Chapter 3

Results

1. Purification of lectin

1.1 By Fetuin-agarose column

In previous study (Paijit, 2001), lectin founded in the hemolymph of *P. merguiensis* could agglutinate erythrocytes from rabbit and rat including human types A, B, AB and O. Among these the highest hemagglutinating titer was observed in the presence of rabbit and rat erythrocytes. Since bleeding from rabbit is more convenient than that from rat, thus rabbit erythrocytes were used for hemagglutinating determination throughout the present study. In addition, hemolymph lectin showed high specificity to *N*-acetyl neuraminic acid (Neu5Ac) and fetuin. Fetuin-agarose affinity colmn was therefore used as one step in the purification of *P. merguiensis* lectin from the hemolymph.

When the hemolymph from banana shrimps was chromatographed on a Fetuin-agarose column, two peaks were obtained (Fig. 9). The first peak, F1, a broad peak that eluted with the equilibrating buffer had no hemagglutinating activity (HA) which contained most of the serum proteins. The second smaller peak (F2) obtained after elution by 100 mM GlcNAc had HA that increased by 1,062-fold when compared to that of the initial serum (Table 7).

1.2 By Superose 12 HR column

Active fractions (peak F2) with high HA from the Fetuin-agarose column were further purified on a FPLC Superose 12 HR column. Again two peaks of protein appeared (Fig. 10), with the HA present only in peak S1. The HA of the S1 peak fractions increased by 1,890-fold comparing to that of the initial serum (Table 7).

1.3 By preparative PAGE

Altermatively, active fractions from the Fetuin-agarose column were further purified by preparative PAGE. Using this technique, lectin was removed from other proteins with

and increasing in the HA to 1,850-fold of the initial serum (Table 7). One protein band of the lectin appreared in nondenaturing gradient PAGE (Fig. 11, lane 5).

Table 7 Summarising the purification of the lectin from *P. merguiensis* hemolymph

Fraction	Protein (mg)	HA[a]	Specific activity[b]	Purification fold	Recovery (%)
Hemolymph	845	70,980	84	1	100
Fetuin-agarose eluate peak F2	0.485	43,298	89,208	1,062	61.0
Superose 12 eluate peak S1*	0.070	11,257	158,760	1,890	15.9
Preparative PAGE elutate*	0.140	21,756	155,400	1,850	30.6

[a] Hemagglutinating activity units tested in the presence of a 2% rabbit erythrocyte suspension.

[b] HA/mg protein.

***** Further purification of Fetuin-agarose eluate peak F2**.**

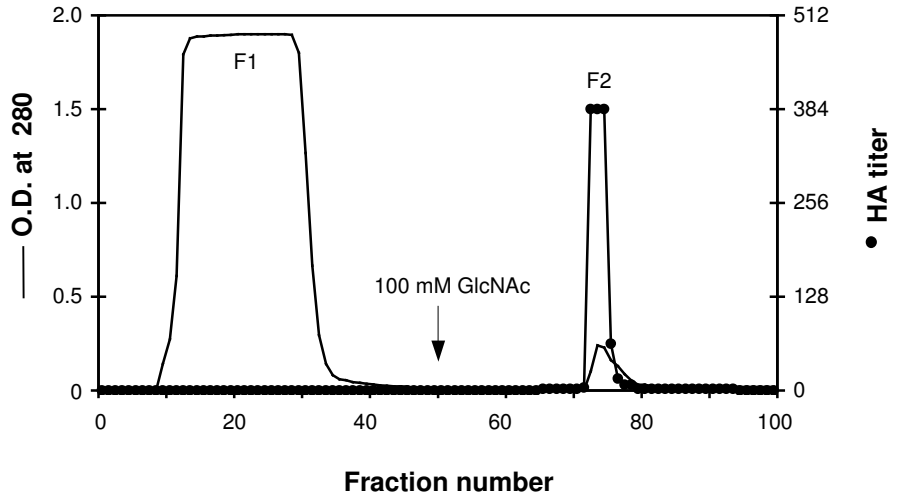


Fig. 9 Purification of lectin from the hemolymph by affinity chromatography on Fetuin-agarose column

Serum (845 mg protein) was applied onto the column (1.2 x 17 cm) equilibrating with TB-NaCa. After a period of 12 h for binding, unbound material was washed out with TB-NaCa, bound lectin was then eluted with the same buffer containing 0.1 M GlcNAc with a flow rate of 15 ml/h. Each fraction (1 ml) was measured for O.D.280 and HA in the presence of 2% rabbit erythrocytes after dialysis against TBS.

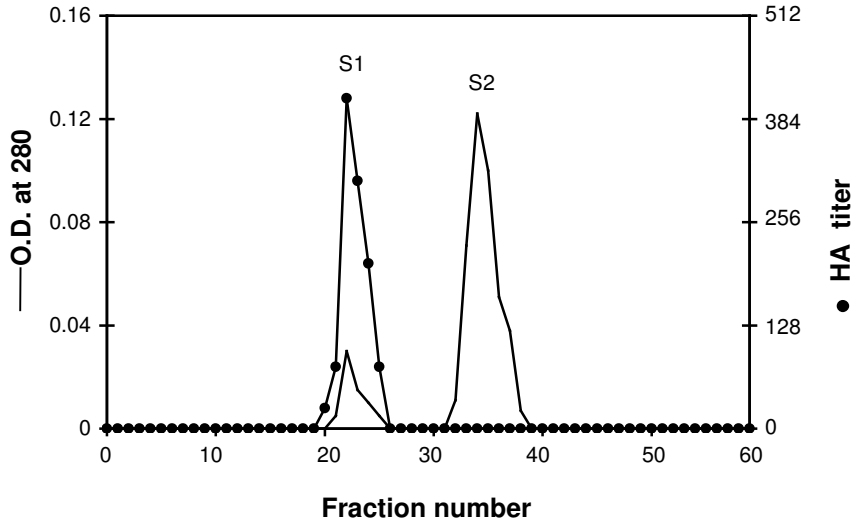


Fig. 10 Purification of high HA fractions from the Fetuin-agarose column on FPLC Superose 12 HR column

The dialyzed fractions with high HA (peak F2) from the Fetuin-agarose column were loaded on a Superose 12 HR column, pre-equilibrated and washed with TBS with a flow rate of 0.5 ml/min. The O.D.280 and HA of 0.5 ml fractions were determined. Fractions 22-28 (peak S1), containing purified lectin, were pooled and concentrated.

The purity of the lectin preparations during the purification steps was assessed by means of nondenaturing gradient PAGE (Fig. 11). Peak F2 from the Fetuin-agarose column showed one major protein band and a faint protein band (lane 3). The single major protein band (lane 4) was also present in peak S1 from the Superose 12 HR column, indicating that the peak S1 represented purified lectin. The same lectin band was also present in the eluate following fractionation of active fractions from the Fetuin-agarose column in preparative PAGE (lane 5). The eluate was therefore free of other proteins, indicating that it represented purified lectin.

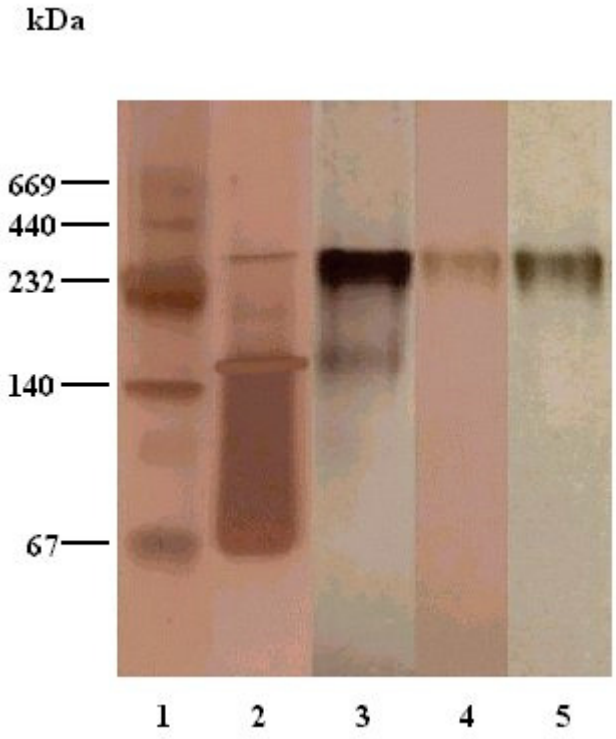


Fig. 11 Nondenaturing 4-10% gradient PAGE of lectin at each purification step

The gel was stained with a silver staining kit. Lane 1, molecular weight markers; lane 2, hemolymph; lane 3, peak F2 from Fetuin-agarose column; lane 4, peak S1 from Superose 12 HR column; lane 5, eluate from preparative PAGE.

2. Characterization of purified lectin

2.1 Electrophoretic analysis

Purified lectin was characterized by nondenaturing PAGE. It showed a single protein band (Fig. 11, lane 4).

After SDS-PAGE on a 6-18% SDS-polyacrylamide gel, purified lectin treated with β-mercaptoethanol produced two bands with molecular masses of 32.3 and 30.9 kDa, after staining with Coomassie Blue (Fig. 12). Analysis of purified lectin in the presence (lane 2) or absence (lane 3) of β-mercaptoethanol produced the same protein patterns, indicating that the 2 subunits were not bound by disulphide linkages.

In addition to staining with glycoprotein staining kit (Fig. 13) after SDS-PAGE, both subunits of purified lectin showed two positively bands. This indicated the presence of bound carbohydrate moiety.

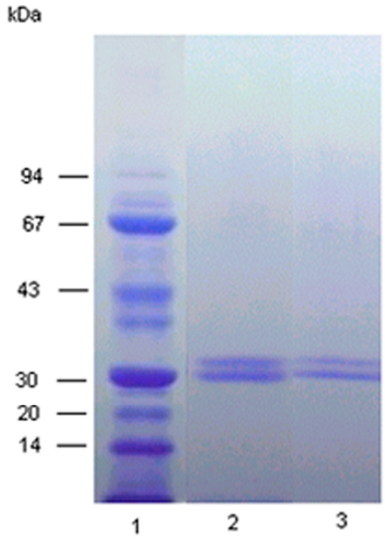


Fig. 12 SDS-PAGE of purified lectin on a 6-18% gradient gel

The protein bands were visualized by staining with Coomassie Blue. Lane 1, molecular weight markers; lane 2, purified lectin in the presence of β-mercaptoethanol; lane 3, purified lectin in the absence of β-mercaptoethanol**.**

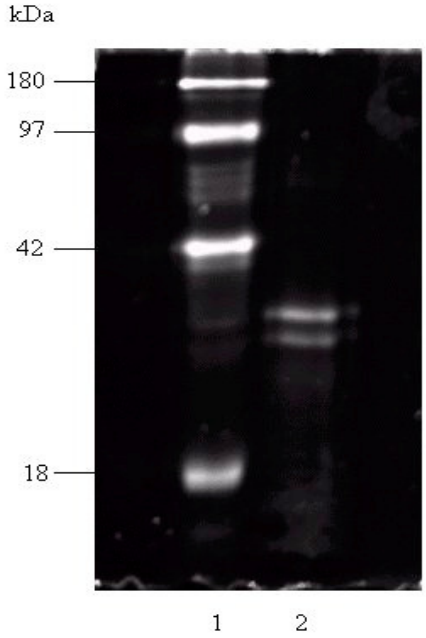


Fig. 13 Characterization of purified lectin staining for carbohydrate moiety

Two μg of purified lectin and 250 ng of CandyCane standards were loaded on 10% SDS-PAGE. The gel was then stained with glycoprotein staining kit.Lane 1, CandyCane standards containing $\propto_2$-macroglobulin (180 kDa), glucose oxidase (97 kDa), $\propto_1$-acid glycoprotein (42 kDa), avidin (18 kDa); lane 2, purified lectin.

2.2 Two-dimensional PAGE (2-D-PAGE) analysis of purified lectin

Two-dimensional PAGE using the SDS gel for the second dimension showed that purified lectin was composed of two protein subunits with the M_r of 32.3 and 30.9 kDa (Fig. 14). From IEF analysis, both subunits of the lectin were focused at the same pI values of 6.0 as estimated by calibration with known standard pI markers.

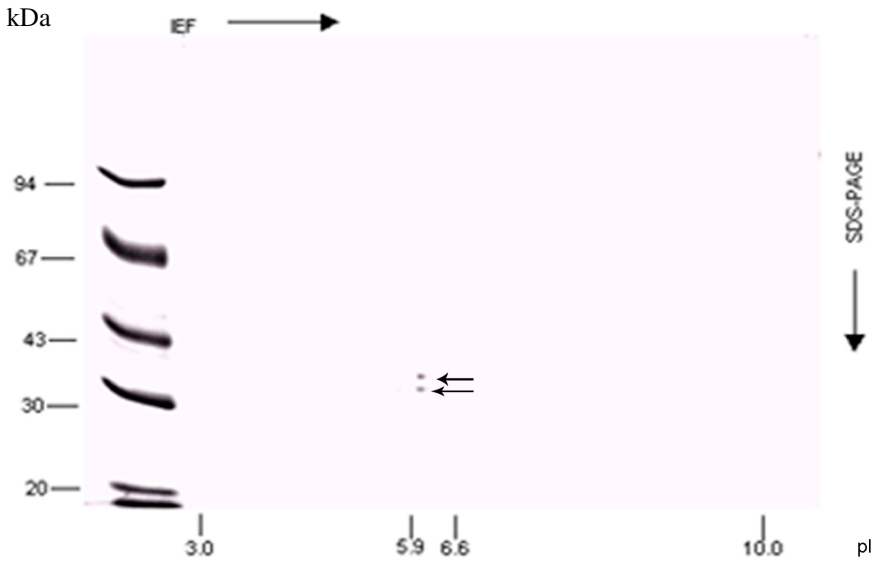


Fig. 14 Two-dimensional electrophoretic analysis of purified lectin

Purified lectin was run in the first-dimension for IEF (pH gradient 3-10). The protein markers used were: carbonic anhydrase, pI 7.0; ovalbumin, pI 5.1; glucose oxidase, pI 4.2; amyloglucosidase, pI 3.8. Afterwards, the gel was subjected to a 12% SDS-PAGE under reducing conditions. Molecular weight markers were: phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and trypsin inhibitor (20 kDa). The arrows indicated the 2 subunit bands of purified lectin.

2.3 Analysis of Amino acid composition

Purified lectin has 16.81% mole of acidic amino acids Asx plus Glx. Also present as high % mole of total amino acids were serine, glycine, histidine and leucine residues, with minor amounts of tyrosine, and methionine residues. It also contained cysteine residues as showed in Table 8.

Table 8 Amino acid composition of purified lectin

Amino acids	%Mole of all amino acids
Asx[a]	6.73
Glx[b]	10.08
Ser	12.19
Gly	13.40
His	10.38
Arg	4.96
Thr	4.91
Ala	6.84
Pro	3.87
Tyr	0.97
Val	3.25
Met	0.49
Cys	2.72
Ile	3.21
Leu	9.68
Phe	2.37
Lys	3.96
Trp	Not determined

[a] Asx: Asp + Asn.

[b] Glx: Glu + Gln.

2.4 Analysis of carbohydrate composition

2.4.1 Composition analysis of neutral amino sugars by HPAEC-PAD

Purified lectin was treated with trifluoroacetic acid (TFA) to cleave all glycosidic linkages. After drying, the hydrolysate was dissolved in water and injected onto a CarboPac PA-1 anion-exchange HPLC column, the fractions were collected according to the retention times of the monosaccharides standards, fucose (Fuc, 6.33 min), galactosamine ($GalNH_2$, 10.83 min), glucosamine ($GlcNH_2$, 12.67 min), galactose (Gal, 13.58 min), glucose (Glc, 15.08 min) and mannose (Man, 16.67 min) (Fig 15). After 1% of the lectin was injected to a column, it was eluted with a low concentration of sodium hydroxide. The elution profiles showed the presence of $GlcNH_2$, Glc and Man in the sample at retention times of 12.58, 15.08 and 16.67 min, respectively (Fig. 16) corresponding to the retention time of external monosaccharide standards ($GlcNH_2$ 12.67 min, glucose 15.08 min and Man 16.67 min) while that of the internal standard (sample blank) $GalNH_2$, $GlcNH_2$ and Glc were 10.75, 12.67 and 10.58 min, respectively. The sugar content of samples was monitored by a pulsed amperometric detector (PAD). The printout of the results showed the profile and individual monosaccharide content expressed in nanomoles present in the volume injected. Blanks (that represent background from the method used for sample preparation) are required for optimum quantitative results. Purified lectin contained the sugars Man, Glc and $GlcNH_2$ but no content of Fuc, Gal and $GalNH_2$ (Table 9). The sugar components in the lectin were identified as $GlcNH_2$: Glc : Man in a molar ratio of 3:0.6:1.

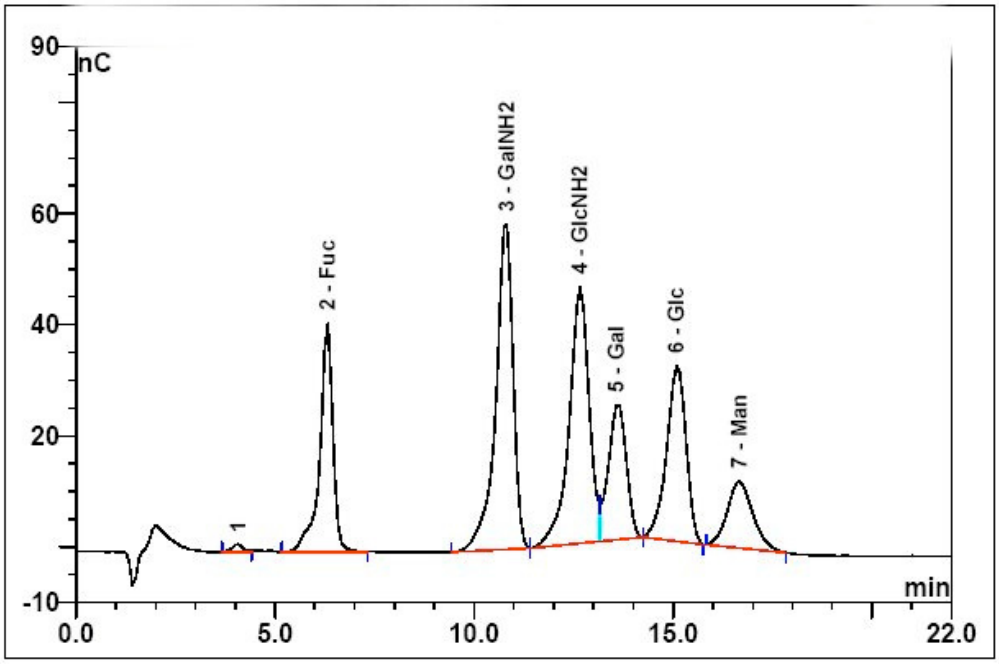


Fig. 15 HPAEC-PAD elution profile of monosaccharide standards

A mixture of monosaccharides standards was separated by CarboPac PA-1 anion-exchange HPLC. Effluent was eluted by a low concentration of sodium hydroxide at a flow rate of 1.2 ml/min. The sugars were detected by a pulsed amperometric detector (PAD). The retention times of fucose (Fuc), galactosamine ($GalNH_2$), glucosamine ($GlcNH_2$), galactose (Gal), glucose (Glc) and mannose (Man) were 6.33, 10.83, 12.67, 13.58, 15.08 and 16.67 min, respectively.

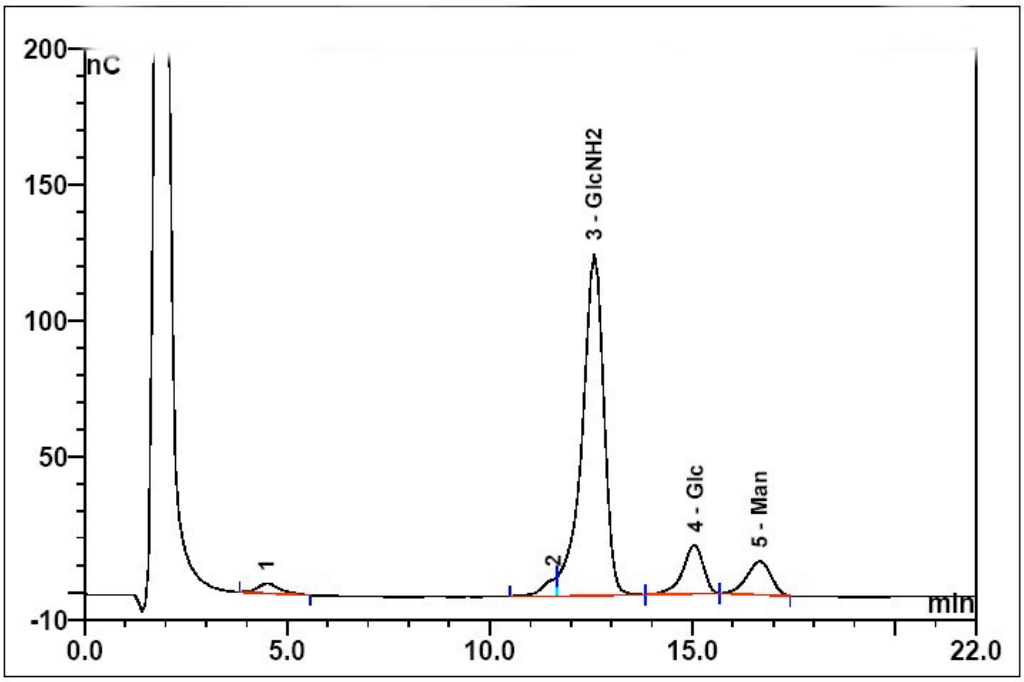


Fig. 16 HPAEC-PAD profile of purified lectin

The fractions containing the sugar contents of purified lectin were eluted from CarboPac PA-1 anion-exchange HPLC. The profile showed the presence of $GlcNH_2$, Glc and Man in the sample at the retention time of 12.58, 15.08 and 16.67 min, respectively.

Table 9 Neutral and amino sugar analysis of purified lectin

Sugar	Content nmol/μg protein	Ratio relative to mannose
Fucose	0	0
Galactosamine	0	0
Glucosamine	11.604	3.040
Galactose	0	0
Glucose	2.316	0.607
Mannose	3.816	1.000

2.4.2 Sialic acid determination by RP-HPLC

The fluorescent sialic acid derivatives from purified lectin were analyzed by RP-HPLC with on-line fluorescence detection. The fractions were collected according to the retention times of the sialic acid derivatives standards, N-glycolyl neuraminic acid (Neu5Gc) (13.60 min) and N-acetyl neuraminic acid (Neu5Ac) (17.30 min) (Fig. 17A). After purified lectin (1:10 dilution) was hydrolyzed and derivatized, it was injected into the RP-HPLC column. The elution profile showed the presence of Neu5Ac in the sample at the retention time of 17.0 min (Fig. 17B) corresponding to that of Neu5Ac standard (17.30 min). The concentration of released sialic acid from purified lectin was displayed in Table 10.

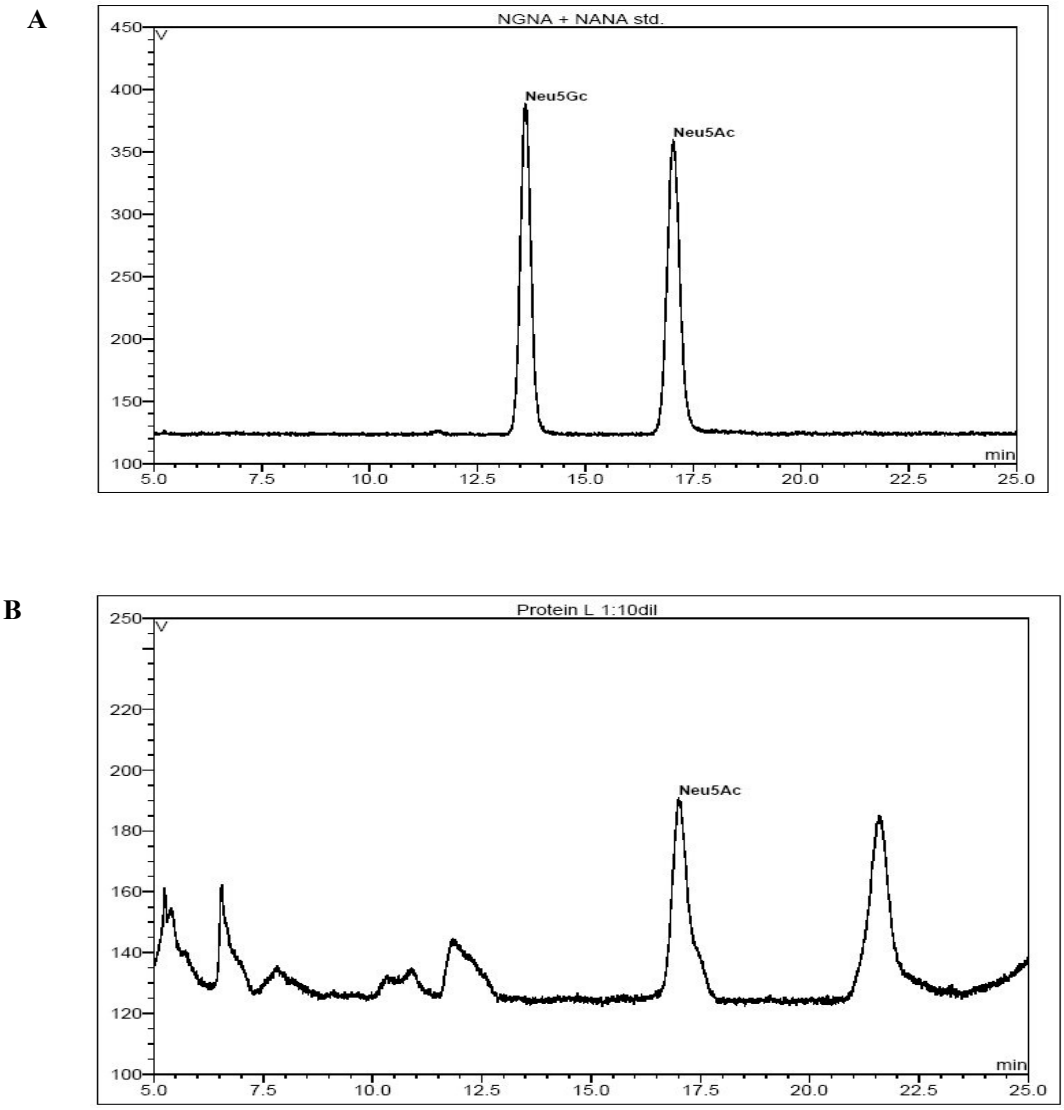


Fig. 17 RP-HPLC elution profile of salic acid standards (A) and purified lectin (B)

(A) A mixture of Neu5Gc (N-glycolyl neuraminic acid) and Neu5Ac (N-acetyl neuraminic acid) derivative standards was separated by RP-HPLC. Fluorescent DMB derivatives were eluted from the column and detected by an on-line fluorescent detector. The retention times of Neu5Gc and Neu5Ac were 13.6 and 17.3 min, respectively. (B) The fluorescent DMB derivatives from purified lectin were separated by RP- HPLC in the same manner. The profile showed the presence of Neu5Ac in the sample at the retention time of 17.0 min.

Table 10 Sialic acid composition of purified lectin

Sialic acid	Content pmol/μg protein
Neu5Gc	0
Neu5Ac	29.84

2.5 Deglycosylation of purified lectin

Prior to treatment of purified lectin by enzymatic and chemical digestion, native lectin oligomer had M_r of 30.9 and 32.3 kDa (Fig. 18 and Fig. 19, lane 2). After treatment by either PNGase F (Fig.18, lane 3) or trifluoromethanesulfonic acid (Fig. 19, lane 3), the lectin showed a single 28 kDa band which was of lower molecular mass and less protein band than that of the untreated lectin.

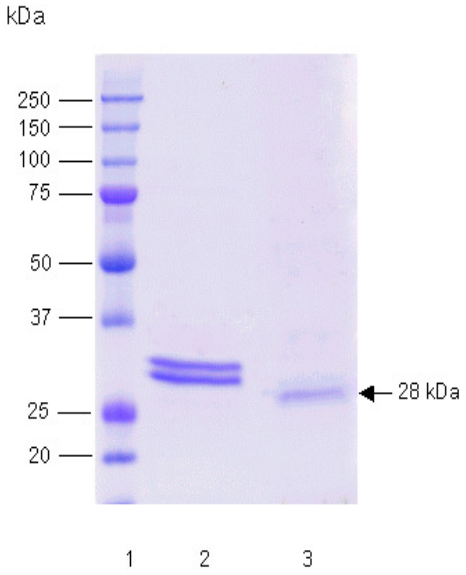


Fig. 18 Deglycosylation of purified lectin by PNGaseF

Purified lectin was treated with peptide N-glycosidase F (PNGase F) and subjected to10% SDS-PAGE. The gel and stained with Coomassie Blue. Lane 1, molecular weight protein markers; lane 2, purified lectin without treatment; lane 3, purified lectin with treatment.

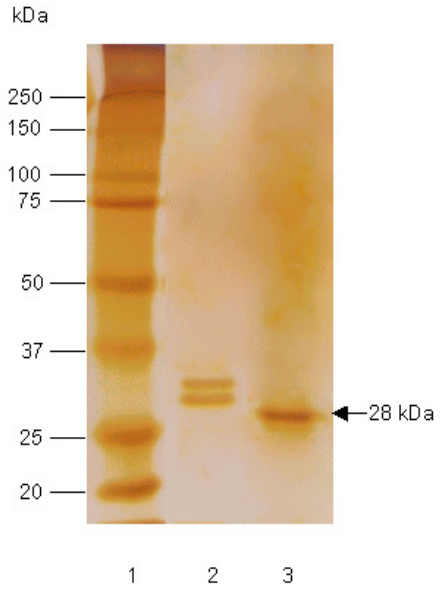


Fig. 19 Chemical deglycosylation of purified lectin by TFMS

Purified lectin was treated with trifluoromethanesulfonic acid (TFMS) and subjected to10% SDS-PAGE. The gel and stained with silver. Lane 1, molecular weight protein markers; lane 2, purified lectin without treatment; lane 3, purified lectin with treatment.

2.6 Affinity blot analysis

An affinity blot test using biotinylated lectins revealed that both subunits of purified lectin composed carbohydrate moieties that were strongly recognized by the lectin from tomato, red kidney bean and snowdrop bulb (Fig. 20). The other lectins also recognized sugar residues in purified lectin molecule, although the reaction was weaker than those from tomato, red kidney bean and snowdrop bulb. The lectin from Maackia, coral tree, Japanese wisteria, winged bean, Jequirity bean and Osage orange were not recognized sugar residues in purified lectin. When the analysis was carried out by using partially purified lectin (peak F2 from the Fetuin-agarose column) and separating by 10% SDS-PAGE, the biotinylated lectin from soy bean, peanut, asparagus pea, wheat germ and pea could react with both protein bands of lectin and that of minor contaminated one (Fig. 21 lane2, 3, 4, 5 and 6). Only biotinylated lectin from tomato, red kidney bean and snowdrop bulb react specially with the lectin band (Fig. 21 lane 7, 8 and 9).

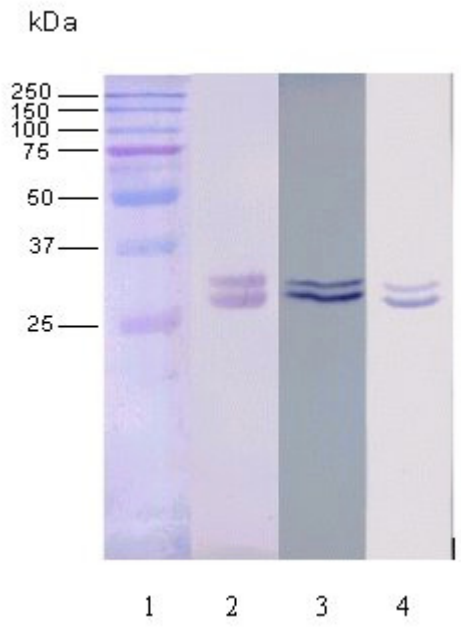


Fig. 20 Affinity blot analysis of purified lectin

After SDS-PAGE on 10% gel, purified lectin was blotted onto nitrocellulose membrane and incubated with 30 μg/ml biotinylated lectins. Immunoreactive band was developed with streptavidin peroxidase conjugated and 3,3′,5,5′ tetramethylbenzidine/ H_2O_2. Lane 1, molecular weight protein markers; lane 2, purified lectin developed with biotinylated *Lycopersicon esculentum* (tomato) lectin; lane 3, purified lectin developed with biotinylated *Galanthus nivalis* (snowdrop bulb) lectin; lane 4, purified lectin developed with biotinylated *Phaseolus vulgaris* (red kidney bean) lectin.

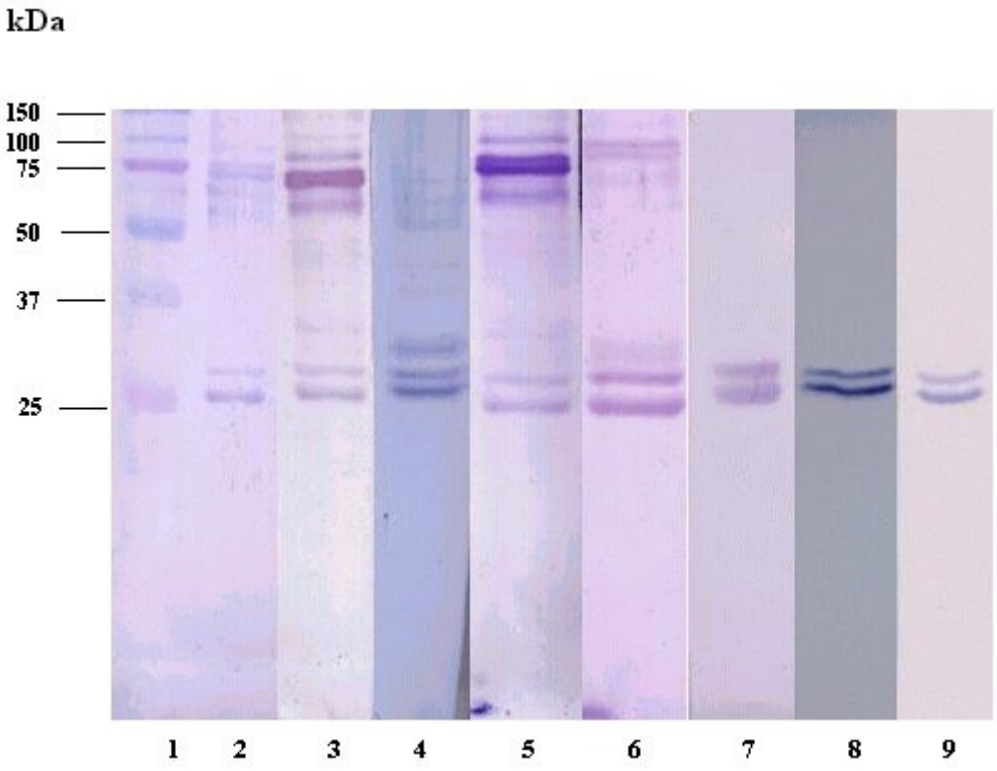


Fig. 21 Western blot analysis of partially purified lectin

After SDS-PAGE on 10% gel, partially purified lectin (peak F2 from the Fetuin-agarose column) was blotted onto nitrocellulose membrane and incubated with 30 μg/ml biotinylated lectins. Immunoreactive band was developed with streptavidin-peroxidase conjugated and TMB/H_2O_2. Lane 1, molecular weight protein markers; lane 2, partially purified lectin developed with biotinylated *Glycine max* (soy bean) lectin; lane 3, partially purified lectin developed with biotinylated *Arachis hypogaea* (peanut); lane 4, partially purified lectin developed with biotinylated *Lotus tetragonolobus* (asparagus pea) lectin; lane 5, partially purified lectin developed with biotinylated *Triticum vulgaris* (wheat germ) lectin; lane 6, partially purified lectin developed with biotinylated *Pisum sativum* (garden pea); lane 7, partially purified lectin developed with biotinylated *Lycopersicon esculentum* (tomato) lectin; lane 8, partially purified lectin developed with biotinylated *Galanthus nivalis* (snowdrop bulb) lectin and lane 9, partially purified lectin developed with biotinylated *Phaseolus vulgaris* (red kidney bean) lectin.

3. Sugar specificity of purified lectin

The sugar specificity of purified lectin was investigated by competitive inhibition of various monosaccharides and glycoproteins on the HA of purified lectin against rabbit erythrocytes. Four N-acetylated sugars were inhibitory with Neu5Ac being the most effective (Table 11). A 1.0 mM concentration of this sugar inhibited purified lectin (titer 1:128), while a four-fold higher concentration of GalNAc and GlcNAc and a sixteen-fold higher concentration of ManNAc, was required to inhibit a similar activity of the lectin. D-galactose, D-glucose, or D-mannose had no inhibitory activity at 200 mM. From the glycoproteins tested, porcine stomach mucin and fetuin were inhibitors with a similar extent. Fetal calf serum asialofetuin and bovine submaxillary gland mucin at concentrations up to 5 mg/ml showed no effect on the HA (Table 11).

Table 11 Inhibition of hemagglutinating activity of purified lectin 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
D-Glucose	NI[b]
D-Galactose	NI[b]
D-Mannose	NI[b]
Bovine submaxillary mucin	NI[b]
Fetal calf serum asialofetuin	NI[b]

[a] Minimum concentration to completely inhibit purified lectin (titer 1:128) in the presence of a 2% rabbit erythrocyte suspension.

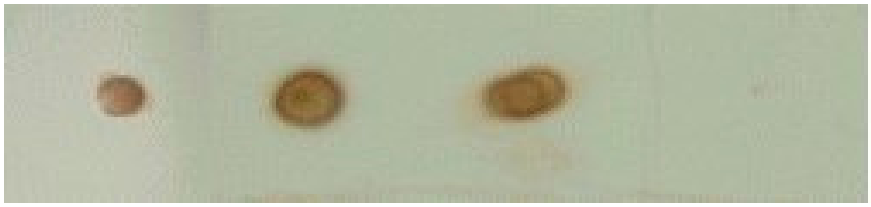
[b] NI: no inhibition of agglutination at 200 mM of sugars or at 5 mg/ml of glycoproteins.

4. Immunological study of purified lectin

4.1 Specificity of anti-lectin antibody

An anti-lectin antibody was successfully raised against purified lectin in a rabbit. Specificity of the antibody was demonstrated by dot blot analysis as shown in Fig. 22. It showed strong cross reactivity with purified lectin and the hemolymph. No reaction was found between the antibody and BSA.

The anti-lectin antibody specificity was also confirmed by Western blot analysis. It revealed that anti-letin antibody could recognize purified lectin in native form in nondenaturing PAGE (Fig. 23, lane 3) and denatured form in SDS-PAGE of both 32.3 kDa and 30.9 kDa subunits as well (Fig. 24, lane 2).



1 2 3 4

Fig. 22 Dot blot analysis of lectin

Same amount of proteins (1.5 μg/spot) was spotted on the nitrocellulose membrane. After reaction with anti-lectin antibody (1:2,000 dilution) and rinsing, HRP-conjugated goat anti-rabbit IgG (1:15,000 dilution) was employed. 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 TBS. Spot 1, purified lectin; spot 2, fraction F2 from Fetuin-agarose column; spot 3, hemolymph; spot 4, BSA.

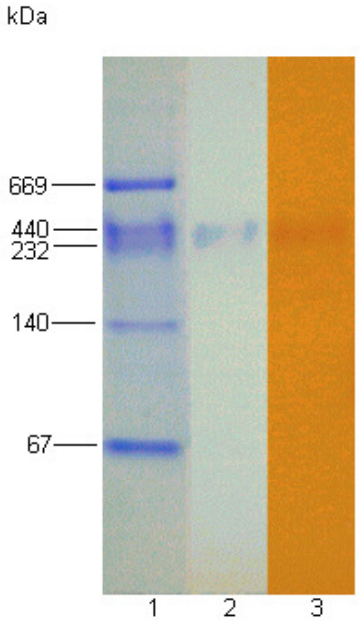


Fig. 23 Western blot analysis of purified lectin in native form

Purified lectin was separated by 4-10% gradient nondenaturing PAGE. After blotting to nitrocellulose membrane, the lectin was reacted with the antibodies and visualized in the presence of peroxidase substrate DAB. Lane1, molecular weight protein markers; lane2, purified lectin in gel staining by Commassie Blue; lane 3, purified lectin blotted to the membrane and reacted with the antibodies.

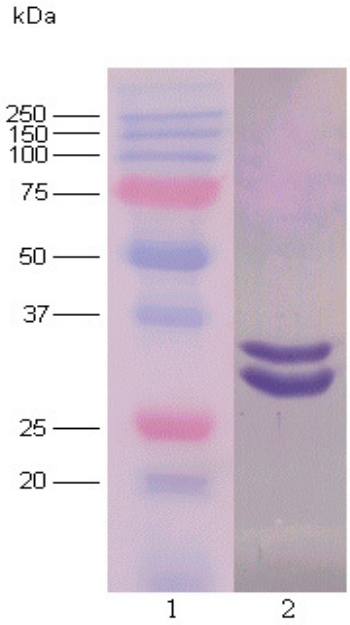


Fig. 24 Western blot analysis of purified lectin in denatured form

Purified lectin was separted by 10% SDS-PAGE. After blotting to nitrocellulose membrane, the lectin was reacted with the antibodies and visualized in the presence of peroxidase substrate TMB. Lane1, molecular weight protein markers; lane2, purified lectin blotted to the membrane and reacted with the antibodies.

4.2 Cross-reactivity of anti-lectin antibody with hemolmph proteins from other crustaceans

In this study, the hemolymph of the following animals were tested, *M. rosenbergii*, *P. monodon*, *P. vannamei* and also *P. merguiensis*. By using dot blot analysis, the proteins from *P. vannamei* and *P. monodon* were positively reacted with the anti-lectin antibody as well as that of *P. merguiensis* (Fig. 25, spot 2 and 3). The hemolymph of *M. rosenbergii* showed no reactivity (spot 4) as BSA (spot 4).

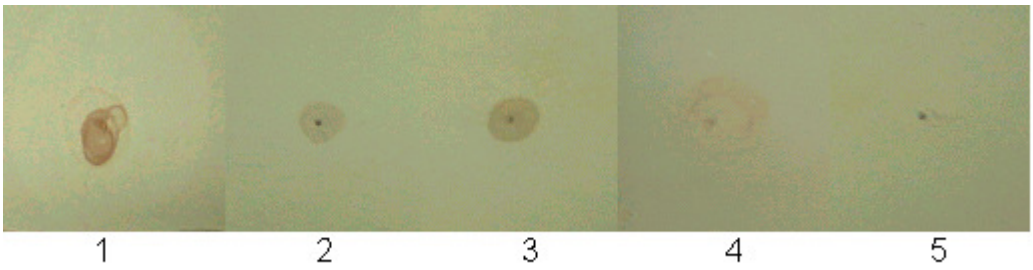


Fig. 25 Dot blot analysis of hemolmph proteins from some crustaceans

Same dilution of the hemolymph (1:100**,** 5 μl/spot) from some crustaceans was investigated by dot blotting with the anti-lectin antibody. Spot 1, purified lectin; spot 2, *P. vannamei* hemolymph; spot 3, *P. monodon* hemolymph; spot 4, *M. rosenbergii* hemolymph; spot 5, BSA.

5. Development of ELISA

The anti-lectin antibody was used to develop an ELISA for quantitating lectin and other immunological related proteins. The effect of varying the concentrations of antibodies during the assay was showed in Fig. 26 and Fig. 27. With purified lectin, there was an increase in the absorbance with the higher concentrations of both antibodies. For the appropriate values of O.D. $_{492}$, 1^{o}Ab (anti-lectin antibody) and 2^{o}Ab (HRP conjugated goat anti-rabbit IgG) were used respectively at 1:3,000 and 1:25,000 dilution as the optimal concentration of the antibodies. The optimal condition of peroxidase reaction by varying the concentrations of peroxidase substrates, OPD and H_2O_2, were showed in Fig. 28 and Fig. 29. The reaction with purified lectin showed a steady increase in the absorbance up to 0.6 mg/ml, which slowly increased with the higher concentration of OPD (Fig. 28). The increasing in the absorbance was observed with the higher concentration of H_2O_2 (Fig. 29). For the practical assay, 0.4 mg/ml OPD and 0.012% H_2O_2 were selected as the optimum concentrations of peroxidase substrates. The reaction time of conjugated peroxidase was investigated by varying the incubation time from 0 to 60 min (Fig. 30). The reaction showed an increase in the absorbance up to 30 min, which a steady increase after 30 min. Therefore, 30 min was selected as the optimal incubation time. From these results, the optimum condition of ELISA was developed by using 1^{o}Ab at 1:3,000 dilution, 2^{o}Ab at 1:25,000 dilution, 0.4 mg/ml OPD, 0.012% H_2O_2 and the incubation time from 30 min. With this optimum condition, a standard curve was obtained with purified lectin.

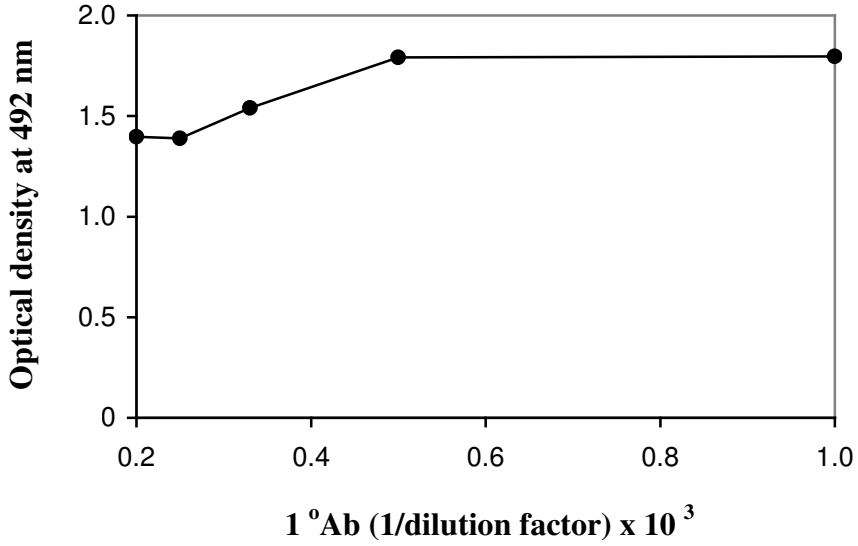


Fig. 26 Effect of anti-lectin antibody concentrations on ELISA

Purified lectin (350 ng/well) was coated on a microtiter plate. After incubation with anti-lectin antibody (1^{o}Ab) at a concentration ranging from 1:1,000 to 1:5,000 , 2^{o}Ab (HRP conjugated goat anti-rabbit IgG) (1:25,000 dilution) was added. The reaction was initiated by 0.4 mg/ml OPD and 0.012% H_2O_2 and $O.D._{492}$ was measured. Each point is an average of two measurements.

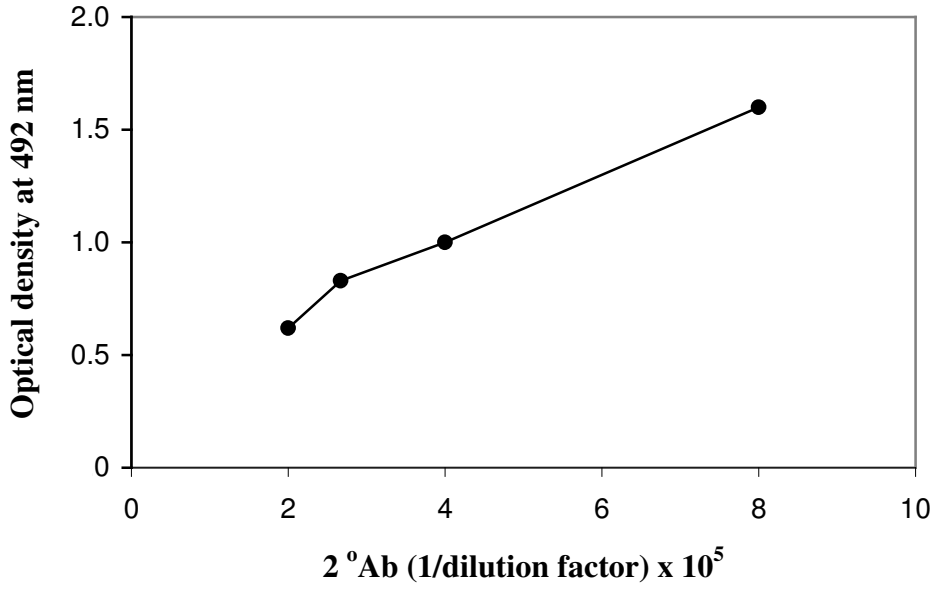


Fig. 27 Effect of 2^{o}Ab concentrations on ELISA

By using purified lectin (350 ng/well) and 1^{o}Ab at 1:3,000 dilution, an addition of HRP conjugated goat anti-rabbit IgG (2^{o}Ab) at a concentration ranged from 1:12,500 to 1:50,000 was carried out. The reaction was initiated by 0.4 mg/ml OPD and 0.012% H_2O_2. The $O.D._{492}$ was measured. Each point is an average of two measurements.

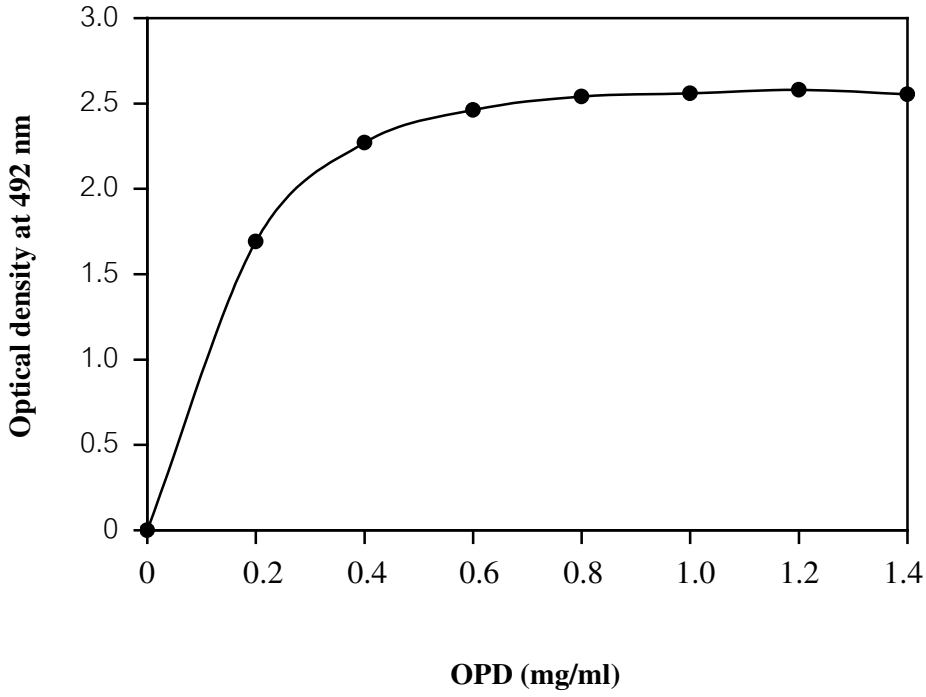


Fig. 28 Effect of OPD concentrations on ELISA

ELISA was performed in the presence of purified lectin (350 ng/well), 1^{o}Ab at 1:3,000 dilution and 2^{o}Ab at 1:25,000 dilution. The reaction was initiated by various concentrations of OPD ranging from 0 to 1.4 mg/ml. After addition of 0.012% H_2O_2 and left for 30 min, the $O.D._{492}$ was measured. Each point is an average of two measurements.

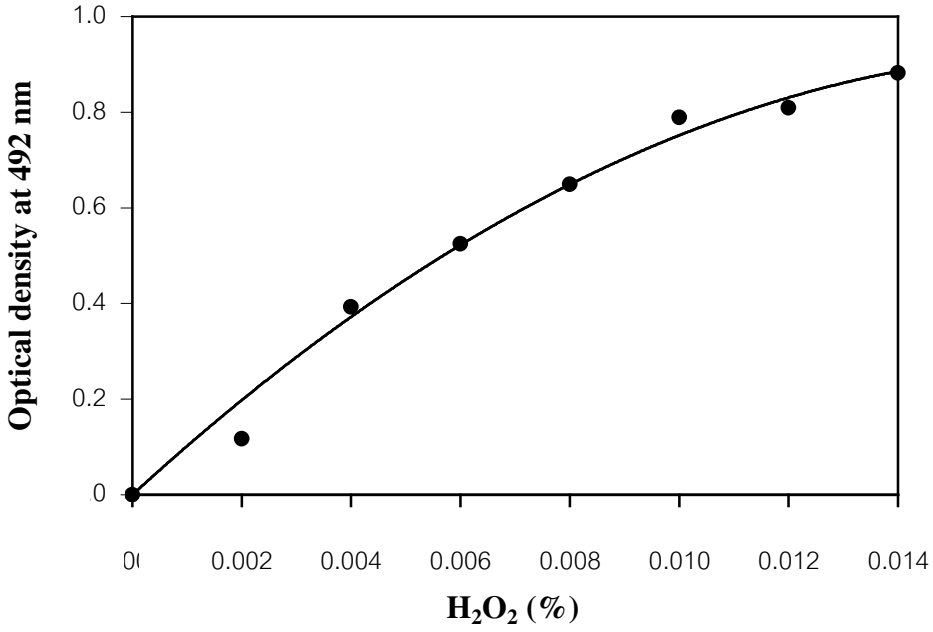


Fig. 29 Effect of H_2O_2 concentrations on ELISA

ELISA was performed in the presence of purified lectin (350 ng/well), 1^{o}Ab at 1:3,000 dilution and 2^{o}Ab at 1:25,000 dilution. The reaction was initiated by 0.4 mg/ml OPD. After addition of H_2O_2 at various concentrations ranging from 0 to 0.014%, the reaction was left for 30 min and the $O.D._{492}$ was measured. Each point is an average of two measurements.

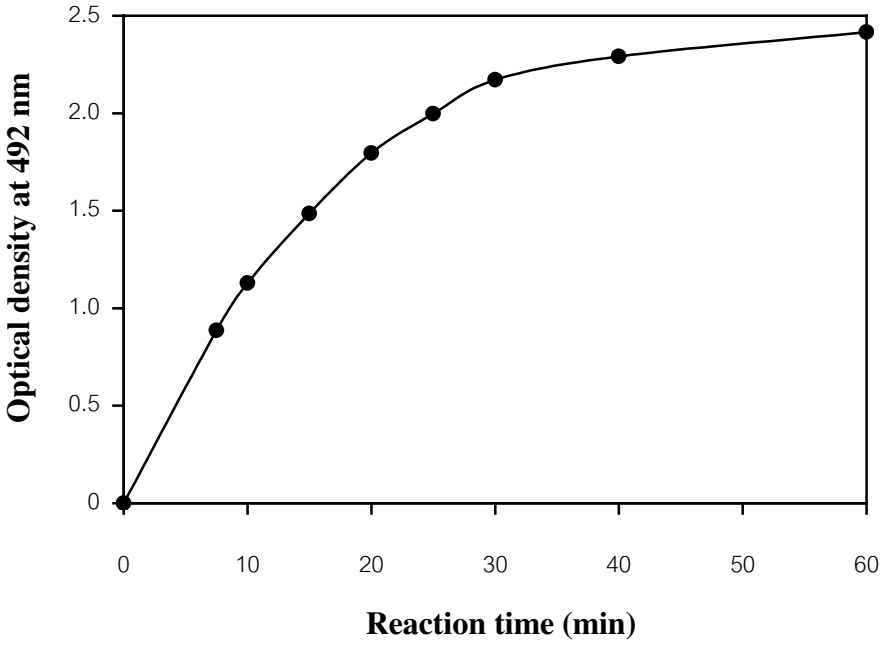


Fig. 30 Effect of reaction time of peroxidase on ELISA

The experiment was carried out in the presence of purified lectin (350 ng/well), 1^{o}Ab at 1:3,000 dilution and 2^{o}Ab at 1:25,000 dilution. The reaction was initiated by 0.4 mg/ml OPD and 0.012% H_2O_2. After left for 0 to 60 min, the $O.D._{492}$ was measured. Each point is an average of two measurements.

Standardization of ELISA was achieved by using purified lectin as the reference antigen. The standard curve was linear over the range of 12.5-350 ng with a correlation coefficient R^2= 0.988 as showed in Fig. 31. The sensitivity of the assay to detect lectin concentration was very low as 83.3 ng/ml (Fig. 32). To determine whether the ELISA is able to quantify hemolymph lectin, the hemolymph was serially diluted and determined by ELISA. The linear curve was obtained over the dilution range of 1:50 to 1:400 (Fig. 33) To ovoid the dilution factor that may have, all of the hemolymph samples were thus diluted to 1:100 dilution and then quantitated for lectin content by ELISA in this study.

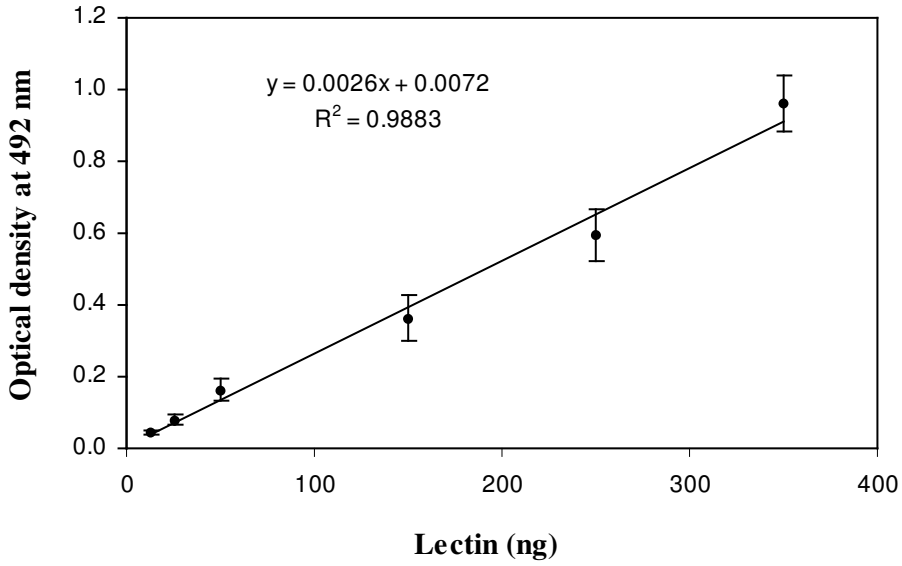


Fig. 31 ELISA standard curve of purified lectin

Each well on microtiter plate was coated with various amounts of purified lectin ranging from 12.5 to 350 ng. ELISA was performed at the optimum condition by using 1^{o}Ab (1:3,000 dilution), 2^{o}Ab (1:25,000 dilution), 0.4 mg/ml OPD and 0.012% H_2O_2 and incubated for 30 min. Each point is a mean $\pm$ SD of 6 measurements.

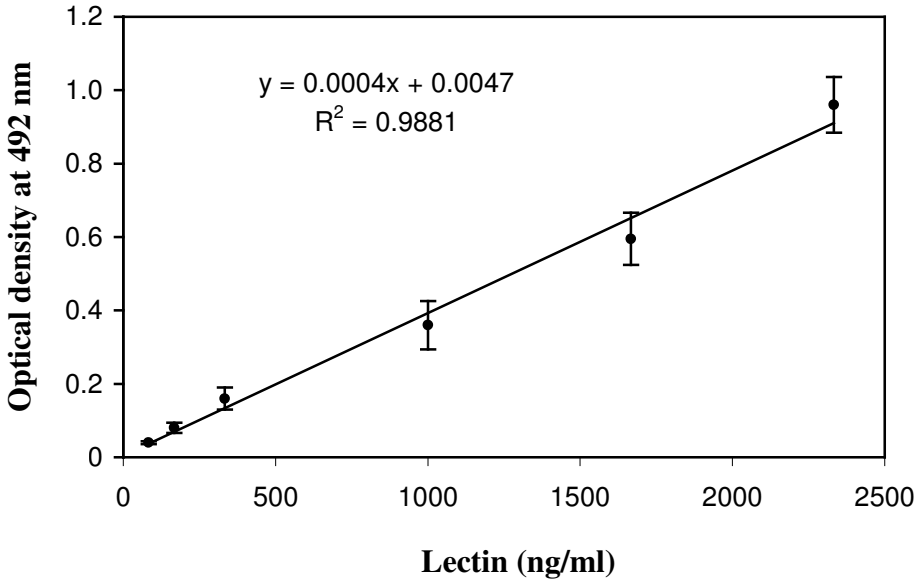


Fig. 32 Sensitivity of ELISA for quantifying purified lectin

Purified lectin at various concentrations ranging from 83.3 to 2,300 ng/ml was coated in a microtiter plate. ELISA was carried out at the optimum condition in a similar manner as in Fig. 31. Each value represents a mean ± SD of 6 determinations.

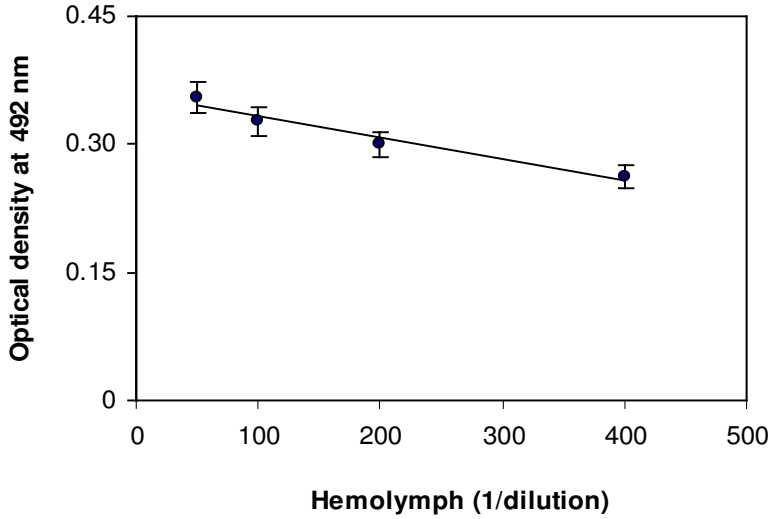


Fig. 33 Dose-response titration of lectin in the hemolymph using ELISA

Microtiter plate was coated with serially diluted hemolymph. An anti-lectin antibody and secondary antibody (HRP conjugated goat anti-rabbit IgG) were used as the same concentration as in Fig. 31. Each point value represents a mean±SD of 3 measurements.

6. Agglutination of bacteria by purified lectin

Three *Vibrio* spp. were isolated from infected penaeid shrimps, *V. vulnificus*, *V. parahemolyticus* and *V. harveyi*, with *V. harveyi* being the predominant species. To establish if lectin had any possible role in protecting the infected shrimps, its ability to agglutinate these pathogenic bacteria was investigated. *V. vulnificus*, *V. parahemolyticus* and *V. harveyi* were strongly agglutinated by purified lectin (Fig. 34), whereas *V. cholerae*, *E. coli* and *S. typhi* were not (data not shown). A lectin concentration of 1 μg/ml was sufficient to agglutinate 5×10^7 cells of *V. harveyi* and *V. parahemolyticus*. Agglutinating activity against *V. vulnificus* was also observed but to a four-fold lower extent, while *V. cholerae*, *E. coli* and *S. typhi* were not agglutinated (Table 12).

Because *V. harveyi* was a major pathogen of *P. merguiensis*, the interaction between purified lectin and *V. harveyi* was further studied. The sugar specificity of the interaction between them was investigated by performing the sugar inhibition test. In this instance, Neu5Ac at 6.25 mM completely inhibited the lectin-induced agglutination of *V. harveyi*. Porcine stomach mucin and fetuin at 1 mg/ml and 3 mg/ml, respectively also caused inhibition. In addition, the activity of purified lectin to induce the agglutination of *V. harveyi* was neutralized by anti-lectin antibody. Complete inhibition required 86 ng/ml of the antibody.

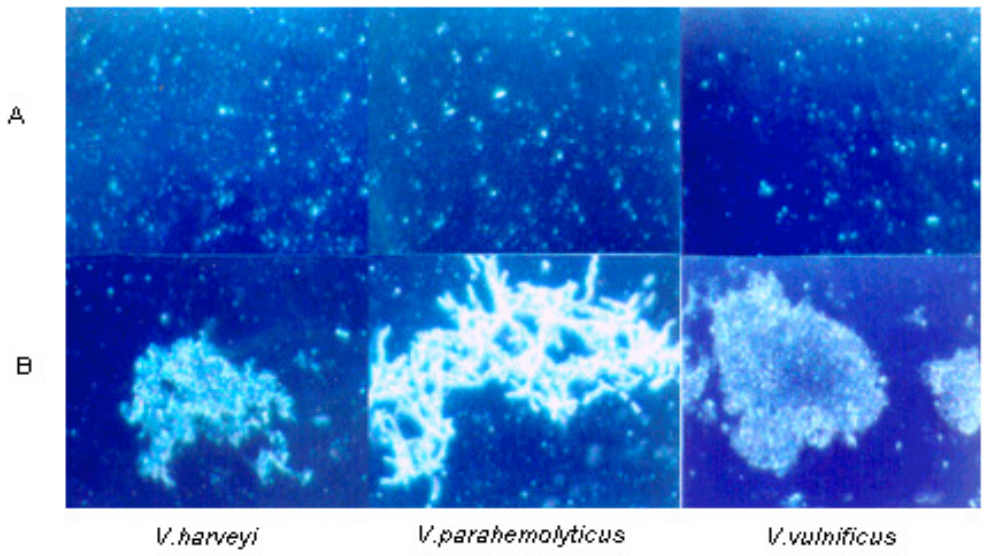


Fig. 34 Agglutination of purified lectin against three species of *Vibrio*

The bacterial samples (5×10^7 cells/ml) were incubated in TBS (A) and with purified lectin (B).

Table 12 Agglutinating activity of purified lectin against various strains of pathogenic and non-pathogenic bacteria

Bacterial strain	Agglutination (BAU/μg protein)[b]	Minimum concentration required (μg/ml)[c]
Vibrio harveyi[a]	40	1.0
Vibrio parahemolyticus[a]	40	1.0
Vibrio vulnificus[a]	10	4.0
Vibrio cholerae	0	No agglutination
Escherichia coli	0	No agglutination
Salmonella typhi	0	No agglutination

[a] Pathogenic bacteria.

[b] Aglutinating activity units tested in presence of each strain of bacteria.

[c] Minimum concentration required for complete agglutination of bacteria by purified lectin.

7. Changes in lectin levels in the hemolymph of *V. harveyei* injected shrimps

7.1 Determination of hemolymph lectin by ELISA

ELISA was used to determine lectin in the hemolymph of shrimps injected by *V. harveyi*. In order to verify whether the hemolymph lectin increased in the hemolymph after pathogen challenge, the hemolymph from 10 individual shrimps collected at time intervals (0, 6 and 12 h) after *V. harveyi* injection was analyzed by ELISA using the specific anti-lectin antibody. Control shrimps were injected by saline (0.85% NaCl) instead of bacterial pathogen. By the end of the experimental time, all of them showed no any infection and survived well whereas death began to occur in the infected shrimps at 12 h after *V. harveyi* administration. From ELISA analysis, lectin levels in the hemolymph of the control shrimps were no significant different (*P* value $>$ 0.05) at any time after the saline injection (Fig. 35A). In contrast, lectin concentrations in the hemolymph of *V. harveyi* injected shrimps were found to increase from 48.55 μg/ml at hour 0 to 73.12 μg/ml at hour 6 and reach the highest as 99.23 μg/ml at hour 12 post-injection (Fig. 36A). In similar, specific lectin contents in the hemolymph of pathogen injected shrimps increased from 0.37 μg/mg protein at hour 0 to 0.55 μg/mg protein at hour 6 and reach the highest as 0.75 μg/ml at hour 12 post-injection (Fig. 36B) while their no significant differences were detected at any time after the saline injection (Fig. 35B).

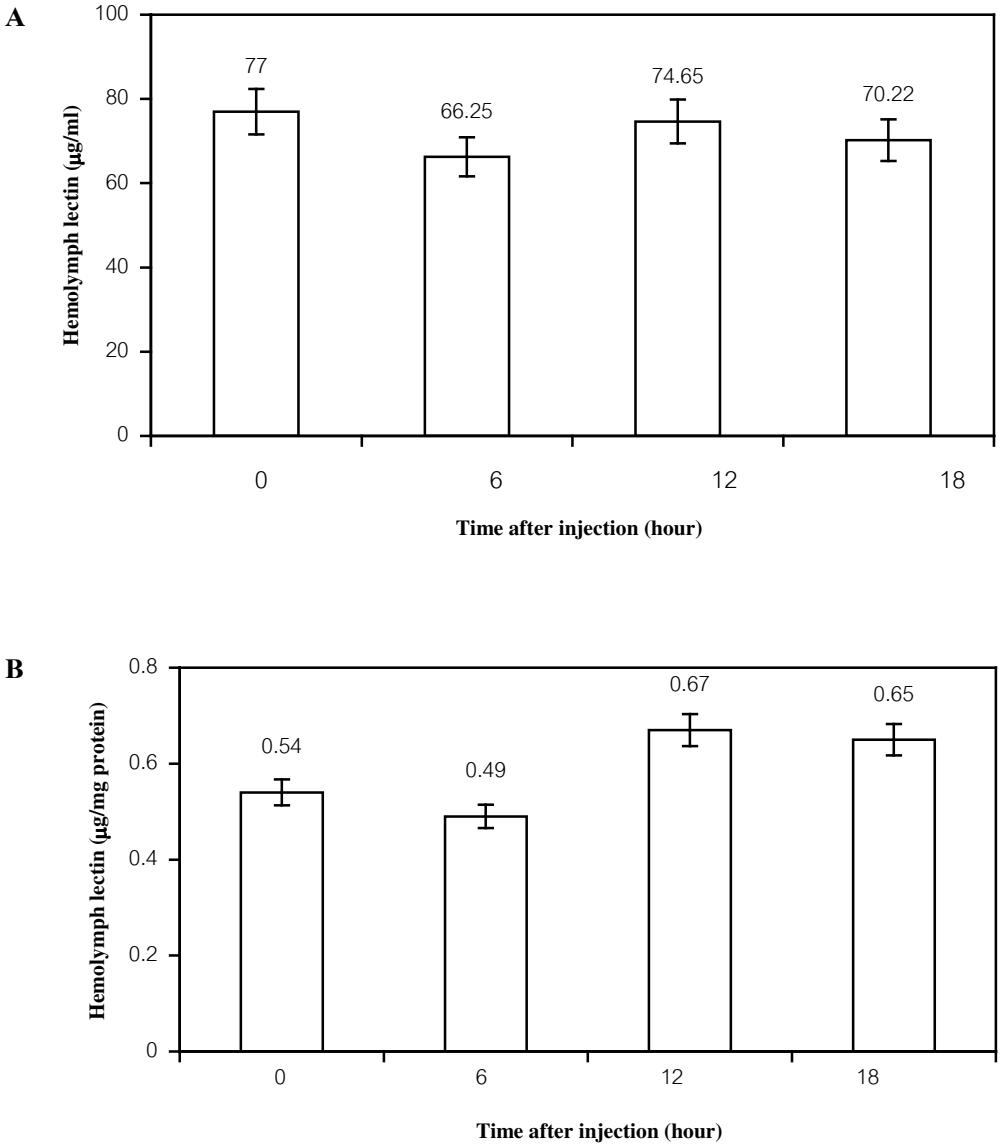


Fig. 35 ELISA quantification of lectin levels in the hemolymph of shrimps injected by saline.

Hemolymph from 14 shrimps was collected at different time intervals after saline injection and then subjected to ELISA analysis. The values were expressed as lectin concentrations (A) or specific lectin contents (B). Bars represent standard errors of mean. Statistics were carried out by SISA program. The values were not different at *P* value $>$ 0.05.

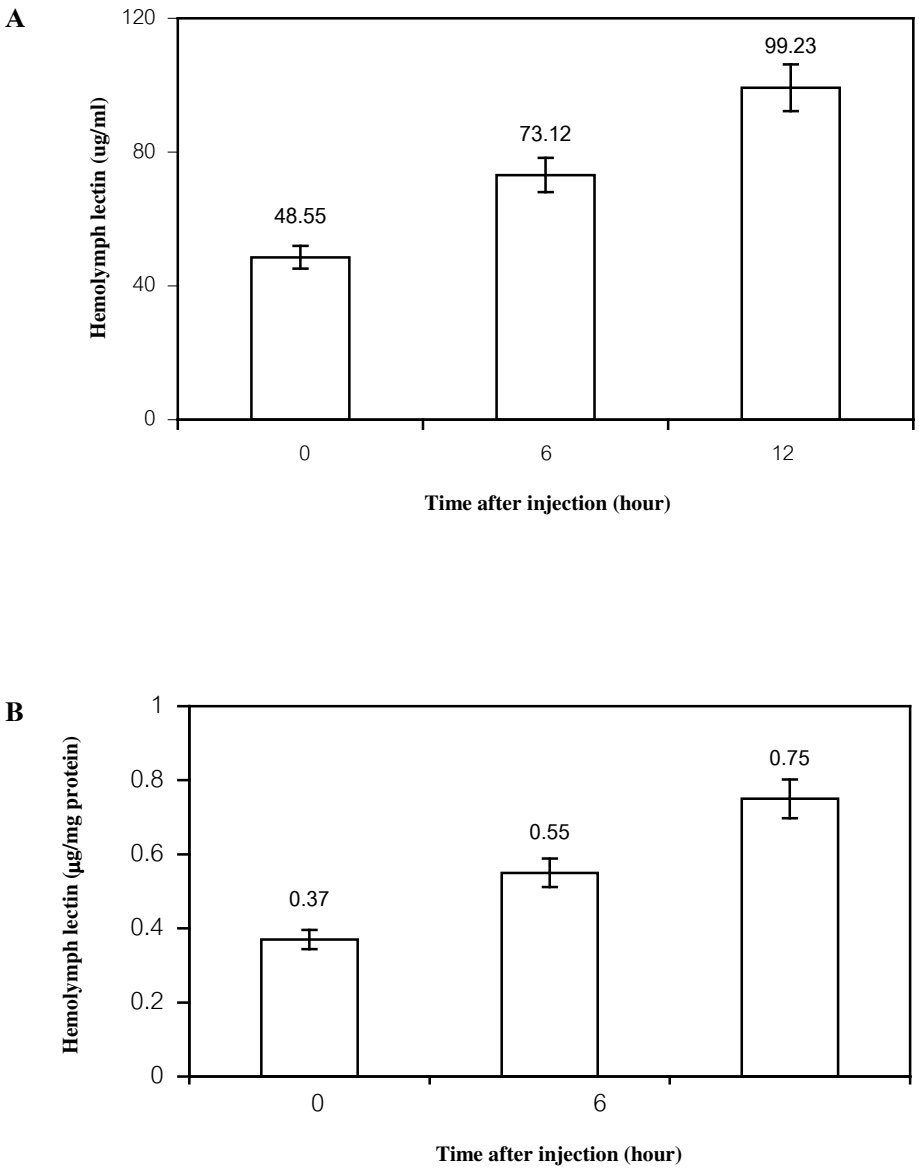


Fig. 36 ELISA quantification of lectin levels in the hemolymph of shrimps injected by *V. harveyei*.

Hemolymph from 10 shrimps was collected at different time intervals after *V. harveyi* injection and then subjected to ELISA analysis. The values were expressed as lectin concentrations (A) or specific lectin contents (B). Bars represent standard error of mean. Statistics were carried out by SISA program. The values were significant different at P value < 0.05 ($P = 0$).

7.2 Determination of hemolymph lectin by hemagglutination assay

In parallel to section 7.1, HA of lectin in the hemolymph of same shrimps either injected by saline or *V. harveyi* was investigated. No significant differences (P value $>$ 0.05) in HA of control shrimps were detected at any time after the saline injection (Fig. 37A). In contrast, the HA in the hemolymph of *V. harveyi* injected shrimps were found to increase from 17,261 unit/ml at hour 0 to 12,420 unit/ml at hour 6 and reach the highest as 472,562 unit/ml at hour 12 post-injection (Fig. 38A). In similar, specific HA in the hemolymph of bacterial injected shrimps increased from 135.17 unit/mg protein at hour 0 to 1044.4 unit/mg protein at hour 6 and reach the highest as 3096.94 unit/mg protein at hour 12 post-injection (Fig. 38B) while they were not significant different at any time after the saline injection (Fig. 37B).

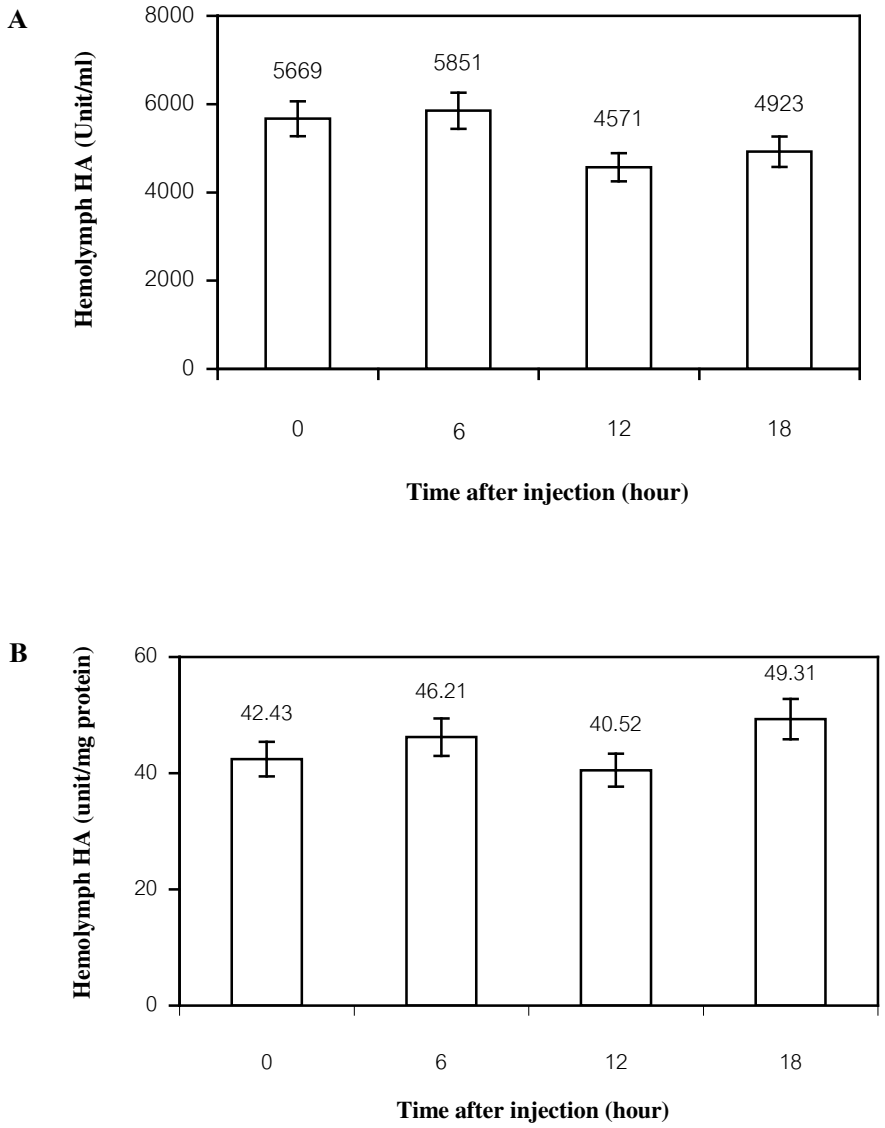


Fig. 37 Hemagglutination test of lectin levels in the hemolymph of shrimps injected by saline

Hemolymph from 14 shrimps was collected at different time intervals after saline injection and then subjected to hemagglutination test. The values were expressed as HA (A) or specific HA (B) of hemolymph lectin. Bars represent standard error of mean. Statistics were carried out by SISA program. The values were not different at *P* value $>$ 0.05.

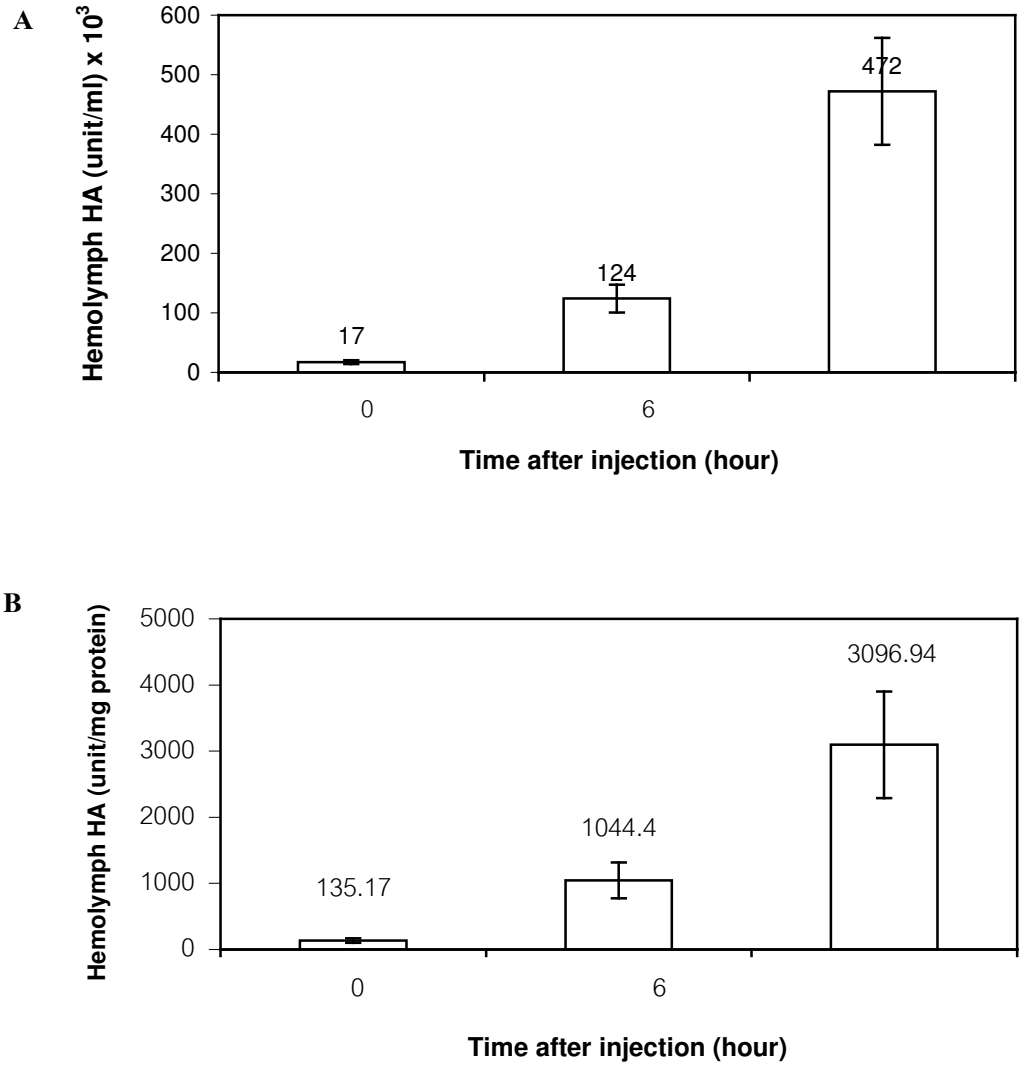


Fig. 38 Hemagglutination test of lectin levels in the hemolymph of shrimps injected by *V. harveyei*

Hemolymph from 10 shrimps was collected at different time intervals after *V. harveyi* injection and then subjected to hemagglutination test. The values were expressed as HA (A) or specific HA (B) of hemolymph lectin. Bars represent standard error of mean. Statistics were carried out by SISA program.The values were significant different at P value < 0.05.

8. Changes in lectin levels in the hemolymph of *P. merguiensis* females at different stages of ovarian development

8.1 Determination of hemolymph lectin by ELISA

Determination of lectin concentrations at different stanges of ovarian development showed that the hemolymph of shrimps at stage 2 to 4 contained the similarly concentrations of lectin of 62.62 ± 4.4, 58.37 ± 4.5, and 60.02 ± 4.4 μg/ml, respectively (Fig. 39A, Table 13). No significant differences ($P > 0.05$) among lectin concentration determined at stage 2 to 4 of ovarian development but they were significant higher than that of stage 1. When comparing to total protein present in the hemolymph, they were 549 ± 11.9, 540 ± 12.6 and 504 ± 12.2 ng/mg protein, respectively for shrimps stage 2 to 4 (Fig. 39B, Table 13). They were significant differences ($P < 0.05$) among lectin contents between stage 1 to 4 of ovarian development. Otherwises, the hemolymph from vitellogenic females (stage 3-4), non-vitellogenic females (stage 1) and male shrimp were serially diluted and determined by ELISA (Fig. 40). Parallel curves were observed in all three cases and lectin in the hemolymph of vitellogenic females showed higher concentration than those of other two cases.

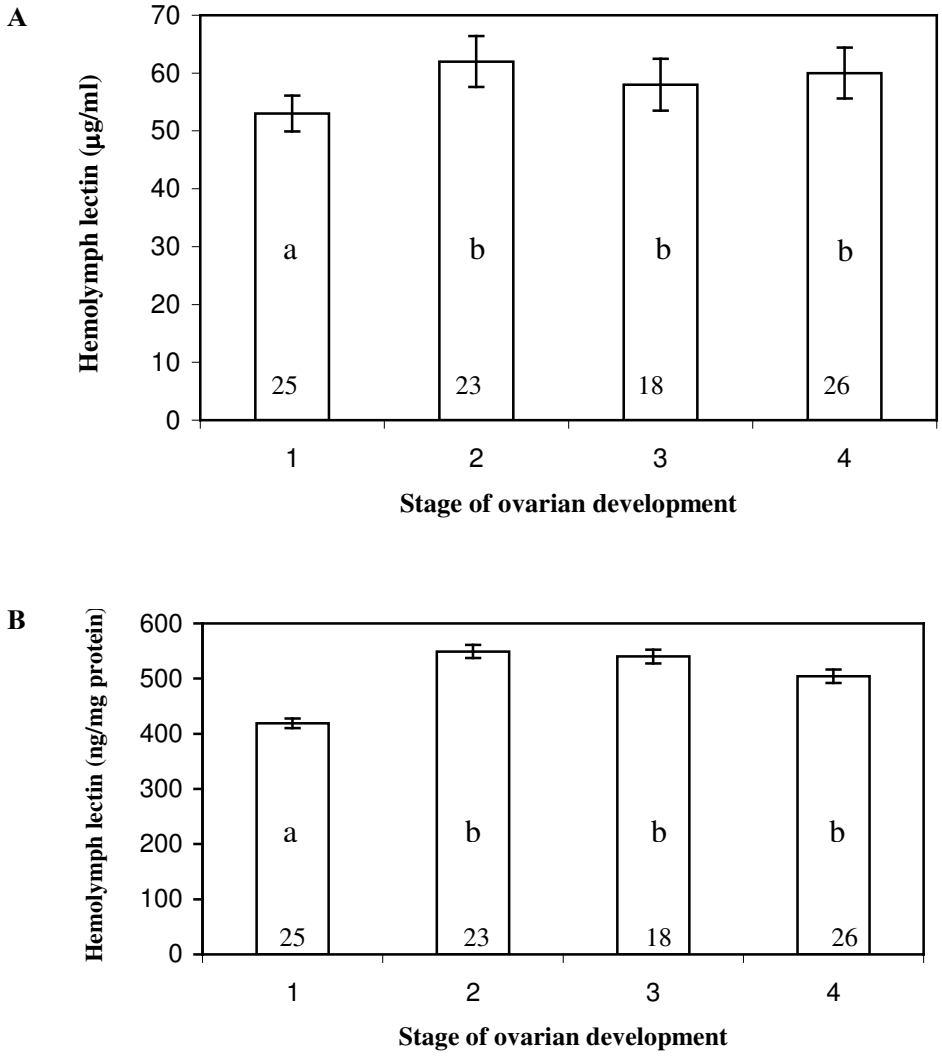


Fig. 39 ELISA quantification of lectin levels in the hemolymph of shrimps at different stages of ovarian development

Microtiter plates were coated with the hemolymph of shrimps at different stages of ovarian development. ELISA was performed in the same manner as in Fig. 31. The values were expressed as concentrations (A) or specific concentrations (B) of hemolymph lectin. Bars represent the standard errors of mean. Numbers of measurements at each stage are shown. Statistics were carried out by SISA program. The values marked by the same letter were not different at P value > 0.05 while by the different letters were different at P value $P < 0.05$.

Table 13 Concentrations of protein and lectin in the hemolymph of female shrimps at different stages of ovarian development determined by ELISA

Ovarain development	Number of samples	Protein (mg/ml)	Lectin (μg/ml)	Lectin (ng/mg protein)
Stage 1	25	127 ± 8.4	53.25 ± 3.1	419 ± 8.8
Stage 2	23	114 ± 6.5	62.62 ± 4.4	549 ± 11.9
Stage 3	18	108 ± 7.4	58.37 ± 4.5	540 ± 12.6
Stage 4	26	119 ± 5.9	60.02 ± 4.4	504 ± 12.2

Each value represents mean ± standard error.

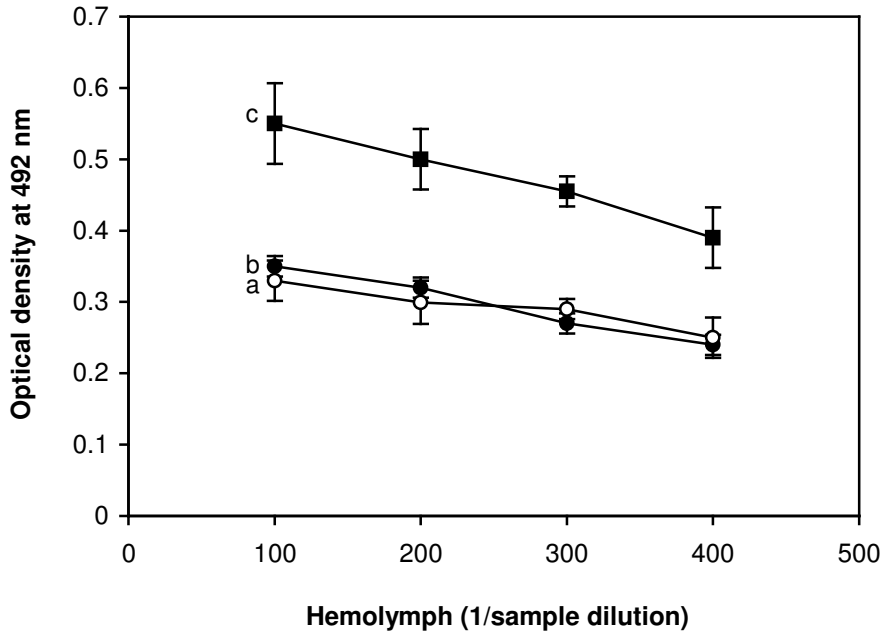


Fig. 40 Dose-response titration of the hemolymph by ELISA

Hemolymph from males (a), hemolymph from non-vitellogenic females (b), hemolymph from vitellogenic females (c). Each point is mean ± standard error of three measurements.

8.2 Determination of the hemolymph lectin by hemagglutination assay

In shrimps stage 1 and 2, their hemagglutinating activities in the hemolymph were not different (Fig. 41A, Table 14). The activities increased continuously when ovarian development reached to stages 3 (18,062 $\pm$ 111 unit/ml) and 4 (20,676 $\pm$ 102 unit/ml). Likewise, specific hemagglutinating activities of hemolymph lectin from shrimps at stages 1 (123 $\pm$ 7.9 unit/mg protein) and 2 (124 $\pm$ 8.2 unit/mg protein) were similar but they increased pronouncedly at stage 3 (167 $\pm$ 10.7 unit/mg protein) and 4 (174 $\pm$ 9.3 unit/mg protein) (Fig. 41B, Table 14).

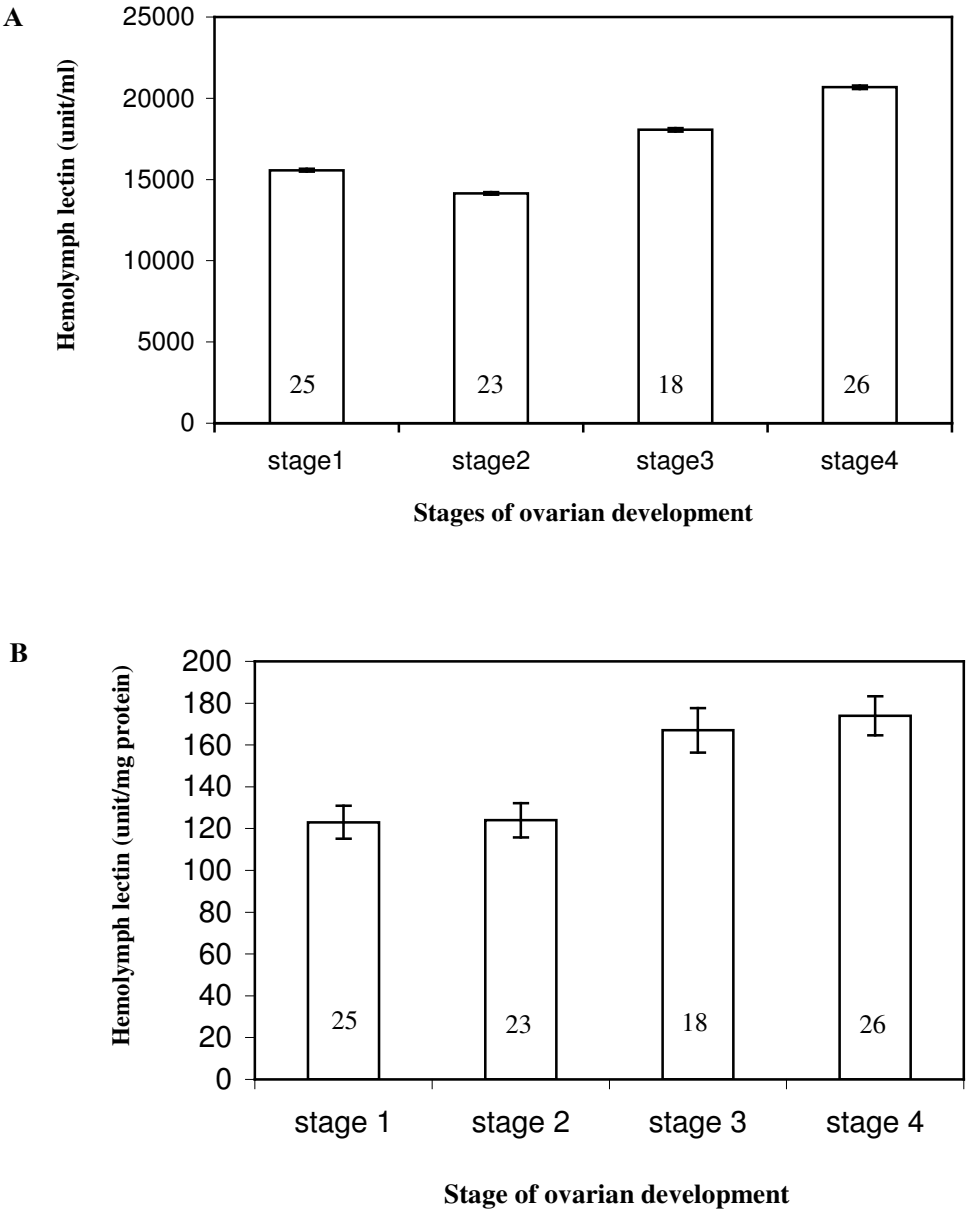


Fig. 41 Hemagglutination assay of lectin in the hemolymph of shrimps at different stages of ovarian development.

Hemolymph from female shrimps at different stages of ovarian development was subjected to hemagglutination test. The values were expressed as HA (A) or specific HA (B) of hemolymph lectin. Bars represent the standard errors of mean. Numbers of measurements at each stage are shown. Statistics were carried out by SISA program at value < 0.05.

Table 14 HA and protein concentrations in the hemolymph of female shrimps at different stages of ovarain development

Ovarain development	Number of samples	Protein (mg/ml)	HA (U/ml)	Specific HA (U/mg protein)
Stage 1	25	127 ± 8.4	15,564 ± 89	123 ± 7.9
Stage 2	23	114 ± 6.5	14,136 ± 78	124 ± 8.2
Stage 3	18	108 ± 7.4	18,062 ± 111	167 ± 10.7
Stage 4	26	119 ± 5.9	20,676 ± 102	174 ± 9.3

Each value represents mean ± standard error.