การผลิตและลักษณะสมบัติของโมโนโคลนอลแอนติบอดีจำเพาะต่อเม็ดเลือดกุ้งกุลาดำ *Penaeus monodon*

นางสาวพอจิต วิโนทพรรษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES SPECIFIC TO HAEMOCYTES OF THE BLACK TIGER PRAWN *Penaeus monodon*

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นางสาวพอจิต วิโนทพรรษ์ : การผลิตและลักษณะสมบัติของโมโนโคลนอลแอนติบอดีจำเพาะต่อเม็ดเลือดกุ้งกุลาดำ *Penaeus monodon* (PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES SPECIFIC TO HAEMOCYTES OF THE BLACK TIGER PRAWN *Penaeus monodon*) อ. ที่ปรึกษา : รศ.ดร.อมร เพชรสม อ.ที่ปรึกษาร่วม : รศ.ดร. ไพศาล สิทธิกรกุล 83 หน้า. ISBN 974-17-6056-6

โมโนโคลนอลแอนติบอดีจำเพาะต่อเม็ดเลือดกุ้งกุลาดำ ผลิตโดยการปลูกภูมิคุ้มกันหนูขาวด้วยส่วนผสมของเม็ดเลือดกุ้งกุลาดำชนิดทำให้เสียสภาพด้วย SDS และชนิดตรึงด้วยฟอร์มาลิน คัดเลือกโดยใช้เทคนิค immuno-cytochemistry ของเม็ดเลือดชนิดตรึงด้วยฟอร์มาลิน และ immunohistochemistry ของเนื้อเยื่อเหงือก หัวใจ lymphoid organ และ haemopoietic tissue ได้โมโนโคลนอลแอนติบอดี 2 กลุ่ม ได้แก่

โมโนโคลนอลแอนติบอดีจำเพาะต่อเม็ดเลือดกุ้งกุลาดำ ซึ่งแบ่งตามความจำเพาะต่อโปรตีนของเม็ดเลือดและการจับของโปรตีนโดยเทคนิค Western blot analysis ได้ 6 กลุ่ม คือ กลุ่มที่ 1 ประกอบด้วย MAbs จำนวน 4 โคลน (HC47d, 126d, 178d และ 425d) จับกับโปรตีนขนาด 102 kDa จำเพาะต่อเม็ดเลือดชนิดไฮยาลิน (hyalinocyte) และ เซมิ-แกรนูโลไซต์ (semi-granulocyte) ที่มีเม็ดแกรนูลขนาดเล็ก และพบทำปฏิกิริยาอย่างอ่อนกับเม็ดเลือดชนิดแกรนูโลไซต์ (granulocyte) นอกจากนี้ยังจับกับเซลล์ใน haemopoietic tissue และ เซลล์ชั้นในบริเวณปลายท่อของ lymphoid organ กลุ่มที่ 2 ประกอบด้วย MAbs 3 โคลน (HC54, 58d และ 249d) จับกับโปรตีนขนาด 43 kDa สามารถจับกับเม็ดเลือดชนิดแกรนูโลไซต์ และเซมิ-แกรนูโลไซต์ที่มีเม็ดแกรนูลขนาดใหญ่ และทำปฏิกิริยากับเซลล์ชั้นนอกของท่อใน lymphoid organ กลุ่มที่ 3 (HC111, 114, 245 และ 327) จับกับโปรตีนขนาดประมาณ 20 kDa สามารถจับเม็ดเลือดชนิดแกรนูโลไซต์ทั้งหมดและเซมิ-แกรนูโลไซต์บางส่วน แต่ไม่จับกับไฮยาลิน กลุ่มที่ 4 (HC201d) กลุ่มที่ 5 (HC200, 136 และ 239) และ กลุ่มที่ 6 (HC55) จับกับโปรตีนขนาด 61 kDa, 175 kDa และ ~230 kDa ตามลำดับ สามารถจับกับเม็ดเลือดทุกกลุ่มในสัดส่วนเท่า ๆ กัน และแอนติบอดีทุกตัวไม่จับกับองค์ประกอบอื่น ๆ ในน้ำเลือด (haemolymph)

โมโนโคลนอลแอนติบอดีจำเพาะต่อเนื้อเยื่ออื่น ๆ ของกุ้งกุลาดำ ประกอบด้วย MAbs 3 กลุ่ม คือ กลุ่มที่ 1 HC32 จับกับเนื้อเยื่อของท่อใน lymphoid organ และเซลล์บางกลุ่มของ haemopoietic tissue และยังจับกับแกรนูลขนาดเล็กในเซมิ-แกรนูโลไซต์ และไฮยาลิน กลุ่มที่ 2 (HC39 และ HC62) จับกับเนื้อเยื่อ lymphoid organ haemopoietic tissue เซลล์ประสาท ตับ-ตับอ่อน เหงือก และหัวใจ กลุ่มที่ 3 (HC31 และ HC179d) จำเพาะต่อโปรตีนขนาด 116 kDa ในเม็ดเลือด จับกับเนื้อเยื่อของท่อ และ spheroid cell ใน lymphoid organ และเม็ดเลือดทุกชนิด

การจับของ MAbs กับ lymphoid organ และ haemopoietic tissue อาจแสดงถึงความสัมพันธ์ในแง่ของพัฒนาการของอวัยวะในการสร้างเม็ดเลือดทั้ง 3 ชนิด ซึ่ง haemopoietic tissue เป็นอวัยวะที่เป็นแหล่งสร้างเม็ดเลือดและอาจส่งไป differentiate ที่ lymphoid organ เป็นเม็ดเลือดชนิดต่าง ๆ

ลายมือชื่อนิสิต.....

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่ออาจารย์ที่ปรึกษา.....

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KEY WORD: GRANULOCYTE/ HAEMOCYTE/ HAEMOPOIETIC TISSUE/ HYALINOCYTE/ LYMPHOID ORGAN/ MONOCLONAL ANTIBODY/ *Penaeus monodon*/ SEMI-GRANULOCYTE

PHOCHIT WINOTAPHAN : PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES SPECIFIC TO HAEMOCYTES OF THE BLACK TIGER PRAWN *Penaeus monodon.* THESIS ADVISOR : ASSOC. PROF. AMORN PETSOM, PhD. THESIS COADVISOR : ASSOC. PROF. PAISARN SITHIGORNGUL, Ph.D. 83 pp. ISBN 974-17-6056-6

Monoclonal antibodies (MAbs) specific to haemocytes of black tiger prawn were generated from mouse immunized with a mixture of SDS-treated and formalin-fixed haemocytes. Hybridoma clones were selected by immunohistochemistry against fixed haemocytes, heart, lymphoid organ, haemopoietic tissue, and Western blot against haemocyte extract and haemolymph. Two parts of MAbs were produced ; MAbs specific to haemocyte and MAbs specific to other tissues.

MAbs specific to haemocytes were obtained and could be divided into 6 groups according to their binding capacities to various haemocyte proteins in Western blot analyses and their differences in recognition of haemocyte sub-populations. The first group of antibodies consists of 4 MAbs (HC47d, 126d, 178d and 425d) reacted with 102 kDa haemocyte protein and strongly recognized a small subset of semi-granulocytes (SG) and hyalinocytes (H) but occasionally stained lightly a very small population of granulocytes (G). The antibodies also bound to a group of cells in haemopoietic tissue as well as cells located at the end of the tubules in the lymphoid organ but not in the spheroid. The second group of antibodies consists of 3 MAbs (HC54, 58d and 249d) strongly bound to 43 kDa and recognized a large sub-population of G and SG with coarse granules but did not bind to most of the H. This group of antibodies also cross-reacted with cells in the outer layer of the tubules in the lymphoid organ. The third group of antibodies (HC111, 114, 245 and 327) reacted with ~20 kDa and recognized all G and only a small portion of SG. The fourth (HC201d), fifth (HC136, 200 and 239) and sixth groups (HC55) reacted with 61 kDa, 175 kDa and ~230 kDa, respectively. They bound to sub-populations of G, SG and H in similar proportions. None of the antibodies showed any cross-reactivity to other components in haemolymph.

MAbs specific to other tissues could be divided into 3 groups. First, HC32 recognized cells of the tubules in lymphoid organ and group of cell in haemopoietic tissue. It is also bound to small granule of SG and H. The second groups (HC39, 62) reacted with spheroid cell and tubule of lymphoid organ, cell in haemopoietic tissue, neuron, hepatopancreas and gill. They also recognized all G, SG and H. The third group (HC31, 179d) bound to 116 kDa haemocyte protein. The MAbs bound to spheroid cell and tubule of lymphoid organ and neuron cell. They also recognize all G, SG and H as well.

The common antigens recognized by MAbs in the haemopoietic tissue and the lymphoid organ may reflect relationships among these organs in the development of the sub-populations of G and SG. Haemopoietic tissue may be the site for haemocyte production and the lymphoid organ may be the site for further differentiation of at least two different lines of haemocytes.

Student's signature……..

Field of study Biotechnology Advisor's signature…….

Academic year 2004 Co-advisor's signature……

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LIST OF ABBREVIATIONS

CNS	=	Central nervous system
CP	=	Clotting Protein
d	=	day
DHC	=	Differential Haemocyte Count
DMSO	=	Dimethylsulfoxide
diam.	=	diameter
EM	=	Electron Microscope
Epi	=	Epicardium
g	=	gram
GAM-HRP	=	goat anti mouse-horseradish peroxidase conjugated
GAR-HRP	=	goat anti rabbit-horseradish peroxidase conjugated
Ge	=	Gill epithelium
h	=	hour
H	=	Hyalinocyte
Hep	=	Hepatopancreas
Hpns	=	Haemopoietic nodules
Hpt	=	Haemopoietic tissue
Hrt	=	Heart
Ig	=	Immunoglobulin
kDa	=	Kilo Dalton
LG	=	Large Granulocyte
LM	=	Light Microscope
Lo	=	Lymphoid organ

MAb	=	Monoclonal Antibody
MAbs	=	Monoclonal Antibodies
mg.	=	milligram
min	=	minute
ml	=	millilitre
nm	=	nanometre
PBS	=	Phosphate Buffer Saline
proPO	=	Pro-Phenoloxidase
RAM-IgG	=	Rabbit anti mouse- IgG
RER	=	Rough Endoplasmic Reticulum
RT	=	room temperature
sec	=	second
SEM	=	Scanning Electron Microscope
SG	=	Semi-Granulocyte
Sp	=	Spheroid cell
TEM	=	Transmission Electron Microscope
THC	=	Total Haemocyte Count
Vnc	=	Ventral nerve cord

CHAPTER I

INTRODUCTION

Shrimp aquaculture has grown into a major industry worldwide. However, outbreaks of various diseases, especially viral infections have led to severe losses in shrimp culture (Flegel et al., 1997). Since there is no therapeutic treatment available, the possibility of prevention and control of shrimp diseases by the shrimp's own immune system has been examined. Shrimp has non-self recognition comprising the recognition of invading microorganisms (Vargas-Albores and Yepiz-Plascencia, 2000), the interaction of haemocytes with foreign molecules (Johansson et al., 2000), and the activation of immediate defence systems (Sritunyalucksana and Söderhäll, 2000). Those processes may consist of both humoral or cellular response (Söderhäll and Cerenius, 1992).

Based on the presence of cytoplasmic granules in the cells, crustacean haemocytes can be divided by morphology and cytochemistry criteria into 3 basic cell types. (Martin et al, 1987; Tsing et al, 1989; Hose et al., 1990; Rodriguez et al., 1995; Van de Braak et al., 1996; Gargioni and Barracco, 1998). Hyaline cells (the smallest haemocytes with minute cytoplasmic granules), semi-granular (cells with small granules in cytoplasm) and granular (the biggest haemocytes with large eosinophilic cytoplasmic granules) (Tsing et al, 1989 ; Rodriguez et al., 1995; Van de Braak et al., 1996)

Each type of crustacean haemocyte is active in defence function (Söderhäll and Cerenius, 1992, Johansson et al, 2000). Fifty to eighty percent of the circulating haemocytes are hyaline cells (Tsing et al., 1989; Le Moullac et al., 1997; Sung et al.,

1999), responsible for phagocytosis (Johansson et al., 2000). Nine to thirty percent of the circulating haemocytes are semi-granular cells (Tsing et al., 1989; Le Moullac et al., 1997; Sung et al., 1999) involved in encapsulation, degranulation, and the prophenoloxidase (proPO) activating system (Johansson et al., 2000). Four to twenty five percent of the circulating haemocytes are granular cells (Tsing et al., 1989; Le Moullac et al., 1997; Sung et al., 1999) participating in storage, cytotoxicity and release of proPO (Johansson et al., 2000). Even though similar criteria on haemocyte classification have been used, different proportions of circulating haemocyte types have been reported, according to species, molting stage and physiological condition of the animals. (Martin and Graves, 1985; Le Moullac et al., 1997; Tsing et al., 1989; Owens and O' Neill, 1977; Sung et al., 1999; Van de Braak et al., 2000)

The morphological features or staining criteria traditionally used to identify the haemocyte categories are insufficient to clarify the precise type of haemocytes and are unreliable criteria to correlate with cellular functions (Van de Braak et al., 1996). In order to elucidate the role of identifiable haemocyte sub-populations, an accurate means based on molecular approaches will be a more useful analytical tool for discriminating haemocyte sub-populations (Sung et al., 1999) and as probes for monitoring haemocyte-associated functions (Scapigliati et al., 1997). In vertebrate systems, monoclonal antibodies (MAbs) are highly specific molecular probes, which have been very effective in characterizing cell types and functions according to antigenic differences (Dyrynda et al., 1997).

Monoclonal antibodies solved the problems of specificity and reproducibility associated with traditional antisera. In 1975 Köhler and Milstein reported the first successful fusion between an antibody-producing spleen cell and an immortal

myeloma cell. They named the resulting cell a “hybridoma.” These hybridomas are adapted to grow in tissue culture and are capable of producing antibodies. A hybridoma cell will secrete only one type of antibody, that is an antibody against only one antigenic determinant. The specific antibody produced by the hybridoma cell is referred to as a “Monoclonal antibody.” (Barrett, 1994)

Following fusion, the myeloma cell lines can be killed in a selective tissue culture medium. The most commonly used selection system is the hypoxanthine-aminoptherin-thymidine (HAT) system. The folic acid analog aminopterin will block *de novo* synthesis of thymidylate and purine nucleotides, the main biosynthetic pathway for nucleic acid. Mutant myelomas used are lacking the enzyme HGPRT (hypoxanthine guanine phosphoribosyl transferase) cannot multiply in the presence of aminopterin and will die in media supplemented with HAT. Hybrid cells with functional HGPRT and thymidine kinase can continue to synthesize nucleic acids using an alternative salvage pathway in which the nucleotide metabolites hypoxanthine or guanine are converted to guanosine monophosphate via the enzyme HGPRT (Barrett, 1994; Goding, 1996)

Several studies have obtained some degrees of success using specific antigenic markers in penaeid shrimps (Rodriguez et al., 1995; Sung et al., 1999; Van de Braak et al., 2000 and 2001). Mice immunized with whole haemolymph of *P. japonicus* (Rodriguez et al., 1995) generated ten MAbs that recognized various components in *P. japonicus* haemolymph but not particular haemocyte sub-populations. However, most of the monoclonal antibodies cross-reacted to components in haemolymph.

More specific results were obtained from Sung et al. (1999) in which their four hybridomas produced from mice immunized with formalin-fixed haemocytes of

P. monodon produced antibodies that bound to 29 kD, 163 kD and 205 kD proteins of SG and G haemocytes.

Van de Braak et al. (2000) immunized mice with membrane lysates of *P. monodon* haemocytes. Four MAbs reacted with different features of *P. monodon* haemocytes such as cell membrane, some granules in all fixed haemocytes, some haemolymph components and also haemocytes in tissues.

From previous reports, production of MAbs that can clearly classify each type of *P. monodon* haemocytes has not yet been achieved. Our attempt to establish more specific MAbs to clarify all three types of *P. monodon*, haemocyte without having cross-reactivity to other components in haemolymph was performed.

The objectives of this study are to produce monoclonal antibodies specific to haemocytes of *P. monodon* and characterize the MAbs for further uses as an immunological tool to study shrimp health and immunity.

CHAPTER II

LITERATURE REVIEW

2.1 Biology of *Penaeus monodon* Fabricius, 1978

The penaeid life cycle includes several distinct stages found in a variety of habitats. Juveniles often prefer brackish waters of estuaries and coastal wetlands, while adults are usually found offshore at higher salinity and greater depths. Larval stages inhabit plankton-rich surface water off-shore, with an on-shore migration as they develop. (Bailey-Brock and Moss, 1992)

2.2 Morphology of *P. monodon*

P. monodon is the largest of the commercial species, reaching 330 mm or more in length. Rostrum has 7-8 dorsal teeth and 3-4 ventral teeth and curves down very slightly. Rostral ridge lacks a distinct groove behind it, and the hepatic ridge is long and curved. Telson has a groove but without lateral spines. Carapace and abdomen have black bands giving a tiger-striped appearance to this species. Pereiopods may be red. (Bailey-Brock and Moss, 1992) .The carapace is carinated with the adrostral carina almost reaching the posterior margin of the carapace. The gastro-orbital carina occupies the posterior one-third to one half distance between the post-orbital margin of the carapace and hepatic spine. The hepatic carina is prominent and almost horizontal. The antennular flagellum is subequal to or slightly longer than the peduncle. Exopods are present on the first four pereiopods but absent in the fifth. The abdomen is carinated dorsally from the anterior one-third of the fourth, to the posterior end of the sixth, somites. (Solis, 1988) (figure 2.1)

Figure 2.1 Male (top) and female (bottom) of black tiger prawn *Penaeus monodon*

2.3 Crustacean circulatory system

Decapod crustaceans have a well-developed open circulatory system for distributing oxygen, nutrients and hormones throughout the body. Arteries leaving the heart branch to form progressively smaller arterioles, which eventually terminate, allowing the fluid to circulate and bathe the internal organ. The fluid from the sinuses is channeled through the gills into the pericardium and back into the heart. The heart is a single-chambered sac that lies in the pericardial chamber. Haemolymph, the fluid circulating within the vessel, contained plasma protein including haemocyanin as the respiratory pigment in decapod. Cells transported within the haemolymph called haemocytes. (Martin and Hose, 1992)

2.4 Crustacean immunity

In vertebrate, the immune defense includes adaptive memory, specific immunoglobulins and specialized cells, as well as non-specific response through phagocytic cells and NK cells. (Vargas-Albores and Yepiz-Plascencia, 2000) Crustacean immune responses are based on both cellular and humoral components, which cooperate to eliminate potentially infectious microorganisms. (Gargioni and Barracco, 1998; Muñoz et al, 2000). A good non-self recognition system should also stimulate defensive responses, including those mediated by cell. (Vargas-Albores and Yepiz-Plascencia, 2000)

2.4.1. Cellular defences

Crustaceans are not different from other animals in that the host defense is largely based on activities of the blood cells or haemocytes. These cells can remove foreign particles in the crustacean haemocoel by phagocytic or encapsulating activites.

2.4.1.1 Phagocytosis

Phagocytosis, which represents an important way to eliminate microorganism or foreign particles, is accomplished by the granulocytes, primarily the small granule haemocytes. These cells are distinguished by the presence of numerous small granules, of which contain lysosomal enzymes, in addition, these cells contain numerous cytoplasmic vesicles that contain acid phosphatase, esterases and β-glucuronidase, the hydrolases that apparently degrade the microorganism within phagosome. (Hose et al., 1987; Hose and Martin, 1989; Martin and Hose, 1992; Muñoz et al, 2000)

In the ridgeback prawn, *Sicyonia ingentis*, two major categories of haemocytes are found, hyaline cells and two types of granulocytes. After 30 minute incubation of haemocytes with gram-negative bacteria *Cytophaga* sp., phagocytosis was accomplished primarily by small granule haemocytes, rarely by large granule haemocytes, and never by hyaline cell (Hose and Martin, 1989). In the American lobster, *Homarus americanus*, phagocytosed particles have been observed inside both hyaline and granular haemocytes. In contrast, only hyaline cells were actively phagocytic in crayfish (Paterson and Stewart, 1974). Phagocytosis was enhanced by prior opsonization of bacteria with cell-free shrimp haemolymph. In adddition, both granulocyte but not hyaline cells attach to and establish capsule around hyphae of the fungus *Fusarium solani*. (Paterson and Stewart, 1974; Hose and Martin, 1989)

2.4.1.2. Nodule formation

When the body cavity is invaded by a large number of microorganisms, in excess of those that can be removed by phagocytosis, nodule or cell clumping occurs in several invertebrates, including the crustaceans. The end result of nodule formation is that the microorganisms become entrapped in several layers of haemocyte, and normally the nodule becomes heavily melanized because of the host's phenoloxidase activity. Bacteria are rapidly removed from the circulation and localized in haemocyte clumps, usually in the gills, but also in the sinuses between the hepatopancreatic tubules. (Johansson and Söderhäll, 1989; Söderhäll and Cerenius, 1992)

2.4.1.3 Encapsulation

When the parasite is too large for each haemocyte to become engulfed by phagocytosis several haemocytes will then collaborate by sealing off the foreign particle from circulation. (Johansson and Söderhäll, 1989; Söderhäll and Cerenius,

1992) Most of haemocytes in this capsules appear to be large granule haemocytes and a lesser degree, small granule haemocytes preferentially attach the fungal hyphae. Hyaline cells were absent or rarely seen in these aggregations. (Götz, 1986)

2.4.1. Haemolymph clotting

Since arthropods have an open circulatory system, wound must be sealed rapidly to prevent blood loss and also to hinder pathogenic microorganisms from entering through the wound and causing infection (Söderhäll and Cerenius, 1992) The clotting reaction of crustaceans is both an extremely rapid and efficient defence reaction.

The coagulation in arthropods has been studied in greatest detail in the horseshoe crabs, *Limulus polyphemus* and *Tachypleus tridentatus.* Bacteria that enter the body cavity through a wound will release LPS from their cell walls, and these cell wall polysaccharides induce an exocytotic release of the clotting cascade into the plasma. Once released from the amoebocytes, LPS will induce activation of the clotting system, which comprises a cascade of serine proteinase and a clottable protein, a coaggulogen. In the clotting system, LPS appears to bind directly to the first cascade serine proteinase, factor C which will induce activation of a second proteinase, factor B which in its active form can cleave and activate the proclotting enzyme, the third proteinase in the cascade. These three serine proteinases are all of the serine type and the complete activation of the cascade will result in the formation of an insoluble clot, which will seal wounds and also entrap parasites. (Söderhäll and Cerenius, 1992)

2.4.1.5 Prophenoloxidase system

Prophenoloxidase is most abundant in large granule haemocytes, a small about is present in some small granule haemocytes and it is absent in hyaline cells (Söderhäll and Smith, 1983 ; Hose and Martin, 1989; Martin and Hose, 1992).

It has long been recognized that the cellular defence reactions in invertebrates are most often accompanied by melanizaton, a dark pigmented spot appears after an animal is injured. The key enzyme in the synthesis of the pigment melanin is phenoloxidase or tyrosinase, which is present in arthropod blood as an inactive pro-enzyme, prophenoloxidase (proPO). ProPO is activated in a stepwise process by microbial cell wall components such as the β-glucans form fungi (Vargas-Albores et al, 1996; Vargas-Albores and Yepiz-Plascencia, 2000; Chang et al., 2003) or the lipopolysaccharides (LPS) (Sung et al, 1998; and peptidoglycans of Gram-negative and Gram-positive bacteria (Sung et al, 1998). The active enzyme, phenoloxidase, oxidizes phenols to quinones, which then polymerize non-enzymatically to melanin which are fungistatic.

The prophenoloxidase activating enzyme (ppA), a serine proteinase catalyzing the conversion of prophenoloxidase to an active phenoloxidase, has a molecular mass of about 36 kDa in its active form. (Johansson and Söderhäll, 1985, 1989; Wang et al, 2001) In crustaceans, both proPO and inactive ppA are stored in the secretory granules of the semi-granular and the granular haemocyte. (Johansson and Söderhäll, 1989)

In crayfish, a protein, 76 kDa cell adhesion factor, released by the haemocytes, amplifies the generation of the proPO system by inducing degranulation of semi- and large granular cells (Söderhäll and Cerenius, 1992).

Two protease inhibitors, α2-macro-globulin (190 kDa subunit) and a trypsin inhibitor (155 kDa), can block ppA activity. (Johansson and Söderhäll, 1989; Söderhäll and Cerenius, 1992; Vargas-Albores and Yepiz-Plascencia, 2000)

In *P. monodon* and *M. rosenbergii*, the proPO system plays an important role in shrimp defense mechanisms which are similar to those that occur in crayfish. In contrast to crayfish and giant freshwater prawn, the proPO of tiger shrimp cannot be activated by trypsin but the addition of the two cations, calcium or magnesium ion, led to an increase in enzyme activity. The cytochemical analysis revealed that proPO system exists in the granulocytes of both tiger shrimp and giant freshwater prawn. (Sung et al, 1998)

PO activity was stimulated *in vivo* of *P. monodon*, by each of the three immunostimulants; heat-killed *Vibrio vulnificus* , β-1,3-1,6-glucan and zymosan. The levels of PO activity varied considerably according to stimulant type; the stimulative effects of beta-glucan and zymosan were stronger than that of the *Vibrio* antigen. It was seemingly increased before either antibacterial activity or O_2^- production. (Sung et al., 1996)

2.4.2 Humoral defense

2.4.2.1 Antibacterial activity

Bactericidal activity can be induced in the haemolymph of several crustaceans by injection of bacteria (Söderhäll and Cerenius, 1992)

Penaeidins are members of a new family of antimicrobial peptides isolated from a crustacean, which present both Gram-positive antibacteria and antifungal activities. The peptides are localized in granulocyte cytoplasmic granules. In *P. vannamei*, 3 hours after microbial challenged, the penaeidin mRNA levels

decreased in circulating haemocytes while increasing in plasma penaeidin concentration occurs. (Destoumieux, Muñoz, Bulet et al, 2000; Destoumieux, Muñoz, Cosseau et al, 2000) Furthermore, penaeidins were shown covering bacterial surfaces suggesting the peptides could be involved in opsonic activity. Penaeidin-positive bacteria were observed to be phagocytosed mainly by hyaline cells, a population that does not express penaeidins. Using *in situ* hybridization and immunohistochemical analyses, penaeidin transcripts and peptides localized in a few haemocytes of larvae from mysisII stage and penaeidin expression could not be detected in other cells than haemocytes. (Muñoz et al, 2002; 2003).

2.4.2.2 Agglutinin

The particular recognition factors reside in invertebrate blood has persisted and popular candidates for these factors have been the agglutinin. These agglutinin, correctly lectins, comprise a heterogenous group of glycoproteins or proteins and bivalent then it can bind two cells specifically on surface polysaccharide and an agglutination reaction occurs. (Söderhäll and Smith, 1986; Söderhäll and Cerenius, 1992; Marques and Barracco, 2000) Agglutinin molecules may occur at the surface of viruses bacteria, yeast, protozoan and throughout all animal and plant kingdoms. (Sharon and Lis, 1989) 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) Thus, lectins can agglutinate microorganisms and second they mediate the binding between haemocyte surfaces and foreign bodies and thus function as an opsonin. (Söderhäll and Cerenius, 1992)

2.4.2.3 Clotting Factors

Clotting protein (CP) was reported as a lipoglycoprotein with a molecular mass of approximately 400 kDa. In *P. vannamei*, CP composed of two identical (210 kDa) subunits that are bound by disulfide bridges. In addition to its participation in the clotting process, CP has been reported to have other functions such as pigment, lipid and carbohydrate transport and protein reserves during starvation (Montaño- Pérez et al, 1999).

2.4.2.4 Other plasma proteins

Beta glucan binding protein (BGBP), a monomeric protein with a molecular mass of 100 kDa. This protein is capable of enhancing the prophenol-oxidase system activation induced by laminarin. (Vargas-Albores et al., 1996)

Peroxinectin, a cell adhesive protein in haemolymph, contains both cell adhesion and peroxidase activity. In crayfish, peroxinectin is present as a biologically inactive protein in HLS (membrane lysate supernatant) and becomes converted into its active form, a cell adhesion molecule, concomitant with activation of the proPO system. With the activation of proPO system by laminarin in *P. monodon*, a cell adhesion activity has been generated in haemolymph as well. (Sritunyalucksana et al, 2001)

2.5 Haematopoiesis

In crustaceans, the haemocyte production and the location of haemopoietic tissue have been investigated.

In *Carcinus maenus,* Ghiretti-Magaldi, Millanesi and Tognon, (1977) investigated that haemopoiesis occurred in the lymphocytogenic nodules of the gizzard walls. Supporting to the Kollmann's hypothesis (Kollmann, 1908 cited in

Ghiretti-Magaldi et al, 1977) that all blood cells derived from a single cell line which proliferates in the nodules. The stem cells are called haemoblasts: they differentiate into circulating haemocytes and into cyanoblasts. Four types of haemocytes have been identified as one agranulated and three different granulated cell types.

In *P. monodon*, Van de Braak et al. (2002) described that Hpt is located in different areas in the cephalothorax, mainly at epigastric region and the onset of the maxillipeds and to a lesser extent, towards the antennal gland. The cells in Hpt were classified into four main types; clusters of densely packed type 1 cells, identified by the highest N/C ratio. The more detached type 2, containing up to 10 relatively large homogenous electron dense granules per section, and type 3 cells, containing more granules The type 4 cells are less abundant, vary in N/C ratio. Type 1 and type 4 cells were observed only in the Hpt, never in the circulatory system. Type 1 cells may be the precursors of the type 2 and 3 cells that might be the young maturing large- and small-granular haemocyte, respectively. The type 4 cells have typical features of interstitial cells. When released into haemolymph, the two types of young haemocytes, H cells, either develop into G and/or SG cells. SG then was transported to the connective tissue, where they finally mature into the G cell.

2.6 Crustacean haemocyte: Haemocyte type

Circulating haemocytes play important role in the host immune reaction. They thought to be involved in hardening of the exoskeleton, prevention of blood loss and the confinement of invasive organisms by clotting formation, recognition of non-self, phagocytosis, melanization, encapsulation, cytotoxicity and cell-cell communication. (Hose et al., 1990; Johansson et al., 2000) Identification and classification of crustacean haemocytes are essential to elucidate their specific immune functions and

to allow comparison among the different crustacean species. (Gargioni and Barracco, 1998)

Several types of haemocytes have been identified based on morphological using light and electron microscope, cytological using the specific staining for detection of carbohydrate, lipid or enzyme and physiological criteria such as phagocytosis. As summarized in table 2.1

Martin and Grave (1985) examined the structure of haemocytes from two species of penaeid shrimp (*Sicyonia ingentis* and *Penaeus californiensis*) by LM and TEM. Haemocytes from two species were indistinguishable and were classified as agranular, small granule or large granule haemocytes. Agranular haemocytes were the smallest, lack of granule and were non-refractile when examined by LM. Small granule haemocyte were the most abundant type of haemocyte appear non-refractile, and contain a variable number of granules (0.4 μm diam.). Large granule haemocytes compose 10-20% of the haemocytes. They were filled with granules (0.8 μm diam.) that were highly refractile (LM) and were electron dense (TEM)

Tsing, Arcier and Brehélin (1989) studied the hemocyte ultrastructure, cytochemistry and haemograms of penaeid shrimp, *Penaeus japonicus , P. monodon* and palaemonid shrimp, *Macrobrachium rosenbergii* and *Palaemon adspersus*. The haemocyte were separated into three cell type according to their ultrastructural features including small granule haemocyte, large granule haemocyte and undifferentiated haemocyte in penaeid shrimp and two for the palaemonid by lacking of UH. For the haemogram of *P. japonicus* , the total haemocyte count (THC) is high in the stage A-B1 and decrease to a minimum in B2 then increases in stage C, remains stable in D2 and increases again and reaches a maximum in D3 and D4.

Hose et al. (1990) examined the haemocyte of three decapod crustaceans, *Homarus americanus, Panulirus interruptus* and *Loxorhynchus grandis* using combination of morphological, cytochemical and functional methods to gain more precisely identification of haemocyte than morphological identification alone. Three types of haemocytes were identified. The hyaline cells and the small- and large granule haemocytes which distinguished by 1) centrally or eccentrically location of the nucleus and 2) the presence of only large granules (>1.2 μm diam.) in large granule haemocytes while small granule haemocytes may contain both large and small granules. With trypan blue experimented for study the lysing cells showed that the percentage of lysing cells in each species corresponded to the percentage of hyaline cells obtained from DHC. DHC which were performed using phase contrast LM and TEM, *P. interruptus* had the highest percentage of hyaline cells at 56%, whereas *L. grandis* and *H. americanus* were considerably lower at 21% and 27%, respectively. Large granule haemocyte constituted between 10% and 13% of the total with small granulocytes comprising about 65% in *H. americanus* and *L. grandis* and 31% in *P. interruptus.*

Vázquez et al. (1997) identified the type of blood cells in the freshwater prawn *Macrobrachium rosenbergii* using morphological criteria. The haemocytes do not fit easily into classification schemes recently presented for marine decapods They were classified in to three types: Hyaline haemocyte, the most abundant type, fusiform shape, have few large cytoplasmic granules, a large nucleocytoplasmic ratio, and lyse spontaneously in the absence of anticoagulant. Granular haemocytes were heterogeneous in size and in density of their granules. The third type were identified as undifferentiated haemocytes were the least abundant and the smallest haemocytes..

Gargioni and Barracco (1998) characterized the haemocytes of two palaemonids, *Macrobrachium rosenbergii* , *M. acanthurus* and a penaeid shrimp *Penaeus paulensis* using light and transmission electron microscope. Three haemocytes were classified as hyaline haemocytes (HH), small granule haemocytes (SGH) and large granule haemocytes (LGH). These round, oval or fusiform HH were unstable haemocytes with a characteristic high nucleocytoplasmic ratio, in both palaemonids, the HH contain numerous granules, whereas in *P. paulensis* a small number of these cells had few or no granules. The ovoid or fusiform SGH had numerous granules that were usually smaller than those of the round, ovoid LGH. The SGH was the main circulating blood cell type in both palaemonids whereas HH was predominant in the penaeid. Based on morphological and functional features, it appeared that the hyaline and the granular haemocytes of the three shrimp species represented different cell lineage.

Sung et al (1999) seperated 3 types of *P. monodon* haemocyte using LM and the combination of lectin staining and Percoll gradient centrifugation. 73% were hyaline cell, 9% were semi-granular cells and 17% were granular cells. It has also shown that either concanavalin A (ConA) or peanut agglutinin (PNA) was capable of binding to most of hyaline cells and a few semi-granular cells but not granular cell. To gain high-density granular cell suspension as an antigen source of monoclonal antibodies production, most hyaline cells were removed from the haemocyte samples via negative selection with ConA-linked beads.

Van de Braak (2000) examined the DHC using light microscopy after H&E staining showed the hyaline cells 25-30%, 60-65% of semi-granular cells and 10-15% of the granular cells. The difference between the three cell types was based upon the

nucleus: cytoplasm ratio. By electron microscopy, the number of large electron dense granules per cell, which were 0-3 for hyaline cell, 4-9 for semi-granular cells and 10 or more for the granular cells.

Le Moullac et al. (1997) identified 3 types of haemocytes of *Penaeus stylirostris*: hyaline cells, which were small with a big nucleus. Hyaline cell had a round, oval shape, represent 80% of the total. Semi-granular cells represent 10-13%; and large granular cells represent 4-10% of the total cells, being the largest cells with highly eosinophilic intracytoplasmic granules. Variations of THC during the moult cycle were mainly related to the variations of hyaline cell representing about 80% of THC. Semi-granular cell relative percentage does not vary significantly during the moult cycle, while in contrast, the relative variations of large granular cells show a maximum in intermoult and a minimum in premoult.

Owens and O'Niell (1997) used a clinical cell flow cytometer (FC) and the haemocyte classification scheme of Tsing et al. (1989) to apply for differential counts of *P. monodon* haemocyte. In comparison with LM the FC has potential as a tool in prawn health studies as total, granular and non granular haemocyte counts, the statistically were similar. The THC was 23.3 x 10^6 cells ml^{-1} using a haemacytometer and 21.0 x 10^6 cells ml^{-1} with the FC. However, sex had a significant effect ($P<0.05$) and once sexual dimorphism, as measured by total length, had occurred in the prawn population, females showed increased non-granular haemocyte counts and, consequently, total haemocyte counts. The third sequential bleeding of prawns (0.1 ml of haemolymph $bleed^{-1}$) produced a significant decrease in THC numbers with proportional increases in granular haemocytes.

Table 2.1 Haemocytes sub-populations in Penaeid and Palaemonid shrimps

Species	Methods	Haemocyte types	Percent in circulation	References
Sicyonia ingentis and *P. californiensis*	LM TEM	Large granular haemocyte Small granular haemocyte Agranular haemocyte	10-20% 75% 5-10%	Martin and Graves (1985)
P. monodon	TEM	Large granule haemocyte Small granule haemocyte Undifferentiated haemocyte HL (cell which lysis in vitro)	15-25% 20-30% 8-10% (50-55%)	Tsing et al. (1989)
Panulirus. interruptus	TEM	Large granule haemocyte Small granule haemocyte Hyaline cell	13% 31% 56%	Hose et al. (1990)
Homarus americanus and *Loxorhynchus grandis*	TEM	Large granule haemocyte Small granule haemocyte Hyaline cell	20-28% 62-69% 10-13%	Hose et al. (1990)
Macrobrachium rosenbergii	TEM	Granular haemocyte Hyaline haemocyte Undifferentiated cells	20% 70% 10%	Vázquez et al. (1997)
P. stylirostris	LM	Large granular cell Semigranular cell Hyaline cell	4-10% 10-13% 80%	Le Moullac et al. (1997)
P. monodon	LM	Large granular Small granular Large nongranular Small nongranular	1.7±2.2 15.6±17.4 12.6±15.0 70.1±13.9	Owens and O'Neill (1997)
	Flow Cyto-meter	Large granular Small granular Large nongranular Small nongranular	2.9±3.4 14.5±14.5 5.9±5.6 2.2±3.1 74.5±17.4	
P. paulensis	LM TEM	Large granular haemocyte Small granular haemocyte Hyaline haemocyte	33% 26% 41%	Gargioni and Barracco (1998)

Table 2.1 Haemocytes sub-populations in Penaeid and Palaemonids shrimps (continue)

Species	Methods	Haemocyte types	Percent in circulation	References
M. rosenbergii and *M. acanthurus*	LM TEM	Large granular haemocyte Small granular haemocyte Hyaline haemocyte	20-29% 54-60% 17-20%	Gargioni and Barracco (1998)
P. monodon	LM Percoll	Granular cell Semigranular cell Hyaline cell	17% 9% 74%	Sung et al. (1999)
P. monodon	LM TEM	Granular cell Semigranular cell Hyaline cell	10-15% 60-65% 25-30%	Van de Braak et al. (2000)

2.7 Factors effecting the change in haemocyte number

The circulating haemocyte or total haemocyte count (THC) of decapod crustaceans play an important role in regulating the physiological functions. The factor effecting variable circulating haemocyte has been examined

Moulting cycle Variation of *P. stylirostris* THC during the moult cycle are mainly related to the variations of hyalinocyte representing about 80% of THC. Similar variations of the THC have been observed in *P. japonicus* and *S. ingentis* (Tsing et al., 1989; Hose et al., 1992). Semi-granulocyte relative percentage does not vary significantly during the moult cycle, while in contrast, the relative variations of large granulocyte show a maximum in intermoult and a minimum in premoult. The THC measured in intermoult (stage C) is significantly lower than the THC values observed in postmoult (stage B) and premoult (stages D0, D1, D2) (Le Moullac et al, 1997) In *M. rosenbergii*, the THC is lowest at D3. The percentage of THC of prawns at A12 and A24 stages is significantly lower than that at C stage. (Cheng and Chen, 2001)

Viral infection After infection of white spot syndrome virus (WSSV) in *Penaeus indicus* significant reductions in THC and haemocyanin contents were observed. The haemolymph failed to clot. Some physiological responses such as oxygen consumption and ammonia excretion were decreased compared to healthy shrimp. (Yoganandhan et al, 2003)

Cooling and immersion In spiny lobster, *Panulirus interruptus*, the cooling rates applied to lower water temperature from 19 to 4°C evoked a significant decrease of THC and PO activity at both cooling rates tested and at the cooling rate of 1.5°C h^{-1}, respectively. The observed decrease in THC suggests that cooling at these rates evoked a physiological response as a decrease in THC, and that this was greater at the faster cooling rate. (Gomez-Jimenez et al., 2000)

Heavy metals Lorenzon et al. (2001) investigated the effect of short-term (96h) exposure to dissolved heavy metals on the number of circulating haemocytes in *Palaemon elegans*. It was found that immersion in artificial seawater containing Hg, Cd, Cu, Cr, Zn or Pb caused a decrease in THC during the first 8h exposure. The greatest decrease in haemocyte numbers was induced by Pb followed by Zn, Hg, Cr, Cu and Cd. Animals treated with Pb or Zn survived with a lower number of circulating haemocytes than animals exposed to the other heavy metals.

Dietary Determination of dietary vitamin E requirement in *P. monodon* was studied by Lee and Shiau (2004) who found that weight gains and total haemocyte count (THC) were higher in shrimp fed diets supplemented with 75-100 mg vitamin E kg $diet^{-1}$ than lower supplements fed. Superoxide dismutase (SOD) activity was higher in shrimp fed diets supplemented with 50-200 mg vitamin E kg $diet^{-1}$ than in shrimp fed other diets.

2.8 Monoclonal antibodies applications

The use of monoclonal antibodies (MAbs) to classify cells according to antigenic difference in penaeid shrimp have been studied as summarized in table 2.2

Rodriguez et al. (1995) characterized shrimp haemocytes and plasma components of *P. japonicus* using MAbs and determined by immunofluorescence. The molecular mass of the antigen was analysed by Western blotting. Three groups of MAbs were produced. Group 1 consists of 4 MAbs that recognized a 170 kDa protein, in reducing condition and was presented both in the plasma and in the haemocytes. Group 2, two MAbs, recognized the granular cells were labeled by 40E2-2A which was specific for a protein of 142 kDa also present in plasma. The 40E10-2B MAbs was assumed to be the marker for small hyaline and semi-granular cell. The third group recognized a protein of 75 and 180 kDa under reduced condition

Sung et al. (1999) got four MAbs by immunization mice with formalin fixed haemocytes of *P. monodon.* They were capable of secreting antibodies specific to granular cells and semi-granular cells.. The Z5E10 MAbs reacted with G and SG and recognized 29 kDa. The Z6A6 specifically bound to SG while recognizing 163 kDa and specific to glycoprotein. The other two MAbs (Z6A5 and Z6H8) separately reacted with SG and were able to recognize the antigens, which were larger than 205 kDa under non-reducing condition but not reducing conditions. Following the negative selection with MAb-linked beads, an increase in hyaline cell density in haemocyte suspension was observed. Suggesting that the epitopes recognized by the four MAbs were located on the surface of haemocytes and that these MAbs maybe useful when they are employed to label and separate haemocyte subpopulations for further study of haemocyte function.

For further studied of these MAbs, Sung and Sun (2002) used the double staining of Z6A5 and Z6H8 to classifiy haemocyte subpopulation and showed that all three types of shrimp haemocyte could be divided into two subclasses, one labeled by both MAbs ($Z6A5^{+}Z6H8^{+}$) and the other labeled by neither ($Z6A5^{-}Z6H8^{-}$)

Van de Braak et al. (2000) immunized mice with membrane lysates of *P. monodon* haemocytes and received four MAbs reacted with different features of *P. monodon* haemocytes. The WSH6 recognized a carbohydrate determinant on an 85 kDa molecule. WSH7, WSH8 and WSH16 recognized 50, 35 and 115 kDa molecules, respectively. In comparison among fixed-haemocyte, non-fixed haemocyte in AS and live cell in L15 cell culture medium, WSH6 reacted with the cell membranes of all fixed haemocytes, while WSH7 and WSH16 reacted with the cell membranes of most of fixed haemocytes. WSH8 did not react with the haemocyte membrane. WSH16 also showed labeling in cytoplasmic vesicles as well as in haemolymph plasma on histological section.

In 2001, Van de Braak et al. reported the cross reactivity of monoclonal antibodies with haemolymph component of three freshwater crustaceans, a terrestrial isopod crustacean and with coelomic fluid of an annelid. No reactions were observed with haemolymph of an insect and a mollusc, nor with blood cells of two vertebrates. They suggested that well conserved molecules were recognized, which may indicate functional importance. The MAbs can be used in studies of the crustacean defense system and may finally result in a better insight into this system.

Table 2.2 Specificities of monoclonal antibodies against haemocytes reported previously.

Species	MAbs		Isotype	Antigen (MW)		Haemocyte specificity	Reference
				HL	haemocyte		
P. japonicus	1	35D6-1A	IgG1	170^{rd}	170^{rd}	All cells	Rodriguez et al. (1995)
		40G8-1B	IgG1	170^{rd}	170^{rd}	All cells	
		40G1-1C	ND	170^{rd}	170^{rd}	All cells	
		41B12-1D	IgG1	170^{rd}	170^{rd}	All cells	
	2	40E2-2A	IgG1	142^{rd}	ND	G	
		40E10-2B	IgG2b	250^{rd} 150^{rd} 66^{rd} $27^{nord,rd}$	150^{rd} 66^{rd} $27^{nord,rd}$	SG&H	
	3	41D11-3A	IgG1	180^{rd}	-	(coagulogen)	
		42C11-3B	IgG1	180^{rd}	-	(coagulogen)	
		42E8-3C	IgG1	180^{rd}	-	(coagulogen)	
		44E6-3D	IgG1	75^{rd}	-	(haemocyanin)	
P. monodon	1	Z5E10	IgG2a	ND	29	G&SG	Sung et al. (1999)
	2	Z6A5	IgG1	ND	>205	SG	
	3	Z6A6	IgG1	ND	163	SG (carbohydrate)	
	4	Z6H8	IgG2b	ND	>205	SG	
P. monodon	1	WSH6	IgG1k	+	85	Membrane of all cells (carbohydrate)	Van de Braak et al. (2000; 2001)
	2	WSH7	IgG3k	+	50	Membrane of all cells	
	3	WSH8	IgG1k	+	35	Granule of non fixed cell	
	4	WSH16	IgMk	+	115	Membrane, cytoplasm, HL	

note G = large granular cell
SG = semi-granular cell
H = hyaline cell
HL = haemolymph
rd = reduced condition
nord = nonreduced condition
ND = not done

CHAPTER III

MATERIALS AND METHODS

3.1 Chemical reagents

Alexa Fluor® Goat anti-mouse 546	Molecular probe
Alexa Fluor® Goat anti-rabbit 488	Molecular probe
Foetal Calf Serum	Starrate
Goat antimouse IgG(H+L) - HRP	Sigma
High MW range standard mixture	Sigma
Mouse MonoAB ID kit (HRP)	Zymed laboratories, INC
Prestained SDS-PAGE standard solution	Sigma
RPMI-1640 medium	Gibco

Other chemical reagents used are analytical grade (AR)

3.2 Equipments

Autoclave vertical type	Huxley
Bottle top filter fit 45μm neck, 250 ml	Nalgene
CO_2 water-jacket incubator	NUAIRE
Inverted microscope IX70	Olympus
Gel-air dryer	BioRad
Haemocytometer	Boeco
Hot air oven	Memmert
Laminar flow	NUAIRE
Light microscope	Olympus
Microplate reader EL307	Biotek instrument

Microscope for BF/Fluorescent/Phase contrast BX51	Olympus
Rotary microtome RM 2135	LEICA
Mini trans-blot cell	BioRad
Mini vertical gel electrophoresis	BioRad
Octapette	Costar
Slide warmer	Medax
Small bench centrifuge Centurion 1000 series	Centurion
Vacuum pump	Millipore

3.3 Animals

Juveniles *Penaeus monodon* (30-40 g) were obtained from farm nearby Bangkok, Thailand. They were held in 1x 1x 0.5 m^3, rectangular concrete tanks with 5 ppt seawater at natural photoperiod.

3.4 Haemolymph collection

Haemolymph was obtained from the arthrodial membrane of the fourth walking leg using a 22 gauge needle and was immediately diluted to double the volume with cold modified Alsever's solution (AS; Rodriguez et al, 1995). The haemocytes were washed twice with AS, centrifuged at 1500x g to diminish the amount of haemolymph protein especially haemocyanin from the haemocytes for further preparation.

3.5 Haemocytes preparation

3.5.1 Formalin- fixed haemocytes

The haemocytes from 3.4 were fixed with 10% formalin in AS for 2 h. The fixed haemocytes then washed 3 times in PBS (0.15 M phosphate buffer saline,

pH 7.2). (1500xg, 5 min) to remove formalin and, then adjusted to about 10^8 cells ml^{-1} in PBS and stored in -20°C until use. (Figure 3.1)

3.5.2 Denatured haemocytes

The haemocytes from 3.4 were denatured with 10% SDS in PBS. The denatured haemocytes was dialysed against PBS to remove SDS, aliquot and kept at -20°C until use for immunization. (Figure 3.1)

3.6 Immunization

Four female Swiss mice were injected intraperitoneally with the 1:1 mixture of formalin-fixed and denatured haemocytes with complete Freund's adjuvant and were subsequently injected with the mixture of haemocytes mixed with incomplete Freund's adjuvant 3 times at 2 week intervals. One week after the fourth injection, mouse antiserum was collected and tested against haemocytes by immunocytochemistry. The mouse with the highest titer of antiserum was boosted three days before hybridoma production.

3.7 Hybridoma production

The cell fusion procedure was adapted from the method developed by Köhler and Milstein (1976), with modifications described by Mosmann et al. (1979). The spleen cells were taken from the highest titer immunized mouse (**3.6**) and a P3X myeloma cell line was used as a fusion partner. The fusion was performed in PEG/RPMI medium. Fusion products from one mouse were plated in 40 microculture plates (96 wells/plate). After identification of the positive clones by immunocyto-chemistry on shrimp haemocytes and tissues and by dot blot, the selected clones were re-cloned by the limited dilution method. The hybridoma were cultured in RPMI-1640

medium (Gibco) supplemented with 10-20% fetal calf serum. The positive clones were preserved in freezing medium (12 % DMSO) and kept in liquid nitrogen. (Figure 3.2)

3.7.1 Cell fusion

The spleen from the highest titer immunized mouse (**3.6**) was dissected sterile and separated into the single cell and a healthy log growth phase P3X myeloma cell line was used as a fusion partner. Spleen cell and myeloma cell were mixed and centrifuge at 1500 g for 5 min then washed the pellet once with RPMI medium and centrifuge at 1500 g for 5 min.

Fusion was performed by adding 1 ml of 40% PEG/RPMI into the pellet, thoroughly mixed by hand shaking for 1 min then gently added 39 ml of RPMI medium supplemented with HT, incubated the mixture in 37°C in 5%CO_2 incubator for 2 h. After that, centrifuged at 1500 g for 5 min and resuspended in HAT medium supplemented with 20% FCS and 1% mouse red blood cell as feeder cell. Fusion products (150 μl/well) were then plated in 40 microculture plates (96 wells/plate) and grown at 37°C in 5% CO_2 incubator.

Contamination and colonization were checked under inverted microscope daily after fusion for 7 days. The screening was done by taken the cell culture supernatant during 10-14 days after fusion. The positive clone was selected to re-clone and expansion.

3.7.2 Re-clone and Expansion

After screening for the positive clone, cells were re-cloned by the limiting dilution method (Eshar, 1985). Cells from positive clone were diluted in HT-RPMI medium supplemented with 20% foetal calf serum and 1% mouse red blood

cells, then plated out into 96 wells microculture plate in concentration of 1 cell/well, cultured in 37°C, CO_2 incubator for 7 days. Selected wells that have single clone and rechecked the specificity and cell viability then individually positive clones were propagated in gradually increasing amounts of medium into the sufficient quantities for cryopreservation. The culture fluid from each positive clone were collected for further characterization.

3.7.3 Cryopreservation

Hybridoma cell lines from the healthy cell culture were taken, centrifuged at 1500 rpm for 5 min, and the supernatant was kept. The pellet was gently mixed with 1 ml of cold 10% DMSO in RPMI medium and aliquots in freezing vial. Then the vial was slowly frozen at -70°C in freezer for 24 h. The cells were transferred for permanent storage in liquid nitrogen tank.

3.8 Screening methods

The screening for hybridoma clones that specific to haemocyte were performed in 2 steps. First, screening by immunocytochemistry with formalin-fixed haemocyte, the hybridoma clones that revealed brownish staining with the haemocyte were selected for further screening. The secondary screening was done using immunohistochemistry of shrimps tissue and dot blotting. The hybridoma clone that reacted with haemocytes or tissue but not reacted with haemolymph of *P. monodon* were selected, re-clone and cryopreservation. Hybridoma supernatant was kept for further characterization.

3.8.1 Immunocytochemistry

a) Haemocyte preparation

Formalin fixed haemocytes were washed three times in PBS (1500x g, 5 min) and permeabilized with 1% Triton X-100 in PBS for 30 min at 37°C, then washed and resuspended in BSA (1 mg ml^{-1}) at the final concentration of 1-5 x 10^6 cells ml^{-1}. Haemocytes suspension was spotted on gelatin coated slides and fixed with 1% formalin in PBS for 30 min in moist chamber. The slides were washed three times in PBS with 5 min intervals, then blocked with $P1^+$ (10% calf bovine serum in PBS with 0.1% merthiolate) and stored in a moist chamber at 4°C until use.

b) Immunocytochemistry

Hybridoma culture supernatant was added on a haemocyte slide and then incubated at 37°C for 3 h. The slides were washed four times, 10 min intervals in PBS before immunoperoxidase reaction was carried out using horseradish peroxidase-labelled goat anti-mouse IgG (GAM-HRP, 1:1000) for 3 h at 37°C. After four times washed in PBS, peroxidase activity was revealed by incubation with 0.03% diaminobenzidine (DAB) and 0.006% H_2O_2 in PBS. The preparations were dehydrated in a graded ethanol series, counter-stained with Mayer's hematoxylin and eosin and mounted with permount.

3.8.2 Immunohistochemistry

Various tissues including gills, heart, lymphoid organ and haemopoietic tissue were dissected, fixed in Davidson's fixative and processed for paraffin sectioning. Serial sections (8 μm) were prepared and processed for indirect immunoperoxidase staining as followed.

3.8.2.1 Tissue preparation

The tissues in Davidson's fixative were thoroughly washed in running tap water for 3 h. The dehydration of tissues were processed in graded alcohol series of 70% EtOH for 3 h., 90% EtOH for 3 h., 95% EtOH over night with two changes, and butanol for 1 h.. Two changes of xylene were used for clearing of the tissue for 2 h. with two changes. Then infiltrated with the mixture of xylene and 60°C paraplast at 1:1 ratio for 30 min followed by 3 changes of 60°C paraplast for 30 min intervals. Finally the tissues were embedded in paraplast held with embedded plastic rings.

The embedded tissues were then cut using rotary microtome for 4-8 μ m. thick. The thin sections were mounted on the gelatin coated slide (Appendix A), and baked in 50°C incubator over night. (Figure 3.4)

3.8.2.5 Immunohistochemistry

After deparaffination with 3 changes of xylene and rehydration in ethanol series; 95% to 70% EtOH, followed by distilled water and PBS. Tissues were then blocked with $P1^{+}$ for 30 min before application of hybridoma supernatant as described in ***3.8.1 b)*** (Figure 3.5)

Selected the hybridoma clone that reacted with the tissues and haemocytes but not haemolymph.

3.8.3 Dot blotting

The antigens, the denatured haemocytes from ***3.5.2*** and haemolymph of *P. monodon*, were used for the second step of screening. Approximately 1 μl/spot of both antigens were applied to pieces of nitrocellulose membrane. The membrane was air dried and thoroughly washed in PBS and then blocked with 5% blotto for 30 min. Each piece of membrane was incubated in hybridoma conditioned media from each clone (1: 50 in 5% blotto) at room temperature for 3 h. After extensive washing

(4 times, 5 min. interval) in 0.5% blotto, the membrane was incubated in GAM-HRP (1: 1500 in 5% blotto) at room temperature for 3 h. The membrane was then thoroughly washed in 0.5% blotto and developed in substrate mixture (0.03% diaminobenzidine, 0.006% hydrogen peroxide, 0.05% cobalt chloride in PBS). The dark-brown color will appeared within 5 min. (Figure 3.6)

Selected hybridoma clones that revealed dark-brown color on nitrocellulose membrane on denatured haemocyte but not binding with *P. monodon* haemolymph, were re-cloned and cryopreserved as described above.

3.9 Monoclonal antibodies characterization

3.9.1 Immunofluorescense

The immuno-staining of positive hybridoma clone was done with formalin fixed haemocytes in the same manner as described in ***3.8.1.*** Instead of GAM-HRP, Alexa Fluor®-546 goat anti-mouse IgG H&L conjugate (1: 500 in $P1^+$) was used, then mounted with glycerin jelly after washing and observed under a fluorescence microscope.

3.9.2 SDS-Polyacrylamide gel electrophoresis

Haemocyte and haemolymph were seperated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a slab gel in a mini-PROTEIN II electrophoresis apparatus according to the method described by Leammli (1970). The preparation of slab gels and buffer used in SDS-PAGE were described in Appendix C

3.9.2.1 Sample preparation

Haemocytes from **3.4** were denatured and solubilized with 10% SDS and the cell debris was removed by centrifugation at 1,500 g for 5 min. The resultant supernatant was stored at -20°C until use.

Haemolymph or denatured haemocyte samples were mixed 1:1 with 2x treatment buffer then boiled in 100°C water for 90 sec before electrophoreses.

3.9.2.2 Electrophoresis

The slab gels consists of a 4% acrylamide stacking gel and 10% acrylamide separating gel. Samples (5-10 μl/well) were applied on stacking gel and electrophoresis was performed in 0.025 M tris-glycine, 0.1% SDS, pH 8.3 tank buffer and running at constant voltage of 100 V. When the dye marker moved near the bottom edge of the separating gel, the separation was terminated. Part of the separating gel was cut off and stained for protein with 0.1% Coomassie brilliant blue R-250 for 30 min. at room temperature with gently shaking. Destaining was performed by immersing the gel in destain I solution for 1 h., 1-2 changed with gently shaking followed by destain II solution with gently shaking until the blue color of protein bands were appeared on the cleared gel. The gel was thoroughly washed with distilled water and dried in a gel air dryer (Bio-Rad)

Standard proteins consisted of myosin (205 kDa), β-galactosidase, (116 kDa), phosphorylase B (97.4 kDa), albumin, bovine plasma (66 kDa), albumin, egg (Ovalbumin) (45 kDa), carbonic anhydrase (29 kDa) (BioRad) were used as molecular weight markers. The molecular weight of sample proteins was known by comparing the relative mobility (Rf) of sample proteins to the standard protein markers

3.9.3 Western blot analysis

Another part of the gel was then transferred to nitrocellulose membrane using Transblot apparatus (BioRad) at 50 V for 3 h. The nitrocellulose membrane was dipped into 5% Blotto for 30 min. at room temperature. The membrane was cut into strip and then incubated individually with MAb supernatant diluted at 1:200 for 3 h. After extensive washing in 0.5% Blotto, the membrane was incubated in GAM-HRP (1:1500) for 3 h. The membrane was then washed 4 times for 5 min.interval in 0.5% Blotto and color developing was performed in a substrate mixture containing 0.03% DAB, 0.006% H_2O_2, 0.05% $CoCl_2$ in PBS. (Figure 3.6)

The prestained standard molecular weight markers, β-galactosidase, *E. coli* (126 kDa), fructose-6-P-kinase (rabbit muscle) (102 kDa), pyruvate kinase, (chicken muscle) (81 kDa), ovalbumin (chicken egg) (53.5 kDa), lactic dehydrogenase (37 kDa), triosephosphate isomerase (31.4 kDa) (BioRad). The vitellin subunit of 215 kDa (Longyant et al., 2000) were also used in blotting experiments.

3.9.4 Class and subclass determination

Class and subclass of mouse immunoglobulin produced by hybridomas were determined by sandwich ELISA using Zymed's Mouse MonoAb ID Kit (HRP) as shown in figure 3.7

Approximately 50 μl of IgGAM (1:50) were plated on Maxisorb microtiter plates (NUNC) and incubated at 4°C for overnight in moisture chamber. After incubation, the plate was washed 3 times with 100 μl of 0.5% Blotto for 10 min interval, then blocked with 50 μl of 5% Blotto and incubated for 30 min at room temperature. The MAbs supernatants were diluted and plated 50 μl/well by column

for each MAb and incubated for 4 h. at room temperature. After incubation, the plate was washed 3 times with 100 μl of 0.5% Blotto at 10 min interval. Rabbit anti-Mouse IgG1, IgG2a, IgG2b, IgG3, IgA, IgM, kappa light chain and lambda light chain (1:20) were plated 50 μl/well by row and incubated for 4 h at room temperature. After incubation, the plate was washed 3 times with 100 μl of 0.5% Blotto at 10 min interval. The HRP-Goat anti Rabbit IgG (H+L)(1:1000) 50μl/well were plated and incubated for 2 h at room temperature. After incubation, the plate was washed 3 times as before. The immunoreactivity was revealed by adding substrate consisted of 1 mg/ml of o-phenelene diamine dihydrochoride (OPD), 0.06% H_2O_2 in 0.1M sodium citrate buffer pH 4.5 (100 μl/well) for 5 min. Reaction was stopped with 1N H_2SO_4 (100 μl/well). The optical density of each well was determined at 492 nm with a microtiter plate reader.

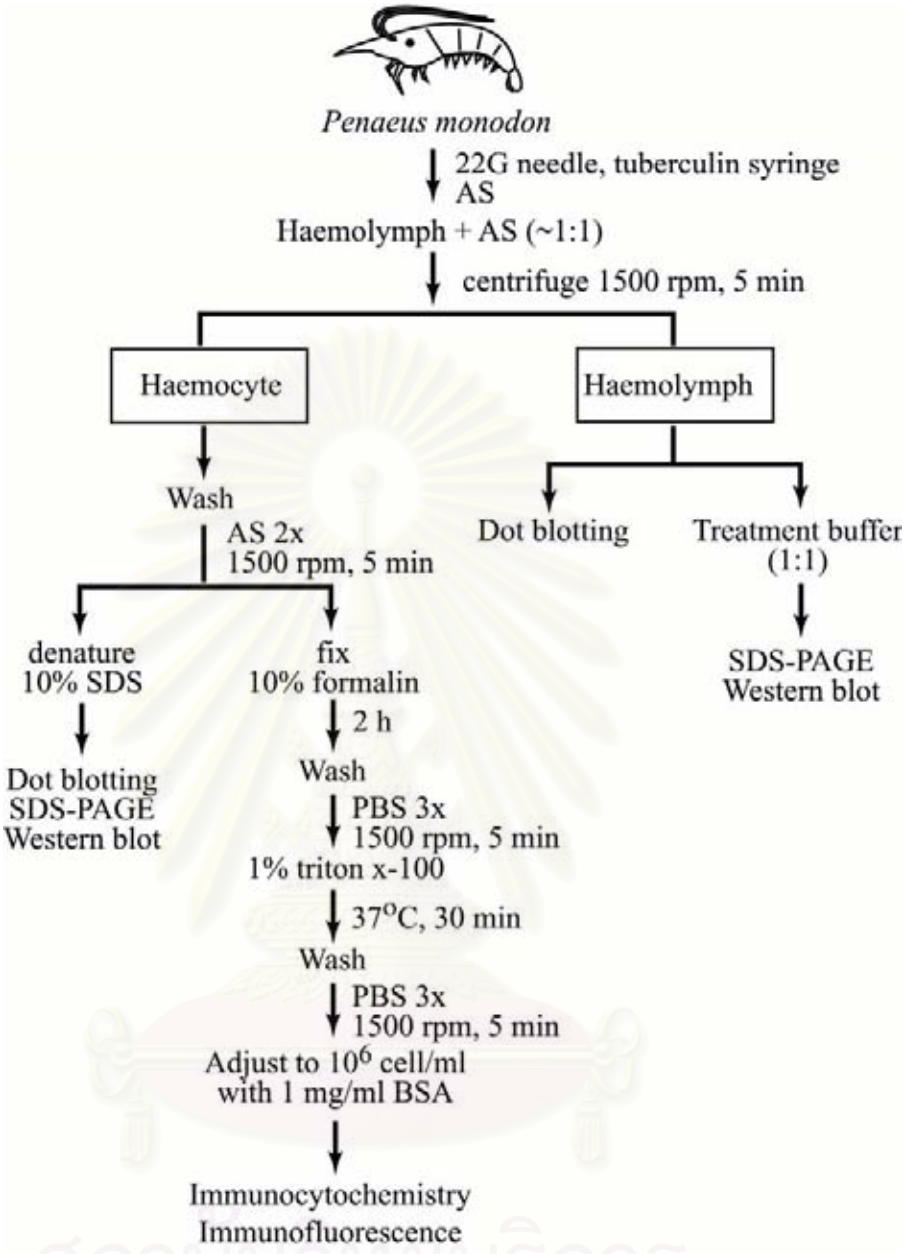


Figure 3.1 Haemocytes and haemolymph collection

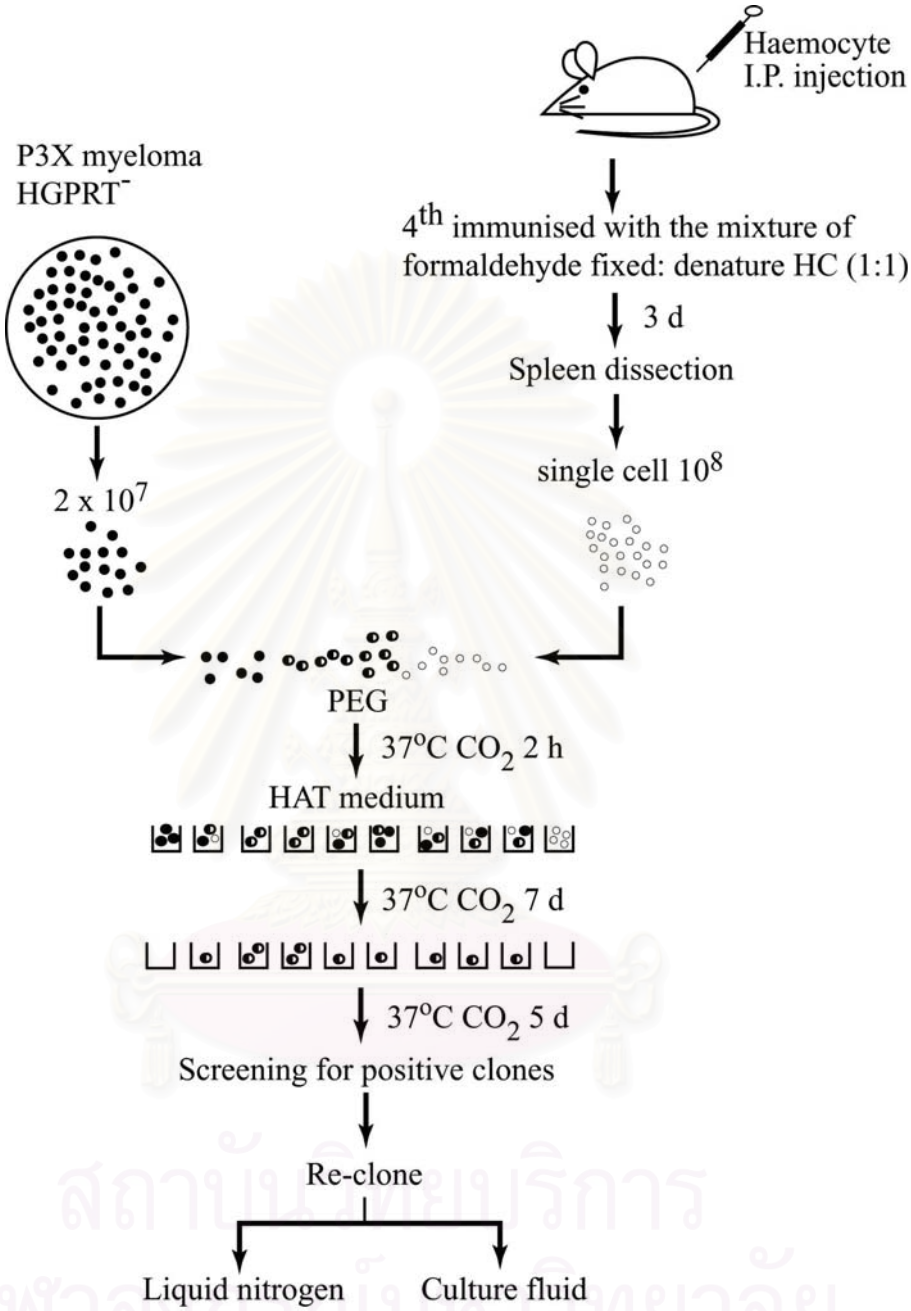


Figure 3.2 Monoclonal antibodies productions

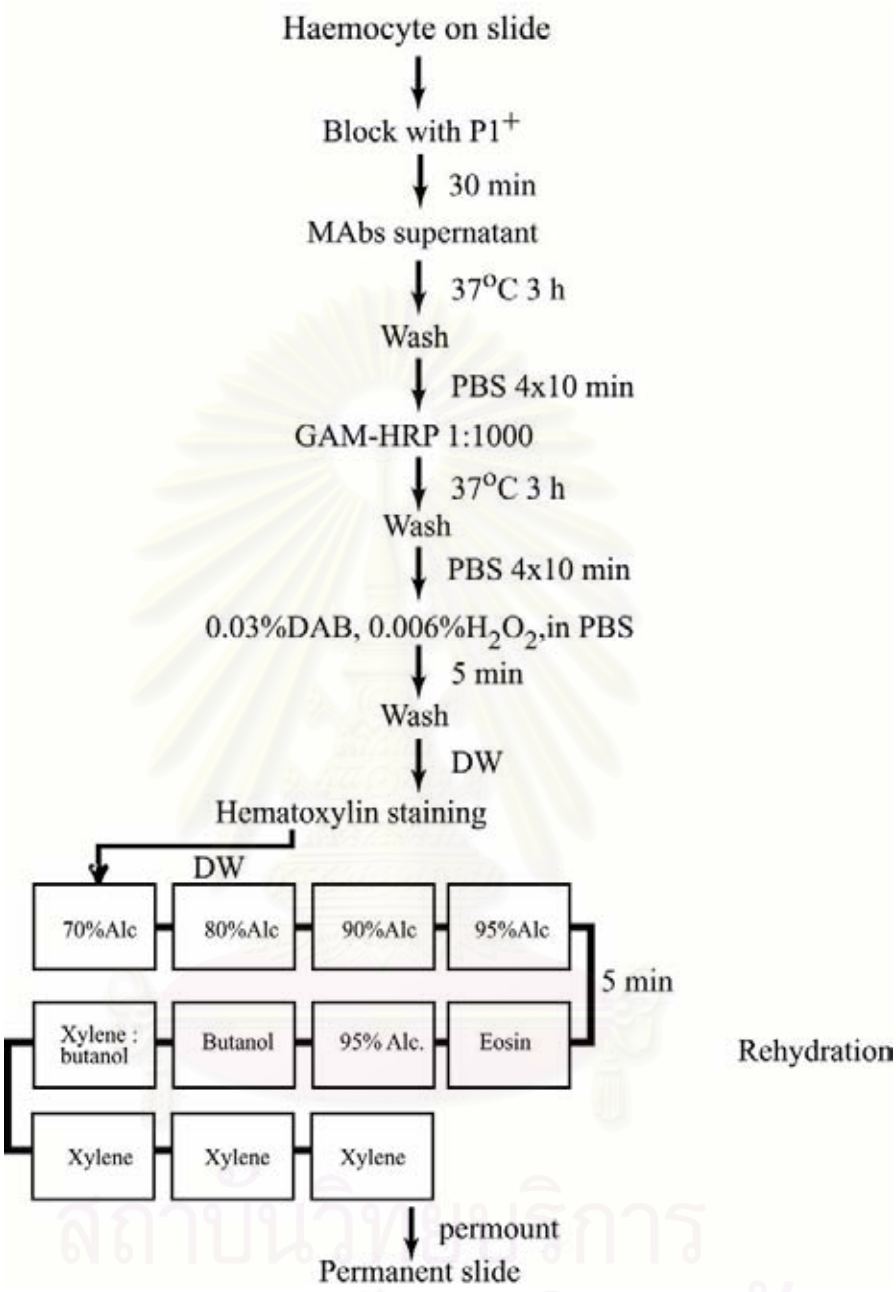


Figure 3.3 Immunocytochemical analysis of haemocyte

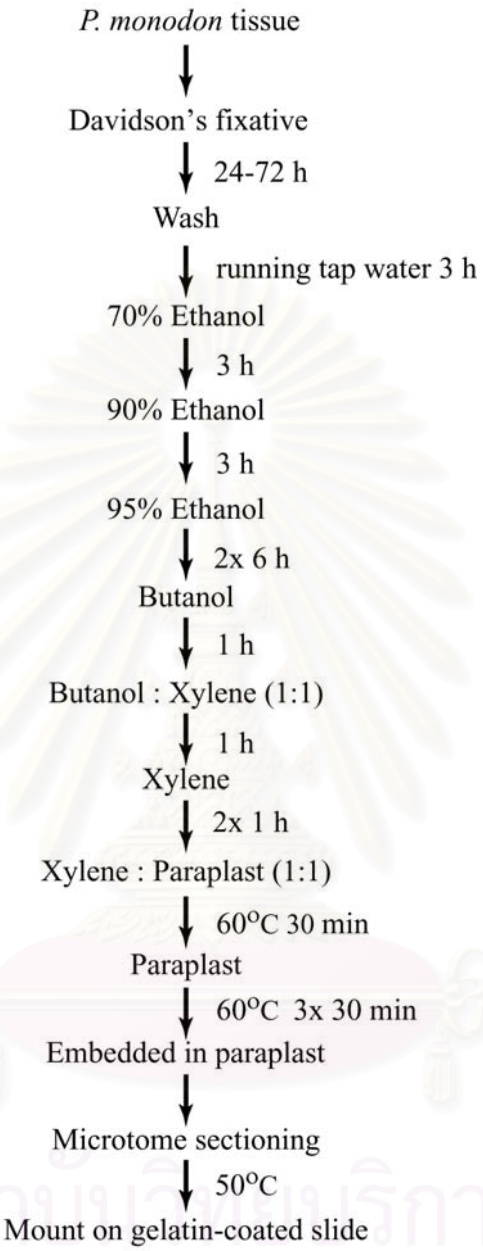


Figure 3.4 Tissue preparation for immunohistochemical analysis

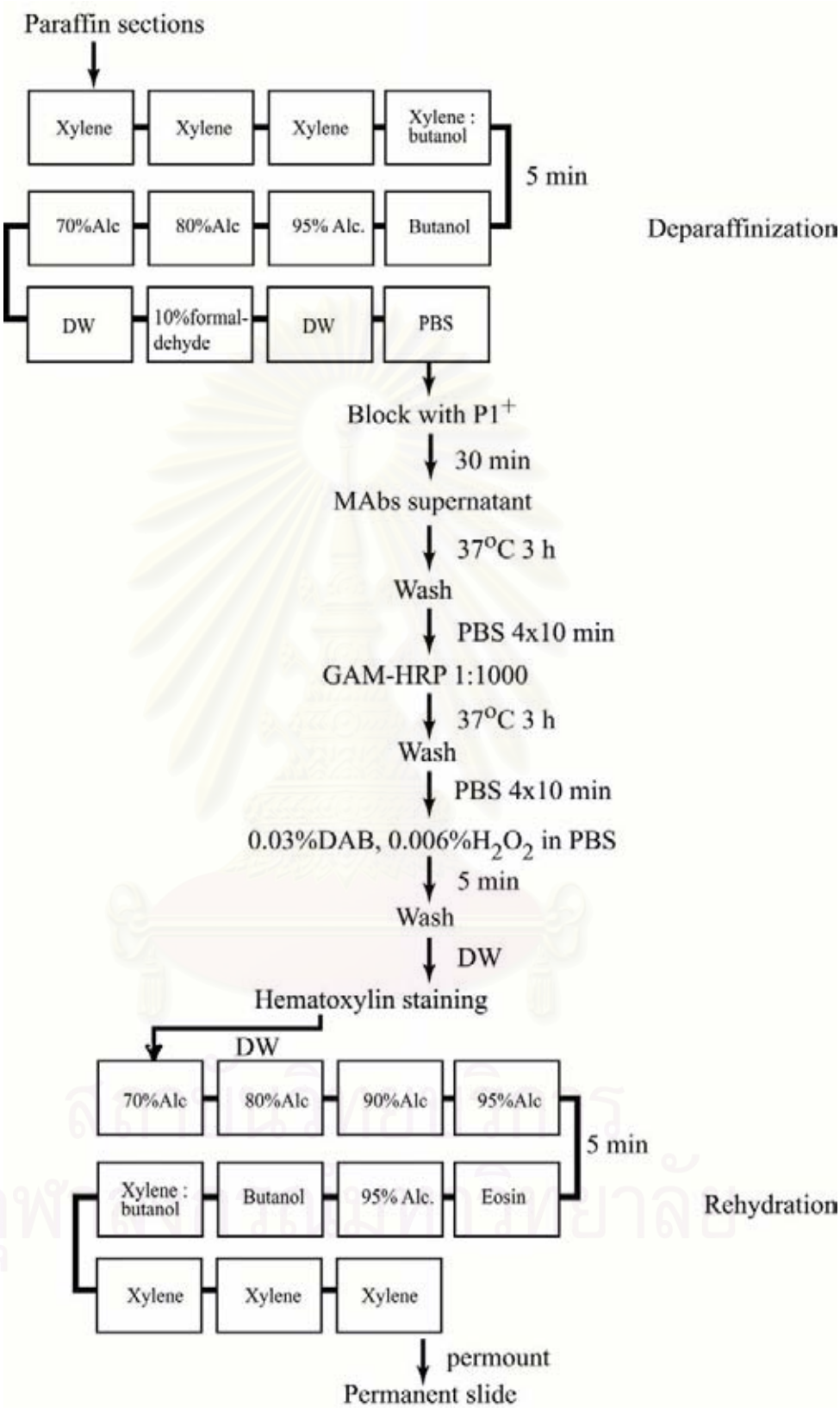


Figure 3.5 Immunohistochemical preparation

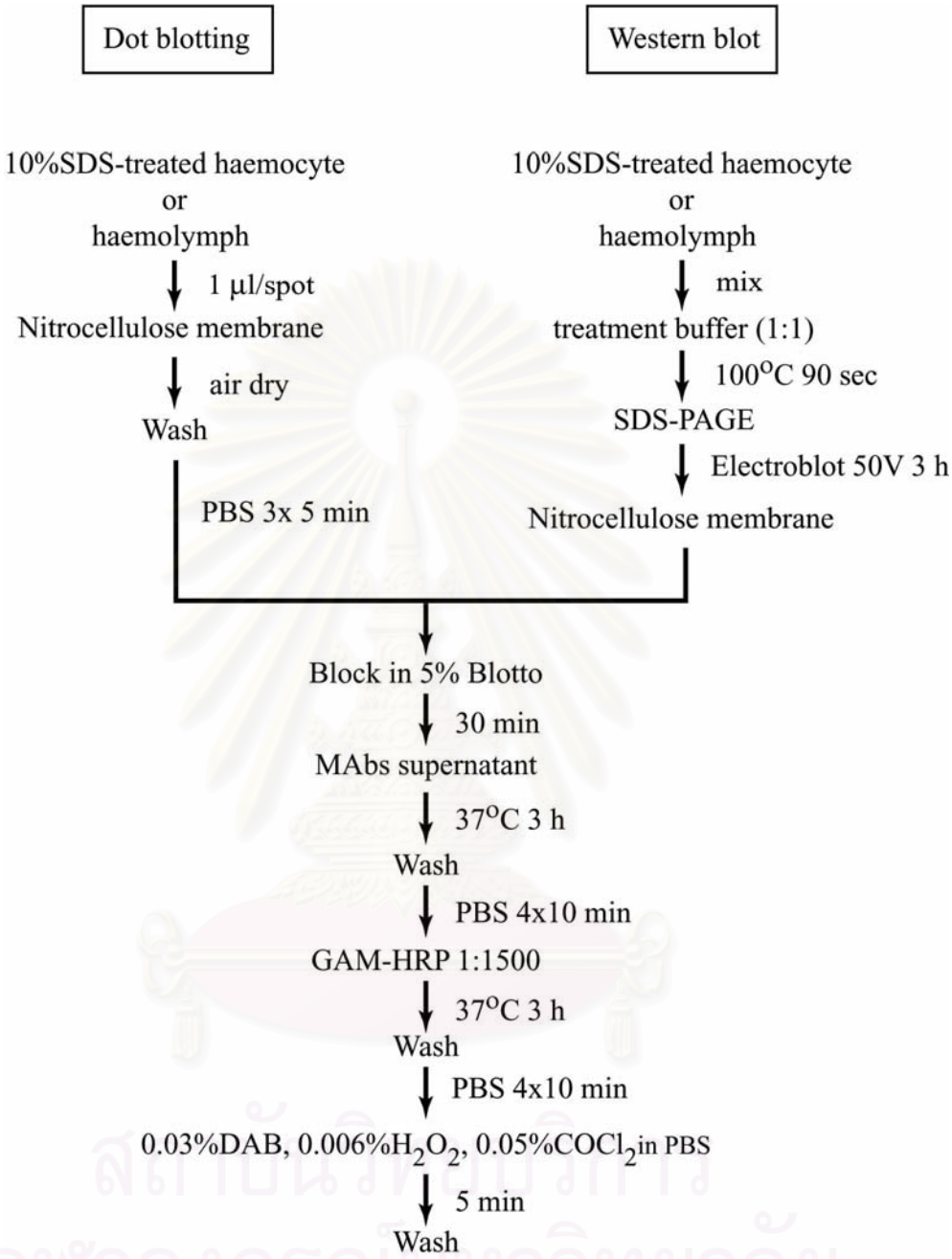


Figure 3.6 Dot blotting and Western blot analysis

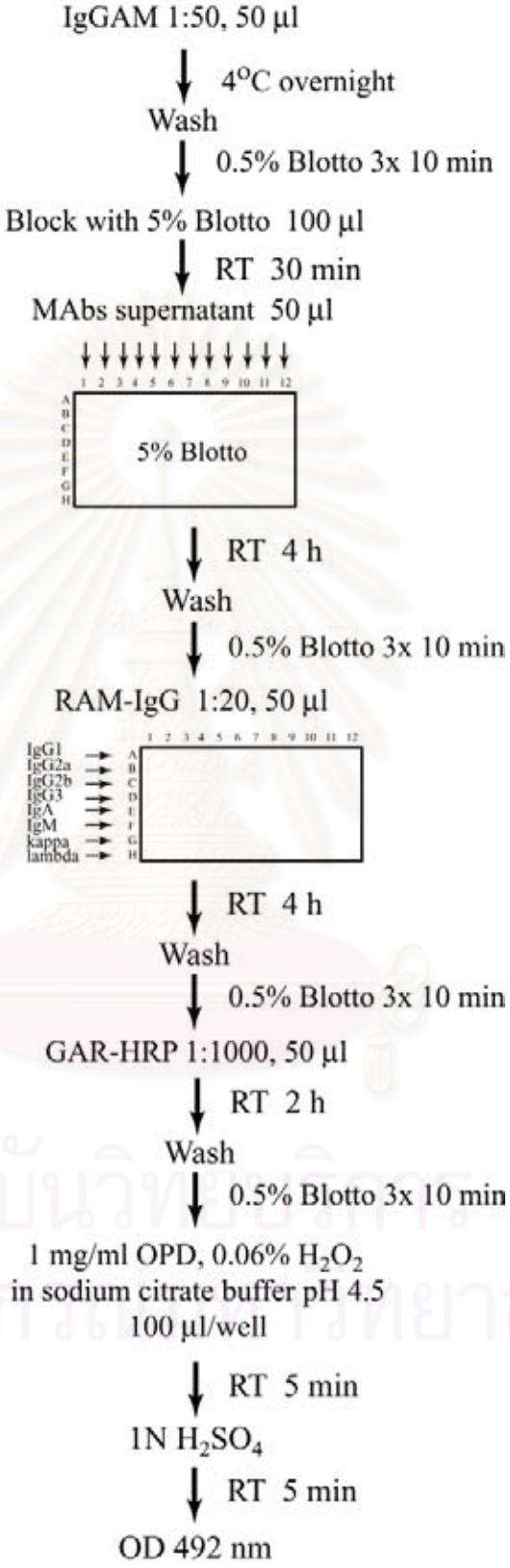


Figure 3.7 Class and subclass determination

CHAPTER IV

RESULTS

4.1 Immunization

After the fourth immunization of 4 mice with the mixed population of fixed and denatured *P. monodon* haemocytes, all mouse antisera from these mice showed brownish immunoreactivity with the fixed haemocytes and tissues of *P. monodon* by immunocytochemistry and immunohistochemistry. Serum from mouse No. 3 seemed to give strong and clear immunoreactivity distinct from other tissues, so this mouse was used as the spleen cell donor for hybridoma production.

4.2 Hybridoma production

In each fusion, from the total of 3500 hybridoma containing wells, 50-200 wells producing antibodies specific to *P. monodon* haemocytes were recognized. After the second screening, most of the antibodies bound to haemocyanin and other components of haemolymph were eliminated. The MAbs were separated into two groups as the MAbs specific to haemocytes and MAbs specific to other tissues of *P. monodon.*

4.2.1 MAbs specific to haemocytes.

From the total of MAbs produced in five fusions, only 16 of these clones did not show any cross-reactivity to other tissues and haemolymph components. The 16 MAbs could be divided into six groups depending on binding capacities to protein components of haemocytes (Figure 4.1) and the recognition of haemocyte sub-populations (Table 4.1).

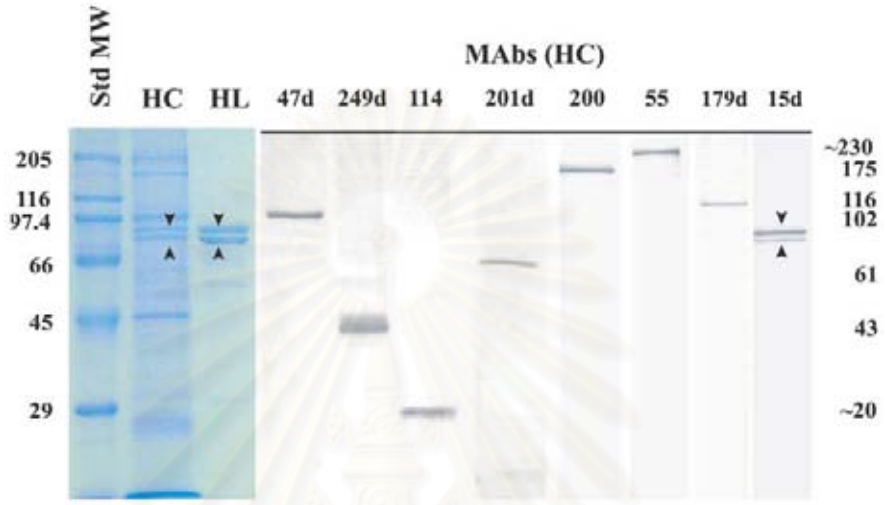


Figure 4.1 SDS-PAGE and Western blot analyses of haemocyte (HC) and haemolymph (HL) of *Penaeus monodon* using various monoclonal antibodies. Coomassie blue staining (Left 3 lanes) of standard protein molecular weight (Std MW) HC and HL proteins. Haemolymph (HL) was shown to identified the presence of two haemocyanin subunits in the haemocyte preparation (arrow head). Western blot analyses showed specific haemocyte proteins using the monoclonal antibodies indicated on top of each lane. The numbers on the right side indicate the molecular mass of the proteins recognized by each antibody.

Table 4.1 Characterization of MAbs specific to *P. monodon* haemocytes. The number of cells (mean ± S.D.) in different sub-populations, recognized by each antibody, were determined from 10 haemocyte smears (n = 10) with a hundred cells in each count. The representative MAb in each group was underlined.

MAb (sub class)	Antigenic protein (kD)	MAb specificity (%)			Other tissues
		G	SG	H	
Group 1. HC47d, 126d, 178d, 425d (IgG1)	102	±2.75±2.1 -6.86±3.5	+8.64±1.4 -18.11±5.6	+5.81±5.1 -57.83±3.4	Cells in haemopoietic tissue, Lymphoid organ (end of tubules)
Group 2. HC54, 58d, 249d (IgG1)	43	+6.35±2.2 ±3.84±2.8	+10.19±4.2 ±13.51±7.1	- -66.11±7.2	Lymphoid organ (outer layer)
Group 3. HC111, 114, 245, 327 (IgG1)	~20	+11.82±3.2 -	+3.45±2.6 -18.54±3.5	- -66.19±3.8	-
Group 4. HC201d (IgG1)	61	+7.54±2.7 ±3.17±2.7	+19.58±7.7 -7.87±2.0	+27.83±7.8 -33.99±8.3	-
Group 5. Hc136, 239 (IgG1), 200 (IgG2a)	175	+8.53±3.6 ±1.12±1.1	+16.80±5.7 -7.32±5.5	+28.45±6.8 -37.78±6.3	-
Group 6. Hc55 (IgG1)	~230	+10.60±2.1 ±2.27±2.0	+21.44±5.4 -6.74±3.8	+16.91±6.3 -42.03±8.1	-

Note G = granulocyte + stained clearly
SG = semi-granulocyte ± Light and inconsistently stained
H = hyalinocyte - Unstained

All of 16 monoclonal antibodies were capable of binding to haemocytes within various tissues such as heart (Fig. 4.2A), hepatopancreas, lymphoid organ, and haemopoietic tissue (Figure4.3 and 4.4). Two groups of MAbs cross-reacted to different layers of tubules in the lymphoid organ and one of these groups recognized sub-populations of cells in the haemopoietic tissue. The specificities of each group of antibodies is summarized in Table 4.1.

The first group consisted of four monoclonal antibodies (HC 47d, 126d, 178d, 425d), that bound to a 102 kD protein (Figure 4.1) and showed strong immunoreactivity with small sub-populations of hyalinocytes (H) and semi-granulocytes (SG) with fine granules (Figure 4.2B and C panel 1). Faint staining of a few granulocytes (G) with coarse granules was occasionally observed (Figure 4.2B and C panel 1). The antibody also showed strong cross-reactivity to cells in the end of the tubules in the lymphoid organ (Figure 4.3A and B panel 1) and sub-populations of cells in the haemopoietic tissue (Figure 4.3 A and B panel 2). However, the hypertrophic cells in the spheroid regions were not stained.

The second group consisted of three monoclonal antibodies (HC 54, 58d and 249d) that bound to a 43 kD protein (Figure 4.1). Most of the G and about half of the SG with coarse granules were stained intensely, but about one third of G and another half of SG with fine granules demonstrated very light staining.(Figure 4.2B and C, panel 2). The antibodies also bound to the cells in the outer layer of the tubules in the lymphoid organ (Figure 4.4A and B). Most of the spheroid cells were not stained. However, very few cells around the peripheral region occasionally showed very light staining (Figure 4.4A).

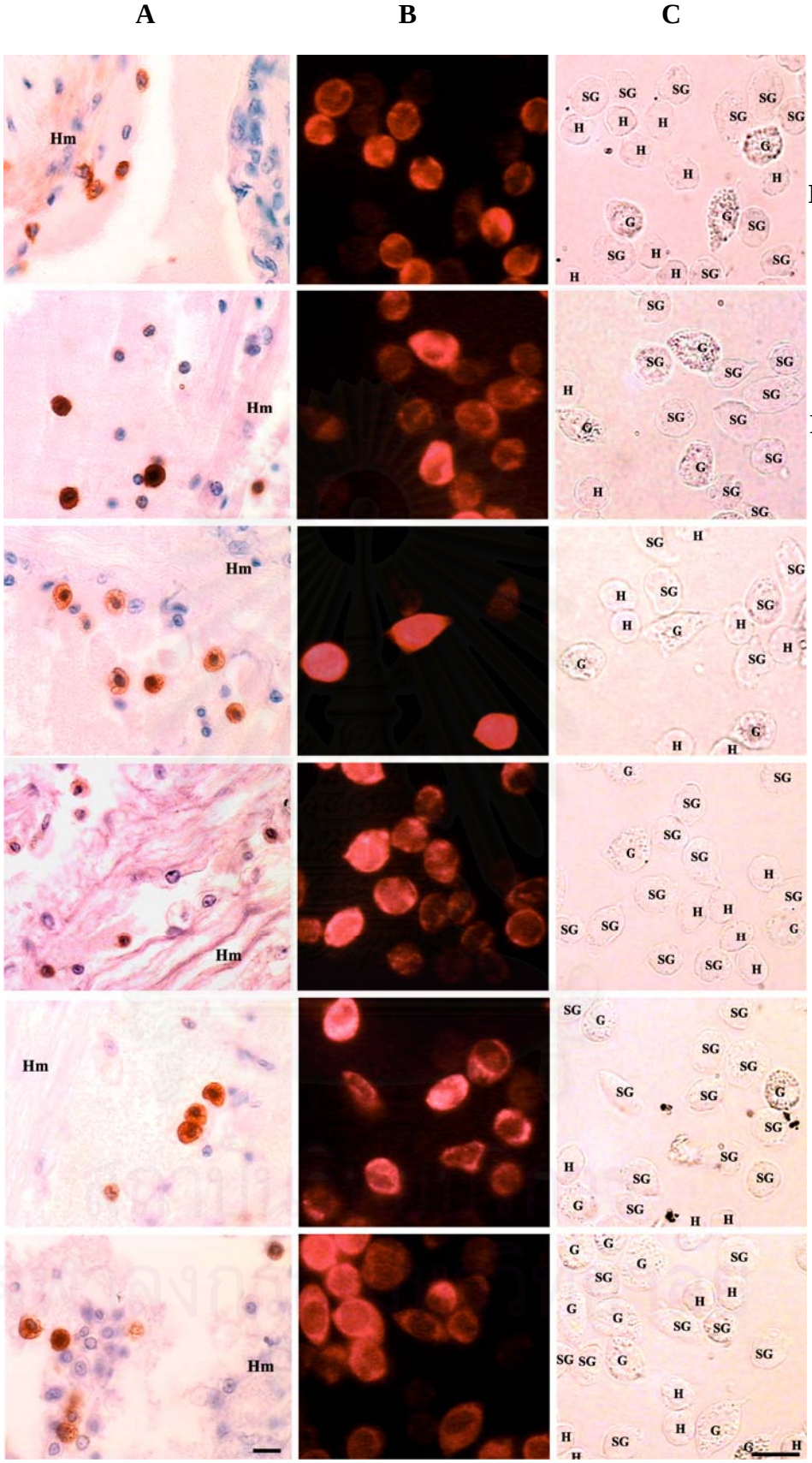


Figure 4.2 Immunohistochemistry of heart tissues (A); and immunofluorescence using various monoclonal antibodies (1-6) (B) and bright field images of haemocytes in the same area of haemocyte smear (C). In column A, Hm = heart muscle, Ge = gill epithelium. In column C, G = granulocyte, SG = semi-granulocyte, H = Hyalinocyte. Scale bar = 10 μm

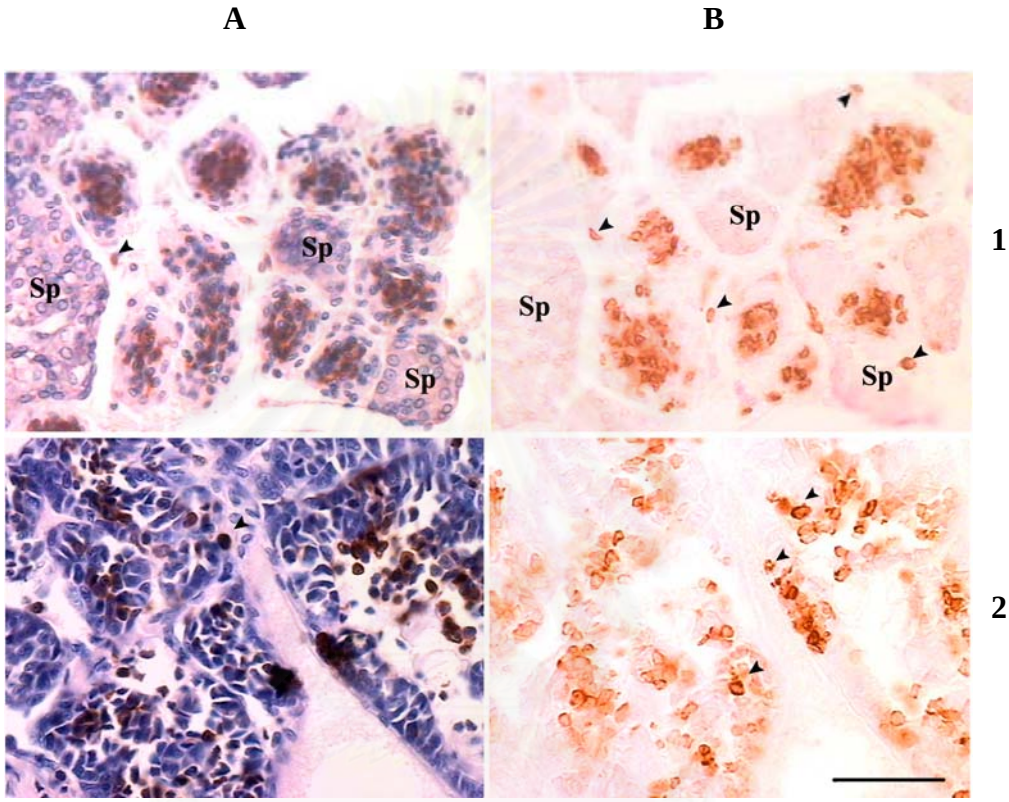


Figure 4.3 Immunoreactive tissue of *P. monodon* stained with MAb HC47d. The pictures on the left (A) are counter-stained with H&E while the picture on the right (B) is from nearby section only counter-stained with eosin-Y. The antibody recognized cell in the end of lymphoid organ tubule Sp = spheroid (1A, 1B) and some population of cell in haemopoietic tissue (2A, 2B). Arrowheads indicated the infiltrated haemocytes. Scale bar = 50 μm

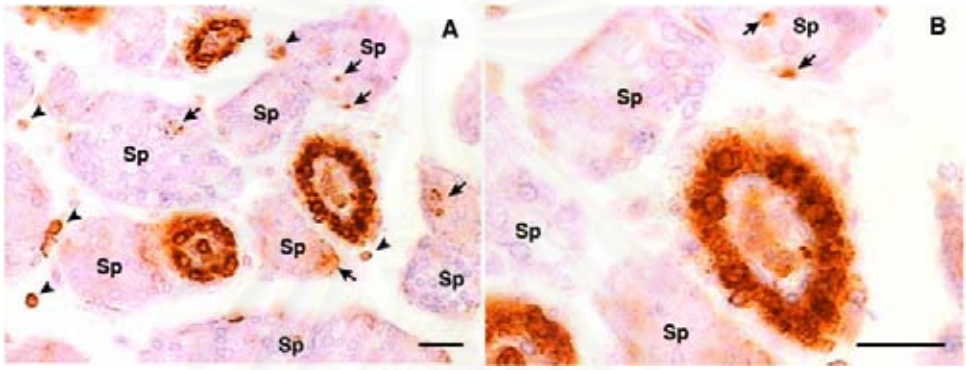


Figure 4.4 Immunohistochemistry of lymphoid organ using MAb HC249d at low (A) and high (B) magnification. The antibody recognized mainly the outer cell layer of the lymphoid tubule. A few cells in outer region of spheroid (Sp) also recognized with very light signal. Arrowheads indicated the infiltrated haemocytes. Arrows indicate the immunoreactivity in peripheral spheroid cells. Scale bar = 20 μm

The third group consisted of four monoclonal antibodies (HC 111, 114, 245 and 327) that bound to an approximately 20 kD protein (Fig. 4.1). They recognized all the G and a small fraction of the SG (Fig. 4.2B and C, panel 3) but did not show binding to H or most of the SG.

The fourth, the fifth and the sixth groups consisted of one (HC 201d), three (HC 200, 136, 239) and one (HC55) MAbs, respectively. They bound to different proteins of molecular masses of 61 kD, 175 kD, and ~230 kD, respectively (Figure 4.1), and strongly recognized similar proportions of sub-populations of G, SG and H. Only small proportions of G and SG were stained lightly or unstained (Figure 4.2B and C panel 4, 5 and 6).

None of the MAbs specific for haemocyte showed any cross-reaction with other components in haemolymph or tissues such as heart muscle, gill epithelium (Figure 4.2A) hepatopancreas and muscle. However, antibodies in the first group occasionally demonstrated very light staining in the wall of large vessels connected to the heart (Figure 4.2A panel 1). The staining of haemocytes within those tissues was observed clearly.

4.2.2 MAbs specific to other tissues of P. monodon

From the total of MAbs produced in five fusions, there are 6 MAbs that showed cross-reactivity to other tissues and haemolymph components. Each MAbs reacted with different tissue except one group of MAbs, HC15d, HC171d and HC383d that reacted with double bands of haemocyanin in haemolymph component as shown in western blot analysis. (Figure 4.1) The rest of MAbs could be stained the tissue, haemocytes and haemolymph as shown in figure 4.5-4.8. The MAbs specificity was summarized in table 4.2 as followed.

Table 4.2 Characterization of MAbs specific to *P. monodon* tissues. The tissues stained with MAbs were described follow Bell and Lightner (1988). The representative MAb in each group was underlined.

MAbs (sub class)	Antigenic protein (kD)	MAbs Specificity				Haemocytes
		Hpt	Lo	CNS	Other tissues	
HC32 (IgM)	-	+	Tubules	-	-	SG, H
HC39, HC62 (IgM)	-	+	Sp Tubules	N	Hrt, Gill, Hep	G, SG, H
HC31, HC179d (IgM)	116	-	Sp	N	Epi	G, SG, H

Note Hpt = Haemopoietic tissue, Lo = Lymphoid organ, Sp = Spheroid cell, CNS = Central nervous system, N = Neuron, Epi = Epicardium, Hrt = Heart, Hep = Hepatopancreas, G = Granulocyte, SG = Semi-granulocyte, H = Hyalinocyte, + = stained, - = Unstained

The first 2 groups of MAbs did not bind to the denatured protein of haemocyte in SDS-PAGE in western blot analysis but it also reacted with some specific tissue.

First MAb, HC32 (IgM) stained the tubular cell of lymphoid organ and some group of cells in haemopoietic tissue (figure 4.5). HC32 also reacted with some population of haemocytes as shown in figure 4.8.

The second MAbs consist of 2 clones HC39 and HC62 (IgM) reacted with cells in haemopoietic tissue, giant cell and ventral nerve cord and some nucleus of cell in heart, gill and hepatopancreas (figure 4.6). It also bound to all types of haemocyte with different level of intensity in immunofluorescense as shown in figure 4.8.

The third group of MAbs consists of 2 clones, HC31 and HC179d (IgM). The MAbs bound to haemocyte protein with molecular mass of 116 kD (figure 4.1). The MAbs stained the spheroid cell and tubular cells in lymphoid organ, the neuron in central nervous system. (figure 4.7) They also bound to the epicardium tissue in heart and stained with all types of haemocytes observed by immunofluorescense and immunocytochemistry. (figure 4.8).

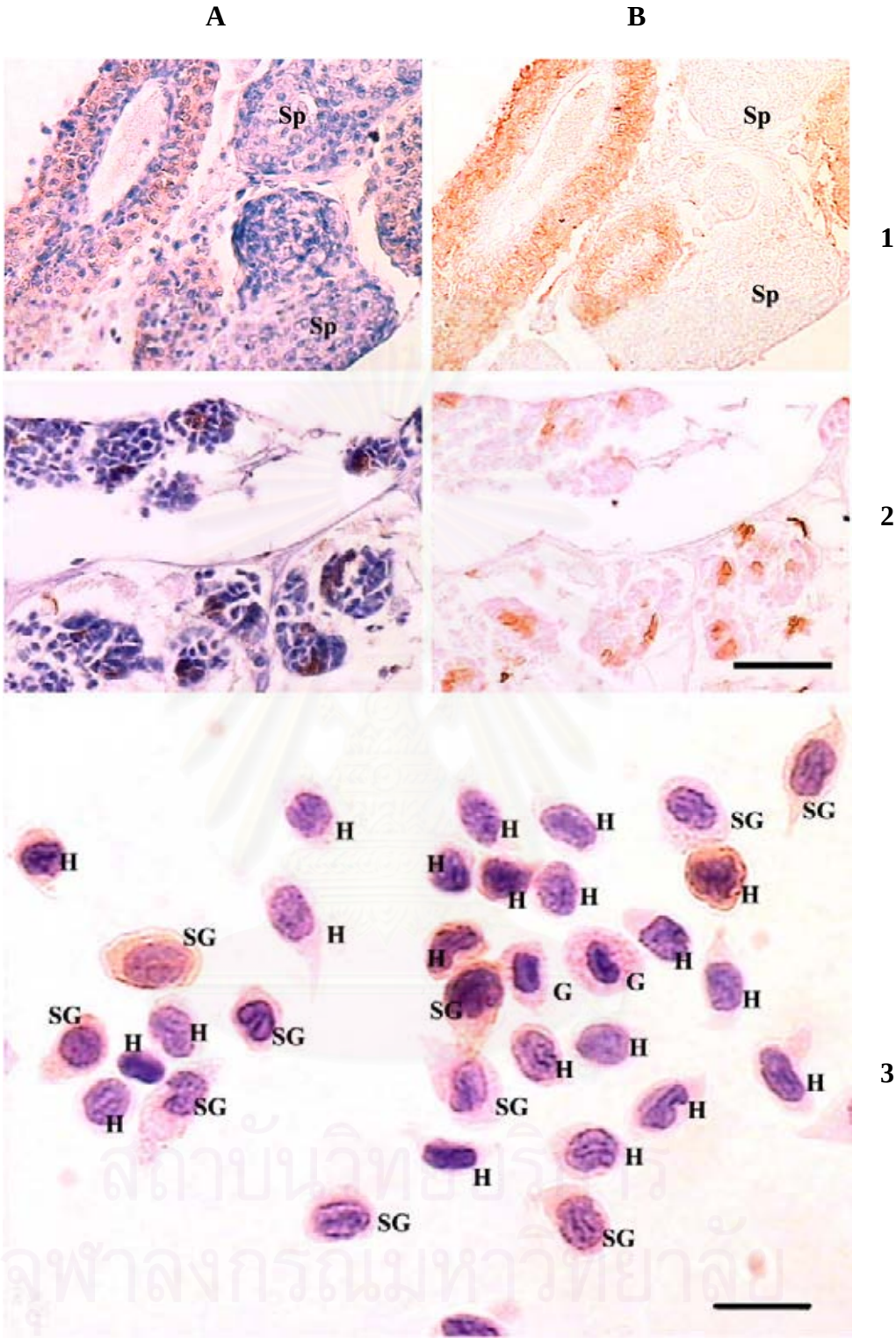
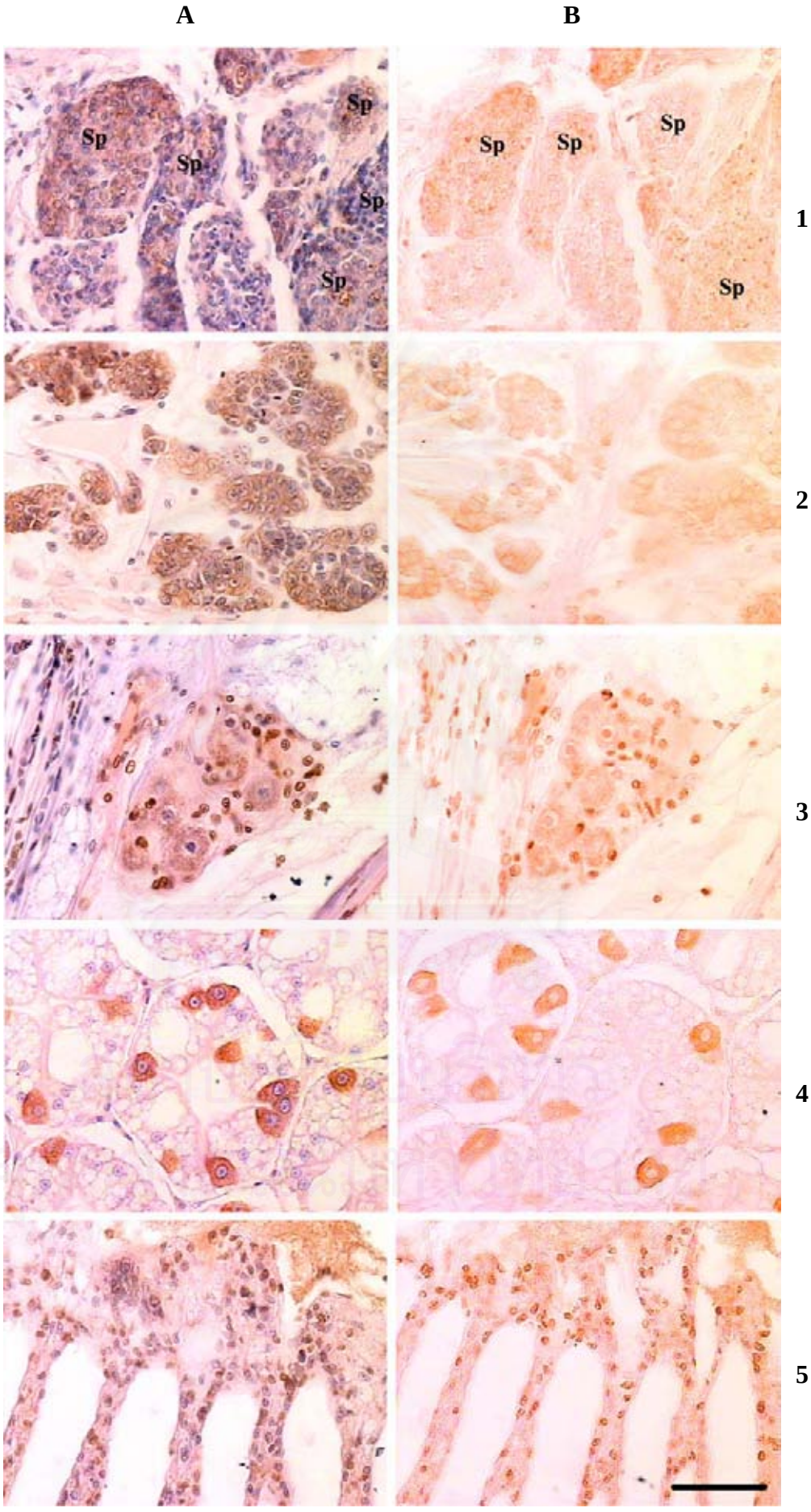


Figure 4.5 Immunoreactive tissues of *P. monodon* stained with MAb HC32. The pictures on the left (A) is counter-stained with H&E while the picture on the right (B) is from nearby section only counter-stained with eosin-Y. The antibody recognized tubular cell in lymphoid organ (1) Sp = spheroid and haemopoietic cell (2) Scale bar = 50 μm and immunocytochemistry of haemocytes (3) counter-stained with H&E, G = granulocyte, SG = semi-granulocyte, H = hyalinocyte scale bar = 10 μm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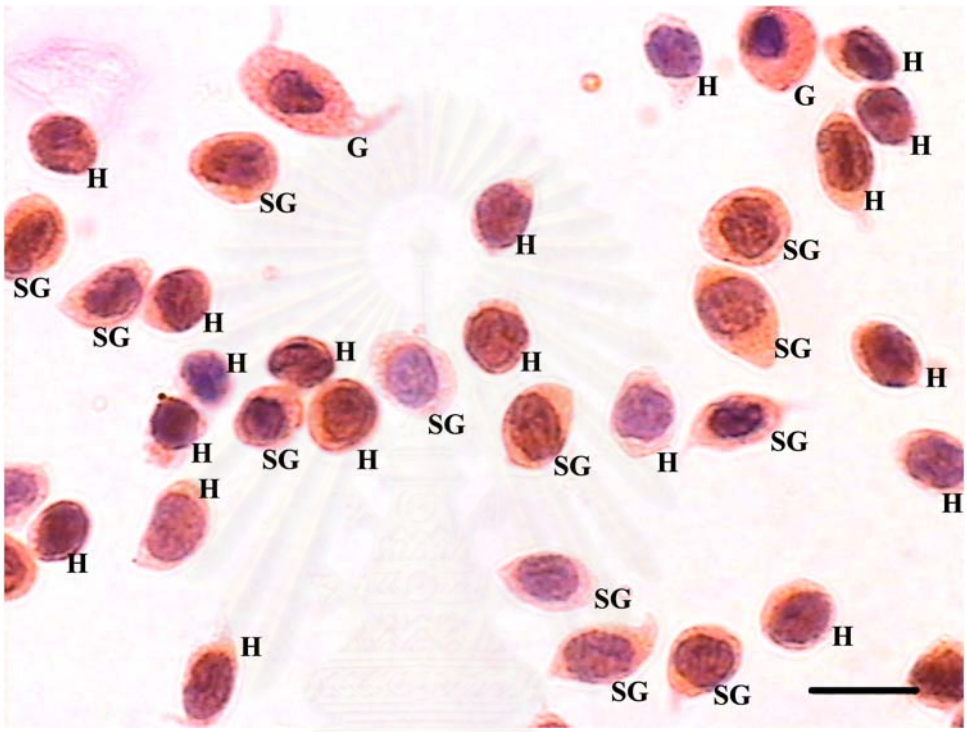

Figure 4.6 Immunoreactive tissue of *P. monodon* stained with MAb HC39. The pictures on the left (A) is counter-stained with H&E while the picture on the right (B) is from nearby section only counter-stained with eosin-Y. The antibody recognized cells in lymphoid organ (Sp) and lightly stained in tubule cell (1), haemopoietic tissue (2), neurons (3), F-cell of hepatopancreatic tubule (4), nucleus of gill (5) Scale bar = 50 μm and immunocytochemistry of haemocytes counter-stained with H&E (6) G = granulocyte, SG = semi-granulocyte, H = hyalinocyte scale bar = 10 μm

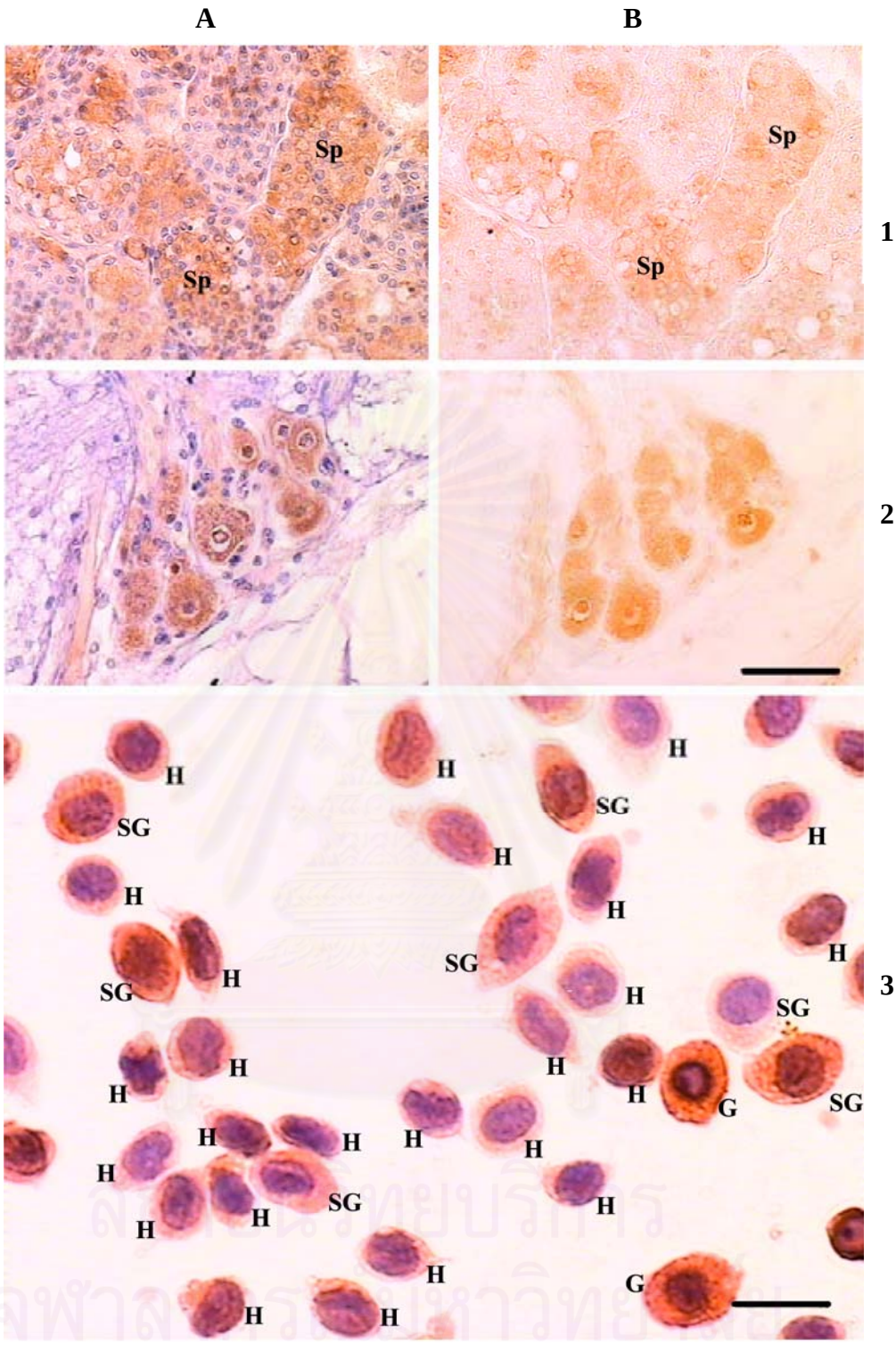

Figure 4.7 Immunoreactive tissue of *P. monodon* stained with MAb HC179d. The pictures on the left (A) are counter-stained with H&E while the picture on the right (B) is from nearby section only counter-stained with eosin-Y. The antibody recognized spheroid cell (Sp) in lymphoid organ (1) and neuron (2). Scale bar = 50 μm and immunocytochemistry of haemocyte (3) counter-stained with H&E G = granulocyte, SG = semi-granulocyte, H = hyalinocyte scale bar = 10 μm

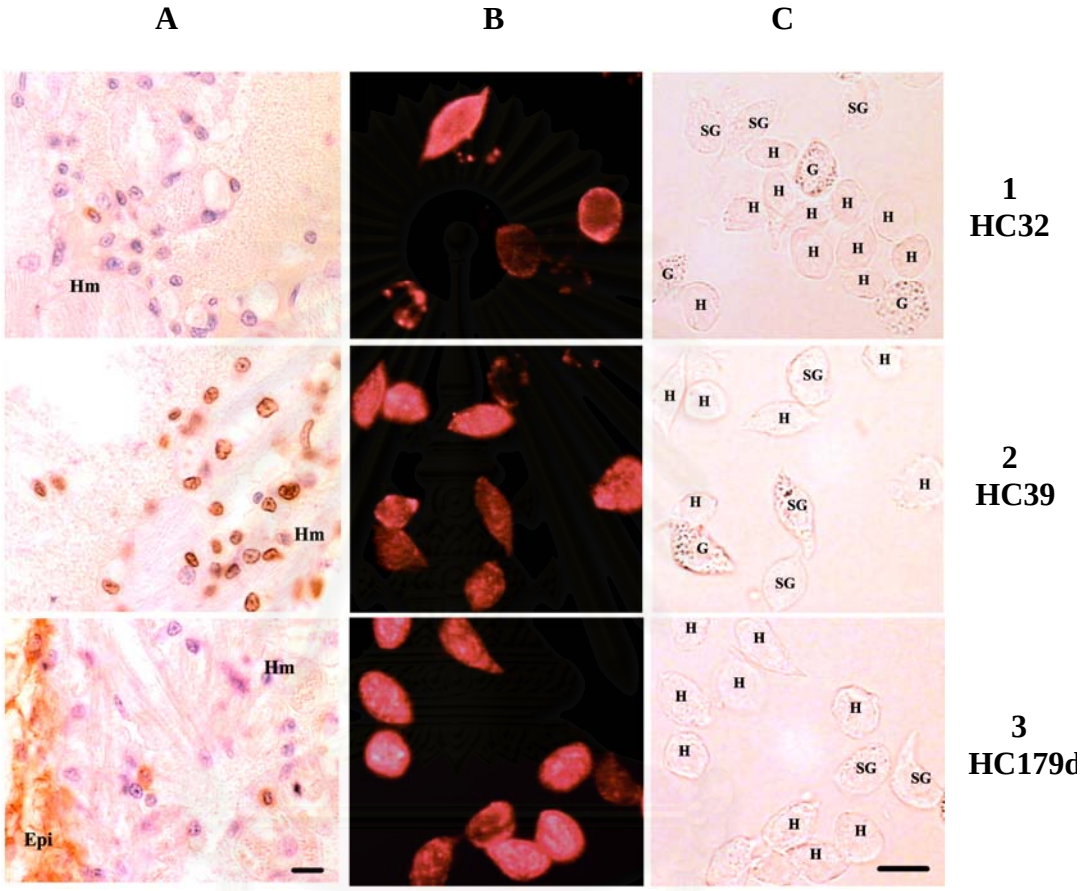

Figure 4.8 Immunohistochemistry of heart tissues (A); and immunofluorescence using various monoclonal antibodies (1-3) (B) and bright field images of haemocytes in the same area of haemocyte smear (C). In column A, Hm = heart muscle, Epi = Epicardium. In column C, G = granulocyte, SG = semi-granulocyte, H = hyalinocyte. Scale bar = 10 μm

CHAPTER V

DISCUSSION AND CONCLUSION

5.1 Haemocyte collection

A special care for haemocytes preparation in this study was developed in order to enhance the interaction of monoclonal antibodies and their target epitopes. Prior to formaldehyde fixation it was necessary to wash the haemocytes with anti-coagulants to reduce the haemolymph protein especially haemocyanin in which the covering of these molecules can conjugate to the surface of haemocyte while fixing. These can block the penetration of the antibodies to the target molecules in the cytoplasm of haemocytes.

Crustacean haemolymph can be collected in a low pH, citrate-EDTA anticoagulant buffer (citric acid serves to delay cell breakdown while EDTA inhibits proPO activation and prevents the clotting reaction) and the haemocytes can be separated by density gradient centrifugation in Percoll, which yields different proportions of the cell population depending on the species. (Johansson and Söderhäll, 1989; Johansson et al, 2000)

5.2 Monoclonal antibodies production

Six groups of monoclonal antibodies specific to haemocytes of *P. monodon* were isolated from a mouse immunized with a mixture of formaldehyde-fixed and SDS-treated haemocytes. The properties of these monoclonal antibodies are different from those of previous reports (Rodriguez et al., 1995; Sung et al., 1999; Van de Braak et al., 2000) in that all monoclonal antibodies bound to different proteins of

haemocytes and did not show any cross reaction to other components of haemolymph. Most of the antibodies specifically bound to sub-populations of G, SG and H. Only antibodies in group 1 do not recognize or recognized G very faintly, and antibodies in group 2 and 3 do not recognize H. Moreover, the two groups of antibodies, group 1 and 2, cross-reacted with different layers of cells in the tubules of the lymphoid organ and antibodies in group 1 cross-reacted with sub-populations of cells in the haemopoietic tissues.

The accurate identification of haemocyte sub-populations could not be done with high confidence by light microscopy even though various investigators have used similar criteria on haemocyte classification including nuclear to cytoplasm ratio and the presence of, size and number of granules in cytoplasm. Different ratios of haemocyte sub-populations have been reported (Martin and Graves, 1985; Le Moullac et al., 1997; Tsing et al, 1989; Owen and O' Neill, 1997; Sung et al., 1999; Van de Braak et al., 2000), even with samples from the same species of shrimps, using different methods (Owens and O'Neill, 1997). The haemocyte ratio in our experiment was close to a previous report by Tsing et al.(1989). The difference in haemocyte sub-populations is postulated to be due to the difference in physiological stage such as molting cycle (Le Moullac et al., 1997). The difficulty of haemocyte classification may be due to the complexity of haemocyte sub-populations. In the 3 sub-populations (H, SG and G), there were at least two haemocyte subsets in SG and G identified by monoclonal antibodies Z6A5 and Z6H8 (Sung et al., 2002). Most of the monoclonal antibodies obtained in this study recognized different ratios of H, SG and G sub-populations. Therefore, it is possible that circulating haemocytes may consist of different maturation stages of haemocyte sub-populations as previous estimates state

that about 8-10% of circulating haemocytes are undifferentiated haemocytes (Tsing et al.,1989).

Haemopoietic tissue was proposed to be the site for haemocyte production and supply, however, the classification of the cells and lines of haemocyte differentiation were not clear (Johansson et al., 2000). Electron microscopic studies and immunohistochemistry with WSH8 monoclonal antibody, recognized sub-populations of cells in haemopoietic tissue and granules of non-fixed haemocytes (Van de Braak, 2000). Four types of cells could be identified; type 1: a precursor of type 2: (large granular), type 3: (small granular) young haemocytes, and type 4: interstitial cell. The WSH8 (bound to a 35 kDa protein) and reacted with type 2 and type 3 cells. Type 2 cells were morphologically and antigenically indistinguishable from hyaline cells and it was suggested that H cells were the young and the immature cells of both G and SG that were released from haemopoietic tissues (Van de Braak et al., 2002a). The first group of monoclonal antibodies in this study recognized a 102 kDa protein present in 2 sub-populations of haemocyte (SG and H) and sub-populations of cells in haemopoietic tissue and cells at the end of the tubules in the lymphoid organ. The second group of antibodies recognized a 43 kDa protein of G and SG, and only the cell layer of the tubular portion in the lymphoid organ. This evidence agrees with the electron microscopic study of the lymphoid organ (incorrectly called haemopoietic nodules) in *Sicyonia ingentis* which demonstrated that there were two distinct developmental cell lines of agranular and granular pathways (Martin et al., 1987).

The MAb HC32 bound to most sub-populations of cell in the haemopoietic tissue and most cells in lymphoid tubules (Figure 4.5, 5.1 and 5.2). However, it bound strongly to semi-granulocytes sub-population and lightly to hyalinocytes but

not recognized granulocyte. It is possible that this MAb bind the antigen that express during the early and middle stage of development (hyalinocyte and semi-granulocyte), then disappeared during the later stage of development (granulocyte).

The MAb HC39 bound to all haemocytes and many cell types such as neurons, hepatopancreas and gill epithelium which may show in special function of those cell while HC179d is more specific which recognized most haemocyte and neurons except few percent of hyaline cells. This antibody may show higher stage of development than HC39.

Strikingly, no immunoreactivity was observed with these two groups of antibodies, group 1 and 2, in spheroid cells. Moreover, this study demonstrated that the common antigens shared among haemocytes, haemopoietic cells and lymphoid organ may indicate the developmental relationship in which the lymphoid organ may serve as the differentiation site for sub-populations of granulocyte and semi-granulocyte after being released from the haemopoietic tissue. Characterization of the cells in different regions of the lymphoid organ revealed that the cells in lymphoid tubules exhibited distinctive lower phenoloxidase and peroxidase activities than the cells in the spheroid region that had similar characteristics to G and SG (Anggraeni and Owen, 2000). The different cell portion in lymphoid tubules may reflect the different stages of haemocyte development from H to SG and G in that the immature cells from the circulation appeared to migrate from the tubule lumen into the stromal matrix, underwent differentiation and migrated out to the circulation via the haemal sinus behind the stromal matrix during maturation to SG and G. On the other hand, the cells in both portions of the lymphoid tubules were clearly distinguished by two sets of MAbs that recognized different sub-populations of circulating haemocytes.

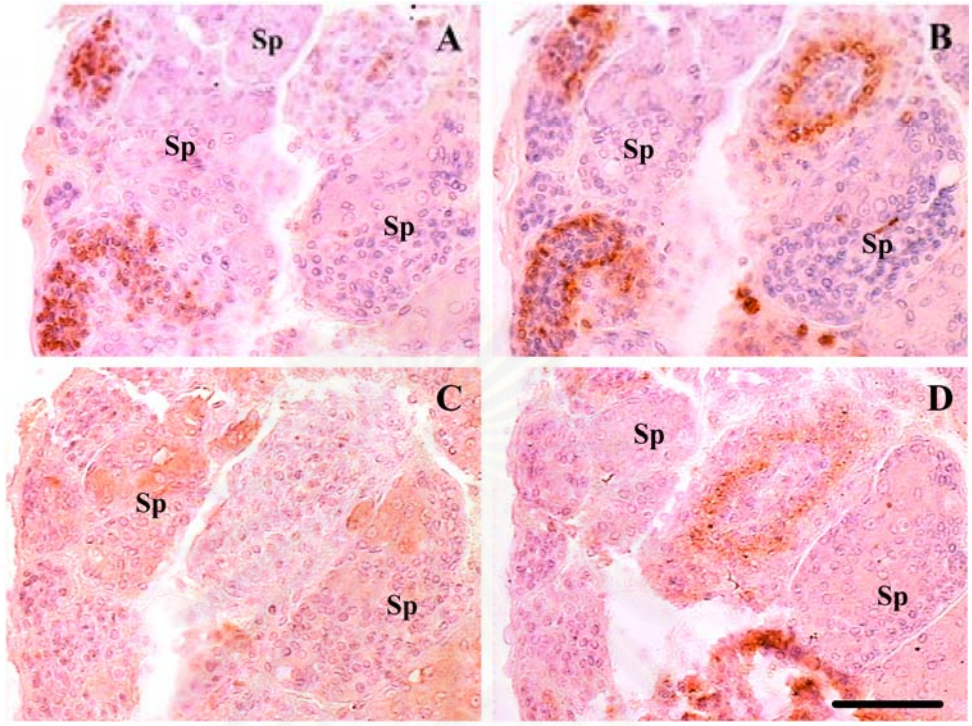

Figure 5.1 Immunoreactive serial section of lymphoid organ of *P. monodon* stained with MAbs HC47d (A), HC249d (B), HC179d (C) and HC32 (D). The tissues are counter-stained with H&E. Sp = spheroid Scale bar = 50 μm.

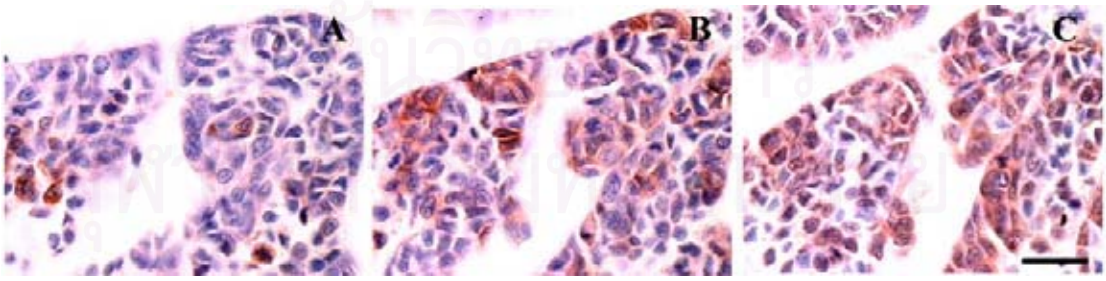

Figure 5.2 Immunoreactive serial section of haemopoietic tissue of *P. monodon* stained with MAbs HC47d (A), HC32 (B) and HC39 (C). The tissues are counter-stained with H&E. Scale bar = 20 μm.

Therefore, two portions of the lymphoid tubule may serve as the differentiation sites for different sub-populations of haemocytes where the end of tubule is for H and fine granular SG and the outer layer is for G and coarse granular SG as suggested by Martin et al. (1987).

On injection of *Vibrio* bacteria into *P. monodon*, the bacteria were phagocytosed by WSH8 antibody positive cells in the tubular lumen of the lymphoid organ, then in the tubular wall and finally in the outer wall of the spheroid. This was accompanied by the presence of WSH8 positive haemocytes that appeared to move into the tubular wall and then dispersed in the outer wall with gradual loss of WSH8 immunoreactivity (Van de Braak et al, 2002b). From this observation, it is possible that the spheroid may be the site of pre-maturation of undifferentiated haemocytes in response to various antigens leading to apoptosis of the cells. Spheroid cells had similar characteristics to G and SG in prophenoloxidase and peroxidase activities (Anggraeni and Owen, 2000). The number and size of spheroids in the lymphoid organ was observed to increase in chronic infections with various kinds of microorganisms including bacteria (Van de Braak et al., 2002b) and viruses such as Taura virus (Hasson et al., 1999); Lymphoid organ virus (Spann et al., 1995), spawner-isolated mortality virus (Fraser and Owen 1996).

Haemocyte identification based on observable morphology with light and electron microscopy may not be sufficient to distinguish the sub-populations since undifferentiated sub-populations of haemocytes may include and cause uncertainty, especially in distinguishing between H and SG. The development of more specific monoclonal antibodies to sub-populations of cells in the haemopoietic tissue, lymphoid organ and circulating haemocyte would help to accurately identify and

determine the relationship among haemocyte sub-populations throughout development. An immunoelectron microscopic and co-localization of MAb specific antigens study would help to elucidate the haemocyte sub-populations as well.

REFERENCES

Anggraeni, M.S., and Owens, L. 2000. The haemocytic origin of lymphoid organ spheroid cells in the penaeid prawn *Penaeus monodon*. Disease of Aquatic Organism 40 : 85-92.

Bachére, E., Mialhe, E., Noël, D., Boulo, V., Morvan, A. and Rodríguez, J. 1995. Knowledge and research prospects in marine mollusc and crustacean immunology. Aquaculture 132 : 17-32.

Bailey-Brock, J.H. and Moss, S.M. 1992. Penaeid taxonomy, biology and zoogeography. In A. W. Fast and L. J. Lester (eds), Marine shrimp culture: principles and practices, pp 9-27. London : Elsevier Science Publishers.

Barrett, C.H.. 1994. Hybridomas and monoclonal antibodies. In V.S. Malik and E.P. Lillehoj (eds), Antibody technique, 71-102. London : Academic Press,

Bell, T.A. and Lightner, D.V. 1988. A handbook of normal penaeid shrimp histology..World Aquaculture Society. Lawrence. Kansas : Allen Press Inc. 114 pp.

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72 : 248-254.

Chang, C.F., Su ,M.S., Chen, H.Y. and Liao, I.C. 2003. Dietary β-1,3-glucan effectively improves immunity and survival of *Penaeus monodon* challenged with white spot syndrome virus. Fish & Shellfish Immunology 15 : 297-310.

Cheng, W. and Chen, J.C. 2001. Effects of intrinsic and extrinsic factors on the haemocyte profile of the prawn, *Macrobrachium rosenbergii*. Fish & Shellfish Immunology 11 : 53-63.

Destoumieux, D., Muñoz, M., Bulet, P. and Bachére, E. 2000. Penaeidins, a family of antimicrobial peptides from penaeid shrimp (Crustacea, Decapoda). Cellular and Molecular Life Science 57 : 1260-1271.

Destoumieux, D., Muñoz, M., Cosseau, C., Rodriguez, J. Bulet, P., Comps, M. and Bachére, E. 2000. Penaeidins, antimicrobial peptides with chitin-binding activity, are produced and stored in shrimp granulocytes and released after microbial challenge. Journal of Cell Science 113 : 461-469.

Dyrynda, E.A., Pipe R.K.and Ratcliffe, N.A. 1997. Sub-population of haemocytes in the adult and developing marine mussel, *Mytilus edulis*, identified by use of monoclonal antibodies. Cell and Tissue Research 289(3) : 527-536.

Eshhar, Z. 1985. Monoclonal antibody strategy and technique. In T.A. Springer (ed), Hybridoma Technology in Bioscience and Medicine, 1-42. New York : Plenum Press.

Flegel, T.W., Boonyaratpalin, S.and Withyachumnarnkul, B. 1997. Progress in research on yellow-head virus and white-spot virus in Thailand. In: T.W. Flegel. and I.H. MacRae (eds), Diseases in Aquaculture III, pp. 285-302. Manila Phillippines : Asian Fisheries Society.

Fraser, C.A. and Owen L. 1996. Spawner-isolated mortality virus from Australian *Penaeus monodon*. Disease of Aquatic Organism 27 : 141-148.

Gargioni, R. and Barracco, M.A. 1998. Haemocytes of the Palaemonids *Macrobrachium rosenbergii* and *M. acanthurus*, and of the Penaeid *Penaeus paulensis*. Journal of morphology 236 : 209-221.

Ghiretti-Magaldi, A., Milanesi, C. and Tognon, G. 1977. Hemopoiesis in crustacea decapoda: origin and evolution of hemocytes and cyanocytes of *Carcinus maenus*. Cell Differentiation 6 : 167-186.

Goding, J.W. 1996. Monoclonal antibodies: principles and practice. 3rd ed.. Sandiego, CA : Academic Press Inc 492 pp.

Gomez-Jimenez, S., Uglow, R.F. and Gollas-Galvan, T. 2000. The effects of cooling and emersion on total haemocyte count and phenoloxidase activity of the spiny lobster *Panulirus interruptus*. Fish & Shellfish Immunology 10 : 631-635.

Götz, P. 1986. Encapsulation in arthropod. In M. Brehélin (ed), Immunity in Invertebrate, pp. 153-169. Berlin : Springer Verlag Heidelberg.

Hasson, K.W., Lightner, D.V. Mohney, L.L., Redman, R.M. Poulos, B.T., and White, B.M. 1999. Taura syndrome virus (TSV) lesion development and the disease cycle in the pacific white shrimp *Penaeus vanamei*. Disease of Aquatic Organism 36 : 81-93.

Hose, J.E. and Martin, G.G. 1989. Defense functions of granulocytes in the ridgeback prawn *Sicyonia ingentis*. Journal of invertebrate pathology 53 : 335-346.

Hose, J.E., Martin, G.G. and Gerard, A.S. 1990. A decapod hemocyte classification scheme integrating morphology, cytochemistry and function. Biological Bulletin 178 : 33-45.

Hose, J.E., Martin, G.G., Tiu, S and McKrell, N. 1992. Patterns of hemocyte production and release throughout the molt cycle in the penaeid shrimp *Sicyonia ingentis*. Biological Bulletin 183 : 185-199.

Johansson, M.W., Keyser, P., Sritunyalucksana, K., Söderhäll, K. 2000. Crustacean haemocytes and haematopoiesis. Aquaculture 191 : 45-52.

Johansson, M.W. and Söderhäll, K. 1985. Exocytosis of the prophenoloxidase activating system from crayfish haemocytes. Journal of Comparative Physiology 156B : 175-181.

Johansson, M.W. and Söderhäll, K. 1989. Cellular immunity in crustaceans and the proPO system. Parasitology Today 5 (6) : 171-176.

Köhler,G., and Milstein, C. 1976. Derivation of specific antibody-producing tissue culture and tumor cell fusion. European Journal of Immunology 6 : 511-519.

Le Moullac, G., Le Groumellec, M., Ansquer, D., Froissard, S., Levy, P. and Aquacop. 1997. Haematological and phenoloxidase activity changes in the shrimp *Penaeus stylirostris* in relation with the moult cycle: protection against Vibriosis. Fish & Shellfish Immunology 7 : 227-234.

Leammli, U.K. 1970. Cleavage of the structure proteins during the assembly of the head of T4. Nature 227 : 680-685.

Lee, M.H., Shia, S.Y. 2004. Vitamin E requirements of juvenile grass shrimp, *Penaeus monodon*, and effects on non-specific immune responses. Fish & Shellfish Immunology 16 : 475-485.

Longyant, S., Sithigorngul, P., Thamapalerd, N., Sithigorngul, W. and Menasveta, P. 2000. Characterization of vitellin and vitellogenin of giant tiger prawn *Penaeus monodon* using monoclonal antibodies specific to vitellin subunit. Invertebrate reproductive and development 37(3) : 211-221.

Lorenzon, S., Francese, M., Smith, V.J. and Ferrero, E.A. 2001. Heavy metals affect the circulating haemocyte number in the shrimp *Palaemon elegans*. Fish & Shellfish Immunology 11 : 459-472.

Marques, M.R.F. and Barracco, M.A. 2000. Lectins, as non-self-recognition factors, in crustacean. Aquaculture 191 : 23-44.

Martin, G.G. and Graves, B.L. 1985. Fine structure and classification of shrimp haemocytes. Journal of Morphology 185 : 339-348.

Martin, G.G. and Hose, J.E. 1992. Vascular elements and blood (hemolymph). Microscopic Anatomy of Invertebrate Volume 10, 117-146. New York : Wiley-Liss, Inc.

Martin, G.G. and Hose, J.E. 1992. Vascular elements and blood (haemolymph). Microscopic Anatomy of Invertebrates 10 : 117-146.

Martin, G.G., Hose, J.E. and Kim, J.J. 1987. Structure of haematopoietic nodules in ridgeback prawn *Sicyonia ingentis*; light and electron microscopic observations. Journal of Morphology 192 : 193-204.

Montaño-Pérez, K., Yepiz-Plascencia, G., Higuera-Ciapara, I. And Vargas-Arbores, F. 1999. Purification and characterization of the clotting protein from the white shrimp *Penaeus monodon*. Comparative Biochemistry and Physiology 122B : 381-387.

Mosmann, T.R., Bauman, R., and Williamson, A.R. 1979. Mutations affecting immunoglobulin light chain secretion by myeloma cells I. European Journal of Immunology 9 : 511-516.

Muñoz, M., Cedeño, R., Rodríguez, van der Knaap, W.P.W., Mialhe, E. and Bachère, E. 2000. Measurement of Reactive oxygen intermediate production in haemocytes of the Penaeid Shrimp, *Penaeus vannamei*. Aquaculture 1991 : 89-107.

Muñoz,, M., Vandenbulcke,F., Gueguen, Y. and Bachére, E. 2003. Expression of penaeidin antimicrobial peptides in early larval stages of the shrimp *Penaeus vannamei*. Developmental and Comparative Immunology 27 : 283-289.

Muñoz,, M., Vandenbulcke, F., Saulnier, D. and Bachére, E. 2002. Expression and distribution of penaeidin antimicrobial peptides are regulated by haemocyte reactions in microbial challenged shrimp. European Journal of Biochemistry 269 : 2678-2689.

Owens, L. and O'Neill, A. 1997. Use of a clinical cell flow cytometer for differential counts of prawn *Penaeus monodon* haemocytes. Diseases of Aquatic Organisms 31 : 147-153.

Paterson, W.D. and Stewart J.E. 1974. In vitro phagocytosis by hemocytes of the American lobster (*Homarus americanus*). Journal of fish reasearch board Canada 31 : 1051-1056.

Rodriguez, J, Boulo, V., Mialhe, E. and Bachere, E. 1995. Characterisation of shrimp haemocytes and plasma components by monoclonal antibodies. Journal of Cell Science 108 : 1043-1050.

Scapigliati, G., Pecci, M., Piermattel, A., and Mazzini, M. 1997. Characterization of a monoclonal antibody against a 180 kDa haemocyte polypeptide involved in cellular defence reactions of the stick insect *Bacillus rossius*. Journal of Insect Physiology 43(4) : 345-354.

Soderhäll, K and Cerenius, L. 1992. Crustacean immunity. Annual Review of Fish Diseases : 3-23.

Söderhäll, K and Smith, V.J. 1986. The prophenoloxidase activating system: the biochemistry of its activation and role in arthropod cellular immunity, with special reference to crustacean. In M. Brehélin (ed), Immunity in Invertebrate, pp. 228-240. Berlin : Springer Verlag Heidelberg.

Solis, N.B. 1988. Biology and ecology., Biology and Culture of *Penaeus monodon*, pp.3-36. Philippines : Brackishwater Aquaculture Information System, Aquaculture Department of the Southeast Asian Fisheries Development Center.

Spann, K.M., Cowley, J.A., Walker, P.J. and Lester, R.J.G. 1995. A yellow-head-like virus from *Penaeus monodon* cultured in Australia. Disease of Aquatic Organism 31 : 169-179.

Sritunyalucksana, K., Söderhäll, K. 2000. The proPO and clotting system in crustacean. Aquaculture 191 : 53-69.

Sritunyalacksana, K. Wongsuebsantati, K., Johansson, M.W. and Söderhäll, K. 2001. Peroxinectin, a cell adhesive protein associated with the proPO system from the black tiger shrimp, *Penaeus monodon*. Developmental and Comparative Immunology 25 : 353-365.

Sung, H.H., Chang, H.J., Her, C.H., Chang, J.C. and Song, Y.L. 1998. Phenoloxidase activity of haemocytes derived from *Penaeus monodon* and *Macrobrachium rosenbergii*. Journal of Invertebrate Pathology 71 : 26-33.

Sung, H.H. and Sun, R. 2002. Use of monoclonal antibodies to classify haemocyte subpopulations of tiger shrimp (*Penaeus monodon*). Journal of Crustacean Biology 22(2) : 337-344.

Sung, H.H. Wu, P.Y. and Song, Y.L. 1999. Characterisation of monoclonal antibodies to haemocyte subpopulations of tiger shrimp (*Penaeus monodon*): immunochemical differentiation of three major haemocyte types. Fish & Shellfish Immunology 9 : 167-179.

Sung, H.H., Yang, Y.L. and Song, Y.L. 1996. Enhancement of microbicidal activity in the tiger shrimp *Penaeus monodon* via immunostimulation. Journal of Crustacean Biology 16(2) : 278-284.

Tsing, A.,Arcier, J.M. and Brehélin. 1989. Hemocytes of Penaeid and Palaemonid Shrimps: morphology, cytochemistry and hemograms. Journal of Invertebrate Pathology 53 : 64-77.

Van de Braak, C.B.T., Botterblom, M.H.A., Lie, W., Taverne, N.,van der Knapp, W.P.W. and Rombout, J.H.W.M. 2002. The role of the haematopoietic tissue in haemcyte production and maturation in the black tiger shrimp (*Penaeus monodon*). Fish & Shellfish Immunology 12 : 253-372.

Van de Braak, C.B.T., Botterblom, M.H.A., Taverne, N., van der Knapp, W.P.W. and Rombout, J.H.W.M. 2001. Monoclonal antibodies against haemocyte molecules of *Penaeus monodon* shrimp react with haemolymph components of other crustaceans and disparate Taxa. Developmental and Comparative Immunology 25 : 279-283.

Van de Braak, C.B.T., Botterblom, M.H.A., Taverne, N., van Muiswinkel, W.B., Rombout, J.H.W.M. and van der Knapp, W.P.W. 2002. The roles of haemocytes and the lymphoid organ in the clearance of injected *Vibrio* bacteria in *Penaeus monodon* shrimp. Fish & Shellfish Immunology 13 : 293-309.

Van de Braak, C.B.T., Taverne, N., Botterblom, M.H.A., van der Knapp, W.P.W. and Rombout, J.H.W.M. 2000. Characterisation of different morphological features of black tiger shrimp (*Penaeus monodon*) haemocytes using monoclonal antibodies. Fish & Shellfish Immunology 10 : 515-530.

Vargas-Albores, F. and Yepiz-Plascencia, G. 2000. Beta glucan binding protein and its role in shrimp immune response. Aquaculture 191 : 13-21.

Vargas-Albores, F., Jiménez-Vega, F. and Söderhäll, K. 1996. A plasma protein isolated from brown shrimp (*Penaeus californiensis*). Developmental and Comparative Immunology 20(5) : 299-306.

Vázquez, L., Perez, A., Millan, D. Agundis, C., Martin, G. Cooper, E.L., Lascurain, R. and Zenteno, E. 1997. Morphology of haemocytes from the freshwater prawn *Macrobrachium rosenbergii*. Journal of Morphology 234 : 147-153.

Wang, R., Lee, S.Y., Cerenius, L. and Söderhäll, K. 2001. Properties of the prophenoloxidase activating enzyme of the freshwater crayfish. European Journal of Biochemistry 268 : 895-902.

Yoganandhan, K., Thirupathi, S. and Sahul Hameed, A.S. 2003. Biochemical, physiological and hematological changes in white spot syndrome virus-infected shrimp, *Penaeus indicus*. Aquaculture 221 : 1-11.

APPENDICES

APPENDIX A

BUFFER AND REAGENT PREPARATION

1. Phosphate buffered saline (PBS) 0.15 M pH 7.2

NaCl	8.00	g
KCl	0.20	g
KH_2PO_4	0.20	g
Na_2HPO_4	1.15	g
or $Na_2HPO_4 . 7H_2O$	2.15	g
Distilled water adjust volume to	1,000.0	ml

2. Bradford solution (Bradford. 1976)

Coomassie brilliant blue G-250	100.0	mg
Methanol	50.0	ml
85% Phosphoric acid	100.0	ml
Distilled water adjust volume to	1,000.0	ml

3. 1% Merthiolate

Thimerosal (Sigma)	1.0	g
Distilled water adjust volume to	100.0	ml

4. 5% Blotto

Skim milk	5.0	g
1% Merthiolate	1.0	ml
Triton X-100	0.1	ml
PBS adjust volume to	100.0	ml

5. Davidson's fixative

95 % Ethyl alcohol	300.0	ml
40 % Formaldehyde	100.0	ml
Glacial acetic acid	100.0	ml
Distilled water	300.0	ml

6. Gelatin coating solution.

Chrome alum	0.05	g
Gelatin	1.0	g
Distilled water	100.0	ml

Dissolved chrome alum in distilled water followed by gelatin and warmed up the temperature to 50-70°C for gelatin melting. Filtered with filter paper no. 1. Kept the filtrate in 60°C until use. To coat the slide, glass slide were washed clearly with distilled water. Dipped the slide once into the warmed gelatin coating solution then air-dried and kept in slide box until use.

7. Modified Alsever's solution (anticoaggulant)

27 mM	Sodium citrate	7.94	g
336 mM	Sodium chloride	19.64	g
9 mM	EDTA	3.35	g
Distilled water adjust volume to		1000.0	ml

APPENDIX B

REAGENT PREPARATION FOR HYBRIDOMA PRODUCTION

1. RPMI medium

RPMI 1640 (Roswell Park Memorial Institute - Gibco BRL, USA)

	10.4	g
D-glucose (Sigma)	3.6	g
L-glutamine (Sigma)	0.2923	g
Sodium pyruvate ($C_3H_3O_3Na$) (Sigma)	1.1005	g
$NaHCO_3$	2.0160	g
HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Sigma)		
	5.9525	g
H_2O (Milli Q water)	1,000.0	ml

The solution of penicillin G, streptomycin and kanamycin were added to the final concentration of 20,000 units, 200 mg and 200 mg per liter, respectively. The medium was sterilized by millipore membrane (pore size 0.22 μm) filtered and stored at 4^oC.

2. RPMI medium with serum

RPMI medium (1)	80.0	ml
Fetal calf serum (FCS, Starrate, Australia)	20.0	ml
or Calf bovine serum (CBS, Starrate, Australia)		
100 X HT supplement (Gibco BRL, USA)	1.0	ml
-10 mM sodium hypoxanthine		
-1.6 mM thymidine		

3. Hybridoma selective medium (HAT medium)

RPMI medium (1)	80.0	ml
FCS	20.0	ml
HT supplement	1.0	ml
50 X Aminopterin (Sigma)	2.0	ml
1 % Mouse red blood cell		

4. Fusion solution (40 % polyethylene glycol)

Polyethylene glycol (PEG)	4.0	g

The solution was prepared by dissolving 4.0 g of polyethylene glycol in 6 ml of RPMI medium (1). The solution was incubated at 37 ^{o}C in CO_2 incubator before use.

5. Freezing medium (12 % DMSO)

Dimethylsulfoxide (DMSO, Sigma)	12.0	ml
RPMI medium (2.1)	88.0	ml

The medium was stored in 4 ^{o}C before use.

APPENDIX C

BUFFER AND SOLUTION FOR SDS-PAGE AND WESTERN BLOT ANALYSIS

1. Stock solution :

1.1 Monomer solution (30 % T, 2.7 % C_{Bis})

Acrylamide (BIO-RAD)	58.4	g
Bis (N,N'-methylene-bis-acrylamide, BIO-RAD)	1.6	g
Distilled water adjust volume to	200.0	ml

Stored at 4^{o}C in the dark bottle.

1.2 4 X Running gel buffer (1.5 M tris-Cl pH 8.8)

Tris (hydroxymethyl) aminomethane (BIO-RAD)	36.3	g
Distilled water adjust volume to	200.0	ml

adjusted pH with HCl

1.3 4 X Stacking gel buffer (0.5 M tris-Cl pH 6.8)

Tris	3.0	g
Distilled water adjust volume to	50.0	ml

adjusted pH with HCl

1.4 10 % SDS

SDS (sodium dodecyl sulfate, BIO-RAD)	50.0	g
Distilled water adjust volume to	500.0	ml

1.5 10 % Ammonium persulfate (freshly prepared)

Ammonium persulfate (BIO-RAD)	0.1	g
Distilled water	1.0	ml

1.6 Running gel overlay (0.375 M tris-Cl pH 8.8, 0.1 % SDS)

1.5 M Tris (3.1.2)	25.0	ml
10 % SDS (3.1.4)	1.0	ml
Distilled water adjust to	100.0	ml

1.7 2 X Treatment buffer (0.125 M tris-Cl pH 6.8, 4 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol)

0.5 M Tris (3.1.3)	2.5	ml
10 % SDS (3.1.4)	4.0	ml
Glycerol	2.0	ml
2-Mercaptoethanol	1.0	ml
Distilled water	0.5	ml

2 Preparation of separating gel and stacking gel

2.1 Stacking gel for SDS-PAGE 4 % gel (4 % T 2.7 % C_{Bis})

Monomer solution (3.1.1)	2.66	ml
0.5 M tris-Cl pH 6.8 (3.1.3)	5.0	ml
10 % SDS (3.1.4)	0.2	ml
Distilled water	12.2	ml
10 % Ammonium persulfate (3.1.5)	100.0	µl
TEMED	10.0	µl

2.2 Separating gel for SDS-PAGE 7.5 % gel (7.5 % T 2.7 % C_{Bis})

Monomer solution (3.1.1)	7.5	ml
1.5 M tris-Cl pH 8.8 (3.1.2)	7.5	ml
10 % SDS (3.1.4)	0.3	ml
Distilled water	14.55	ml
10 % Ammonium persulfate (3.1.5)	150.0	µl
TEMED	20.0	µl

Table 1D Preparation of separating gel and stacking gel for SDS-PAGE

	Separating gel		Stacking gel
	7.5 % T 2.7 % C_{Bis}	10 % T 2.7 % C_{Bis}	4 % T 2.7 % C_{Bis}
30 % T 2.7 % C_{Bis} (1.1)	2.5 ml	3.35 ml	0.67 ml
1.5 M tris-Cl pH 8.8 (1.2)	2.5 ml	2.5 ml	-
0.5 M tris-Cl pH 6.8 (1.3)	-	-	1.25 ml
10 % SDS (1.4)	0.1 ml	0.1 ml	0.05 ml
Distilled water	4.85 ml	4.2 ml	3.05 ml
Mixed and deaerated using vacuum pump			
10 % Ammonium persulfate (1.5)	50 µl	50 µl	25 µl
TEMED	3.5 µl	3.5 µl	2.5 µl
Mixed and rapidly poured between the glass plate			

3. Running buffer :

3.1 SDS-PAGE Tank buffer

(0.025 M tris pH 8.3, 0.192 M glycine, 0.1 % SDS)

Tris	12.0	g
Glycine	57.6	g
10% SDS (3.1.4)	40.0	ml
Distilled water	4,000.0	ml

4. Staining and destaining solution

4.1 Staining solution for protein (Coomassie blue)

4.1.1 Stain stock (1% Coomassie blue R-250)

1% Coomassie blue R-250	1.0	g
Distilled water	100.0	ml

4.1.2 Stain (0.1 % Coomassie blue R-250, 50% methanol, 10% acetic acid)

Stain stock (4.1.1)	50.0	ml
Methanol	250.0	ml
Acetic acid	50.0	ml
Distilled water	500.0	ml

4.2 Destaining solution for Coomassie blue

4.2.1 Destain I (50% methanol, 10% acetic acid)

Methanol	500.0	ml
Acetic acid	100.0	ml
Distilled water	1,000.0	ml

4.2.2 Destain II (5% methanol, 7% acetic acid)

Methanol	50.0	ml
Acetic acid	70.0	ml
Distilled water	1,000.0	ml

Method of protein staining

A gel was stained with 0.1 % Coomassie blue R-250, 50% methanol, 10% acetic acid for 5-6 hrs. The gel was then washed in destain I for 1 hr. with 1-2 changes and followed by destain II until the gel was cleared. After washing in distilled H_2O for a few times, the gel was dried in a gel air dryer (BIO-RAD)

6 Towbin transfer buffer pH 8.8

6.1 25 mM tris, 192 mM glycine, 20 % methanol pH 8.8

The buffer consisted of the following ingredients :

Tris	3.03	g
Glycine	14.4	g
Methanol	200.0	ml
Distilled water adjusted to	1,000.0	ml

The buffer was pre-chill before use

APPENDIX D

REAGENT FOR DETERMINATION OF ISOTYPES AND SUBISOTYPES OF MONOCLONAL ANTIBODIES

Hybridoma sub-isotyping kit, mouse (Zymed) contents :

A) Rabbit anti-Mouse IgG1 (γ_1 chain specific)

B) Rabbit anti-Mouse IgG2a (γ_{2a} chain specific)

C) Rabbit anti-Mouse IgG2b (γ_{2b} chain specific)

D) Rabbit anti-Mouse IgG3 (γ_3 chain specific)

E) Rabbit anti-Mouse IgA (α chain specific)

F) Rabbit anti-Mouse IgM (μ chain specific)

G) Rabbit anti-Mouse kappa light chain

H) Rabbit anti-Mouse lambda light chain

I) Normal Rabbit Serum, (Negative Control)

J) Positive Control, Monoclonal Mouse IgG1(Mouse IgG1 is in RPMI-1640 with 10 % FBS)

K) Substrate Buffer, Concentration (10 X) (1 M citrate, pH 4.2, containing 0.03 % H_2O_2)

L) ABTS Substrate, Concentrated (50 X) (2,2-azino-di[3-ethylbenzthiazoline sulfonic acid])

M) Blocking Solution, Concentration (50 X) (25 % BSA in PBS and 0.05 % NaN_3)

N) HRP-Goat anti-Rabbit IgG (H+L), Concentrated (50 X)

O) Goat anti-Mouse IgGAM, Concentrated (50 X) (0.5 mg/ml in PBS containing 10 % glycerol and 0.05 % NaN_3)

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ACADEMIC PRESENTATION :

Phochit Winotaphan, Nanthika Panchan, Paisarn Sithigorngul, Poolsiri Prukkanone, Siwaporn Longyant, Parin Chaivisuthangkura, Weerawan Sithigorngul and Amorn Petsom. Identification of five crustacean hyperglycemic hormones (CHH) isoforms and a truncated form in *Penaeus monodon.* Invertebrate Neuropeptide Conferences. February 15-19, 2004. Huatulco; Mexico.