ANTIBACTERIAL ACTIVITY OF LECTINS FROM HEMOLYMPH OF BANANA PRAWN (*Penaeus merguiensis* De Man)

INTRODUCTION

Invertebrate defense system is dependent on an innate immune system by a complex of cellular and humoral factors. The cellular immune responses includes phagocytosis, encapsulation and nodule formation (Franc and White, 2000), whereas the humoral immunity which is a relatively short-term protective memory included 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 which agglutinates erythrocytes, bacteria and other cells through interaction with carbohydrate on the cell surface (Goldstein, 1980; Kocorek and Hořejši, 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; Vázquez *et al*., 1993), echinoderms (Giga *et al*., 1987; Matsui *et al*., 1994) and clam (Bulgakov *et al*., 2004). Due to the fact that lectins have the ability to bind carbohydrate and promote the agglutination of different cells, such as bacteria and other invading pathogens, it is reasonable to assume that these molecules may be regarded as having a potential role in invertebrate non-self-recognition reactions (Marques and Barracco, 2000). In crustacean, lectins may protect these animals against infection by acting as opsonin and enhancing phagocytosis or playing a role in larval development (Vargas-Albores *et al*., 1992; Vasta and Cohen, 1984) as well as being an antibacterial agent (Tunkijjanukij and Olafsen, 1998). However, compared to other arthropod groups, such as insects and horseshoe crabs, the current knowledge on lectin involvement in crustacean non-self-recognition is still much less well established. In contrast, studies on the prophenoloxidase activating system and related molecules (Söderhäll and Cerenius, 1992) and more recently, on clotting proteins in crustaceans have progressed well.

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 ensure the sustainability of shrimp culture, the understanding in shrimp immunology is necessary to control the disease. In order to investigate the lectin 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, I have preliminary elucidated the role of lectin from hemolymph and hemocyte lysate supernatant of banana prawn in the term of antimicrobial protein and clearance activity. Additionally, the proteomic pattern of affinity purified lectin from hemolymph of banana prawn was observed 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, *V. harveyi*.

The specific aims of the study were:

1. To purify and characterize lectin from the hemolymph of *P. merguiensis*
2. To study the antimicrobial activity of the hemolymph, hemocyte lysate supernatant and purified lectin
3. To study the proteomic approach for identification of lectin from hemolymph of *P. merguiensis* after *Vibrio harveyi* infection

LITERATURE REVIEW

1. General background

Banana prawn, *Penaeus merguiensis* is distributed across the Indo-West Pacific, from the Persian Gulf to Thailand, Hong Kong, the Philippines, Indonesia , New Guinea, New Caledonia and Northern Australia (Figure 1). The most important shrimp fishing grounds in Thailand are the Gulf of Thailand and the Andaman Sea. The coastline and shrimp farming areas of Thailand is shown in Figure 2 (Viboonkit, 2002).

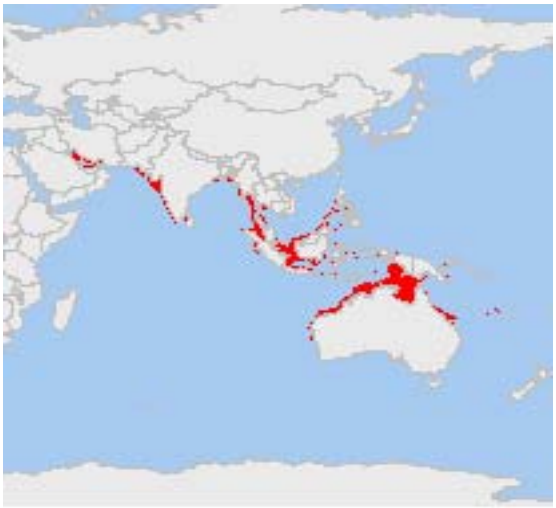
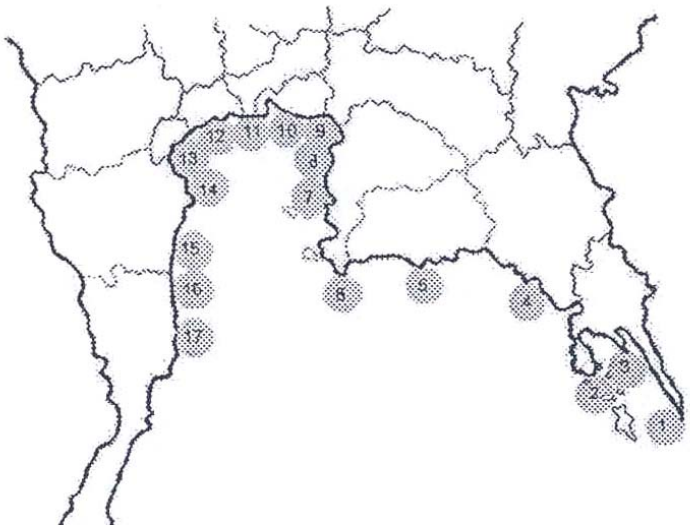


Figure 1 Geographical distribution for *Penaeus merguiensis* (Fisheries and Aquaculture Department, Food and Agriculture Organization [FAO], 2007)



1-3 Trad
4 Chantaburi
5 Rayong
6-8 Chonburi
9 Chachoengsao
10 Samut Prakarn
11 Bangkok
12 Samut Sakorn
13 Samut Songkarm
14-15 Petchaburi
16-17 Prachup Khiri Khan

Figure 2 Geographical distribution for *Penaeus merguiensis* in the Upper Gulf of Thailand (Viboonkit, 2002)

Shrimp production

Penaeid shrimp is one of the fastest growing crustacean aquaculture production sectors. Commercial shrimp farming is mainly based on tiger shrimp *P. monodon*, white shrimp *Litopenaeus vannamei*, *P. indicus*, *P. merguiensis* and kuruma shrimp *Marsupenaeus japonicus*. World cultured shrimp production levels reached 1.48 million metric tones in 2002, accounting for more than 49 percent of global capture and cultured shrimp production. The contribution of *P. monodon* has remained stable at around 600,000 metric tones from 1994 through 2002, whilst its contribution to world shrimp production has declined from over 63 percent to 40 percent in 2002, as *P. chinensis* and *P. vannamei* productions have increased to more than 500,000 metric tones. From 1980 to 2004, the production of capture *P. merguiensis* increased more than two-fold (Figure 3), while aquaculture production for *P. merguiensis* increased more than four-fold as shown in Figure 4 (Briggs *et al*., 2005).

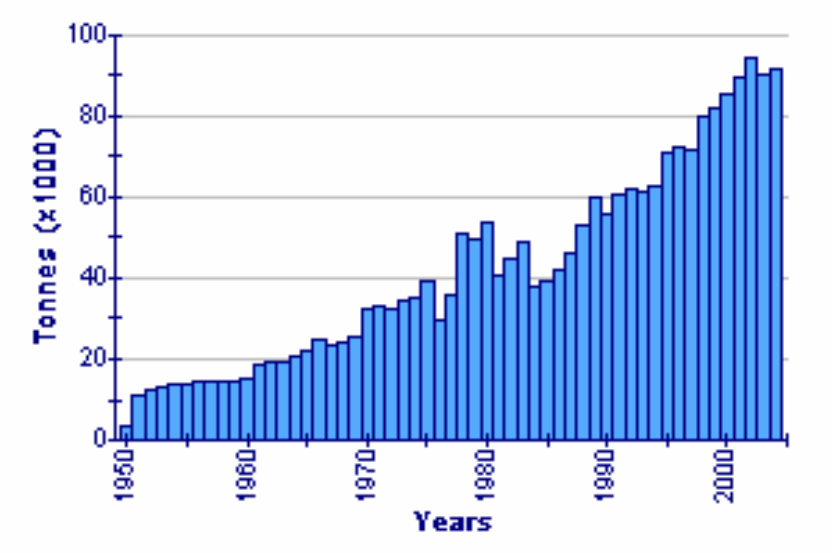


Figure 3 Global capture production for *Penaeus merguiensis* (FAO Fishery Statistic, FAO, 2006)

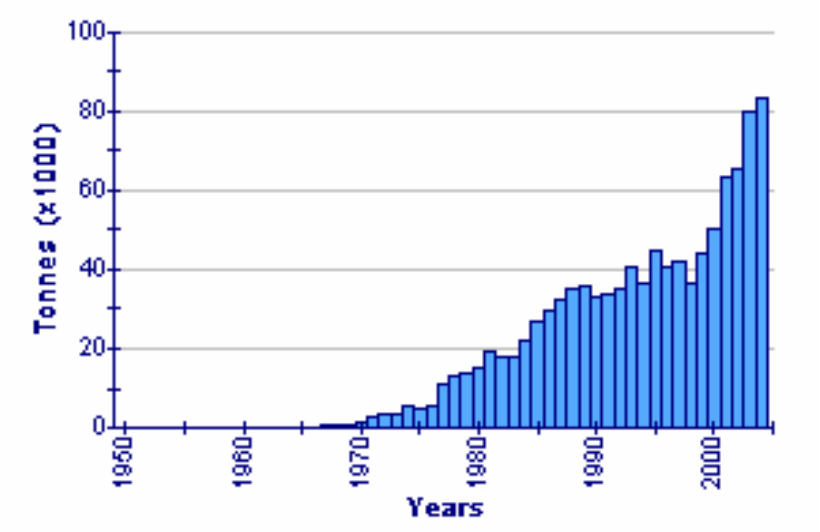


Figure 4 Global aquaculture production for *Penaeus merguiensis* (FAO Fishery Statistic, FAO, 2007)

Thailand shrimp production by capture includes several species*: P. merguiensis*, *P. indicus*, *P. semisulcatus*, *P. latisulacatus*, *P. monodon* and *Metapenaeus* spp. (Viboonkit, 2002). The production of marine shrimp culture in

Thailand during 1985-2004 is shown in Figure 5. Shrimp culture production was steadily from 300,000-330,000 MT during 2000 to 2004.

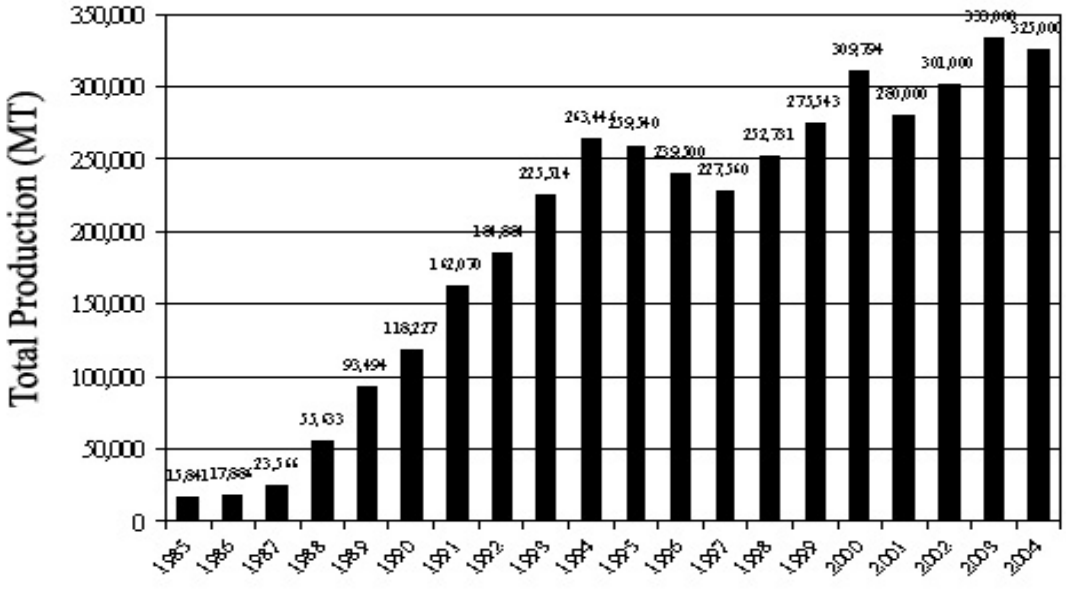


Figure 5 Thailand marine shrimp culture production during 1985-2004 (Fisheries Statistics Division, National Center for Genetic Engineering and Biotechnology, 2007)

2. Biology of *Penaeus merguiensis*

2.1 General morphology of shrimp

Shrimp constitute a large group of crustaceans varying in size from 2 cm to over 35 cm in length. The maximum total length of female *P. merguiensis* is 24 cm. The exterior of penaeid shrimp is distinguished by a cephalothorax (carapace) with a characteristic hard rostrum, and by segmented abdomen. The abdomen is longer than the cephalothorax. The organs such as gills, digestive system and heart are located in the cephalothorax, while the muscles located in the abdomen. The antennales, or first pair of feelers, in species bear a small scale or spine, the stylocerite, at their bases. The antennal scales of the second pair of feelers, the antennae, are generally large and plate-like. The pereopods (walking legs) are slender,

with the first three pairs of pereopods ending in chelae. The five pairs of pleopods (swimming legs) used for swimming are well-developed and present on the abdominal somites as shown in Figure 6 (Chaitiamvong and Supongpan, 1992). The *P. merguiensis* is similar to *P. indicus* Milne Edwards, but adults have distinctive rostra, the progressive relative shortening of the rostrum during development makes immature specimens of these 2 species difficult to distinguish.

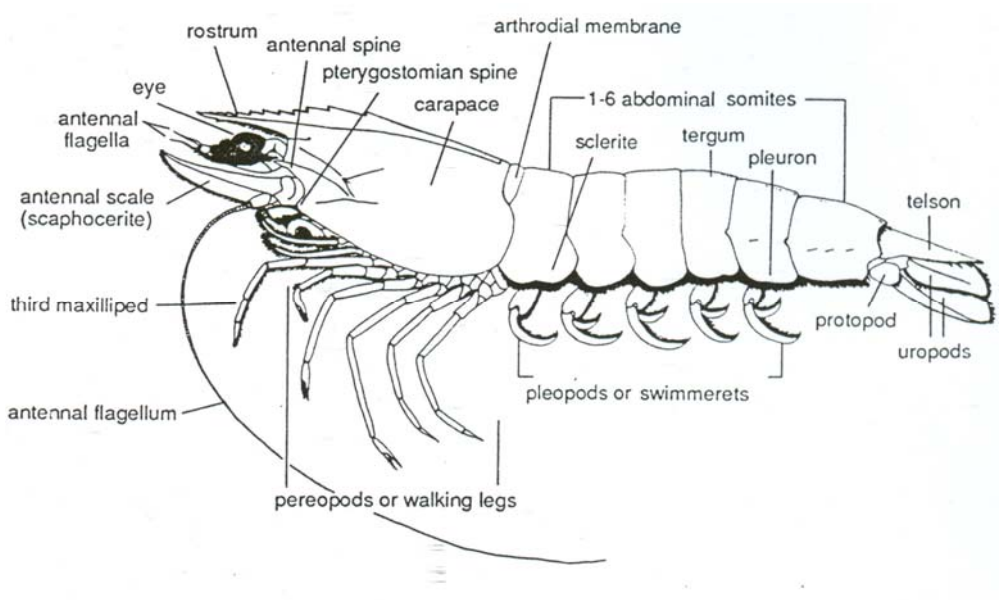


Figure 6 General morphological features of Penaeid shrimp (*Penaeus latisulcatus*)

Taxonomy

Penaeus is a genus of prawns, the most important species of farmed crustacean is the giant tiger prawn (*P. monodon*). Base on the morphological differences, the genus has been reorganized following a proposition of Pérez farfante and Kensley 1997.

Phylum Arthropoda
Superclass Crustacea Pennant, 1777
Class Malacostraca Latreille, 1806
Order Decapoda Latreille, 1803
Suborder Dendrobranchiae Bate, 1888
Superfamily Penaeoidea Rafinesque-Schmaltz, 1815
Family Penaeidae Rafinesque, 1815
Genus *Penaeus* Fabricius, 1798
Species *merguiensis*

Commercial names:
Australia: Banana prawn, White prawn
Japan: Tenjikuebi, Bananaebi
Malasia: Udang kaki merah, Udang pasir
Iran: Banana shrimp, Brow tiger shrimp
Thailand: Kung chaebauy

2.2 Life cycle of shrimp

Shrimp mature and breed only in a marine habitat. The females lay 50,000 to 1 million eggs, which hatch a few hours after spawning. Each larva is left to fend for itself as it develops to about twelve free-swimming planktonic stage of nauplius, protozoea and mysis before metamorphosing into a post larva (Figure 7). The nauplii feed on yolk reserves within their body and then undergo a metamorphosis into zoea. The third larval stage, mysis stage already look like to tiny shrimp and feed voraciously on zooplankton such as rotifers (*Artemia* nauplii) and algae. After another three to four days they metamorphose a final time into postlarvae, young shrimp having all the characteristics of adults. The whole process take about 12 days from hatching. In the wild, the postlarvae then migrate into estuaries, where the nutrients are rich and salinity is lower. They grow rapidly, develop into juveniles and, as size increases and eventually migrate back into open waters when they mature. Adult shrimp are benthic animals living primarily on the sea bottom (Chaitiamvong and Supongpan, 1992).

The banana prawn is widely distributed and important Indo-West Pacific species lives in shallow water between 10 and 45 meters on muddy bottoms. Juveniles are estuarine, adults mostly marine.

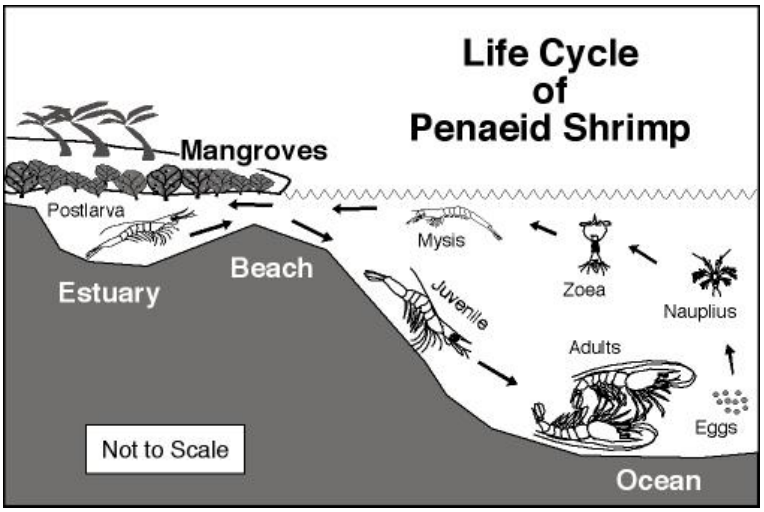


Figure 7 Life cycle of shrimp showing distribution with depth, a) shrimp eggs b) nauplius c) protozoea d) mysis e) postmysis f) juvenile and g) mature adult shrimp.

3. Vibrio disease of cultured shrimp

In shrimp farming, there are also a number of bacterial infections that lethal to shrimp. Vibriosis or luminous disease caused by luminous bacterial infection is a universal problem in a coastal or estuarine animal populations. Vibrio infections have been observed frequently in cultured penaeid shrimp. The isolation of *Vibrio* species from water or diseased shrimp are *V. parahaemolyticus, V. harveyi, V. alginolyticus, V. mimicus* and *V. damsela* (Liu *et al.*, 2004; Vandenberghe *et al*., 1999). Among them, *V. harveyi* is the causative agent associated with shrimp mortality (Ruangpan and Kitao, 1991; Hisbi *et al*., 2000; Nakayama *et al*., 2006). These bacterial isolates were Gram-negative, motile rods, oxidase- and catalase-positive, and produced colonies on Thiosulfate Citrate Bile Salts (TCBS) agar. A number of bacteria have been implicated as causes of disease and mortality in shrimp cultured, especially in the larval, post

larval and juvenile stages. The shrimp become weak and disoriented and may have dark wounds on the cuticle. The mortality can exceed 70%. Most such bacterial infections are strongly correlated to stressful conditions such as overcrowded ponds, high temperatures and poor water quality, factors that positively influence the growth of bacteria (Lightner and Redman , 1998). Vibriosis has also been reported to cause economic losses to the shrimp industry (Liu *et al*., 1996; Moriarty, 1999).

Important aspects of vibriosis in penaeid shrimp were summarized as follows: 1) Most outbreaks are consequences of extreme stresses and opportunistic pathogens. 2) Isolates of *Vibrio* spp. from shrimp may not always produce experimental infection, except when massive doeses are injected. 3) The bacteria are not fastidious and will grow on a wide variety of nutrient agar media including the selective madia Thiosulfate Citrate Bile Salts (TCBS) Agar. 4) Larval, postlarval, juvenile, and adult shrimp may be infected. 5) *Vibrio* spp. which infect shrimp are ubiquitous, and have been reported from all major shrimp culture regions. 6) Vibrio species and strains differ markedly in their virulence for penaeids, as they do for other hosts and large numbers of bacteria in the hemolymph (Saulnier *et al*., 2000). Detailed procedures and culture characteristics of several species of *Vibrio* were described in Berggy's Mannual of Determinative bacteriology 9th edition (Holt *et al*., 1994).

4. Crustacean Immunity

Immunity to infection is mediated by two systems, namely the acquired or adaptive immune system and the innate or natural immune system. The acquired immune system evolved about 400 million years ago and is found only in vertebrates, at some point between the divergence of the cyclostomes (lamprey) and cartilaginous fishes (sharks) (Fujita, 2002). The responses are mediated by many different agents: macrophages and other phagocytic cells, B and T lymphocytes, antibodies and multitude of other participating proteins.

The innate immune system is an evolutionarily ancient form of immunity and offers the main resistance to microbial pathogens within the first minutes, hours or

days of an infection. Invertebrate animals, which lack an adaptive immune system, have developed various defense systems that make-up their so-called " innate immunity" and response to common antigens on the surface of potential pathogen. Invertebrate defense system is dependent on an innate immune system by a complex of cellular and humoral factors. These systems are already active in the body before an invader appears . Although they cannot produce antibodies and hence have no immune memory, innate immune mechanisms are sufficient to protect and preserve themselves from microorganisms (Beck and Habicht, 1996; Lee and Söderhäll, 2002). 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 included lectin, lysins and other killer substances which are capable of inactivating potentially pathogenic material (Cooper and Lemmi, 1981).

Both marine and freshwater crustaceans, live in an environment often rich in different parasites and pathogens. Therefore, crustaceans must be able to mount an efficient defense against invading pathogenic organisms. Knowledge of crustacean immunity is mainly related to the crayfish *Pacifastacus leniusculus*, some marine decapods such as lobster, *Homarus vulgaris*, and crab, *Carcinus maenas.* Few data concern shrimp species, the most species studied are the ridgeback prawn, *Sicyonia ingentis*, the kuruma prawn, *P. japonicus* , the giant tiger prawn *P. monodon* and the brown shrimp, *P. californiensis*.

4.1 Cellular immune response

The cellular defense system includes phagocytosis, encapsulation, nodule formation, melanization and coagulation (Franc and White, 2000; Johansson *et al*., 2000, Montaño-Pérez *et al*., 1999). For Crustaceans, hemocytes are divided into three types, hyaline semigranular and granular cells (Persson *et a*l., 1987; Hose *et al*., 1990; Bachére, 1995).

1. The hyaline cell refers to the smallest hemocytes that contain no or only a few cytoplasmic granules which are considered as phagocytes. The hyaline cells are involved in the initiation of the hemolymph coaglulation. (Hose *et al*., 1990).

2. The semigranular cells which contain small granules and display some phagocytic capacities, would be specialized in particle encapsulation by recognized lipopolysaccharides and β-1,3-glucans (Persson *et al*., 1987).

3. The large granule haemocytes which did not respond to lipopolysaccharides and β-1,3-glucans (Söderhäll and Cerenius, 1992).

However, the size and number of granules in hyaline cells and granulocytes vary greatly between species and therefore are not useful criteria for identifying these cells.

In crustacean immune defense, the haemocytes play a role in:

1. They remove foreign particles in the hemocoel by phagocytosis, encapsulation and nodular aggregation (Söderhäll and Cerenius, 1992).

2. They take part in wound healing by cellular clumping and initiation of coagulation processes through the release of factor required for plasma gelation (Johansson and Söderhäll, 1989).

3. They carry and release of the prophenoloxidase (proPO) system (Johansson and Söderhäll, 1989).

4. They are also involved in the synthesis and discharge in the hemolymph of important molecules, such as α2-macroglobulin (Armstrong *et al*., 1996) agglutinin and antimicrobial peptides (Destoumieux *et al*., 1997).

Hyaline cells are involved in the initiation of hemolymph coagulation whereas granulocytes are involved in defense against foreign material by phagocytosis and encapsulation. Agglutinin or lectins are protein or glycoprotein from hemolymph of crustaceans as potential molecules that involved in immune recognition in phagocytosis through opsonization (Marques and Barracco, 2000).

4.2 Humoral immune response

4.2.1 Lectin

Lectins are antibody-like molecules and have multivalent by bearing at least two sugar-binding sites but have no enzymatic activity (Goldstein, 1980; Kocorek and Hořejši, 1981).

Detection of lectin activity

Lectins have been isolated from various biological sources. They occur in viruses and bacteria to vertebrates and probably in all living organisms. 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).

Although the first invertebrate lectin reported was identified in extracts of snail albumin gland, the early work was done using body fluids or secretions, such as hemolymph, coelomic and seminal fluids and mucus, or whole body extracts obtained by the use of blenders, mortars and presses. Then, the solutions were assayed for lectin activity. Agglutination of mammalian or animal erythrocytes is the most commonly used method of detecting the presence of lectin extract solution with untreated or enzyme-treated erythrocytes or precipitation reactions with glycoproteins and polysaccharides. Hemagglutination is generally performed in 96 wells microtiter plates, usually at room temperature, in volumes of 50 µl/well, using a panel of erythrocytes, untreated or enzyme-treated, representing as many vertebrate species as possible. The lectin extract is a two-fold serial dilution in saline or physiological buffer before incubate with 0.5-2% erythrocytes (Vasta and Ahmed, 1995). Activity is expressed as an agglutination titer which is defined as the reciprocal of the highest dilution of the lectin that gives visible agglutination.

Classification of animal lectins

Lectins can be divided into at least six families by sequence comparison of the carbohydrate-recognition domain (CRD); (i) legume lectins, (ii) cereal lectins, (iii) P-, (iv) S- and (v) C-type lectins, and (vi) pentraxins. Based upon amino acid and nucleotide sequence similarities, three-dimentional structures, and information about CRD that are available, animal lectins are practically categorized into a number of four groups and subgroups as summarized in Table 1 (Arason, 1996).

The C-type lectins are a family of extra cellular carbohydrate recognition proteins characterized by a common sequence motif of 115 to 130 amino acid residues. This domain, the carbohydrate-recognition domain contains two α-helixes as well as two β-sheets and some loops and usually shows specificity. Calcium is essential for carbohydrate binding but weak calcium-dependent binding to a variety of monosaccharide. The different specificities are accounted by monomer structural variations near the sugar-binding site.

P-type lectins have been described both are intracellular transmembrane proteins with towards mannose-6-phosphate and function in the intracellular targeting of lysosomal enzyme.

S-type lectins or galectins are small, soluble proteins with Ca^{2+} - independent affinity for lactosamine and other β-galactosides.

Pentraxins show Ca^{2+} - dependent binding to saccharides on bacterial cell surfaces and exist in serum as acute phase proteins, indicating their role in defence. Pentraxins and C-type lectins are ubiquitous in metazoan animals, but S-type lectins have not been described from advanced protostomes. P-type lectins have been studied only in mammals.

Animal lectins are a heterogeneous class of molecules, which exhibit a high structural diversity. They have been initially classified in two different groups: C- and S-type lectins, based on amino acid sequence similarities, particularly in the CRD, along with overall domain organization and physico-chemical properties, such as divalent cation dependence and free thiol requirement. Lectins mediate many different types of interaction including adhesion between animal cells within a single organism, targeting of bacterial toxins to animal cells and immune recognition of bacteria and fungi by animals. Hence, the structures of lectins in complex with sugars provide reasonably satisfying explanations for these different reactin (Drickamer, 1997).

Calcium dependence is the main property associated with C-type lectins, whereas thiol dependence is the S-type group's characteristic. Recently, as a consequence of three-dimensional structure studies and the availability of additional primary sequence data, a more complete classification has emerged. Therefore, C-type lectins have been further characterized into distinct subgroups, and S-type lectins reclassified as galectins; but the existence of new categories was also established (Vasta *et al*., 1996; Marques, 2000).

Table 1 Classification of lectins by Gudmundur Johann Arason (1996)

Type of lectins	Subunits	Subunit MW (kDa)	CRDs per subunit	Ca^{2+} depen-dent	Disulphide bonds	Carbohydrate Specificity
Legume lectins	2 or4	25-30	1	+	-	Diverse
Cereal lectins	2	18	2	-	++	GlcNAc, NeuAc
P-type lectins	1-4	46 or 275	1 or 15 Similar CRD	-	+	Man-6-P
S-type lectins	1 or 2	14-35	1 or 2 Conserved CRD	-	-	Gal
C-type lectins	Variable	14-165	1 or 8 Conserved CRD	+	+	diverse
Pentraxins	5 or 6 Pentra-meric subunits	20-25	-	+	+	diverse

GlcNAc: N-acetylglucosanine; NeuAc: N-acetylneuraminic acid; Man-6-P: Mannose-phosphate; Gal: Galactose

Dodd and Drickamer (2001) have classified lectins that contain CRDs in Table 2. Lectins recognize more complex structures at the cell surface, such as C-type lectins and galectins, are also found in invertebrate organisms as well as vertebrates, but the functions of these proteins have evolved differently in the different animal lineages.

Table 2 Summary of lectin categories by Roger B. Dodd and Kurt Drickamer (2001)

Lectin group	Structure of CRD	Length	Typical ligands	Examples of functions
Calnexin	Unknown	-	Glc_1Man_9 oligosaccharides	Protein sorting in the endoplasmic reticulum
L-type	β-sandwich	230+	Various	Protein sorting in the endoplasmic reticulum
P-type	Unique β-rich structure	130+	Man 6-phosphate	Protein sorting post Golgi
C-type	Unique mixed α/β structure	115+	Various	Cell adhesion (selectin) Glycoprotein clearance Innate immunity (collectins)
Galectins (S-type lectins)	β-sandwich	125+	β-Galactosides	Glycan crosslinking in the Extracellular matrix
I-type	Immunoglobulin Superfamily	120+	Sialic acid	Cell adhesion (siglecs)
R-type	β-trefoil	125+	Various	Enzyme targeting Glycoprotein hormone turnover

Calnexin and calrecticulin are from the part of the quality control system for glycoproteins in the endoplasmic reticulum. They bind to terminal glucose residuces on N-linked oligosaccharide and retain misfolded glycoproteins in the endoplasmic reticulum. Calnexin is a transmembrane protein and calreticulin is a soluble protein retained in the lumen by a C-terminal retention signal. The luminal N-terminal portion of calnexin is very similar to calreticulin, although one of the repeated segments of calnexin is absent from calreticulin.

L-type, plant and animal lectins have divergent sequences and different molecular properties. The plant lectins are secreted, soluble proteins and are found at high level in specialized tissues while the animal L-type lectins are membrane-bound luminal proteins and are found at low levels in many different cell types. These differences reflect the fact that plant and animal L-type lectins are likely to serve different functions.

I-type CRDs, the siglec family of cell surface adhesion receptors are sialic acid-binding protein that contain I-type CRDs derived from the immunoglobulin fold.

R-type CRDs or the ricin-like are the only sugar-binding protein modules from animal lectins that have also been found in bacteria.

Carbohydrate specificity

In decapods, the specificity of lectins towards carbohydrates is mainly related to N-acetylated carbohydrates, such as NeuAc, GlcNAc and N-acetyl-D-galactosamine (GalNAc), as can be depicted by the data partially summarized in Table 3. Although the recognition of NeuAc is a common feature among crustacean lectins some of them exhibit a particular pattern of specificity towards O-acetylated sialic-acid derivatives (Vagas-Albores *et al*., 1993; Middleton *et al*., 1996). The marine crab *C. antennarius* hemolymph lectin has shown the specificity to 9-O/4-O-acetyl sialic acid (Ravindranath *et al*., 1985), whereas the hemolymph lectins of the

freshwater prawn *Macrobrachium rosenbergii* and the marine crab *Liocarcinus depurator* have been shown to specifically recognize 9-O-acetyl sialic acid (Vàzquez *et al*., 1993; Fragkiadakis and Stratakis, 1995). The agglutination of the bacterium *Bacillus cereus* by *M. rosenbergii* hemolymph lectin can be related to the recognition of these O-acetylated sugars on the bacteria cell surface (Vàzquez *et al*., 1996)

Invertebrate lectins

Most invertebrate lectins are oligomers that consist of subunits of equal or different size, held together by disulfide bonds or non-covalent interactions. These subunits may be constituted by one or more polypeptide chains bound by interchain disulfide or non-covalent interactions as well, each polypeptide chain may be folded and stabilized by interchain disulfide bonds. Examination of some biochemical characteristics of shrimp lectins are summarized in Table 3.

In penaeid *P. monodon*, Ratanapo and Chulavatnatol (1992) reported the agglutination of the highly pathogenic bacteria *V. vulnificus* by a purified lectin called monodin. In the *P. californiensis* has been investigated the ability of the purified lectin to react with different marine species of *Vibrio* (Vargas-Albores *et al,* 1993). They demonstrated that the agglutinin of this penaeid was able to react to at least three different *Vibiro* species such as *V. vulnificus, V. fisheri* and *V. parahaemolyticus*. This reaction was specific and the agglutination of *V. parahaemolyticus* could be inhibited by GalNAc and lipopolysaccharide (LPS). The inhibition by LPS suggested that this natural ligand of the penaeid lectin could be one effective sign that triggered the shrimp immune system.

Table 3 Properties of purified lectins from Penaeid shrimp

Shrimp	Molecular Weight (kDa)	Subunit (kDa)	Divalent Cation dependence	Sugar specificity	Biological properties	Ref.
Penaeus monodon	420	27	Yes	GlcNAc, GalNAc, ManNAc, NeuAc, BSM, Fetuin	Bacterial agglutinating activity; *Vibrio vulnificus*, *V. parahaemolyticus*	Ratanapo and Chulavatanatol 1990; 1992
P. japonicus	330	33	Yes	GlcNAc, GalNAc, NeuAc, BSM, PSM Ribose, Fetuin	Opsonic activity	Kondo *et al*., 1992
P. californiensis	175	41	Yes	GlcNAc, GalNAc, NeuAc, BSM, Fetuin, LPS	Bacterial agglutinating activity; *V. fischeri* Opsonic activity	Vargas-Albores *et al*., 1993; Vargas-Albores, 1995
P. longirostris	440 210	27 36	Yes	NeuAc, Gal, GalNAc, BSM, Fetuin	Bacterial agglutinating activity; *E. coli*	Fragkiadakis and Stratakis, 1995
P. indicus	181	97 84	No	GlcNAc, GalNAc, ManNAc NeuAc, BSM, Fetuin, LPS	Inhibition of *Vibrio* V-5	Maheswari *et al.*, 1997

ManNAc: N-Acetylmannosamine; NeuAc: N-Acetylneuraminic acid;
BSM: Bovine submaxillary mucin; GalNAc: N-Acetylgalactosamine;

Table 3 (Continued)

Shrimp	Molecular Weight (kDa)	Subunit (kDa)	Divalent Cation dependence	Sugar specificity	Biological properties	Ref.
Fenneropenaeus indicus	220	27	Yes	GlcNAc, GalNAc, ManNAc BSM, Fetuin Ovalbumin	Bacterial agglutinating activity;	Maheswari, 2002
Litopenaeus schmitti	220	31 34	No	NeuAc, BSM, Fetuin, LPS	Not report	Cominetti, 2002

Biological role of lectin in invertebrates

1. Lectin plays role as non-self recognition molecule relies on the molecular basis for the generation of binding diversity and specificity to account for an efficient immunorecognition (Marques and Barracco, 2000). The examples in arthropods appear to support the view that invertebrate lectins may exhibit the required binding diversity to efficiently discriminate non-self-particles.

The cockroach *Blaberus discoidalis*, which contains multiple plasma lectin, BDL1, BDL 2, BDL3 and GSL each with different carbohydrate-binding specificities and consequently, is able to potentially recognize different invading pathogens. It was demonstrated that each of these purified molecules was capable to induce a specific and enhance phagocytic response towards different microorganisms, such as yeast (*Saccharomyces cerevisiae*) and bacteria (*Escherichia coli* and *Bacillus cereus*). This response was related to the carbohydrate exposed on the microorganism surface and to the sugar specificity of each lectin (Wilson *et al*., 1999).

Arthropod lectins must be able to specifically bind to non-self-particles surface and also to adhere to virtually specific receptors on the phagocyte (hemocyte) surface, the true opsonic factors.

2. Lectins exhibit the antimicrobial protein

Saito *et al*., (1995) showed that the Japanese horseshoe crab *Tachypleus tridentatus,* several lectins, named tachylectins, were purified from hemolymph. Tachylectin 1 had a broad specificity to S and R types of lipopolysaccharides (LPS) from gram-negative bacteria cell walls and was also capable to inhibit the growth of gram-negative bacteria.

Tachylectin 2 had a binding specificity for GlcNAc and GalNAc and promoted the agglutination of certain strain of gram-positive *Staphylococcus* and recognized several kinds of LPS (Beisel *et al*.,1999).

Tachylectin 3 was specific to the human blood group A antigen and recognized with highly specific the sugar moiety O-antigen from LPS of several gram-negative bacteria (Inamori *et al*., 1999).

Tachylectin 4 had a binding specificity for fucose and specifically recognized S-type of LPS from several gram-negative bacteria through O-specific polysaccharides (O-antigen) (Saito *et al.,* 1997).

Tachylectin 5 was identified in the horseshoe crab plasma (and not in hemocyte extracts) and might probably be the primary lectin to recognize microbes. This lectin type had a broad specificity to N-acetylated substances and promoted the strongest agglutinating activity against both gram-positive and gram-negative bacteria (Kawabata and Iwanaga, 1999). It was proposed that the innate immune system of the horseshoe crab, *T. tridentatus* may recognize invading pathogens through a combinatorial method by using broad and highly specific lectins against molecules exposed on pathogen surface and function synergistically to ensure an effective host defense (Iwanaga, 2002).

4.2.2 Antimicrobial peptides and proteins

Antimicrobial peptides represent an essential alternative first line of defense. These molecules, universally distributed in metazoans, are usually amphipathic, carry a net positive charge, and can form α-helical or β-sheet structures in membrane-like environments (Zasloff, 2002).

Antibacterial peptides and proteins have been well studied in arthropods , mainly in insects, horseshoe crab, marine crustacean decapods and marine bivalves (Gillespie *et al.* 1997; Tunkijjanukij and Olafsen, 1998; Haug *et al*.; 2002; Iwanaga, 2002) where the families of antimicrobial molecules have been isolated and characterized. Much of the research on the immune strategies of crustaceans has been stimulated by the increasing importance of decapods in aquaculture, and over 10 years great studies have been made in our understanding of the cellular and humoral defenses in these animals.

In crustacean, the reports of antibacterial activity have been concerned with economically important species, such as spiny lobster (Evans *et al.*, 1969) and shrimps (Adams *et al.*, 1991; Chisholm and Smith, 1995; Noga *et al*., 1996). Crustacean hemolymph and hemocytes have been shown to inhibit bacterial growth *in vitro* against gram-positive and gram-negative bacteria.

The hemocytes of the shore crab, *C. maenas*, have been shown to have potent antimicrobial activity against a range of gram-positive and gram-negative bacteria *in vitro* (Chisholm and Smith, 1992). The activity resides chiefly in the granular cells, is not requirement of divalent cations and is stable at 100°C for 10 min or -20°C for one month. Antimicrobial activity is also exhibited by the hemocytes of a variety of other marine crustaceans (Chisholm and Smith, 1995) and for most species the response does not appear to entail bacterial agglutination, direct lysis of the bacterial cell wall or phenoloxidase activity.

The fractionation of *C. maenas* hemocyte lysate supernatants (HLS) by gel filtration and PAGE shows that the hemocytes of this crab contain several constitutive antibacterial proteins which inhibit the growth of both gram negative and gram positive bacteria. The molecular weights of these proteins have >70 kDa, ~45 kDa, ~14 kDa and 6.5 kDa (Schnapp *et al*., 1996). Low molecular weight antibacterial proteins are known to be key components in the non-specific defenses of both vertebrates and invertebrates and are believed to represent an ancient form of tissue protection against opportunistic microbial exploitation (Boman, 1991)

In addition, lectins from invertebrates play a role in defense molecules such as antibacterial activity or bacterial agglutination (Arason, 1996; Tunkijjanukij and Olafsen, 1998). Bactericidal activity against gram-negative bacteria has been clarified in the hemolymph of *P. monodon* using the colony-forming units inhibition assay (Adam, 1991). Antibacterial activities in marine crustacean decapods are reported in *Pandalus borealis* (northern shrimp), *Pagurus bernhardus* (hermit crab), *Hyas araneus* (Spider crab) and *Paralithodes camtschatica* (king crab) (Haug *et al*. 2002).

The penaeidins are the first antimicrobial peptides found in penaeid shrimp. Penaeidins were isolated from the hemocytes of the Pacific white shrimp (*Litopenaeus vannamei*) that the structures are characterized by their antimicrobial activities (Destoumieux *et al*., 1997). The antimicrobial lectin, named scyllin was isolated from the edible crab (*Scylla serrata*) (Chattopadhyay *et al*., 1996). So far, the production of antimicrobial peptides and proteins is important for host defense. The larger antimicrobial proteins, containing more than 100 amino acids, are often lytic enzymes or contain sites that target specific microbial macromolecules. Almost all antimicrobial peptides are cationic and amphipathic. Antimicrobial peptides have been found in the epithelial layers, phagocytic cells and body fluids of multicellular animals (Ganz, 2003).

Penaeidins

In penaeid shrimp, the most studied family of antimicrobial peptides are the penaeidins, penaeidins 1, 2 and 3. The molecular weight of peptides range from 5.5-6.6 kDa, are characterized by an over-representation of proline residues in their NH_2-terminal domain and by 6 cysteine residues engaged in three intramolecular disulfide bridges concentrated in their COOH-terminal domain. Penaeidins from *L. vannamei* are mostly predominant active against gram-positive bacteria (Destoumieux *et al*., 1997). More recently, Cuthbertson *et al*., (2004) have reported the identification of new penaeidin, penaeidin 4 from *L. setiferus*. Chiou *et al*., (2005) have reported the molecular cloning and characterization of a penaeidin-like antimicrobial peptide complementary DNA from the hemocytes of black tiger shrimp *P. monodon*. The mature peptide contains a proline-rich domain at the N terminus and 6 cysteine residues at the C terminus, and it shares less than 50% amino acid sequence identity with the mature penaeidins of *L. vannamei.*

4.2.3 Prophenoloxidase activity system

The importance of the prophenoloxidase system in invertebrate defense system has been highly documented (Gollas-Galván *et al*., 1999). The enzyme responsible for initiating the biosynthesis of melanin called melanization is the phenoloxidase. Phenoloxidase, a copper-containing enzyme, is widely distributed not only in animals but also in plants and fungi. The melanin will physically shield an intruder and therefore prevent or retard the parasites and microorganisms growth, but perhaps even more importantly during melanin formation, highly reactive and toxic quinone intermediates are produced. Phenoloxidase will catalyze the early steps in the pathway to melanin formation. The enzyme catalyzes the oxygenation of monophenols to o-diphenols and further oxidation of o-diphenols to o-quinones (Cerenius and Söderhäll, 2004).

4.2.4 Lysozyme

Lysozymes are group of proteins defined as 1,4-β-N-acetylmuramidases (EC 3.2.1.17). These enzymes cleave the glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine of peptidoglycan, a major component of gram positive bacterial cell walls (Gillespie *et. al*., 1997). Lysozyme is one component of the humoral defense system of insects, the Mediterranean cricket, *Gryllus bimaculatus* (Schneider, 1985). The I-type invertebrate lysozyme has been increasing interest in recent years in the distribution and characterization which include lysozymes of bivalve mollusks such as lysozyme from plasma of the eastern oyster, *Crassostrea virginica* (Xue *et al*., 2004).

Lysozyme in hemocyte of *P. vannamei* shows broad antimicrobial lytic activity. The lysozyme cDNA of white shrimp *P. vanamei* has also been cloned and characterized for studies on the molecular mechanisms of immunity in marine invertebrates (Sotelo-Mundo *et al.*, 2003).