DISCUSSION

1. Identification of Antibacterial Affinity Purified Lectin from Hemolymph of *Penaeus merguiensis* after *Vibrio harveyi* Infection

The hemolymph of marine invertebrates contains biological active substance 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*), *P. vannamei* and white prawn (*P. indicus*). Hemolymph of these crustacean possesses antibacterial activity which inhibited to Gram-negative bacteria, *Vibrio* spp. (Noga *et al.*, 1996; Destoumieux *et al*.,1997; Jayasree, 2001). From this study, lectin from banana prawn has demonstrated antibacterial effect against gram-negative bacteria more than gram-positive bacteria. The antimicrobial lectin might inhibit bacteria by agglutinating activity. The agglutinin isolated from *P. californiensis* could bind to lipopolysaccharide (LPS) which is a component of the gram-negative bacterial cell wall and agglutinated *Vibrio sp.* (Vargas-Albores *et al*., 1993). The lectin monodin purified from *P. monodon* agglutinated *V. 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 are still not clear with the exception of the antibacterial activity against *V. cholerae*, *P. aeruginosa* and *S. aureus.* The gel filtration purified lectin has lower than affinity purified lectin at 1 h. There 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 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 involved in recognition and defense by interaction with carbohydrate on cell surface of bacteria and binding site of lectin to decrease bacterial infection (Ratanapo and Chulawatnatol, 1992; Sritunyalucksana *et al*., 1999 and Zenteno *et al*, 2000). This means that some of subunits of PML recognized the lipopolysaccharide (LPS), the composition of carbohydrate on Gram-negative microbial cell wall pattern. The hemagglutinin from plasma of the crayfish *Pacifastacus leniusculus* also reacts with LPS (Kopàcek *et al*., 1993). My 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 weight of 30.09 and 28.01 kDa and with different isoelectric points. 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 a 175 kDa oligomer, made up to subunits of 41 kDa, whereas in *P. monodon*, a 420 kDa hemolymph lectin, called monodin, comprises subunits of 27 kDa (Vargas-Albores *et al*., 1993; Ratanapo and Chulavatnatol, 1990).

Recent developments in technology and equipment 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 the database. The lectin subunit spot 2 and 3 with molecular weight 30.09 kDa and spot 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 number six was homologous to hemocyanin (100% identity) from *L. 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 had antiviral property (Zhang *et al*., 2004). In the study of Lee *et. al.* (2004) it was indicated that arthropod hemocyanin could be converted to phenoloxidase and also be processed to produce antimicrobial peptides possibly when animal is wounded or subjected to an infection. These reports might be support our results that showing the protein profile by SDS-PAGE and 2D-gel of hemolymph from banana prawn after *V. harveyi* infection. At 15 min, hemocyanin (Mr~78 kDa) may be degraded to lectin or other proteins which involved antibacterial activity.

2. Characterisation of Hemocyte of *Penaeus merguiensis*

The circulating hemocytes of invertebrates are essential in immunology. In general, crustacean hemocytes were classified as hyaline, semigranular and granular cells (Söderhäll and Smith, 1983). In order to clarify hemocyte was based on size and number of intracellular granules. A hemocyte classification system was developed, which relates cellular morphology at the light and electron microscope levels, cytochemistry, and three essential functions: clotting, phagocytosis and encapsulation. However, this classification is confusing and contains a very high number of name and description of different cell types (Hose *et al*., 1990; Azumi *et al*., 1996). According to Martin and Graves (1985), the penaeid shrimp hemocytes are often classified into agranular cell (hyaline cell), small-granular (semigranular) and large-granular (granular). In the present study, *P. merguiensis* hemocytes were classified in three types under light microscope as hemocyte from *P. californiensis* and *P.*

zonangulus (Martin and Graves, 1985; Cardenas *et al*., 2000). Like other crustaceans, *Farfantepenaeus californiensis*, *Litopenaeus vannamei* and *L. stylirostris* have three main populations differing in presence and size of cytoplasmic granules. These results are different among the previously report of *P. monodon* that the hemocytes were classified in five types which the cells were stained with Grunwald-Giemsa and detected under light microscope (van de Braak, 2002).

On the surface of all eukaryote cell are plasma membrane proteins attached to carbohydrate. This carbohydrate-rich cell coat may has an affinity for lectins. Thus, lectins are powerful tools for the study of carbohydrate and their derivatives, both in solution and cell surfaces (Lis and Sharon, 1986). In two decapods, the ridgeback prawn *Sicyonia ingentis* and the American lobster *Homarus americanus* hemocytes were classified by using wheat-germ agglutinin (WGA) which specifically binds N-acetyl-D-glucosamine. Fluorescence and electron microscopy were used to show that WGA stains the cytoplasmic granules in the granulocytes but not the hyaline cells (Martin *et al*., 2003). My results showed that the extract from hemocyte of banana prawn contained an agglutinin which strongly agglutinated rabbit erythrocyte. Therefore, the lectin from HLS may be different from serum and henolymph.

3. Total Hemocyte Count

THC of banana prawn was $4.74\pm1.32x10^6$ cell/ml. and consisted of 56±15% viable cells. The numbers of circulating hemocytes were different between penaeid shrimps. *P. californiensis, P. ingentis, P. monodon, F. californiensis, L. vannamei* and *L. stylirostris* exhibited wide rang of THC ranged from $11x10^3$-$50.9x10^6$ cells/ml. by using hemocytometer (Martin and Graves, 1985; van de Braak, 2002; Vargas-Albores, 2005). When comparing between the *F. californiensis, L. vannamei, L. stylirostris* and *P. merguiensis* (in this study), the total hemocyte counts were 4.04 ± 1.63, 9.35 ± 2.10, $14.16\pm5.30 \times 10^6$ cells/ml(Vargas-Albores *et al.*, 2005) and $4.74\pm1.32x10^6$ cells/ml respectively. Sequeira *et al*. (1995) reported that the *P. japonicus* hemocyte cell population related to sex and molt cycle. There are studies which suggest that a number of invertebrate hemocyte cells depend on

environmental factors such as temperature, pH and salinity as well as chemical need to maintain the hemocyte during *in vitro* study (Paterson and Stewart, 1974; Oliver and Fisher, 1995). In addition to traditional methods such as microscopy and protein chemistry, flow cytometry has been used to investigate the circulating hemocytes for evaluation of invertebrate hemocyte responses to immunological stimuli (Cardenas *et al*., 2000). To date, the freshwater crayfish *Astacus leptodactylus* ultrastructural features were automatically analysed by means of image-analysis software and combined with traditional THC by using light-/electron microscopy (Giulianini *et al*., 2007).

The invertebrate *Tachypleus tridentatus* (Japanese horseshoe crab), *F. californiensis, L. vannamei* and *L. stylirostris* contain granular hemocyte (small-granular and large-granular) which comprise 99, 72, 75 and 66% of the circulating hemocyte respectively (Iwanaga, 2002; Vargas-Albores *et al*., 2005) whereas, *P. merguiensis* contain 88% of THC. Granular hemocytes are involved in defense against foreign material by phagocytosis and encapsulation instead of hyaline cells are involved in the initiation of hemolymph coagulation (Hose *et al*., 1990).

4. Hemagglutinin and Antibacterial activity of Hemocyte Lysate Supernatant

It is well-known fact that invertebrates appear to lack important substance of the immune system as defined in vertebrate including, immunoglobulins, thus the antibacterial activity of humoral and cell-associated lectins from banana prawn were studied. Serum hemolymph and HLS from the prawn contained agglutinin for human and animal erythrocytes. The HLS demonstrated highest agglutinating activity against rabbit erythrocyte and gave three major bands, Mr 69.5, 57.0 and 43.5 kDa by SDS-PAGE that different from the protein bands of serum and hemolyph. Hemolymph of the banana prawn gave two lectins subunit, PML1 (Mr 30.09 kDa) and PML2 (Mr 28.01 kDa). That means, the agglutinin from HLS may different from the serum and hemolymph agglutinin. The previous study by reported that five types of lectins named tachylectin (TL)-1 to 5 and several bacterial agglutinin were

identified in circulating hemocytes and hemolymph plasma of horseshoe crab (limulus) (Iwanaga, 2002).

For *P. merguiensis*, my results showed that serum hemocyte lysate supernatant and affinity purified lectin had antimicrobial activity *in vitro*(Table 11). Here, we have been attempted with respect to location of antibacterial activity in hemolymph of the prawn. HLS protein at concentration of approximately 2 μg/100 μl reduced colony counts of *Vibrio* spp., *E. coli* and *S. aureus* ranged from 24.94-57.83% while serum was exhibited effect against all test strains ranged from 17.64-45.32% when using approximately 1 μg protein in100 μl of reaction mixture. Alpuche-Osorno *et al.* (2005) indicated that antibacterial substance related to hemocyanin that subunit (Mr 84 kDa) was the major band in serum and hemolymph by SDS-PAGE. Nagai *et al.* (2001), showed that the clotting enzyme of the Japanese horseshoe crab functionally converts hemocyanin to phenoloxidase. HLS antibacterial activity was higher against gram-negative bacteria than gram-positive bacteria, *S. aureus*. The HLS antimicrobial properties of shore crab, *C. maenas* exhibited heat stable and was independent of divalent cation. Also, HLS were not due to phenoloxidase activity (Chisholm and Smith, 1992). Antibacterial activity resides exclusively in the granular cells and non-lytic in character mainly exhibited against gram-negative organisms (Chisholm and Smith, 1992). As same as HLS from *C. maenas*, the hemocyte from *Galathea strigosa, Nephrops norvegicus, Crangon crangon* and *Glyptonotus antracticus* contain antibacterial factors and antibacterial potency varies from species to species (Chisholm and Smith, 1995). However, phenoloxidase activity is weak in *G. strigosa* and absent in *G. antracticus* (Smith and Söderhäll, 1991). The resistance of live *Micrococcus luteus* suggested that the blue crab *Callinectes sapidus* antibacterial activity was not a lysozyme (Noga *et al*., 1996). Therefore, the lectin in the hemocyte of *P. merguiensis* may be a recognizable molecule that play a role in the antibacterial activity.