Chapter 1

Introduction

Introduction

The aquaculture of penaeid shrimp in the world has developed rapidly to a major industry. The producing countries are mainly in Asia and South America. Thailand is one of the leading shrimp producing and exporting countries. However, the rapid expanding shrimp industry started to face problems in 1992. Diseases have showed as a major problem to the sustainable growth of shrimp aquaculture. Under poor farming condition, it is often probable diseased caused by bacteria, fungi and protozoa which are consistently present in the pond environment that caused death of the shrimp. Due to bacterial and viral disease incidence in farmed penaeid shrimp in China, industrial productions dropped to 55,000 tons in 1994 which is only 25% of the 1992 production level (Wang *et al*., 1997). The collapse was caused by the 'white spot syndrome virus' also referred to as WSSV, SEMBV, WSBV or RVPJ which became epizootic due to a deteriorated ecological environment (Wang *et al*., 1997). For nearly as long as penaeid shrimp were cultured, reports of bacterial infections and diseases caused by *Vibrio* spp. were by far the most numerous (Lightner, 1996). Numerous *Vibrio* species were reported as causal agents of diseases in various penaeid shrimp species: *Vibrio harveyi* infections in *Penaeus indicus* (Prayitno and Latchford, 1995) and *Penaeus monodon* larvae (Lavilla-Pitogo *et al*., 1990), *V. campbellii* infections in *Penaeus orientalis* larvae (Xu *et al.*, 1994) and *V. splendidus* infections in *P. monodon* larvae (Lavilla-Pitogo *et al*., 1990; Prayitno and Latchford, 1995). *V. parahaemolyticus* infections in juvenile and adult *P. monodon* (Ruangpan and Kitao, 1991; Nash *et al*., 1992; Chanratchakool *et al*., 1995) and *P. orientalis* (Xu *et al*., 1994). *V.* v*ulnificus* (Song *et al*., 1990; Ruangpan and Kitao, 1991; Nash *et al*., 1992; Chanratchakool *et al*., 1995) and *V. damsela* (Nash *et al*., 1992) infections in juvenile and adult *P. monodon.*

In view of the economic importance of shrimp aquaculture and the number of pathological problems in industry is now suffering. Therefore, studying and developing tools for the rapid recognition and control of pathogens are needed. Correct diagnosis, including knowledge of the life cycle and ecology of the pathogen, is obviously a critical step in any control program. Epidemiological surveys of pathogens are still marginally performed, partly due to a lack of suitable diagnostic methods. However, technologies for quick recognition of pathogens in shrimp culture are developing rapidly and diagnostic probes, which can be used in screening of captured broodstock and their postlarvaes prior to their stocking, are now available for many of the severe shrimp pathogens (Lightner, 1996).

Control of diseases is an essential importance and can be reached in different ways, however, until now chemotherapy and management practices are the only methods available to decrease the infection stress in shrimp farming. Therefore, research on quantitative assays to control the defense system of penaeid shrimp and accordingly the health status has a high priority. Disease prevention is more important than treatment. Studying about defense molecules in shrimp is one approach to overcome disease problem. Studies related to invertebrate immunity are infrequent mainly due to the idea that they do not require an efficient immune system because of their short life period and high reproductive rate. However, in recent years, the defense response of penaeid shrimp has surprisingly formed subject of minor interest. The economical importance of shrimps has prompted the necessity to study their immune components. Immunity in invertebrates is believed to lack adaptive and to reply on innate immune system including both humoral and cellular immune reactions (Loker *et al*., 2004). In the shrimp defense mechanism, lectins are believed to take part in humoral defense reactions (Cooper et al., 1987; Renwrantz, 1986; Sima and Vetvicka; 1993) and structure-function relationships relevant to their reaction with non-self and effect on phagocytosis were reviewed (Olafsen, 1986; 1995; 1998; Vasta, 1990; 1991; Vasta and Marchalonis, 1987).

Lectins are carbohydrate-binding proteins that agglutinate erythrocytes, bacteria, and other cells through interaction with appropriate complementary ligands (Goldstein *et al*., 1980; Nesser *et al*., 1986; Sharon *et al*., 1984). The defense role of lectins in invertebrates against infective microbes and parasites was a subject of interest for a long time (Anderson and Good, 1976; Ratcliffe *et al.,* 1985; Olafsen, 1988). For example, stimulation of hemolymph lectin

activity by pathogenic bacteria has been demonstrated in Pacific oyster, *Crassostrea gigas* (Hardy *et al.,* 1977). Similar events have also been reported in earthworm, *Lumbricus terrestris* (Stein *et al.,* 1986) and silk moth, *Antheraea pernyi* (Qu *et al.,* 1987).

In the present work, I purified and characterized lectin from hemolymph of banana shrimp, *Penaeus* (*Fenneropenaeus*) *merguiensis*. *P. merguiensis* was selected to be a model for study of the lectin due to these factors. *P. merguiensis* shrimps tolerate in low water quality, can be grown at high densities and are readily available in the wild resources. In addition, *P. merguiensis* brood stock is cheapter than that of *P. monodon* and both of them are the most important economically fishery penaeids in Thailand. However, the specific role of lectin in this species has not been clearly defined yet. The difficulties in assessing a specific role of this protein in the shrimp should be due to lack of its accurate quantification. Initiation of my currently investigations required purification of lectin from *P. merguiensis* hemolymph. This study aimed to characterize purified lectin, to generate specific and reliable anti-lectin antibody that was used for identification and characterization of lectin in the hemolymph and also to development an enzyme linked immunosorbent assay (ELISA). ELISA was then applied to quantitate lectin level in *P. merguiensis* hemolymph at different stages of ovarian development including that of bacterial infectous of *P. merguiensis*. The ELISA quantification will be useful in future measurement of *P. merguiensis* lectin against potential shrimp pathogens, which would also be helpful in controlling shrimp diseases.

Review of literatures

Since the 1880's, lectin was known that extracts from certain plants could agglutinate red blood cells. In the 1940's, agglutinins were discovered which could "select" types of cells based on their blood group activities. Today the term of lectin is used more generally and includes sugar-binding proteins from many sources.

Lectin or agglutinin is a sugar-binding protein of non-immune origin that agglutinates cells or precipitates glycoconjugates (Goldstein *et al.*, 1980). A lectin molecule contains at least two sugar-binding sites; sugar-binding proteins with a single site will not agglutinate or precipitate structures that contain sugar residues, so are not classified as lectins. The specificity of lectin is usually defined by the monosaccharides or oligosaccharides that are best at inhibiting the agglutination or precipitation the lectin causes. Lectins have been found in plants, viruses, microorganisms and animals, but despite their ubiquity, their function in nature is unclear. Although lectins share the common property of binding to defined sugar structures, their roles in various organisms are not likely to be the same.

Literally thousands of articles on lectins have been published during the past forty years examining hundreds of different aspects and uses of lectins. But only a few of the uses and properties of each lectin are described. Concurrently, it was shown that lectins function as recognition molecules in cell–molecule and cell–cell interactions in a variety of biological systems (Sharon and Lis, 2004).

1. Structures, properties and classification of lectin

Most lectins studied to date are multimeric, consisting of non-covalently associated subunits. A lectin may contain two or more of the same subunit, such as concanavalin A (Con A) (Agrawal and Goldstein, 1972), or different subunits, such as *Phaseolus vulgaris* agglutinin (Takahashi *et al*., 1980). It is this multimeric structure which gives lectins their ability to agglutinate cells or form precipitates with glycoconjugates in a manner similar to antigen-antibody interactions.

One major property of lectins is their specific saccharide- binding sites. Some lectins are composed of subunits with different binding sites. The specificity of the binding sites of the lectins suggest that there are endogenous saccharide receptors in the tissues from which

they are derived or on other cells or glycoconjugates with which the lectin is specialized to interact. Because of the specificity that each lectin has toward a particular carbohydrate structure, even oligosaccharides with identical sugar compositions can be distinguished or separated. Some lectins will bind only to structures with mannose or glucose residues, while others may recognize only galactose residues. Some lectins require that the particular sugar be in a terminal non-reducing position in the oligosaccharide, while others can bind to sugars within the oligosaccharide chain. The affinity between a lectin and its receptor may vary a great deal due to small changes in the carbohydrate structure of the receptor.

Another property of some lectins is an ability to induce mitosis in cells which are normally not dividing (Reichert *et al.,* 1973). This property has been exploited extensively in an attempt to understand the process of lymphocyte blastogenesis and the biochemical and structural alterations associated with mitogenesis. It is not clear why some lectins are mitogenic since the structures to which mitogenic lectins bind are not necessarily the same, and not all lectins with similar binding specificities are mitogenic. It is likely that binding to the cell surface alone is not sufficient to cause mitosis but that other interactions on the cell surface are equally important.

Most lectins are considered glycoproteins contain no covalently attached carbohydrates. However, non-glycoprotein lectins are believed to be synthesized as glycosylated precursors. All glycoprotein lectins contain a peptide sequence: asparagine-X-threonine/serine, which is characteristic of glycosylation sites. These sequences are different in the non-glycoprotein lectins. Also, peptide sequences, which in one glycoprotein lectin contain the glycosidic side-chains, are not necessarily conserved in another glycoprotein lectin. This may suggest that the biological activity of the lectins may not be determined by carbohydrate part of their structure (Barondes, 1981).

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 ("C" stands for Ca-requiring and "S" stands for SH-requiring) based on amino acid sequence similarities, particularly in the carbohydrate recognition domain, along with overall domain organisation and physico-chemical properties, such as divalent cation dependence and free thiol requirement (Drickamer, 1988).

C-type lectins are calcium-dependent animal lectins that are carbohydrate-binding proteins of animal origin. Carbohydrate-binding activity of C-type lectins is based on the function of the carbohydrate recognition domain whose structure is highly conserved among this family (Drickamer, 1988). Calcium is not only directly involved in the carbohydrate binding itself at the binding site (Weis *et al*., 1992) but contributes to the structural maintenance of the lectin domain that is essential for the lectin activity (Kimura, 1995).

Galectins are defined as lectins having both galactose-binding ability and amino acid sequences which characterize galectins. Since these lectins usually require a thiol reducing reagent for the maintenance of their activity, Drickamer (1988) designated them as "S-type" lectins.

C-type lectins and galectin that recognize complex structures at the cell surface found in invertebrate organisms as well as vertebrates but the functions of these proteins have evolved differently in different animal lineages (Dodd and Drickamer, 2001).

Lectins are often complex, multi domain proteins, but sugar-binding activity can usually be ascribed to a single protein module within the lectin polypeptide. Such a module is designated a carbohydrate recognition domain (CRD). Lectins can be divided by sequence comparison of the CRD. Some of the best characterized of these CRD groups are summarized in Table 1.

Table 1 Summary of lectin categories (Drickamer and Taylor, 1998)

Lectin group	Structure of CRD	Typical ligands	Examples of functions
Calnexin	Unknown	Glc_1Man_9	Protein sorting in the endoplasmic reticulum
M-type lectins		Man_8	Endoplasmic reticulum-associated degradation of glycoproteins
L-type lectins	β-sandwich	Various	Protein sorting in the endoplasmic reticulum
P-type lectins	Unique β-rich structure	Man 6-P	Protein sorting post Golgi
C-type lectins	Unique mixed α/β structure	Various	Cell adhesion (Selectins), Glycoprotein clearance, Innate immunity (Collectins)
Galectins	β-sandwich	β-Galactosides	Glycan crosslinking in the extracellular matrix
I-type lectins	Immunoglobulin superfamily	Sialic acid	Cell adhesion (Siglecs)
R-type lectins	β-trefoil	Various	Enzyme targeting, Glycoprotein hormone turnover

Glc, glucose; Man, mannose; Man-6-P, mannose-6-phosephate.

The functions listed are primarily those that have been identified in vertebrates.

The lectins that contain CRDs listed in the Table 1 fall broadly in two categories. Lectins that contain CRDs in three of the structural groups are located mostly intracellularly, in luminal compartments. They function in the trafficking, sorting and targetting of glycoproteins in the secretory and other pathways. CRDs in the remaining structural groups are found in lectins that function largely outside the cell and are either secreted or localised to the plasma membrane (Dodd and Drickamer, 2001).

Relatively simple invertebrates organism may serve as useful model for some of the functions of sugar-binding the protein in mammals. The early intracellular sorting events involving calnexin and L-type lectins as well as the role of R-type CRDs in glycosyltransferases are likely to be quite similar, whereas later sorting events involving the mannose-6-phosephate receptors will probably be different. At the cell surface, the role of some of the galectins may be similar in all animals so that genetic and developmental analysis of the model invertebrates is likely illuminated studies of the vertebrate proteins as well. In contrast, the greater diverstion of vertebrates and invertebrates proteins containing C-type lectin like domains (CTLDs) suggests that these proteins probably participate in more specialized functions of glycan that are unique to different groups of animals (Dodd and Drickamer, 2001).

2. Functional aspects of lectins

In subsequent years numerous lectins have been isolated from plants as well as from microorganisms and animals, and during the past two decades the structures of hundreds of them have been established. Concurrently, it was shown that lectins function as recognition molecules in cell –molecule and cell–cell interactions in a variety of biological systems. In a broader sense, the foregoing discussion implies that lectins possess the ability to act as recognition molecules inside cells, on cell surfaces, and in physiological fluids (Fig. 1 and Table 2).

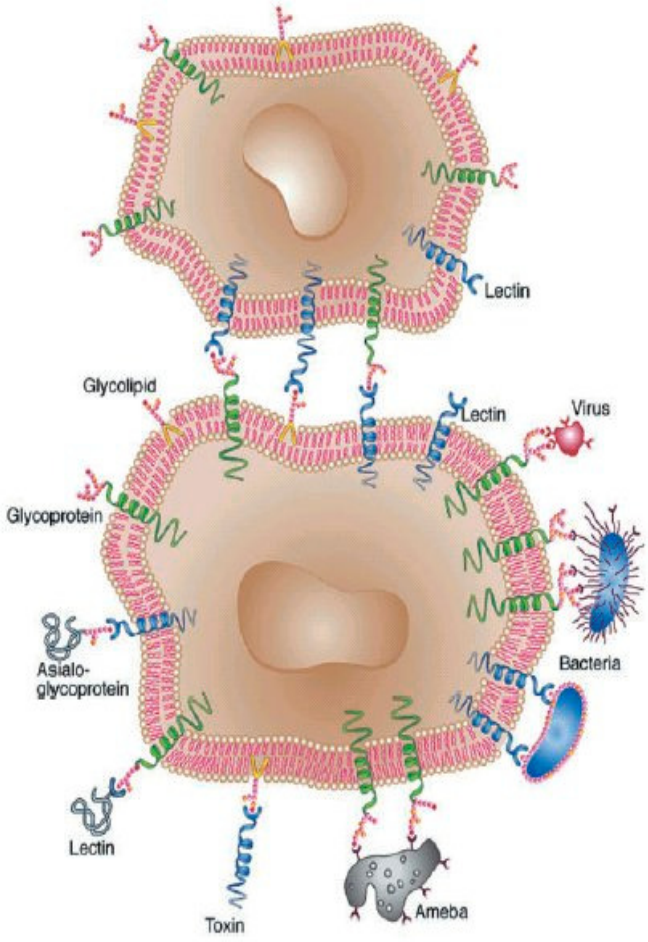

Fig. 1 Cell surface lectin–carbohydrate interactions (Sharon and Lis, 2004)

Lectins serve as means of attachment of different kinds of cell as well as viruses to other cells via the surface carbohydrates of the latter. In some cases, cell surface lectins bind particular glycoproteins (e.g., asialoglycoproteins), whereas in other cases the carbohydrates of cell surface glycoproteins or glycolipids serve as sites of attachment for biologically active molecules that themselves are lectins (e.g., carbohydrate-specific bacterial and plant toxins, or galectins).

Table 2 Functions of lectins (Sharon and Lis, 2004)

Lectin sources	Roles
Microorganisms	
Amoeba	Infection
Bacteria	Infection
Influenza virus	Infection
Plants	
Various	Defense
Legumes	Symbiosis with nitrogen-fixing bacteria
Animals	
Calnexin, calreticulin, ERGIC-53	Control of glycoprotein biosynthesis
Collectins	Innate immunity
Dectin-1	Innate immunity
Galectins	Regulation of cell growth and apoptosis; regulation of the cell cycle; modulation of cell–cell and cell–substratum interactions
Macrophage mannose receptor	Innate immunity; clearance of sulfated glycoprotein hormones
Mannose-6-phosephate receptors	Targeting of lysosomal enzymes
L-selectin	Lymphocyte homing
E- and P-selectins	Leukocyte trafficking to sites of inflammation
Siglecs	Cell-cell interactions in the immune and neural system
Spermadhesin	Sperm-egg interaction

From the functional point of view, lectins can functionally be distinguished by whether they recognize endogenous or exogenous ligands. The former appear to play an important role in fertilization and development, and their function often involves cell-to-cell or cell-to-matrix interaction. The latter probably evolved for self/non-self discrimination and they may be soluble or surface bound (Arason, 1996).

Selectins and the asialoglycoprotein receptor can recognize endogenous ligands. Selectins belong to the C-type lectin family and a similarity in the domain organization allows them to be recognized as a family of cell-cell adhesion molecules. They interact with carbohydrate ligands on leukocytes and endothelial cells. These operate in the vascular and hematologic systems. Selectins are essential for leukocyte recruitment into inflamed tissue (Graves *et al*., 1994) and the function of lymphocyte selectin plays a role in the homing of lymphocytes to the peripheral lymph nodes (Arason, 1996).

The high affinity of selectins for their ligands is probably achieved by forming dimers or oligomers in the cell membrane. This is a common theme in mammalian lectins and is known to result in increased affinity for multivalent ligands. A point quite well illustrated by the asialoglycoprotein receptor of hepatocytes (Lodish, 1991). This receptor is thought to function in the removal of effete serum glycoproteins with oligosaccharides terminating in galactose.

The macrophage mannose receptor and the collectins can recognize endogenous ligands. The macrophage mannose receptor binds to terminal mannose, fucose or *N*-acetyl glucosamine (GlcNAc). This ligand is common in the glycocalyx of viruses, bacteria, fungi and parasites. But it is not normally found at the termini of mammalian glycoproteins other than intracellular ones (Kornfeld and Kornfeld, 1985). The mannose receptor of mammalian macrophages and hepatic endothelial cells (Stahl, 1992) mediates phagocytosis of pathogens as well as receptor-mediated pinocytosis of potentially harmful high-mannose glycoproteins, released from cells in response to pathological events.

Collectin (collagen-like lectin) is a subgroup of C-type (i.e. Ca^{2+}-dependent) animal lectins characterized by the presence of collagen-like sequences (Gly-Xaa-Yaa triplet). They play important roles in innate immunity without involvement of antibodies (Lu, 1997). The collectins are known to act as opsonin in various circumstances. They recognize and bind to non-host carbohydrates structures present on the surface of a range of microorganisms including

bacteria, yeasts, fungi, parasitic protozoa and viruses on microorganisms and particles and participate in the processing or elimination of such material by interaction with phagocytic cell receptors.

The collectin serum protein called mannan-binding protein, MBP can activate the complement system through the classical pathway. This MBP-mediated complement activation, called the lectin pathway, provides an additional mechanism of microorganism recognition by the complement system in the absence of specific antibodies (Fig. 2).

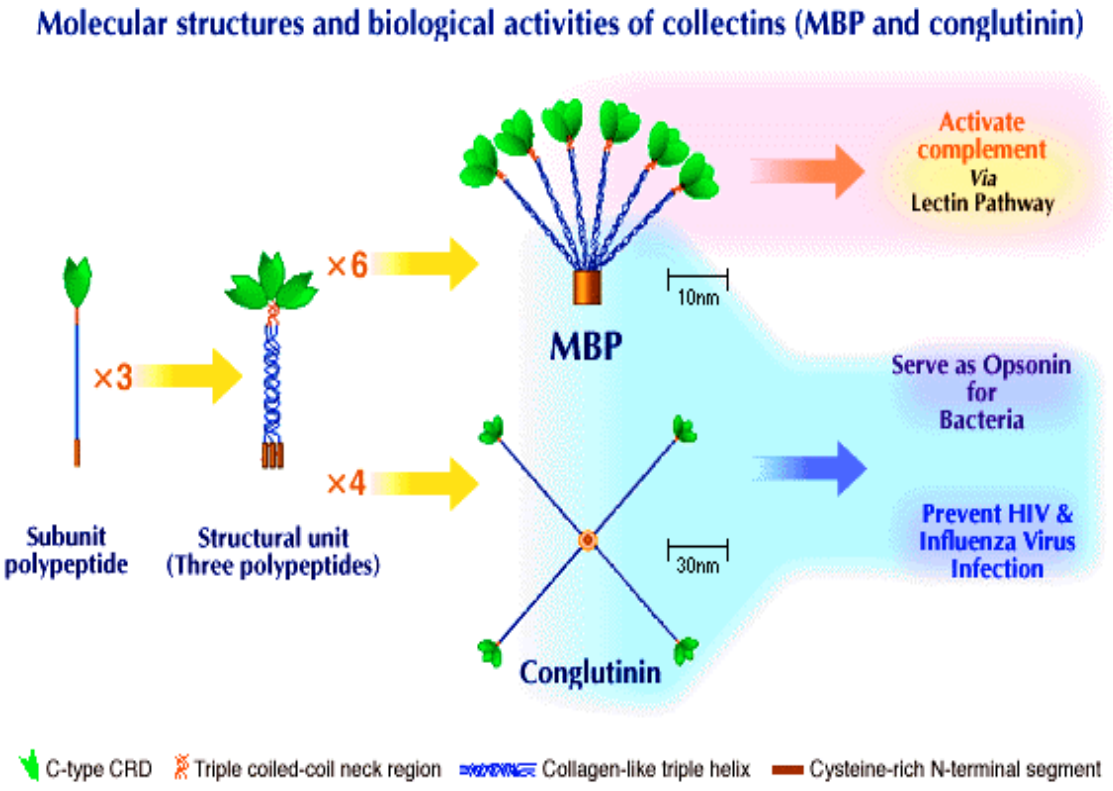

Fig. 2 Molecular structures and biological activities of the collectins (MBP and conglutinin) (Sharon and Lis, 1995; Holmskov *et al*., 1994)

The first substantial evidence of multiple lectins in crustaceans was reported more than two decades ago in the lobster *Homarus americanus* by Hall and Rowlands (1974). The authors purified two different plasma agglutinins and characterized the sugar specificity of both purified lobster lectins. The authors emphasized the fact that multiple lectins with different sugar specificity could constitute an important evidence of agglutinin heterogeneity and thus, support the hypothesis of the role of agglutinins as non-self-recognition molecules in crustacean defense.

Numerous reviews of agglutinin involvement in arthropod defense and recognition mechanisms have been reported (Amirante, 1986; Olafsen, 1986; 1988; Vasta, 1992; Millar and Ratcliffe, 1994, Vargas-Albores, 1995; Vasta *et al*., 1996; Natori *et al*., 1999). The two recent examples in arthropods appear to support the view that invertebrate lectins may exhibit the required binding diversity to efficiently discriminate non-self-particles. The first, concerns the cockroach *Blaberus discoidalis* which contains multiple plasma lectins, each with different carbohydrate-binding specificities (Chen *et al*., 1993; Wilson *et al*., 1999) and consequently, able to potentially recognize different invading pathogens. It was demonstrated that each of these purified molecules was capable to induce a specific and enhanced 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).

Another interesting example of multiple lectins with different sugar specificities and playing distinct functional roles in non-self-recognition was also provided by horseshoe crabs. In the Japanese horseshoe crab *Tachypleus tridentatus*, several lectins were purified from hemolymph. They promoted the agglutination of certain strain of Gram-positive *Staphylococcus* and recognised several kinds of lipopolysaccharides, LPS (Okino *et al*., 1995; Kawabata and Iwanaga, 1999).

Besides lectins, the immune system of arthropods includes also other defense mechanisms involving microorganism-carbohydrate recognition. A complex proteolytic cascade known as prophenoloxidase (proPO) system is triggered by components of microbial cell walls.

(Soderhall and Cerenius, 1992; Soderhall *et al*., 1996; Ashida and Brey, 1997). Recently an interesting connection between lectins and the proPO activating system of arthropods was reported in insects. In the cockroach *B. discoidalis*, Wilson *et al*. (1999) obtained evidences that the level of phagocytosis of the distinct microorganisms mentioned above could be increased by the several lectins, in a proPO-dependent and/or proPO-independent mechanism.

The observations of Vazquez *et al*. (1993; 1996; 1997) on the lectins of the hemolymph of the freshwater prawn *Macrobrachium rosenbergii* are of particular interest. The authors demonstrated that the granulocytes of *M. rosenbergii*, in spite of expressing a surface receptor, which seemed to correspond to the humoral purified lectin, had the ability to recognize foreign cells in an apparently non-mediated sugar recognition basis.

It is important to note that apart from the potential involvement of arthropod lectins in non-self-recognition and opsonization, recent findings are emerging on the role of these molecules as immune effectors in microorganism neutralization.

3. Synthesis and induction of lectins

There are evidences that arthropod lectins might be synthesised by hemocytes. The agglutinating activity in the lobster *H. americanus* was strongly associated to hemocyte extracts and the authors suggested that they should be the main agglutinin source (Hall and Rowlands, 1974). In the horseshoe crab *T. tridentatus*, also referred to above, four of the five purified lectins came from hemocytes and were released from the granules upon LPS stimulation.

In the majority of arthropods, attempts to stimulate their production have met only limited success. The potential inducibility of lectins, especially in species of economic interest, such as shrimps, could be of particular relevance since if these molecules are really involved in immune defense reactions, the increase in their concentration could virtually confer a better protection to the host against invading pathogens. In the black tiger shrimp *P. monodon*, Ratanapo and Chulavatnatol (1992) reported an elevation of the lectin monodin level in most of the shrimps suffering from bacterial *V.* v*ulnificus* infection. However, this finding could not be clearly associated to a possible inducible mechanism, since this increased lectin concentration was not observed in all infected shrimps. On the other hand, in the same shrimp species, Sritunyalucksana *et al*. (1999) failed to induce an increase of lectin concentration by using

components of microorganism cell wall, such as LPS, β-glucans, peptidoglycan and also commercial stimulants.

4. Lectins in marine invertebrates

Although a number of lectins of various molecular weights have been found in marine invertebrates, very limited information concerning their structures has thus far been obtained. One of the most probable roles of marine invertebrate lectins is to act as humoral factors in the defense mechanism, as do immunoglobulins in vertebrates. This is suggested from some observations such as the activation of phagocytes by the binding of lectin to foreign cells (opsonin activity) or the enhancement of lectin production in body fluids after injection of foreign substances. On the other hand, direct hemolytic activity has recently been found for a sialic acid-specific lectin from horseshoe crab *Tachypleus tridentatus* (Miura *et al*., 1992) and a galactose-specific lectin from the sea cucumber *Cucumaria echinata* (Hatakeyama *et al*., 1995). After binding to the specific carbohydrate chains on the erythrocyte surface, these lectins damage the cell membrane, leading to cell lysis (Fig. 3).

These lectins may play an important role against bacterial infections or natural enemies. A lectin with biological activities such as mitogenic and chemotactic activities was found in the venom of the pedicellariae (spines) of the sea urchin *Toxopneustes pileolus*, suggesting its involvement in toxic action. Other functions of marine invertebrate lectins have also been suggested for some C-type lectins: calcium carbonate crystallization (the acorn barnacle, *Megabalanus rosa*), morphogenesis (the tunicate, *Polyandrocarpa misakiensis*), and vitelline coat lysis (the blue mussel, *Mytilus edulis* sperm). These examples suggest that there may be various lectins with different physiological roles in marine invertebrates.

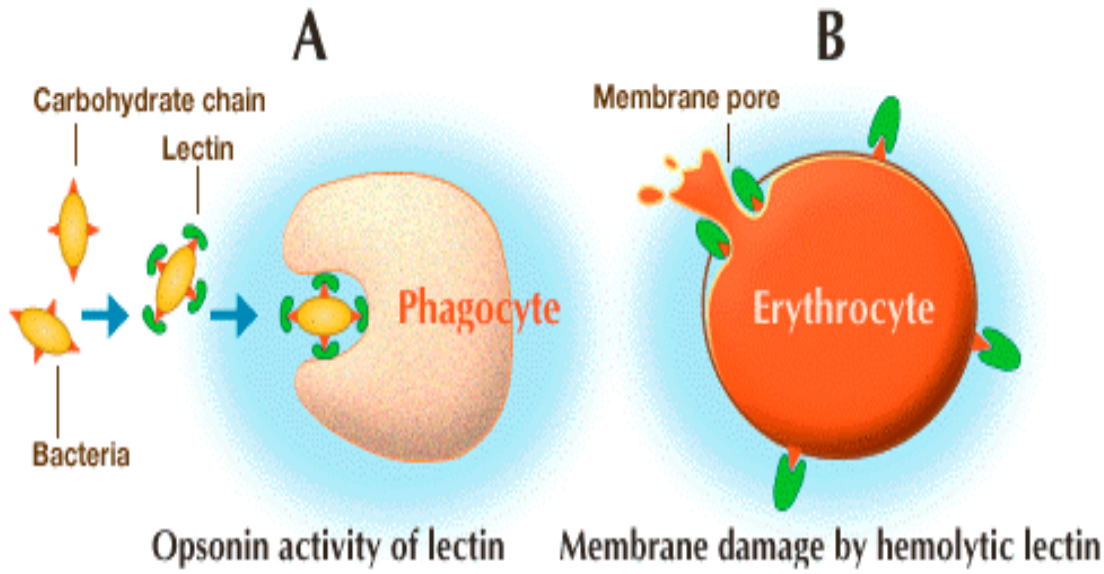

Fig. 3 Binding of lectins to foreign cells (Hatakeyama *et al*., 1995)

Binding of lectins with opsonin activity to foreign substances such as bacteria promote phagocytosis (A). After binding to the carbohydrate chains on erythrocyte surface, hemolytic lectins induce membrane damage (e.g., by forming pores), leading to hemolysis (B).

5. Penaeid shrimp biology

Penaeid shrimp belongs to the largest phylum in the animal kingdom, the Arthropoda, characterized by jointed appendages and an exoskeleton or cuticle that is periodically molted. There are thousands of terrestrial species in this phylum, and a large, predominately aquatic subphylum, the Crustacea. The more highly evolved crustaceans (Class Malacostraca) include the penaeid shrimp (Order Decapoda). The class Malacostraca contains about three-fourths of the known species and includes crayfish, lobsters, shrimps and crabs (Bailey-Brock and Moss, 1992).

Decapods can be distinguished from other higher crustaceans by examining differences in the thoracic appendages. The first three pairs of thoracic appendages, the maxillipeds, are modified for feeding and the remaining five pairs are the walking legs, hence the name Decapoda or "ten-legs". Penaeid appendages typically consist of two branches (biramous), the exopodite and endopodite. These structures are variously developed for feeding, locomotion or burrowing; or they bear feathery gills (modified epipodites) contained beneath the carapace, or sensory structures on the antennae and antennules (Bailey-Brock and Moss, 1992) (Fig. 4).

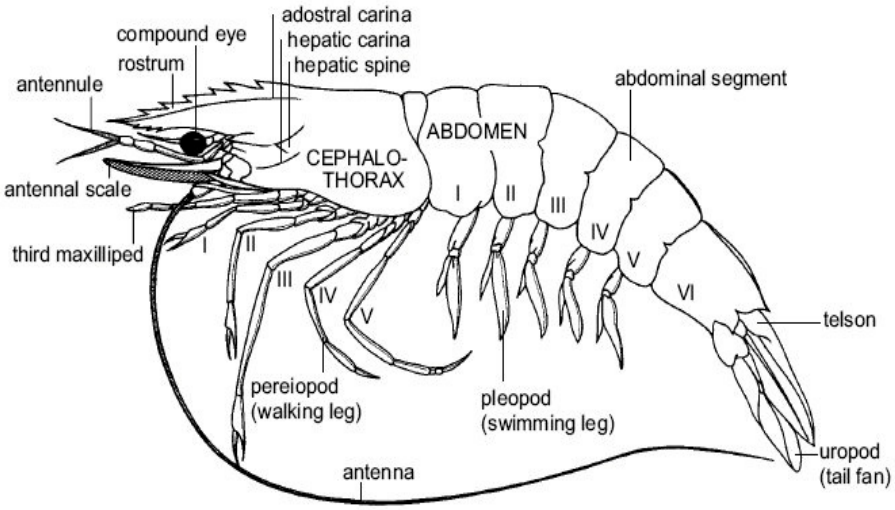

Fig. 4 Lateral view of the external morphology of the penaeid shrimp (Primavera, 1990)

The penaeid life cycle includes several distinct stages found in a variety of habitats. Eggs hatch within 16 h after fertilization. The larval stages comprise nauplius (6 stages in 2 days), protozoea (3 stages in 5 days), mysis (3 stages in 4-5 days) and megalopa (6-35 days). The megalopa and early juvenile are called postlarvae. Transition from juvenile to subadult takes 135-255 days and subsequently completion of sexual maturity occurs within 10 months (Motoh, 1984).

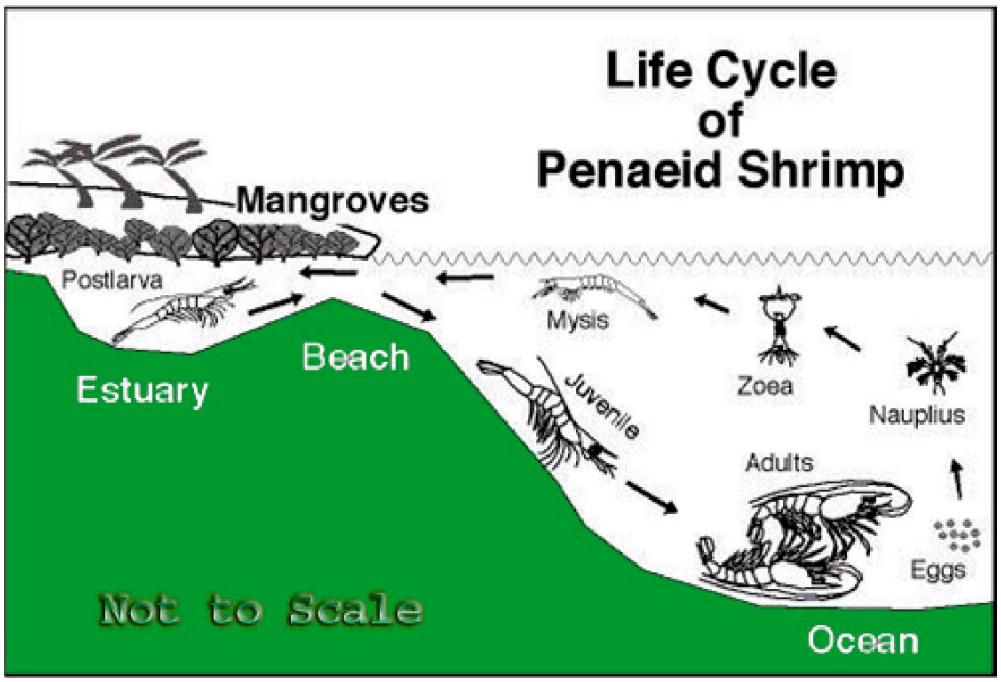

Fig. 5 Schematic of a typical penaeid shrimp life cycle (Bailey-Brock and Moss, 1992)

Maturation of penaeid shrimp refers to process of egg development (oogenesis) in the ovary of the female shrimp. The reproductive system of the female Penaeid consists of paired ovaries, oviducts, genital apertures, and a thylecum. Precursor eggs (oogenia) are produced mitotically from germinal epithelium throughout the reproductive life of the female. The oogonia meiosis, differentiate into oocytes, and become surrounded by follicle cells. The resulting oocyte (egg) then absorbs yolk material from the mother's blood through the follicle cells (Bailey-Brock and Moss, 1992).

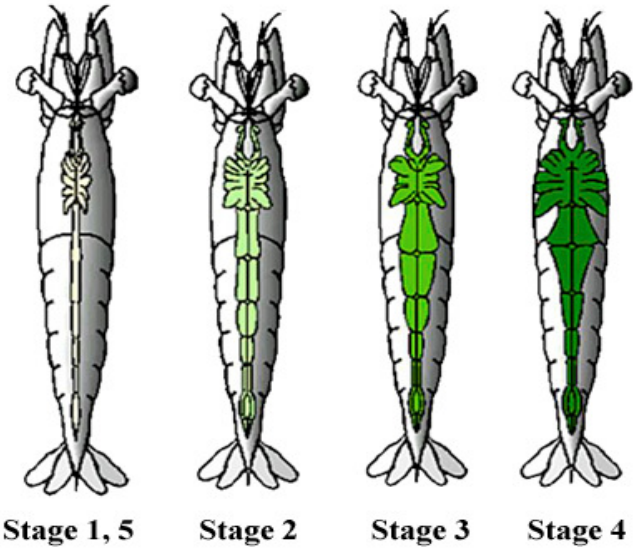

Fig. 6 The view observed by hatchery operators when female broodstocks are graded for ovarian development by torchlight (http://www.aims.gov.au)

Ovarian maturation is accompanied by macroscopic changes in the ovary, which can be estimated without microscopical sectioning. For convenience, the process has been divided into stages that correspond with the external appearance of the ovaries (Dall *et al.*, 1990):

Stage 1 Ovarian lobes translucent and smaller in diameter than the gut; oocytes at beginning of development.

Stage 2 Ovarian lobes opaque and with diameter similar to the gut; oocytes increased in size.

Stage 3 Ovarian lobes yellowish and larger in diameter than the gut; vitellin accumulating in oocytes.

Stage 4 Ovarian lobes deeply pigmented and occupying the dorsum of the body; oocytes mature.

Stage 5 Ovaries spent; lobes flaccid and much convoluted; ova undergoing resorption.

In stages 3 and 4 the ovaries are visible through the dorsum of the live animal. The colour of the ovaries intensifies as the shrimp approaches spawning, but the final colour depends on the species. In heavily pigmented species the colour is often olive-green, but may be a slaty grey; in lightly pigmented species the colour is more often yellow or orange-yellow (Dall *et al.*, 1990).

6. Shrimp aquaculture

The cultivation of aquatic species, such as crustaceans, molluscs and fish, was expanding over the last two decades, contributing to the economic and social development of different Asian and Latin-American developing countries. Among crustaceans, shrimp aquaculture represents more than 75% in quantity and penaeids account for the greatest part of the production of this sector. In Asia, penaeid shrimps have for centuries been grown in traditional systems, with low productivity aimed for domestic markets. Today shrimp farming makes up only 3-4% of global aquaculture production by weight, but almost 15% by value. Around 80 percent of cultured shrimp come from Asia with Thailand, China, Indonesia and India as the top producers. In the Western hemisphere, Ecuador is the major shrimp producing country. The black tiger shrimp (*P. monodon*) accounts for more than half of the total shrimp aquaculture output This species comprised 56% of the total 1999 production, the Asian white shrimps *P. indicus* and *P.*

merguiensis came next with 17%, followed by *P. vannamei* (16%), and *P. chinensis, P. stylirostris, P. japonicus* contributed to the rest. (Rosenberry, 1999)

7. *Penaeus merguiensis*

P. merguiensis are raised on extensive farms throughout Asia. These species have attracted attention recently because they tolerate low water quality better than *P. monodon*, they can be grown at high densities, and are readily available as postlarvae in the wild (Rosenberry, 1999).

Characteristics of *P. merguiensis* has been investigated. Banana shrimps also know as white shrimps are relatively large commercial shrimps and are members of the family Penaeidae. They have poorly defined gastro-orbital ridge. Rostrum extends horizontally and has an elevted crest with 6-10 large teeth dorsally and up to 6 ventral teeth. Median and adostral grooves are shallow and diminish at the middle of the carapace. There is no hepatic ridge so the carapace appears smooth (Bailey-Brock and Moss, 1992). Their body is pale yellow or translucent and speckled with reddish brown dots.

Fig. 7 *Penaeus merguiensis* or banana shrimp

Banana shrimps inhabit tropical and subtropical waters. They inhabit coastal waters from shallow estuaries and intertidal areas to a maximum depth of 45 meters. They live in turbid waters for most of their lives, over muddy substrates in estuaries and muddy sands offshore. Banana shrimps have a larval life phase and live to about 18 months old. Juveniles inhabit small creeks and rivers in a sheltered mangrove environment in waters ranging from almost fresh to high salinity. Adult ones inhabit medium and low energy coastlines, although they can withstand high energy cyclonic events. Banana shrimps can become sexually mature at about 6 months of age. Spawning occurs throughout all of the shallow coastal zone inhabited by adults and older adults may migrate shorewards at the time of spawning. Mating occurs during moulting. Eggs are shed into the water prior to the moult and are fertilized externally by sperm from the male. Females can lay between 100,000 to 400,000 eggs, and can be laid in several batches. The maximum life span is approximately 12-18 months.

8. The crustacean defense system

8.1 An overview of the immune system

The immune system is divided into two parts determined by the speed and specificity of the reaction. These are named the innate (natural) and the acquired (adaptive) immune system (Table 3). The innate immune system can be defined as all immune defenses that lack immunological memory (Walport, 2001; Delves and Roitt, 2000; Medzhitov and Janeway, 2000; Courtney *et al*., 1999), found in all multi-cellular animals and consists of cellular and humoral elements. The most prominent cellular defense reactions against invading microorganisms are phagocytosis, encapsulation, cell-mediated cytotoxicity and clotting. The humoral defense factors, such as clotting proteins, agglutinins (e.g., lectins), hydrolytic enzymes and antimicrobial peptides are often produced by and act in conjunction with the defense cells. The term innate immunity is sometimes used to include physical, chemical, and microbiological barriers, but more usually encompasses the elements of the immune system (neutrophils, monocytes, macrophages, complement, cytokines, and acute phase proteins) which provide immediate host defense. Nowadays, the innate immune response is understood to be essential to the function of the adaptive immune response (Fearon and Lockley, 1996). The adaptive immune

system is phylogenetically younger, consists of antigen-specific reactions through T lymphoctyes and B lymphocytes. However, reactions to unknown foreign contacts are slow, since it takes three to seven days before clonal selection and expansion of lymphocytes ensures a specific immune response. This response is very flexible in its adaptation to molecular changes in varying pathogens. Whereas the innate response is rapid but sometimes damages normal tissues through lack of specificity. The adaptive response is precise, but takes several days or weeks to develop. It has memory, so that subsequent exposure leads to a more vigorous and rapid response, but this is not immediate (Delves and Roitt, 2000).

Table 3 A simplified version of immune system (Walport, 2001; Delves and Roitt, 2000)

Innate immunity	**Cellular**	**Phagocytes**: monocytes, macrophages, neutrophils **Dendritic cells** **Natural killer cells**
	Humoral (soluble)	**Complement**: - Mannose binding lectin or lectin complement activation pathway (LP) - Alternative complement activation pathway (AP) - Classical complement activation pathway (CP) **Acute phase proteins** **Cytokines** e.g. interferon **Chemokines**
Adaptive immunity	**Cellular** **Humoral**	**T lymphocytes, B lymphocytes** **Antibodies** (secreted by B lymphocytes or memory cells)

8.2 Functions of the crustacean defense system

Invertebrate animals, which lack an adaptive immune system, have developed various defense systems that makeup their so-called 'innate immunity' and respond to common antigens on the surface of potential pathogens (Medzhitov and Janeway, 2000). These defense systems include hemolymph coagulation, melanization, complement activation, cell agglutination, antimicrobial action, active oxygen formation and phagocytic action. The hard cuticle, a physical barrier that also may contain antimicrobial factors, can be considered as the external defense in crustaceans. The haemocytes play an important and central role in the internal defense. A schematic overview of the most important factors in the crustacean defense system, which are known until now, is given in Fig. 8.

The first and essential internal defense process is the recognition of invading microorganisms, which is mediated by the haemocytes and plasma proteins (Vargas-Albores and Yepiz-Plascencia, 2000). The immobilized invaders are finally killed by antimicrobial substances released mainly from many types of hemocytes (Aderen and Ulevitch, 2000; Imler and Hoffmann, 2000; Pieters, 2001).

Several types of recognition proteins have been described and are called pattern recognition proteins (PRPs). The PRPs recognize carbohydrate moieties of cell wall components of microorganisms, like LPS or peptidoglycans (PG) from bacteria, or β-1,3-glucans from fungi (Soderhall *et al*., 1996; Vargas-Albores *et al*., 1996; 1997). Some of the PRPs are lectins and can work directly as agglutinins or opsonins (Kopacek *et al*., 1993; Soderhall *et al*., 1996). After binding of the PRP ligand with the microbial component, a second site becomes active for cellular binding. Haemocyte activation is generated after this second binding step (Vargas-Albores and Yepiz-Plascencia, 2000). After detection of foreign material, haemocytes migrate to the site of invasion by a process of chemotaxis that results in inflammation, which also appears a relevant event in vertebrates. The open circulatory system demands a rapid and efficient defense, in which the proteoloytic cascades play an important role (Sritunyalucksana and Soderhall, 2000). The haemocytes are involved in the synthesis, storage and -upon activation- discharge of proenzymes and substrates of the clotting and proPO cascades (Johansson and Soderhall, 1992; Soderhall *et al*., 1996; Sritunyalucksana and Soderhall, 2000).

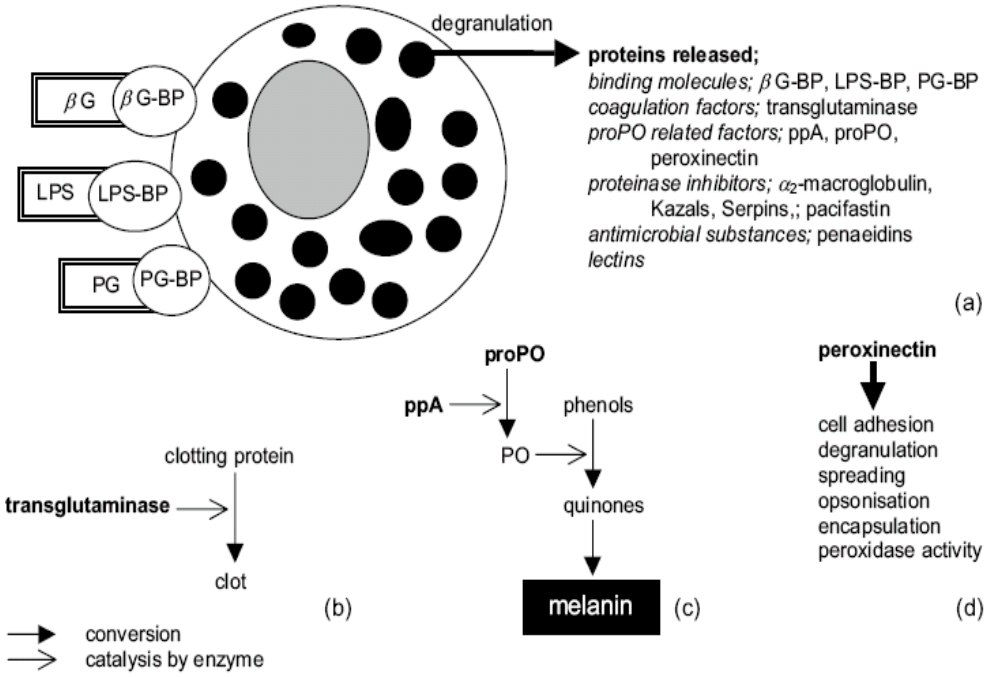

Fig. 8 Simplified overview of the most important defense factors of decapod crustaceans.

(a) Several proteins are pro-enzymes, others are substrates. The proteins that are released are involved in the clotting (b) the prophenoloxidase activating (c) system or in other cellular activation processes (d) The proteins involved in those processes that are released from the haemocytes are indicated in bold letters. Activation of cascade processes is regulated by different proteinase inhibitors. βG, β-1,3-glucan; βG-BP, β-1,3-glucan binding protein; LPS, lipopolysaccharide; LPS-BP, lipopolysaccharide binding protein; PG, peptidoglycan; PG-BP, peptidoglycan binding protein; PO, phenoloxidase; ppA, prophenoloxidase activating enzyme; proPO, prophenoloxidase (Soderhall *et al*., 1996; Soderhall and Cerenius, 1998).

The clotting mechanism entraps foreign material and prevents loss of haemolymph. The transglutaminase (TGase)-dependent clotting reaction of crustaceans is best described in the freshwater crayfish *Pacifastacus leniusculus* (Kopacek *et al*., 1993; Hall *et al*., 1999). The clotting reaction is induced when TGase is released from the haemocytes or tissues. The Ca^{2+}-dependent TGase catalyses polymerization of the clotting protein, found in the plasma, to form a gel (Kopacek *et al*., 1993; Yeh *et al*., 1998).

The proPO-activating system is an enzymatic cascade in many invertebrates. Large amount of information of this system has come from work done on freshwater crayfish *P. leniusculus* (Soderhall *et al*., 1996; Soderhall and Cerenius, 1998). Proteins of the proPO system occupy a very prominent position in non-self recognition, haemocyte communication and the production of melanin. Upon activation and degranulation of the haemocytes, the inactive proPO is converted to the active phenoloxidase (PO) by prophenoloxidase activating enzyme (ppA). The PO enzyme catalyses the stepwise oxidation of phenols to quinones, followed by several intermediate steps that lead to the formation of melanin. During this formation also antimicrobial factors are formed (Soderhall *et al*., 1996; Soderhall and Cerenius, 1998).

An important factor that is associated with the proPO system is peroxinectin. Peroxinectin has two different functions: cell-adhesion and peroxidase activity. The cell-adhesion is involved in attachment, spreading, phagocytosis, encapsulation, nodule formation and agglutination (aggregation), while the antimicrobial properties of the peroxidase activity of the protein might help to kill invading microorganisms (Johansson and Soderhall, 1988; 1989; Kobayashi *et al*., 1990; Tharnqvist *et al*., 1994). Phagocytosis is the internalisation of small foreign particles by individual cells. After ingestion, shrimp haemocytes use cytotoxic oxygen radicals to kill the foreign material (Song and Hsieh, 1994; Munoz *et al*., 2000). If large amounts of particles enter the body or if they are too large to be internalised, several haemocytes will cooperate to seal off the pathogens, these phenomena are called nodule formation and encapsulation, respectively (Soderhall *et al*., 1996).

Enzyme inhibitors are necessary to regulate the proteinase cascades and prevent over-activation and damage to the host tissue. Serine proteinase inhibitors from the Kazal and Serpin families have been identified in crustaceans (Kanost, 1999). Also α_2-macroglobulin, which serves as a broad spectrum protease-binding protein is stored in the haemocyte granules (Armstrong and Quigley, 1999). In addition, haemocytes play an important role in the production and discharge of agglutinins (e.g., lectins) (Kopacek *et al*., 1993), of antibacterial peptides (Destoumieux *et al*., 1997; 2000) and of cytotoxic molecules such as lysosomal enzymes (lysozyme, esterases, phosphatases, phospholipases, peroxidases and proteases) (Millar andRatcliffe, 1994). For an efficient immune defense, all different components of the immune system must work together.

8.3 Lectins as defense molecules in crustaceans

In invertebrates, the mechanism of non-self recognition is still largely unknown. Scientists focus their attention on two systems which could produce recognition molecules: the proPO system and lectins of hemolymph. It is well documented that invertebrate hemolymph contains internal defense factors against potential pathogens, and humoral factors such as lectins may contribute to such defense.

Lectins in invertebrates have been detected in the blood components, namely cell-free hemolymph and hemocytes (Yeaton, 1981; Ratclife *et al*., 1985; Renwrantz, 1986; Richards and Renwrantz, 1991; Smith and Chisholm, 1992), in tissues (Mullainadhan and Renwrantz, 1986; Suzuki *et al*, 1991) and in mucus (Fountain and Campbell, 1984). The actual functions of invertebrate lectin are not yet well understood. However, they have been implicated in several diverse physiological processes, including host immune responses, such as non-self recognition, phagocytosis, encapsulation, and hemocoelic clearance of foreign cells (Renwrantz, 1986; Olafsen, 1988; Vasta, 1991; Mercy and Ravindranath, 1994).

Lectins from the hemolymph of invertebrates, including crustaceans, have been regarded as potential molecules involved in immune recognition and microorganism phagocytosis through opsonization. Opsonization that occurs when a lectin binds bacteria to blood cell (hemocyte) surfaces is considered to be the first step that promotes adherence, ingestion and subsequent elimination of microbes (Martin *et al*., 1993). Concerning carbohydrate specificity, invertebrate lectins can vary from specific molecules to others that exhibit a broader spectrum of recognition. The specificity of a lectin is related to the carbohydrate for which it shows the highest affinity. But most lectins that are considered specific for one monosaccharide may also bind to other carbohydrates which are structurally related. The agglutination of many different types of cells/glycoconjugates may actually reflect the ubiquity of the ligand. A lectin from the horseshoe crab, *Limulus polyphemus* that binds sialic acid can illustrate this statement and be considered as a lectin of a broad spectrum of recognition, since the fact that it agglutinates a variety of cells can be associated to the wide distribution of this monosaccharide among cell surfaces (Cohen *et al*., 1983; Vasta, 1992).

Among decapod crustaceans, the species whose lectins have been examined are the freshwater prawn, *M. rosenbergii* (Huang *et al*., 1981), crayfish, *P. leniusculus* (Kopacek *et*

al., 1993), *P. japonicus* (Muramoto *et al*., 1995), freshwater crab, *Parathelphusa hydrodromus* (Nalini *et al*., 1994) and *P. californiensis* (Vargas-Albores *et al*., 1993). The specificity of lectins towards carbohydrates is mainly related to *N*-acetylated carbohydrates, such as *N*-acetyl neuraminic acid, *N*-acetyl glucosamine and *N*-acetyl-D-galactosamine as can be depicted by the data partially summarised in Table 4.

9. Purification and characterization of lectins from invertebrates

A number of reports regarding the purification and characterization of crustacean lectins are now available (Marques *et al*., 2000). 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 proPO-activating system and related molecules (Soderhall and Cerenius, 1992; Soderhall *et al*., 1996) and, more recently, on clotting proteins (Kopacek *et al*., 1993; Komatsu and Ando, 1998; Hall *et al*., 1999) in crustaceans have well progressed. Most of reported lectins belong to the C-type lectins. The occurrence of C-type lectins has been reported in crustaceans for two species of the acorn barnacles, *M. rosa* and *Balanus rostratus* (Muramoto and Kamiya, 1989; Toda *et al*., 1998). It is relevant to point out that among C-type lectins, little or no homology may be present in domains other than that involved in CRD (Vasta *et al*., 1996).

Most of the literature dealing with crustacean lectins concerns the purification and characterisation of a unique lectin from the hemolymph and very rarely is its biological activity determined. This is quite intriguing, in view of the great economic interest of crustaceans, especially penaeid shrimps. Some examples of crustacean lectins, mainly from shrimps, functioning as potential non-self-recognition factors show in Table 4. In the penaeid *P. monodon*, Ratanapo and Chulavatnatol (1992) reported the agglutination of the pathogenic bacteria *V.* v*ulnificus* by a purified lectin called monodin. The ability of the purified lectin from *P. californiensis* was investigated (Vargas-Albores *et al*. 1993) to react with different marine species of *Vibrio*. They demonstrated that the agglutinin of this penaeid was able to react to at least three different *Vibrio* species, *V.* v*ulnificus*, *V. fischeri and V. parahaemolyticus*. This reaction was specific and the agglutination of *V. parahaemolyticus* could be inhibited by N-acetyl galactosamine and 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 (Vargas-Albores *et al*., 1993). In the shrimp *P. longirostris*, Fragkiadakis and Stratakis (1995) also referred that purified lectins from the hemolymph that recognised *N*-acyl aminosugars strongly agglutinated formalin-fixed bacteria, *Pseudomonas aeruginosa* and *E. coli*.

Bacterial agglutination has been demonstrated with lectins from a wide variety of invertebrates such as the sea hare, *Aplysia* sp. (Zipris *et al*., 1986), solitary ascidian,

Halocynthia roretzi (Azumi *et al*., 1991), black tiger shrimp, *P. monodon* (Ratanapo and Chulavatnatol, 1992), Pacific oyster, *C. gigas* (Hardy *et al*., 1977; Olafsen *et al*., 1992), freshwater prawn, *M. rosenbergii* (Vazquez *et al*., 1996), edible crab, *Scylla serrata* (Chattopadhyay *et al*., 1996) and horse mussel, *Modiolus modiolus* (Tunkijjanukij and Olafsen, 1998).

The report of Ravindranath *et al*. (1985) has shown the specificity of the marine crab *Cancer antennarius* hemolymph lectin to 9-*O*-4-*O*-acetyl sialic acid, whereas the hemolymph lectins of *M. rosenbergii* and the marine crab *Liocarcinus depurator* have been shown to specifically recognise 9-*O*-acetyl sialic acid (Vazquez *et al*., 1993; Fragkiadakis and Stratakis, 1997). 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 (Vazquez *et al*., 1996). On the other hand, a sialic acid-binding lectin with specificity for *N*-glycolyl neuraminic acid was purified from the hemolymph of the marine crab *S. serrata* (Mercy and Ravindranath, 1993). The unique binding specificity of this lectin distinguishes it from other known. Some lectins characterised among crustaceans were summarized in Table 5.

Objectives

1. To purified lectin from the hemolypmh of *P. merguiensis*.
2. To characterize purified lectin.
3. To develop a sensitive ELISA to quantify lectin levels in hemolypmh of *P. merguiensis.*
4. To study changes in lectin levels in the hemolymph of the *Vibrio harveyi* injected *P. merguiensis* by ELISA and hemagglutiniation assay.
5. To study changes in lectin levels in the hemolymph of female *P. merguiensis* at different stages of ovarian development by ELISA and hemagglutiniation assay.