Chapter 4

Discussion

1. Purification and characterization of lectin

In this thesis, purification of lectin was achieved from the hemolymph of *P. merguiensis*. Chromatography on the Fetuin-agarose column could remove most of other serum proteins resulting in putative lectin with a HA increasing to 1,062-fold and showed a major band and one minor band (Fig. 11, lane 3). Further purification of the lectin was successfully obtained by gel filtration on the Superose 12 HR column or preparative PAGE with a similar purity of 1,850-fold when compared to the initial serum. Moreover, using preparative PAGE gave rise a lectin yield (30.6%) approximately twice of that by the gel filtration (15.9%). Purified lectin had a M_r of 316.2 kDa determined by gel filtration on Superdex 200 (Paijit, 2001). By comparing the molecular masses of the native protein and its subunits, indicating that purified lectin is an oligomeric protein of 316.2 kDa made up from two distinct subunits of 32.3 kDa and 30.9 kDa. It seemed to be absent of any covalent disulfide linkages between the subunits since a reductive treatment of the lectin with β-mercaptoethanol produced the same subunit patterns in SDS-PAGE as that without reduction. Lectins of various molecular weights and subunit organizations have been isolated from different species of penaeid shrimps. The lectin purified from the hemolymph of *Litopenaeus setiferus* is a 291 kDa protein, containing a heterotetramer of two 80 kDa and two 52 kDa subunits (Alpuche *et al*., 2005); that of *P. californiensis* (BSH-1) is a 175 kDa protein, containing subunits of 41 kDa (Marques and Barracco, 2000); whereas, the lectin isolated from *P. monodon* is a 420 kDa protein, composed of 27 kDa subunits (Ratanapo and Chulavatnatol, 1990). Lectins from crustaceans, such as *P. japonicus* (Kondo *et al*., 1998), the horseshoe crab *T. tridentatus* (Inamori *et al*., 1999), or from the American lobster *H. americanus* (Hall and Rowlands, 1974), are also formed from different numbers of monomers. The sizes of native and

subunit forms of purified lectin comparing with other lectins from shrimps and crabs were shown in Table 15.

Purified lectin has 29.7% hydrophobic, 42.6% polar, and 27.7% charged amino acids. It contains 2.7% cysteine residues that seemingly do not participate in a disulfide bridge between its subunits. The amino acid composition profile of purified lectin was similar to that found for lectins from other shrimps (Table 16) like *M. rosenbergii* both native (Vazquez *et al*., 1993) and aggregated forms (Pereyra, *et al*, 2004), *P. vannamei* (Montano-Perez *et al*., 1999) and *L. setiferus* (Alpuche *et al*., 2005).

Table 15 Characteristics of lectin from the hemolymph of shrimps and some crabs

Species	Molecular mass (kDa)		References
	Intact	Subunit	
Penaeus merguiensis	316.2	30.9, 32.3	This study
Litopenaeus schmitti	220	31, 34	Cominetti *et al*., 2002
Penaeus japonicus	452	37	Yang *et al*., 2006
Litopenaeus setiferus	291	52, 80	Alpuche *et al*., 2005
Penaeus californiensis	175	41	Marques and Barracco, 2000
Penaeus monodon	420	27	Ratanapo and Chulavatnatol, 1990
Macrobrachium rosenbergii	19	9.6	Vazquez *et al*., 1993
Paratelphusa jacquemontii		34	Denis *et al*., 2003
Scylla serrata	55	30,25	Mercy and Ravindranath, 1994

Table 16 Amino acid composition of purified lectin from *P. merguiensis* and some shrimps

Amino acids (% Mole)	*Penaeus merguiensis* lectin[*]	*Macrobrachium rosenbergii* MrL-I[1]	*Macrobrachium rosenbergii* MrL-III[2]	*Litopenaeus setiferus* LsL[3]
Asx[a]	6.73	7.6	9.2	12.0
Glx[b]	10.08	11.0	11.2	9.54
Ser	12.19	8.2	11.9	7.30
Gly	13.40	13.6	11.5	13. 6
His	10.38	4.5	4.6	3.30
Arg	4.96	3.9	3.5	2.81
Thr	4.91	4.5	4.7	4.90
Ala	6.84	8.0	8.1	9.0
Pro	3.87	5.8	4.6	5.34
Tyr	0.97	1.1	1.6	1.90
Val	3.25	6.6	5.2	6.65
Met	0.49	1.9	1.8	1.0
Cys	2.72	0.3	0.3	0.14
Ile	3.21	4.5	3.7	3.91
Leu	9.68	8.7	7.3	8.0
Phe	2.37	4.8	4.9	4.97
Lys	3.96	5.0	5.8	5.63
Trp	Not determined	Not determined	Not determined	Not determined

[*]From this study, [1,2] From Vazquez *et al*., 1993,[3] From Alpuche *et al*., 2005.

Each value was expressed as % mole of total amino acids.

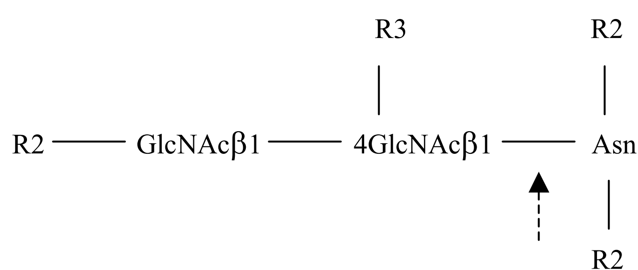
Purified lectin was analyzed by 2-D PAGE with immobilized pH gradients, it showed 2 protein subunit bands with the M_r of 32.3 kDa and 30.9 kDa and the same pI value of 6.0. Lectins from other crustaceans also have a high proportion of polar amino acids, with pI values ranging from 4 to 6.1 (Vazquez *et al*., 1993; Fragkiadakis and Stratakis, 1997; Drif and Brehelin, 1994; Zenteno *et al*., 2000; Tunkijianukij and Olafsen, 1998). For example, lectin from the hemolymph of the crab *L. depurator* had a pI value about 6.0 (Fragkiadakis and Stratakis, 1997) and lectin from the hemolymph of *M. rosenbergii* had a pI value of 5.4-6.1 (Vazquez *et al*., 1993). The pI of purified lectin is therefore within the range found for other crustaceans.

Purified lectin is composed of carbohydrate as shown by positively staining with a glycoprotein staning kit. Glycostaining of SDS-PAGE gel showed that both bands of the lectin (32.3 and 30.9 kDa) were glycosylated. Like many other invertebrate lectins, purified lectin is a glycoprotein containing 4.4% carbohydrate as previously determined by phenol-sulfuric acid reaction (Paijit, 2001). Lectins from different crustaceans such as those from *P. monodon* (Ratanapo and M. Chulavatnatol, 1990) and *M. rosenbergii* (Zenteno *et al*., 2000) were also glycoproteins while that from *L. sertiferus* was not (Alpuche *et al*., 2005). Carbohydrate analysis of purified lectin revealed that it was composed of monnose, glucose, glucosamine and sialic acid, Neu5Ac but not Neu5Gc. The components of the amino sugars in the lectin were identified as $GlcNH_2$:Glc:Man in a molar ratio of 3:0.6:1. This indicated that the main carbohydrate chains conforming the lectin are N-glycosidically linked. The presence of amino sugars such as mannose, glucose, glucosamine and sialic acid (Neu5Ac) was also detected in *M. rosenbergii* lectin (Zenteno *et al*., 2000) and in the freshwater Indian gastropod *Belamyia bengalensis* (Banerjee *et al*., 2003). Sialic acids have been reported in lectins from the Eastern oyster *Crassostrea virginica* (Acton *et al*., 1973) and the silkworm *Bombyx mori* larvae (Kato *et al*., 1994). In contrast, it has been observed that removal of sialic acids may reduce the activity of other immune signaling molecules (Fischer *et al*., 1990). Sialic acids have been demonstrated in *Drosophila melanogaster* (Roth, 1992) and in *P. monodon* (Ratanapo and Chulavatnatol, 1990), but conclusive evidence for the occurrence of sialic acids in Annelida, Arthropoda and Mollusca is missing (Roth *et al*., 1992) and sialic acids are reportedly absent in invertebrates phylogenetically below echinoderms (Schauer, 1982; 1985; Suzuki and Mori, 1990). Sialic acids were suggested to play a role as regulator in lectins from larvae of *B. mori*, due to the fact

that removal of the terminal sialic acid converted the inactive form of the lectins to an active form (Kato *et al*., 1994).

The type of glycosylation of purified lectin was determined by treatment with either the peptide N-glycosidase F (PNGase F) or trifluoromethanesulfonic acid (TFMS). Most glycoproteins contain three major types of glycans: N-linked, O-linked, and glycosylphophatidylinositol (GPI) lipid anchors. N-linked glycans are linked to the protein backbone via an amide bond to asparagine residues in an Asn-Xaa-Ser/Thr motif, where Xaa can be any amino acid, except proline (Tarentino and Plummer, 1994). On the other hands, O-linked glycans are attached to the hydroxyl group of serine or threonine.

In this study, PNGaseF was used to released N-linked glycans from purified lectin. It is one of the most widely used enzymes which is capable to cleave the linkage between GlcNAc and asparagine residues of glycans (Fig.42). In comparison, deglycosylation by TFMS was used to removed a variety of oligosaccharide motifs including N-linked glycans (except the inner most Asn-linked GlcNAc or GalNAc), O-linked glycans, glycosaminoglycans linked to the proteoglycan core, and collagen saccharides (Hyp linkage) (Sojar and Bahl, 1987).



R1 = N- and C-substitution by groups other than H

R2 = H or the rest of an oligosaccharide

R3 = H or α1,6 fucose

Fig. 42 PNGase F cleavage site (Kornfeld and Kornfeld, 1985)

Both PNGase F and TFMS treatment of purified lectin generated a same single protein band with a M_r of 28 kDa in SDS-PAGE, suggesting that purified lectin is N-linked oligosaccharide. The enzyme used was tested to be active by capable digestion of fetuin, N-linked oligosaccharide under the identical conditions (data not shown). Since deglycosylation significantly reduced the size heterogeneity of lectin oligomers, the observed heterogeneity is likely due to structural differences in N-linked oligosaccharides rather than in polypeptides. In addition, the result of amino acid composition, supporting that Asn(x) makes N-glycosidic linkages (-GlcNAc(β)Asn) with GlcNAc of purified lectin. Its binding specificity might be achieved through a combination of hydrogen bonding between the carboxyl groups of amino acid residues and the hydroxyl groups of the sugar with van der Waals packing, often including packing of the hydrophobic sugar face against aromatic amino acid side chains (Weis and Drickamer, 1996). Moreover, purified lectin contained mostly the sugars component typical for N-glycan (mannose, glucose and glucosamine), which found in a common core of carbohydrate components bound by N-glycosidic linkages in the glycoprotein (Yamashita *et al*., 1999; Kornfeld and Kornfeld, 1985).

The biological roles of the N-linked oligosaccharides from many proteins have been studied (reviewed in Varki, 1993; O'Connor and Imperiali, 1996). N-linked oligosaccharides have been shown to enhance the thermal stability of proteins, modulate and stabilize protein secondary structures such as β-turns, mediate intercellular transport of polypeptides, modulate protein half-life, and facilitate protein-protein interactions (Varki, 1993; O'Connor and Imperiali; 1996; Wang et al., 1997; Dwek, 1995). However, the possibility of alternative and untested roles of the N-linked oligosaccharide such as enhanced protein half-life or recognition by endogenous lectins *in vivo* cannot be ruled out.

An affinity blot test of purified lectin by using various biotinylated lectins, revealed the highly positive reaction with lectins from tomato, red kidney bean and snowdrop bulb. Since both tomato and red kidney bean lectins bind specifically to GlcNAc while snowdrop bulb lectin binds with Man, indicating that purified lectin is a glycosylated protein containing mainly mannose, glucose and GlcNAc. The lectin molecule is also composed of other sugars as it can be recognized by other plant lectins. From the result that purified lectin was not recognized by lectins from Maackia, coral tree, Japanese wisteria, winged bean, Jequirity bean and Osage

orange, suggesting that the lectin molecule may not contain some sugars such as αGal, [β 1,3GalNAc], NANA (α2,3) Gal, GalNAcα1,6Gal, LacNAc, Lac and GalNAc.

2. Sugar specificity of purified lectin

By means of the sugar inhibition study, purified lectin has the highest specificity for Neu5Ac. Three other N-acetyl aminosugars, GalNAc, GlcNAc and ManNAc could inhibit the lectin HA but with a lower efficiency. The same preferential affinity is also reported in other penaeids like *L. schmitti* (Cominetti *et al*., 2002), *P. indicus* (Mashewari *et al*., 1977), *P. monodon* (Ratanapo and Chulavatnatol, 1990) and *L. setiferus* (Alpuche *et al*., 2005). In contrast, some crustacean lectins do not seem to discriminate among different N-acetylated aminosugars, e.g., those of *P. californiensis* (Vargas-Albores *et al*., 1993), *M. rosenbergii* (Vazquez *et al*., 1993) and *Diogenes affinis* (Murali *et al*., 1999). However, the lectins of the crayfish *P. leniusculus* (Kopacek, 1993) and the crab *S. serrata* (Kongtawelert, 1998) have a binding affinity only to sialoglycoconjugates, but not to free Neu5Ac. Strong inhibition of the lectin HA by sialoglycoproteins, porcine stomach mucin and fetuin, was observed while bovine submaxillary mucin (BSM) did not inhibit. Unlike that of purified lectin, binding of lectins identified from other decapod crustaceans was inhibited by BSM, including those of *P. monodon* (Ratanapo and Chulavatnatol, 1990), *L. setiferus* (Alpuche *et al*., 2005), *M. rosenbergii* (Vazquez , 1996), *C. antennarius* and *L. depurator* (Vazquez, *et al*. 1998; Fragkiadakis and Stratakis, 1997). However, asialofetuin showed no capacity to interact with purified lectin. These results suggest that sialyl or acetylated moiety may be required for the interaction of the lectin.

3. Characterization of anti-lectin antibody

3.1 Specificity of anti-lectin antibody

Specificity of the anti-lectin antibody was revealed by dot blot and Western blot analysis. Anti-lectin antibody showed strong cross reaction with purified lectin and the hemolymph of *P. merguiensis*. The similar result of dot bloting was shown by Western blot analysis, using the antibody against purified lectin, indicated that the 32.3 kDa and 30.9 kDa bands of the lectin, as well as the bands aggregate in native form, were positively recognized by the antibody. The immunological study of lectin has been previously demonstrated in several

crustaceans, including the freshwater prawn *M. rosenbergii* (Pereyra *et al*., 2004), penaeid shrimps such as *P. monodon* (Ratanapo and Chulavatnatol, 1992), *P. indicus* (Jayasree *et al*., 2000) and the crab *L. depurator* (Fragkiadakis and Stratakis, 1997).

3.2 Cross-reactivity of anti-lectin antibody and immunological proteins from other crustaceans

The anti-lectin antibody of *P. merguiensis* could also react against other penaeid shrimps; *P. monodon* and *P. vannamei* by dot blot analysis. The hemolymph of *M. rosenbergii* or either from *Sicyonia ingentis* showed no reactivity (data not shown). Cross-reactivity using antibody raised against purified β-1,3-glucan-specific lectin was reported in the cockroach, *Blaberus discoidalis* was shown that similar molecules also exist in the plasma of cockroach, *B. craniifer*, *Periplaneta americana*, *Leucophaea maderae* and *Gromphadorhina portentosa* (Chen *et al*., 1998).

4. Development of ELISA

The anti-lectin antibody was used to develop an ELISA for detecting lectin in the hemolymph of *P. merguiensis*. The optimal condition of the assay was validated as follows: 1°Ab at 1:3,000 dilution, 2°Ab at 1:25,000 dilution, 0.4 mg/ml OPD, 0.012% H_2O_2 and the incubation time of HRP for 30 min. Standardization of ELISA was achieved by using purified lectin. The optical density was proportional to the amount of the coated antigen. Using purified lectin, the standard curve for the ELISA had a linear range of 12.5-350 ng and the regression coefficient (R^2) was 0.988. The sensitivity of the assay allowed me to detect lectin concentrations as low as 83.3 ng/ml that was about 2-fold higher than that of *M. rosenbergii* (30 ng/ml) (Agundis *et al*., 2000). A dilution curve of the hemolymph was linear in a range of 1:50 to 1:400 dilutions. A 1:100 dilution of hemolymph samples were practically quantified by this assay. In this work, the ELISA was used to determine the lectin concentrations in the hemolymph of *P. merguiensis* at different stages of ovarian development and the hemolymph of *V. harveyei* injected shrimps. The ELISA developed will be helpful to monitor the antibacterial lectin in the defense mechanism of shrimps. Naturally occurring lectin in the hemolymph varies in its concentration according to the external physiological state (i.e., stress conditions, pH, nutrient status) and susceptibility of the species to various diseases. ELISA assay was reportedly used to determine the lectin

concentration in the serum of the freshwaster prawns, *M. rosenbergii* (Agundis *et al*., 2000) and the hemolymph of *P. indicus* (Jayasree *et al*., 2000).

5. Agglutination of bacteria by purified lectin

P. merguiensis lectin has similar binding specificity to carbohydrate and agglutination activity against both vertebrate's erythrocytes and bateria tested. Shrimps are susceptible to many natural bacterial infections. Concerning the recognition of microorganisms by the binding of hemolymph agglutinins, many reports have studied the specificity of crustacean lectins towards different bacteria. In this study, use of the purified lectin will minimize any chance of error in the conclusions due to the presence of any impurity in the hemolymph. Purified lectin strongly agglutinated the major infective bacteria, *V. harveyi* and *V. parahemolyticus* and also *V. vulnificus* to a lesser degree. It did not show agglutinating activity against the shrimp non-pathogenic strains *E. coli, S. typhi* and *V. cholerae*. The different inhibition pattern of purified lectin for distinct bacterial sources suggests a preferential binding affinity towards certain bacteria strains.The lectin-induced agglutination of the pathogenic bacteria may be similar to the roles of humoral substances that agglutinate foreign agents in other invertebrates (Ratcliffe *et al*., 1985). This type of selective agglutination towards the bacterial cell wall was also noticed among prawns (Jayasree, 2001; Vargas-Albores *et al*., 1993; Fragkiadakis and Stratakis, 1995) and the crayfish (Kopacek *et al*., 1993). A preference for recognition of vibrios was also observed in the agglutination of bacteria by the hemolymph of the Eastern oyster, *Crassostrea virginica* (Tamplin and Fisher, 1989). It has been reported that the blue crab, *Callinectes sapidus* contains lectins that interact with a bacterial pathogen for that species, *V. parahemolyticus.* Its serum apparently contains multiple lectins that recognize the pathogenic strains and mediate their clearance, but lacks lectins for an indigenous *Vibrio* strain (Vasta, 1992). These data suggest to the view that crustacean lectins act against specific microbes or their components (poly-and lipopolysaccharides 3-Deoxy-D-manno-octulosonic Acid, KDO).

The specific inhibition of purified lectin to agglutinate *V. harveyi* by Neu5Ac (at 6.25 mM), and porcine stomach mucin (at 1 mg/ml), which contains Neu5Ac and Neu5Gc in O-glycosidically linked glycans (Schauer, 1982), or fetuin (at 3 mg/ml) that has Neu5Ac in O- and N-glycisidically linked glycans (Cumming *et al*., 1989) indicates that the lectin may bind with

sialyl or acetylated group on the bacterial surface (Kowal *et al*., 2001). This is consistent with the agglutination of *P. monodon* lectin to *V. vulnificus* that was partially inhibited by sialic acid-specifc lectins (Ratanapo and Chulavatnatol, 1992). Furthermore, the inhibition of the lectin-induced bacterial agglutination by anti-lectin antibody should be an additional assurance that the agglutination is due to the lectin itself. In addition, the lectin isolated from *P. monodon* could agglutinate gram-negative bacteria and had an opsonin effect that enhances hemocyte phagocytosis. Thus, it was postulated to prevent and control bacterial injection (Lu *et al*., 2006). Our results of the selective binding of purified lectin to some of the shrimp pathogenic and non-pathogenic bacteria also indicate an immunological role for the shrimp lectin during interaction with these bacteria in natural conditions.

6. Changes in lectin levels in the hemolymph of *V. harveyi* injected *P. merguiensis* determined by ELISA and hemagglutination assay.

P. merguiensis which suffering from bacterial infection, showed discoloration of the body tissue or loss of some anatomical parts. The hemolymph of the *V. harveyi* injected *P. merguiensis* was collected at 6 h intervals. Experimental injections with 0.85% NaCl was used as a control. In this thesis, polyclonal antibody against purified lectin was used in ELISA to determine the concentrations of lectin in the hemolymph of *P. merguiensis* injected by *V. harveyi*. In parallel, the hemolymph was subjected to hemagglutination test. Both the specific lectin contents determined by ELISA and specific HA recorded by hemagglutination test, increased significantly (at P value < 0.05) and continuously as the longer time of post-injection. In comparison, those of the uninfected shrimps showed no differences at any time of post saline-injection. These observations indicated that the elevation of the hemolymph lectin was inducible in response to the pathogenic infection of *P. merguiensis* shrimps. An increase in lectin level observing by Western blotting was also found in *P. monodon* infected with *V. vulnificus* (Ratanapo and Chulavatnatol, 1992) or *F. chinensis* challenged by WSSV (white spot syndrome virus) (Liu *et al*., 2006). The lectin level increased after a challenge by *V. anguillarum* was also observed in the oyster, *C. gigas* (Hardy *et al*., 1977). Hemolymph lectin induction by foreign particles was reported in the blue crab, *C. sapidus* (Pauley, 1973). The defense roles of lectins in invertebrates against infective microbes and parasites was reported for a long time. For example,

stimulation of hemolymph lectin activity by pathogenic bacteria was demonstrated in the earth warm, *L. ferrestris* (Stein *et al*., 1986) and the silk moth, *A. pernyi* (Qu *et al*., 1987). Other lectins including C-type lectin from *B. mori* (Koizumi *et al*., 1999), tachylectin-4 from the Japanese horseshoe crab, lectin L-6 from the American horseshoe crab *T. tridentatus* (Saito *et al*., 1997), and a sialic acid-binding lectin from the mussel (Tunkijjanukij and Olafsen, 1998; Tunkijjanukij *et al*., 1998) demonstrated anti-bacterial activity.

7. Changes in lectin levels in the hemolymph of *P.merguiensis* females at different stages of ovarian development determined by ELISA and hemagglutination assay.

In the present work, lectin in the hemolymph of *P. merguiensis* females at different stages of ovarian development was measured by ELISA and hemagglutination assay. The parallelism between lectin standard curve and diluted hemolymph samples of *P. indicus* was observed (Jayasree *et al*., 2000) which was not performed in my result. By ELISA assay, parallelism was obtained between diluted hemolymph samples of males and females of *P. merguiensis*. It is also important to note that hemolymph lectin of male and female shrimps was immunological in distinguishable. In addition, lectin concentrations in the hemolymph of vitellogenic female shrimps were higher than that of non-vitellogenic females, but the behavior remained unchanged. Shrimps showed no significant differences (at P value > 0.05) of the specific lectin contents determined by ELISA at stages 2 to 4 of ovarian development but these values were higher than that of at stage 1. From results of lectin detected by hemagglutination assay, the similar HAs in the hemolymph of shrimps at stages 1 and 2 were obtained. The HA gradually increased as shrimps reached the ovarian development to stages 3 and 4. It is consistent with the higher detection of HA was observed previously in the hemolymph from vitellogenic female shrimps other than those of non-vitellogenic females and males (Paijit, 2001). These indicated that the hemolymph lectin may involve in ovarian maturation in *P. merguiensis* since the lectin levels and HA were higher detected in the hemolymph of vitellogenic female shrimps. Lectin postulated to involve in ovarian maturation was also reported in the acorn banacle, *M. rosa* as its elevation occurred along ovarian development (Muramoto *et al*., 1991). However, since the increase in the lectin levels in the vitellogenic females were not found in all shrimps studied, the function of lectin in ovarian maturation may be under some unidentified control.