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PARTIAL PURIFICATION OF LECTIN FROM HEMOLYMPH OF *PENAEUS MERGUIENSIS* WITH ANTIBACTERIAL ACTIVITY AND BACTERIAL CLEARANCE ACTIVITY

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ABSTRACT

Lectins play relevant role in humoral defense responses of invertebrates. The hemolymph of banana prawn, *Penaeus merguiensis* contains a lectin (PML). In an attempt to identify the lectin subunits and responsive proteins in banana prawn after infected with *Vibrio harveyi*, lectin was purified by affinity chromatography on mucin-CNBr-activated Sepharose 4B and by gel filtration chromatography on Sephacryl S-200. The native molecular mass of PML was estimated to be 112 kDa and consisted of 30.09 (PML1) and 28.01 kDa (PML2) subunits by SDS-PAGE. Isoelectric point of PML was 5.23. The internal amino acid sequence of purified lectin from *P. merguiensis* by gel filtration was analyzed by 2-D gel electrophoresis and LC-MS/MS and then compared with nrFasta database which it was identified to be DAELLLLR DAEALEVGR and DTDVTTVRSR. The antibacterial activity of gel filtration purified lectin on *Vibrio* spp. was higher than that of affinity purified lectin. Protein profile of affinity purified lectin after the prawns were infected with *V. harveyi* by SDS-PAGE and 2-D gel electrophoresis demonstrated that the lectin expression responded to the bacterial infection. These results indicated that lectin has antibacterial activity and involved in shrimp immune responses.

Keywords: Lectin, *Penaeus merguiensis*, Vibrio species, affinity chromatography, antibacterial activity, 2-D gel electrophoresis, liquid chromatography mass-spectrometry.

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INTRODUCTION

Invertebrate defense system is dependent on an innate immune system by a complex of cellular and humoral factors. The cellular immune responses include phagocytosis, encapsulation, and nodule formation (Franc and White, 2000), whereas the humoral immunity which is a relatively short-term protective memory includes lectin, lysins, and other killer substances which are capable of inactivating potentially pathogenic material (Cooper and Lemmi, 1981). Lectin is a protein or glycoprotein of non-immune origin that agglutinates erythrocytes, bacteria and other cells through interaction with carbohydrate on the cell surface (Goldstein, 1980; Kocourek and Horejsi, 1981). Lectins have been isolated and characterized in various marine invertebrates, including sponges (Pajic et al., 2002), tunicates (Nair et al., 2000), crustaceans (Ravindranath et al., 1985; Vazquez et al., 1993), echinoderms (Giga et al., 1987; Matsui et al., 1994) and clam (Bulgakov et al., 2004). In crustacean, lectins may protect these animals against infection by acting as opsonins and enhancing phagocytosis or playing a role in larval development (Vargas-Albores et al., 1992; Vasta and Cohen, 1984) as well as being antibacterial agents (Tunkijjanukij and Olafsen, 1998).

The cultivation of shrimp is a worldwide economically important activity, especially in Thailand. The most important causes of cultured penaeid shrimp diseases are luminescence bacterial and viral etiologies (Lightner and Redman, 1998). To insure the sustainability of shrimp culture, the understanding in shrimp immunology is necessary to control the disease. In order to investigate the lectins expression in the hemolymph after a bacterial challenge is to identify and determine the protein amounts in the spots of two-dimensional gel electrophoresis (2D-gel). A proteomic approach using mass spectrometry has been proven to be the key technology for the identification of proteins. Recently, the standard method for quantitative analysis of protein mixtures has used 2D-gel in combination with mass spectrometry (MS) or tandem mass spectrometry (MS/MS) and identification of stained spots (Delahunty and Yates, 2005). Therefore, we have preliminary elucidated the proteome pattern of affinity purified lectin from hemolymph of banana prawn after experimentally infected with *Vibrio harveyi*. The identification of responsive lectin in hemolymph using 2D-LC-MS/MS and the efficiency of bacterial clearance would be the methods to a better understanding of the role of lectin to eliminate the pathogenic bacteria, *Vibrio harveyi*.

MATERIALS AND METHODS

Preparation of hemolymph

Adult banana prawns, *Penaeus merguiensis*, were collected from Koh Si Chang, Chonburi, Thailand. Hemolymph was obtained from each animal by inserting a 25-gauge needle which contained AC-1 anticoagulant, pH 7.0 (Soderhäll and Smith, 1983) into the pericardial sinus. The hemolymph was centrifuged at 7,700g for 15 min at 4°C. The supernatant of hemolymph was dialyzed against 0.05 M Tris-HCl buffer [containing 0.15 M NaCl, pH 7.6 (TBS) and 10 mM $CaCl_2$ (TBS-Ca)] and then was applied directly to affinity chromatography.

Lectin hemagglutination assay

The hemagglutination assay was performed by two-fold serial dilutions of test solution in 96-well microtiter plates using TBS-Ca as the diluents. Trypsinized human A erythrocytes were prepared by incubating 4% erythrocytes suspension with 0.2% trypsin in 0.05 M TBS pH 7.6 at 37°C for 1 h. After washing four times, a 2% erythrocyte suspension was prepared in TBS. The results were expressed by the titer value which is the maximum dilution for positive agglutination.

Protein determination

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

Purification of the lectin

Affinity chromatography

The affinity sorbent was prepared by immobilization of mucin type II from porcine stomach (PSM) (Sigma Chemical Co., USA) on CNBr-activated Sepharose 4B (Amersham Phamacia Biotech,1999). The coupling procedure for use with CNBr-activated Sepharose 4B (15 g) was followed the affinity chromatography principles and the methods handbook (Amersham Phamacia Biotech AB, 1999). After swelling and washing the gel, 3.0 g mucin in 75 ml 0.1 M $NaHCO_3$ buffer, pH 8.3 containing 0.5 M NaCl was added, and the suspension was incubated at 25°C for 18 h with gentle shaking. The unbound arm was blocked with 1.0 M ethanolamine in 0.1 M Tris-HCl buffer, pH 8.0. Then, the excess adsorbed protein was washed at least three cycles with coupling buffer followed by 0.1 M acetate buffer containing 0.5 M NaCl, pH 4.0 and 0.1 M Tris-HCl buffer containing 0.5 M NaCl, pH 8.0. The mucin coupled to CNBr-activated Sepharose 4B was packed into a 2.5x15 cm column. The affinity sorbent was equilibrated with TBS-Ca and used for the affinity purification of lectin derived from *Penaeus merguiensis*.

Affinity chromatography was carried out as previously described (Watanachote et al., 2005). The dialyzed hemolymph (20 ml) was applied to the mucin-CNBr-activated Sepharose 4B column at a flow rate of 0.2 ml/min. The column was washed sequentially with TBS-Ca until the absorbance at 280 nm of the effluent was stable at zero, and the lectin was eluted with 25 mM EDTA-TBS. Lectin binding was monitored by the hemagglutination assay. The fractions (1 ml) exhibiting hemagglutination activity were collected and dialyzed against 0.01 M TBS for 48 h and lyophilized using LYOALFA-6 (Telstar Industial, Spain) before storage at -20°C.

Gel filtration chromatography

Affinity purified lectin derived from *Penaeus merguiensis* was applied to a column of Sephacryl S-200 HR (Phamacia Biotech, Sweden), 1.5x100 cm. The column was previously equilibrated with TBS-Ca. Fractionation was carried out at a flow rate of 0.3 ml/min, and fractions of 1 ml were collected. The hemagglutination titer of each fraction was measured. The active hemagglutinating fractions were collected and protein concentration was measured by the Bradford method. Fractions were dialyzed against deionized water for 48 h and concentrated by freeze-drying for SDS-PAGE and 2-D gel electrophoresis.

SDS-PAGE

The purity and approximate molecular mass of the purified lectin was estimated by SDS-PAGE under reduced condition with a 12.5% running gel and a 4% stacking gel in buffer system of Laemmli (1970) using Hoefer miniVE vertical electrophoresis system for 7-cm and Hoefer SE 600 Series for 13-cm (Amersham Pharmacia Biotech, USA). Visualization of protein bands was performed by colloidal Coomassie brilliant blue G-250. The protein molecular mass markers from range 10-200 kDa (Fermentas, USA) were used as standards to calculate molecular mass by using ImageQuant TL V2005 (Amersham Bioscience, USA).

Two dimensional (2-D) gel electrophoresis

One-two ml lyses-buffer (30 mM Tris, 7 M urea, 2 M thiourea, 4% CHAPS, pH 8.5 stored at –15°C to -30°C) was added directly to affinity purified lectin and mixed by using vortex for an additional 30 sec, then centrifuged 13,000g at 4°C for 15 min. The supernatant was subsequently removed and transferred to a clean tube, and any insoluble pellet was discarded. The samples were cleaned by Ettan™ sample preparation kit and Reagents 2D clean-up kit (Amersham Biosciences, USA). The protein was determined by Bio-Rad protein assay kit with BSA as the standard. The unused extract was stored in aliquots at -80°C.

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

Running conditions for pH 3-10: temperature 20°C; current 50 μA per strip.		
Step voltage mode	Voltage(V)	kVh
1. Step and hold	500	0.5
2. Gradient	1000	0.8
3. Gradient	8000	11.3
4. Step and hold	8000	4.4

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

Protein identification by LC-MS/MS

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

Test bacterial species

As representative of test bacteria, nine species were used in this study which six isolates of *Vibrio angillarum*, *V. cholerae*, *V. fluvialis*, *V. harveyi*, *V. parahemolyticus*, and *V. mimicus* were obtained from Microbiology Laboratory, Institute of Marine Science, Burapha University, Chonburi, Thailand. Other bacterial isolates, namely, *Staphylococcus aureus* TISTR 517, *Micrococcus luteus* TISTR 884, *Escherichia coli* TISTR 887, and *Pseudomonas aeruginosa* TISTR 1467 were obtained from Microbiological Resources Center, Thailand Institute of Scientific and Technological Research. Bacteria were grown in tryptic soy broth (TSB). *Vibrio* sp. cultures were grown in 5 ml TSB supplemented with 1.5% NaCl (TSB/NaCl) at 30°C, 16-18 h with continuous shaking. Then, the cultures 0.5 ml were transferred to 5 ml TSB or TSB/NaCl and incubated at 30°C, 1-5 h. The bacterial suspension with 10^6-10^7 cells/ml (~0.3 AU at 600 nm) was used for antibacterial assay.

Antibacterial activity

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

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

Bacterial clearance

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

Determination of bacterial clearance rate was performed on six sets of prawn; 12 prawns of each set were injected intramuscularly at the 6th abdominal segment with 10 µl of the bacterial suspension. Thet welve prawns from each feed-treatment group were removed immediately after injection at 1, 15, 30, 45, 60, and 120 min post-injection to determine the number of bacterial cells/ml in hemolymph. Hemolymph (50 µl) was collected from the pericardial sinus into a syringe containing 100 µl of anticoagulant solution and added immediately to a tube containing 350 µl of TSB. Hemolymph mixture was ten-fold diluted in normal saline, then 100 µl of diluted mixture wer e spread onto plates of thiosulphate citrate bile sucrose agar (TCBS) to obtain the reduction of colony forming unit (CFU). After incubation at 35°C for 10 h, counts were calculated according to dilution and recorded as the mean CFU count ± standard deviation for the triplicate counts (modified from Sritunyalucksana et al., 1999).

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

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

RESULTS

Characterization of the lectin

The lectin from hemolymph of *Penaeus merguiensis* was purified by affinity chromatography on mucin-CNBr activated Sepharose 4B and gel filtration on Sephacryl S-200 HR as previously described (Watanachote et al., 2006). The results are summarized in Figure 1. The native molecular mass of the lectin (PML) was 112 k Da and the protein consisted of 30.09 (PML1) and 28.01 kDa (PML2) subunits by SDS-PAGE, as shown in Figure 2. The native lectin by gel filtration gave one spot with isoelectric points (pI) around 5.23 by 2D-gel with immobilized pH gradients. The affinity purified lectin revealed two subunits of Mr 30.09 and 28.01 kDa with different isoelectric points, pI ~ 6.48 and ~6.70, as shown in Figure 3.

Partial amino acid sequences of lectin subunits

By using 2D-gel, the affinity purified lectin gave six high intensity spots, as shown in Figure 3. Six protein spots were in gel digested and internal amino acid sequence analyzed by mass spectrometry. Table 1 shows the partial amino acid sequences of spot 1, 2, 3, 4, 5, and 6.

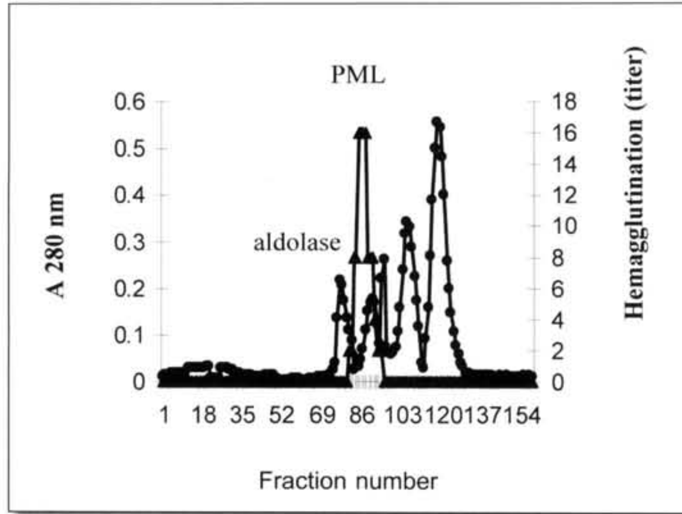

Figure 1. Analysis of lectin from the *Penaeus merguiensis* using gel filtration on Sephacryl S-200, •-• absorbance at 280 nm, ▲-▲ hemagglutinin (titer) against trypsin treated human A erythrocytes. The standard proteins were aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen (25 kDa).

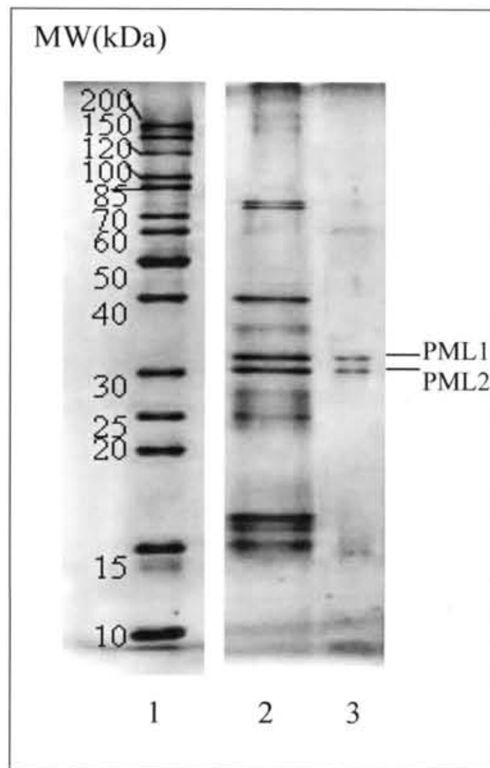

Figure 2. Analysis of the lectin from *Penaeus merguiensis* using 12.5% SDS-PAGE. Molecular weight marker (lane 1), affinity purified lectin (lane 2), and gel filtration purified PML (lane 3).

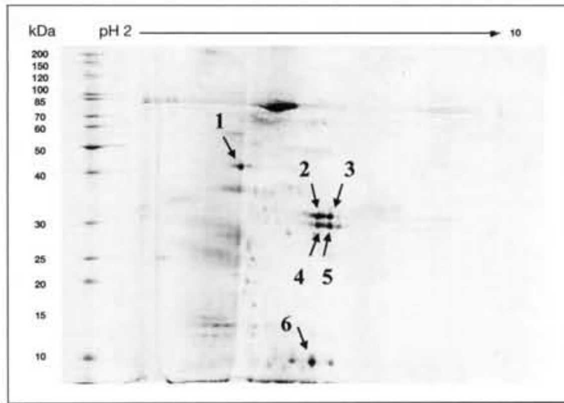

Figure 3. Analysis of affinity purified lectin using 2-D gel performed with 200 µg of affinity purified lectin shows the separation of subunits by using Immobiline DryStrip pH 3-10 (13 cm). Spots of subunits are indicated by arrow and number. Spot number 1 was observed at Mr 45.19 kDa (pI ~ 4.94). Spot number 2 and 3 were observed at Mr 30.09 kDa (pI ~ 6.48 and ~ 6.70), spots number 4 and 5 at 28.01 kDa (pI ~ 6.48 and ~ 6.70), and spot number 6 observed at Mr 29.39 kDa (pI ~ 6.48).

Table 1. Partial amino acid sequences of the purified lectin subunits from hemolymph of *Penaeus merguiensis* by gel filtration and affinity chromatography

Sample	Spot	Rf	pI	MW(kDa)	Amino acid sequence	Accession no.
Gel filtration	PML	0.51	5.33	31.67	DAELLLLR	No similarity
purified lectin	spot				DAEALEVGR	No similarity
					DTDVTTVRSR	No similarity
Affinity	1	0.36	4.94	45.19	(Q/K)(L/I)DT(L/I)(Q/K)R	No similarity
purified lectin					DE(L/I)VEVK	No similarity
					DGV(L/I)S(Q/K)VK	No similarity
					(L/I)(Q/K)(F/MO)G(L/I)GDENSK	No similarity
	2	0.51	6.48	31.67	MQTILYKANSR	No similarity
	3	0.51	6.70	31.67	GG(L/I)ADSDCGASGSG-K	No similarity
					N(L/I)ADSDLGASGSG-K	No similarity
					GGNAGGSDCGASGSG-K	No similarity
					NNAGGSDCGASGSG-K	No similarity
	4	0.53	6.48	29.39	YEELQITAGR	No similarity
	5	0.53	6.70	29.39	GDN(L/I)NGVYG-R	No similarity
	6	0.94	5.54	10.12	HWFSLFNPR	gi 7414468 emb
						CAB85965.1
						Hemocyanin *(Litopenaeus*
						vannamei) Mass: 76502.8
						No. of Amino acids: 671

Antibacterial activity

The antibacterial activity of lectin from the hemolymph affinity and gel filtration purification (1.28 μg protein) from banana prawn is presented in Table 2. The affinity purified lectin exhibited strong antibacterial effect against all tested strains of inhibition, ranging from 21.26-68.75%. The lectin from gel filtration purification showed stronger antibacterial activity on *Vibrio* spp. than lectin from affinity purification except *Vibrio cholerae, Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Serum of the banana prawn showed slight effect against some *Vibrio* spp., with inhibition ranges from 17.64-45.32% while inhibitory effect was not found on *Vibrio harveyi, P. aeruginosa, S. aureus*, and *Micrococcus luteus*.

Table 2. Antibacterial activity in serum, hemolymph affinity and gel filtration purified *Penaeus merguiensis* lectin incubated with *Vibrio* spp. for 1 h. Percentage of inhibition was determined from the appropriate serial dilution as colony forming unit (CFU).

Type of bacteria	% inhibition±SD		
	with serum	with affinity purified lectin	with gel filtration purified lectin
Vibrio fluvialis	34.44±10.05	61.16±6.28	76.02±2.41
V. alginolyticus	45.32±10.47	68.75±6.22	71.41±0.81
V. mimicus	29.66±7.96	33.90±2.05	96.87±1.37
V. harveyi	0	47.38±11.45	69.06±7.85
V. parahemolyticus	40.51±6.43	21.26±2.93	76.74±1.93
V. cholerae	17.64±7.15	62.05±0.29	0
Pseudomonas aeruginosa	0	21.97±2.70	0
Escherichia coli	18.12±3.95	13.01±4.58	75.69±5.36
Staphylococcus aureus	0	42.11±0.38	27.08±3.0
Micrococcus luteus	0	28.09±6.95	43.27±3.46

Protein profile of affinity purified lectin from *Vibrio harveyi* infected *Penaeus merguiensis* hemolymph

The investigation on the relationship between the roles of lectin to eliminate bacterial infection was carried out using *Vibrio harveyi* as test organism. In order to study the lectin expression, PML1 and PML2 were separated by SDS-PAGE (Figure 4). The quantities of the protein bands were determined automatically by the ImageQuant TL software and presented in the intensity, and the result was confirmed by 2-D gel electrophoresis. A 2-D gel was used to investigate differences in the pattern of affinity-purified lectin from hemolymph of banana prawn after challenged with *V. harveyi* for 1, 15, and 120 min. We were able to detect five or six high intensity spots by using the 2-D gel (Figure 5). Table 3 summarizes the hemagglutinating specific activity, the number of bacterial count, the lectin subunits intensity by SDS-PAGE, and the different protein spot intensity by 2-D gel after banana prawn had been exposed to *V. haveyi*. For 2-D gel, the amount of spot 2 plus 3 referred to PML1 and spot 4 plus 5 referred to PML2.

The amount of lectin in hemolymph after post-injection with *V. harveyi* for 1, 15, 30, 45, 60 and 120 min gave hemagglutinating specific activity of 1144.77, 1146.77, 1234.15, 1440.69, 1057.85, and 2143.33 titer/mg protein, respectively. In comparison of lectin expression to the number of bacterial count,

the result showed that the intensity of PML1 and spot no.1 were the highest at 15 min which the bacterial count was decreasing. At the same time, the intensities of spots no. 2 to 5 were in the range of 0.045028-0.133672. On the other hand, after 15 min the number of bacterial count decreased but hemagglutinating specific activity, PML2, intensity of spot 2 plus 3 and spot 4 plus 5 tended to increase. After post-injection (60 min), the bacterial count increased again, this was similar to the hemagglutinating specific activity and the volume of spots no.2 to 5.

The result suggested that lectin might agglutinate bacteria, whereas some bacterial colonies that were not agglutinated could grow up again. Thus, bacterial clearance ability was not due only to lectin, but it might dependent on other immune substances.

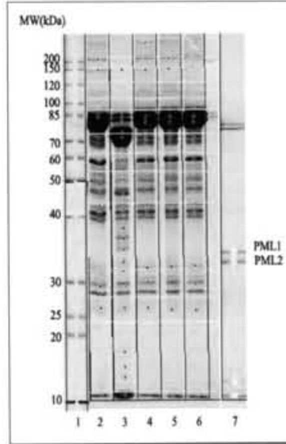

Figure 4. Protein profile of hemolymph from the *Penaeus merguiensis* in 10% SDS-PAGE (13 cm.) after challenged with *Vibrio harveyi*. Molecular weight marker (lane 1), hemolymph (20 μg protein) after post-injection with *V. harveyi* at 1, 15, 30, 45, and 60 min (lanes 2, 3, 4, 5, and 6), and affinity purified lectin (lane 7).

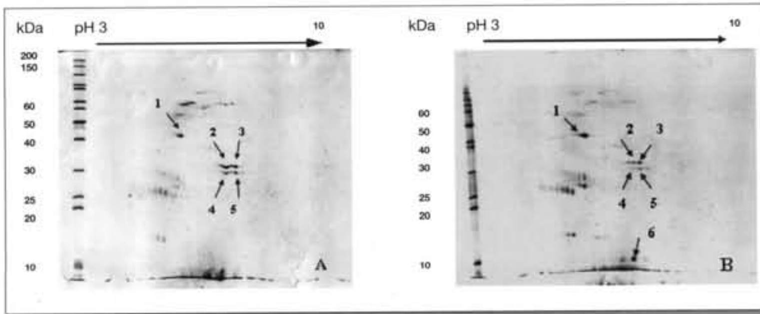

Figure 5. Two dimensional maps of colloidal Coomassie brilliant blue G-250 stained proteins of the affinity purified lectin after post-injection with *Vibrio harveyi* at 1 min (A) and 120 min (B). The protein was separated by 2-Dimensional Gel Electrophoresis. Isoelectric focusing (IEF) was performed in the Immobiline™ DryStrip pH3-10, 13 cm, IPG strips in a disposable cassette. Protein volumes were adjusted in order to analyze the same amount of 200 g for each set of injected samples. Maps were analyzed with ImageMaster 2D Platinum software. Labeled spots are described previously on Figure 3. A comparison was made between the affinity purified lectin after post-injection with *V. harveyi* at 1 min and 120 min. Labeled spots (1-6) are involved humoral defense which response to *V. harveyi* infection.

Table 3. Protein expression in hemolymph of *Penaeus merguiensis* after challenging with Vibrio harveyi.

	Time after bacterial injection (min)					
	1	15	30	45	60	120
Hemagglutinating specific activity (titer/mg protein)	1144.77	1146.77	1234.15	1440.69	1057.85	2143.33
Bacterial count (n=10) mean (CFU)±SD	1318±0.43	372±0.49	117±0.38	32±0.29	2±0.31	776±0.27
SDS-PAGE intensity×10^5 PML1 band	49.9	132.66	74.47	41.05	44.75	44.84
SDS-PAGE intensity×10^5 PML2 band	39.25	41.67	38.90	48.34	52.69	74.22
Spot intensity from 2D-gel spot 2+3	1.121417	0.213166	-	-	-	0.4712425
Spot intensity from 2D-gel spot 4+5	0.573962	0.084577	-	-	-	0.137864
Spot intensity from 2D-gel spot 1	0.535281	2.00586	-	-	-	1.200329
Spot intensity from 2D-gel spot 2	0.669488	0.079494	-	-	-	0.168125
Spot intensity from 2D-gel spot 3	0.451929	0.133672	-	-	-	0.3031175
Spot intensity from 2D-gel spot 4	0.317006	0.039549	-	-	-	0.078455
Spot Intensity from 2D-gel spot 5	0.256956	0.045028	-	-	-	0.0594086
Spot intensity from 2D-gel spot 6	0.239643	0.841620	-	-	-	0.680408

DISCUSSION

The hemolymph of marine invertebrates contains biological active substances, such as complement, lectins, a clotting factor, and antimicrobial peptides. Many invertebrate lectins have been proposed to be involved in binding carbohydrates which are present in microbial cell wall and initiate several immune responses, as well as agglutinate the invading micro-organism (Lee and Söderhäll, 2002). Moreover, Jayasree et al. (2000) demonstrated that the health condition process in *Fenneropenaeus indicus* can be monitored through the concentration of agglutinin in the hemolymph.

Antibacterial activity from hemolymp of crustacean has previously been reported in blue crab *(Callinectes sapidus)*, *Penaeus vannamei*, and white prawn *(Penaeus indicus)*. Hemolymph of these crustaceans possesses antibacterial activity which is inhibited to Gram-negative bacteria, *Vibrio* spp. (Noga et al., 1996; Destoumieux et al., 1997; Jayasree, 2001). In these results, lectin from banana prawn has

demonstrated antibacterial effect against Gram-negative bacteria more than Gram-positive bacteria. The antimicrobial lectin might be inhibiting the bacteria by agglutinating activity. The agglutinin isolated from *Penaeus californiensis* could bind to lipopolysaccharide (LPS) which is a component of the Gram-negative bacterial cell wall and agglutinated *Vibrio* spp. (Vargas-Albores et al., 1993). The lectin monodin purified from *Penaeus monodon* agglutinated *Vibrio vulnificus* (Ratanapo and Chulavatnatol, 1992). Both of the affinity and gel filtration purified lectin from banana prawn exhibited strong antibacterial against some *Vibrio* spp. which are pathogenic bacteria of shrimp. The antibacterial activity demonstrated that gel filtration purified lectin has high recognition to pathogen. However, our results still not clear with the exception of the antibacterial activity against *Vibrio cholerae, Pseudomonas aeruginosa* and *Staphylococcus aureus,* by the gel filtration purified lectin that has more lowly than affinity purified lectin at 1 h. This may be depended on the rate of interaction between lectin molecule and receptor site on pathogens that Tunkijjanukij and Olafsen (1998) have demonstrated the lectin from the horse mussel with agglutinating activity against human and/or horse erythrocytes (modiolin H and/or E activity, respectively). The result showed that the modiolin H activity was less accountable during the purification steps than modiolin E activity. Their explanation was possibly due to random rearrangement of its molecule entities that were separated into populations according to the predominantly expressed binding specificities.

In marine invertebrate, many lectins are oligomers or multimers of homogeneous subunits with carbohydrate-binding moieties (Kondo et al., 1992 and Maheswari et al., 2002). There are many reports of the lectin from crustacean which involve in the recognition and defense by interaction with carbohydrate on cell surface of bacteria and binding site of lectin to decrease bacterial infection (Ratanapo and Chulavatnatol, 1992; Sritunyalucksana et al., 1999 and Zenteno et al, 2000). This means that some of subunits of PML recognize the lipopolysaccharide (LPS), the composition of carbohydrate on Gram-negative microbial cell wall pattern. The agglutinin isolated from the prawn, *P. californiensis,* could bind to LPS and agglutinates *Vibrio* spp. (Vargas-Albores, 1995). The hemagglutinin from plasma of the crayfish, *Pacifastacus leniusculus,* also reacts with LPS (Kopàcek et al., 1993). Our results showed that the banana prawn lectin purified by affinity and gel filtration chromatography displayed an important role in humoral defense of non-specific antimicrobial activity against both Gram-positive and Gram-negative bacteria.

The PML, purified lectin from hemolymph of banana prawn by gel filtration was identified by SDS-PAGE, 2D-gel, and LC-MS/MS. It consisted of two bands with molecular weights of 30.09 and 28.01 kDa and with different isoelectric points' pI ~ 6.48 and ~ 6.70. The molecular structure of crustacean lectins exhibited differences in molecular mass, number and size of subunits. A lectin purified from *P. californiensis* hemolymph BSH-1 was shown to be a 175 kDa oligomer, made up to subunits of 41 kDa, whereas in *P. monodon,* a 420 kDa hemolymph lectin, called monodin, comprised subunits of 27 kDa (Vargas-Albores et al., 1993; Ratanapo and Chulavatnatol, 1990).

Recent developments in technology and instrumentation have made mass spectrometry the method of choice for the protein identification from gel-separation and protein databases (Aebersold and Mann, 2003). Amino acid analysis of gel filtration purified PML by LC-MS/MS and compared to nrFasta database revealed that the 112 kDa lectin was not homologous to any proteins from invertebrate in database. The lectin subunit spots 2 and 3 with molecular weight 30.09 kDa and spots 4 and 5 with molecular weight 28.01 kDa gave different amino acid sequences but not homologous to the proteins in database. Therefore, PML may be a putative lectin from hemolymph of banana prawn. However, the amino acid sequence of lectin subunit spot no. 6 was homologous to hemocyanin from *Litopenaeus vannamei.* Hemocyanin occurs in several classes: Crustacean, Myriapoda, Merostomata, and Arachnida.

The hemocyanin isolated from shrimp hemolymph is composed of three 75-76 kDa structural and functional subunits. Moreover, the shrimp hemocyanin itself has antiviral property (Zhang et al., 2004). Lee et al. (2004) indicated that arthropod hemocyanin could be converted to phenoloxidase and be processed to produce antimicrobial peptides, possibly when animal is wounded or subjected to an infection. These reports support our results showing the protein profile by SDS-PAGE and 2D-gel of hemolymph from banana prawn after *Vibrio harveyi* infection (Figure 4 and Table 2). At 15 min, hemocyanin (Mr~78 kDa) may be degraded to lectin or other proteins that involve immune responses. The relationship between lectin and hemocyanin with antimicrobial activity is one of the interesting aspects to be investigated.

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