 The optimal condition of peroxidase reaction

The optimal condition of peroxidase reaction was investigated in a similar manner by varying the concentration of peroxidase substrate (OPD) from 0 to 1.4 mg/ml and H_2O_2 concentration from 0 to 0.014%. The optimal reaction time was investigated by varying the incubation time from 0 to 60 min. The experiment was obtained by using 350 ng of purified lectin per well, 1^{o}Ab and 2^{o}Ab was used at 1:3,000 and 1:25,000 dilutions respectively.

15.3 Quantitation of hemolymph lectin by ELISA

ELISA was carried out in 96-well microtiter polystyrene plates (Nunc). Standardization of the curve was performed by using purified lectin. The lectin (30-300 ng) in 150 µl of 50 mM carbonate buffer, pH 9.6 was fixed to the bottom of each well at 4^{o}C overnight. The plate was washed three times with PBST. Nonspecific binding sites were blocked by incubating the plate at room temperature for 2 h with 250 µl of the super blocking buffer and washed three times with PBST. Thereafter, 150 µl of anti-lectin antibody (1^{o}Ab) at 1:3,000 dilution was added and incubated for 2 h at room temperature. The plate was then washed three times with PBST followed by addition of 150 µl of 2^{o}Ab (1:25,000 dilution) per well and incubation for 2 h at room temperature. After washing again as described above, then 150 µl of a substrate solution (0.4 mg/ml OPD in 50 mM citrate buffer, pH 5 containing 0.012% H_2O_2) was added to each well. The enzyme reaction was allowed to proceed for 30 min and then stopped by addition of 50 µl of 2 M H_2SO_4. The O.D.492 was immediately read using the ELISA microplate reader.

Lectin concentrations in the serum or hemolymph were calculated on the basis of a standard curve of purified lectin concentrations. The sensitivity of the assay for an immunoreactive protein was 0-350 ng per assay.

16. Preparation of bacteria

Three isolates, *Vibrio vulnificus*, *Vibrio parahemolyticus* and *Vibrio harveyi* from infected *P.* merguiensis 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^{o}C. Other bacterial isolates including *Escherichia coli*, *Salmonella typhi* and *Vibrio cholerae* were obtained from the Fish and Fisheries Product, Quality Control and Research Center, Songkhla province. Each was grown with a Luria-Bertani medium slant at 37^{o}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^{o}C with continuous rotary shaking at 200 rpm. An aliquot (10 ml) 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 0.85% NaCl. The turbid suspensions were adjusted to approximately 10^{9} cells/ml.

17. Bacterial agglutination test

Purified lectin was 2-fold serially diluted with TBS. Fifty μl of the bacterial suspension ($5x10^{7}$ cells) was mixed in each tube with 50 μl of the diluted lectin. The tubes were left at room temperature for 1 h and then shaken twice at 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 examined with a light microscope with dark-field illumination. The bacterial agglutination titer (BAU) was expressed as the highest dilution of purified lectin giving a complete agglutination of the bacterial cells. As control, TBS was used in the same manner instead of the lectin solution.

To test the inhibition of purified lectin-induced agglutination of *V. harveyi* by sugar, the selected sugar was two-fold serially diluted with TBS. Purified lectin of 128 BAU (25 μl) was mixed with an equal volume of the diluted sugar in a microtiter plate. After incubation at

room temperature for 45 min, 50 µl of the *V. harveyi* suspension was added and the mixture was left to stand for an additional 1 h. The agglutination was observed with a dark-field, light microscope. The minimum concentration of the sugar required for complete by inhibition of agglutination was recorded.

To test for the inhibition of agglutination by anti-lectin, the above tests were performed using anti-lectin antibody instead of the sugar.

18. Injection of bacteria

To study the change in lectin levels in the hemolymph of infected *P. merguiensis* shrimps, 100 µl of bacterial suspension (1.38 $x10^9$ cfu/ml) resulting in 1.38 $x10^8$ cells/shrimp was injected into the shrimp abdominal muscle located at the sixth abdominal segment. After injection, they were kept in a separate tank. The hemolymph was collected at 6 h intervals after injection. Shrimps injected with an equal volume of sterile saline solution were used as controls.

19. Statistical analysis

The standard errors of mean (mean $\pm$ S.E.) were calculated. T-test program online from Simple Interactive Statistical Analysis (SISA) was used to compare lectin concentrations in the hemolymph (Steel and Torrie, 1980).