Chapter I
Introduction

1.1 General introduction

The black tiger shrimp, *Penaeus monodon* is the most important penaeid species that is cultured throughout the Southern Indo Pacific region. The world cultured shrimp production in 2000 accounts for 56% of *P. monodon*, 17% of *P. merguiensis*, 16% of *P. vannamei* and 11% of the others (Rosenberry, 2000). Farming of *P. monodon* has achieved a considerable economic and social importance in the region, constituting a significant source of income and employment.

In Thailand, *P. monodon* have been intensively cultured for more than two decades. Approximately 60% of the total harvest shrimp comes from cultivation. Shrimp farms and hatcheries are scattered along the coastal areas of Thailand. Southern provinces (Nakorn Sri Thammarat and Surat Thani) account for the majority while those in the East (Chanthaburi) and Central regions (Samut Sakhon and Samut Songkhran) comprise the minority in terms of number. The intensive farming system (85%) has been used for *P. monodon* farming activity resulting in the consistent increase in the outcome production (Source: Department of fisheries).

Thailand has been regarded as the leader for *P. monodon* production for nearly a decade(Table 1.1). In the year 2001, the shrimp production from Thailand is 280,000 metric tons following by the other major exporting countries China, India and Indonesia. The outbreak of infectious disease has a great impact on the decreasing of shrimp production in several countries including Thailand, Ecuador, Vietnam, Taiwan, etc. The great loss of production was in Ecuador. The production decreased from 155,000 metric tons in 1998 to 80,000, 40,000 and 20,000 metric tons in 1999, 2000 and 2001,

Table 1.1 The world total shrimp production. Estimates on shrimp aquaculture production (in 1,000 metric tons).

Country	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
Thailand	120.0	150.0	225.0	220.0	160.0	150.0	210.0	220.0	250.0	280.0
China	220.0	55.0	35.0	70.0	80.0	80.0	80.0	85.0	85.0	100.0
Indonesia	150.0	80.0	100.0	130.0	90.0	80.0	80.0	85.0	85.0	90.0
India	42.0	60.0	70.0	70.0	70.0	75.0	70.0	75.0	80.0	100.0
Bangladesh	27.0	29.0	30.0	30.0	35.0	34.0	38.0	45.0	45.0	55.0
Ecuador	110.0	90.0	100.0	100.0	120.0	130.0	155.0	80.0	40.0	20.0
Vietman	39.0	41.0	50.0	50.0	30.0	30.0	25.0	35.0	35.0	42.0
Mexico	6.0	6.5	12.0	12.0	12.0	16.0	17.0	20.0	25.0	32.0
Philippines	25.0	20.0	18.0	25.0	25.0	10.0	15.0	20.0	20.0	25.0
Coloumbia	10.0	12.0	18.0	20.0	20.0	18.0	18.0	18.0	20.0	25.0
Taiwan	25.0	20.0	15.0	7.0	6.0	14.0	10.0	9.0	10.0	10.0
Honduras	5.2	5.7	6.5	10.0	10.0	12.0	12.0	10.0	10.0	12.0
Panama	4.2	4.4	4.6	10.0	10.0	10.0	10.0	9.0	8.0	5.5
Guatemala	2.5	2.7	3.0	7.0	7.0	7.0	7.0	6.0	6.0	4.5
Peru	5.6	5.8	6.0	8.0	8.0	6.0	6.0	5.0	5.0	2.5
Japan	3.5	3.5	3.6	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Others	45.0	51.2	50.0	14.0	30.0	35.0	55.0	54.0	50.0	47.0
Total	840.0	636.8	746.7	788.0	718.0	712.0	813.0	781.0	779.0	855.5

Source : Globefish

respectively. Whereas, Thailand had a severe outbreak during 1995-1997 causing the decrease in the shrimp production at that period. Nevertheless, Thailand is still the largest *P. monodon* producer.

The United States of America and Japan are the major shrimp importers (Table 1.2). Approximately 68% of *P. monodon* exported from Thailand are imported to these countries, worthing 38,859 million baht. The remaining markets are Europe, Asian countries, Australia and others.

Table 1.2 Thai Frozen Shrimp Export in 2001

Country	Quantity (metric tons)	Amount (million baht)
United States	66,990	27,203
Japan	24,837	11,656
Canada	5,758	2,245
Singapore	6,610	2,129
Taiwan	6,308	1,762
Australia	3,638	1,406
Republic of Korea	4,121	1,270
China	3,412	1,051
Hong Kong	2,610	971
United Kingdom	1,587	598
France	1,553	497
Germany	1,242	474
Italy	876	162
New Zealand	337	115
Others	5,031	1,680
Total	134,910	53,219

Source : Globefish

The farming activity of *P. monodon* in Thailand has rapidly increased reflecting a large annual production. The reasons for this are supported by several factors including the appropriate farming areas without the serious disturbing from typhoons or cyclone, small variable of seawater during seasons, and ideal soils and terrain for pond construction. Culture of *P. monodon* increases national revenue, therefore this penaeid shrimp species is an economically important species in Thailand.

1.2 Taxonomy of *P. monodon*

The taxonomic definition of the giant tiger shrimp, *P. monodon* is as follows (Bailey-Brook and Moss, 1992):

Phylum Arthropoda
Subphylum Crustacea
Class Malacostraca
Subclass Eumalacostraca
Order Decapoda
Suborder Natantia
Infraorder Penaeidea
Superfamily Penaeoidea
Family Penaeidae Rafinesque, 1985
Genus Penaeus Fabricius, 1798
Subgenus Penaeus
Species monodon

Scientific name: *Penaeus monodon* (Fabricius), 1798

Common name: Jumbo tiger prawn, Giant tiger prawn, Blue tiger prawn, Leader prawn, Panda prawn (Australia), Jar-Pazun (Burma), Bangkear (Cambodia), Ghost prawn (Hong Kong), Jinga (India, Bombay region), Udang windu(Indonesia), Ushi-ebi (Japan), Kamba ndogo (Kenya), Kalri (Pakistan), Sugpo (Phillipines), Grass shrimp (Taiwan), Kung kula-dum (Thailand), Tim

sa (Vietnam). FA.O. Names: Giant tiger prawn, Crevette giante tigre, Camaron tigre gigante.

1.3 Morphology

Externally the shrimp can be divided basically into the thorax and abdomen (Figure 1.1). The thorax (or head) is covered by a single, immobile carapace, which protects internal organs and supports muscle origins. The eyestalks and eyes, the sensory antennules and the antennae arise rostrally. The pereiopods or walking legs are the thoracic appendages. Gills are formed from sac-like outgrowths of the base of the walking legs and sit in branchial chambers on ether side of the thorax. The carapace extends laterally to cover the gills completely. The abdomen has the obvious segmentation of invertebrates. A swimming legs or pleopods are the abdominal appendages. A pair of pleopods arises from each of the G abdominal segments. A tail fan comprises of a telson, which bears the anus, and two uropods attach to the last (6^{th}) abdominal segment. The telson has deep medication groove without dorso-lateral spines. A rapid ventral flexion of the abdomen with the tail fan produces the 0quick backward dart characteristic of prawn (Anderson, 1993).

The cuticle, which is secreted by an epidermal cell layer, consists of chitin and protein in which calcium carbonate and calcium phosphate have been deposited. The epidermis detaches from the inner cuticle layer and begins to secrete a new cuticle, while the old cuticle is moulted. After moulting the new cuticle is soft and is stretched to accommodate the increased sized of the shrimp.

The black tiger shrimp has the following characteristic coloration: carapace and abdomen are transversely banded with red and white, the antennae are grayish brown, and the pereopods and pleopods are brown with crimson fringing setae. In shallow brackishwaters or when cultured in ponds, the color changes to dark and, often, to blackish brown (Moton, 1981.cited in Solis, 1988).

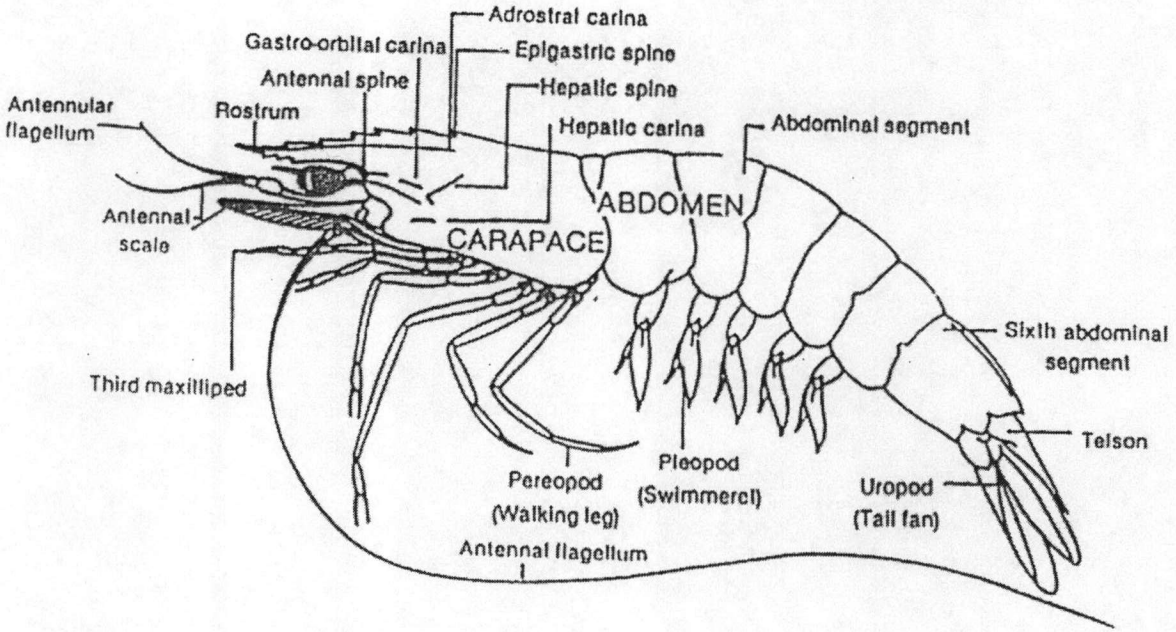


Figure 1.1 Latreal view of *P.monodon* showing important portent parts (Anderson, 1993)

1.4 Life cycle

The development of penaeid shrimps is complex. Larvae hatching from the fertilized eggs pass through a series of moults and metamorphic stages before becoming adulting-like (juveniles). Development begins with a larva hatching from the fertilized egg to the first stage, nauplius, followed by protozoa, mysis and post larval stages (Figure 1.2). These require the development times about 1-5 days, 5 days, 4-5 days and 6-15 days, respectively (Solis, 1988). Shrimp larvae are naturally planktonic in behaviors. Swimming is possible using antennae in nauplii, antennae and thoracic appendages in protozoa and thoracic appendages in mysis larvae. The normal adult slow swimming using the pleopods (abdominal appendages) is seen in the post larvae. Nauplii are about 0.3 mm long at hatching and are characterized by being totally planktonic and positively phototoxic; they exist entirely on their own egg yolk. The larvae begin to feed as protozoa. The second metamorphic change is seen when the third protozoa stage moults into the first mysis stage. Mysids have five pairs of functioning pereiopeds (thoracic appendages). The carapace now covers all the thoracic segments. The mysids swim in a more adult manner and actively seek out phytoplankton and zooplankton to feed on. The final metamorphosis is to the post-larvae stage, where a full complement of functioning appendages is present.

Post-larvae are given a numerical suffix, which indicates the time in days since metamophosis. They continue to moult as they grow. They migrate shoreward and settle in nursery areas close to shore or in estuaries, where they grow quickly to juvenile and sub-adults, tolerating the variable physico-chemical environment. Sub-adults migrate back to sea where they finally mature to mate and spawn. Penaeid shrimps are rarely older two years (Anderson, 1993;Solis, 1998).

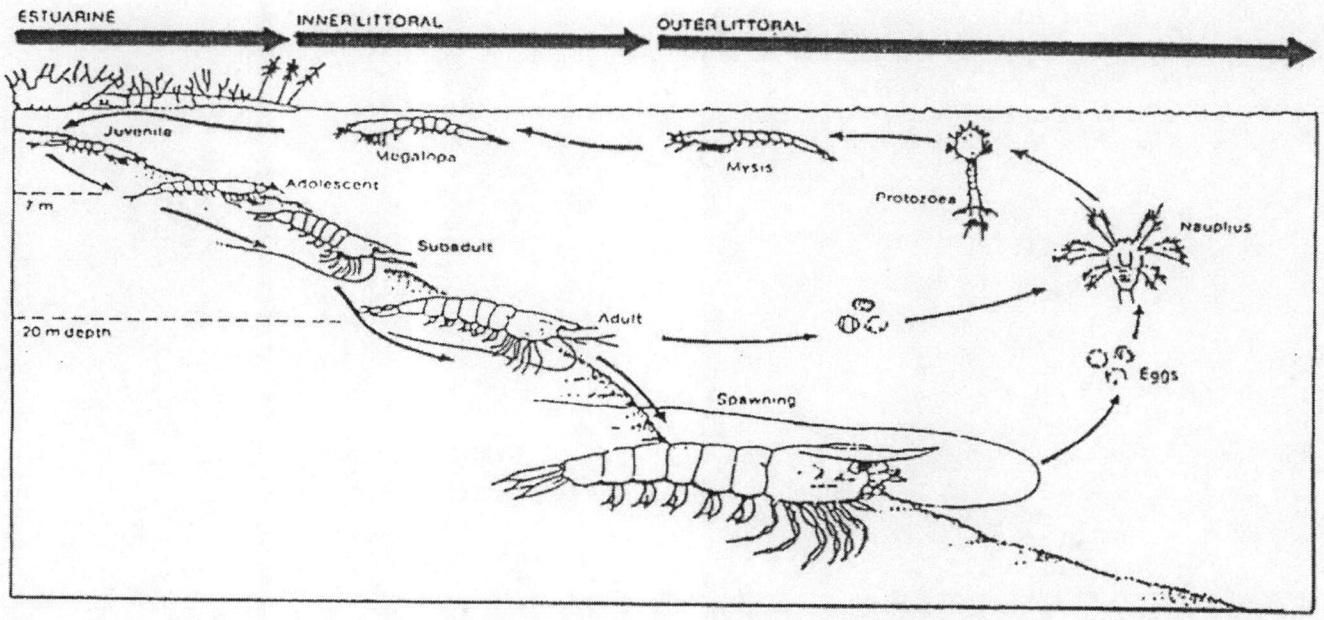


Figure 1.2 Life cycle of penaeid shrimp (Bailey-Brock and Mass, 1992)

1.5 Distribution

The black tiger shrimp *P. monodon* is principally distributed in the major part of the Indo-West Pacific region. It is commonly found in the East and Southeast Africa, through the Red Sea and Arabian Gulf, around the Indian subcontinent, and throughout the Malasian Archipelago to Northern Australia and Japan (Fig 1.3). It is a marine species inhabits in mud or sands bottoms at all depths from shallows to 110 meters (360 feet), so it can be caught from offshore or inshore as well as from tidal zones (or ponds). The species is one of the most important aquaculture shrimp species in Asia (Rosenberry, 1997).

1.6 Exploitation

Thailand has been regarded as the leader of *P. monodon* production for nearly a decade. The success of *P. monodon* industry in Thailand has resulted in the steadily increased income for the nation annually. Nevertheless, the shrimp industry has encountered several problems including environmental degradation, outbreak of diseases, and depletion of the wild broodstock that are used to stock commercial hatcheries (Browdy, 1998).

The outbreaks of infectious diseases have become more serious problem, causing a great loss to the productions (Roch, 1999). The mainly causes of infectious disease are white-spot syndrome virus (WSSV), yellow-head virus (YHV) and luminescent bacteria, *Vibrio* species (Chou et al., 1995;Flegel et al., 1995; Jiravanichpaisal et al., 1994). Although diagnostic methods of pathogenic agent in *P. monodon* are well developed, mechanism and expression of genes responded to pathogenic infection and/or immune systems are not well understood or controllable. Therefore, more knowledge on shrimp immune system and genetics are required.

The farming of *P. monodon* relies entirely on wild caught broodstock for supply of juveniles because breeding of this species in captivity is extremely difficult. The high demand on broodstock lead to overfishing in the sea. The broodstock was also declining in the wild population (Browdy, 1998). The

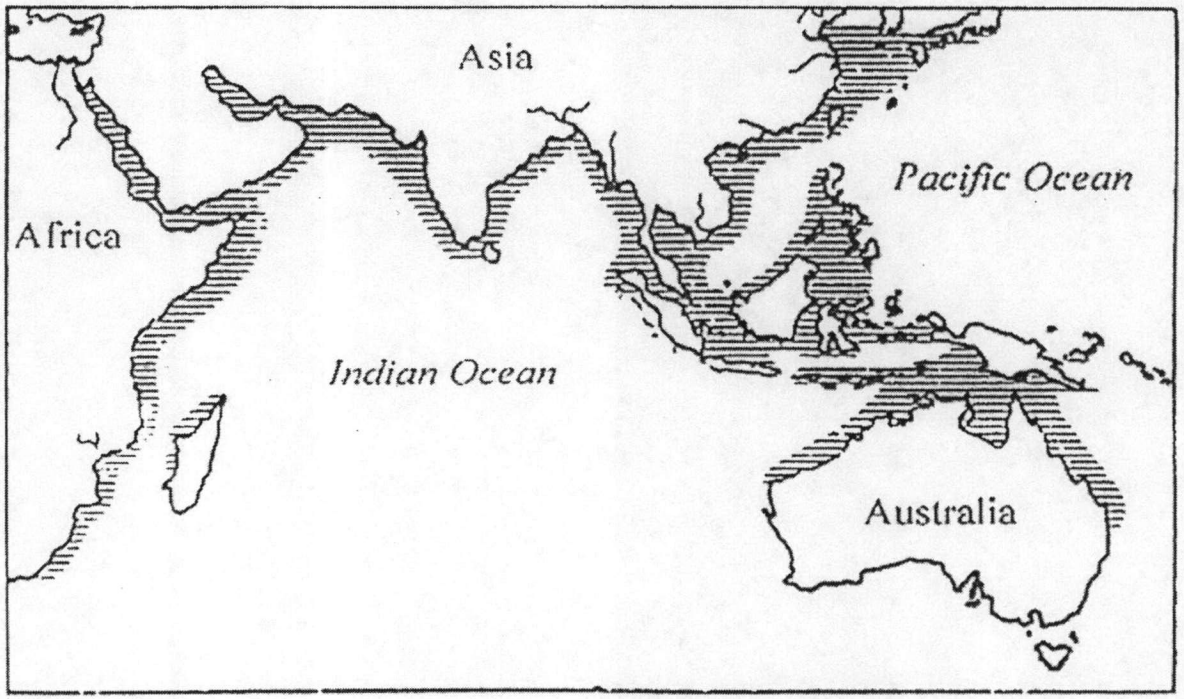


Figure 1.3 Geographic distribution of *P. monodon* in the Indo-West Pacific regions (Grey et al., 1983)

research concerning domestication of this important species has been initiated to overcome this problem by production of high quality pond-reared broodstock. Selective breeding program must be applied to produce broodstock with desired phenotypes for the shrimp industry. Thus, the basic knowledge on the control of growth, reproduction and immune system is required for genetic control and selection of *P. monodon*.

1.7 Shrimp disease

The outbreaks of infectious diseases become serious in the shrimp industry because of increasing shrimp farming and lack of proper knowledge involving shrimp biology, farm management and disease. Moreover, shrimp aquaculture is presently based on wild animals that are adopted to natural conditions and not to the artificial conditions of shrimp hatcheries and farms, where water quality, microbiological flora and nutrition are vastly different from those in the sea. Intensive rearing conditions are stressful for shrimp and lead to physiological disturbances or immunodeficiencies that increase sensitivity to pathogens. The infectious diseases in *P. monodon* are caused mainly by virus and bacteria, belonging to Vibrionacea (Lightner et al., 1983; Kroll et al., 1991; Mohney et al., 1994; Hasson et al., 1995; Flegel, 1997).

Outbreaks of yellow head disease were the most serious problem in central and southern Thailand during 1993-1994, while white spot disease has been the most serious problem from 1994 to 1996. Also, from mid 1996 until now, luminescent bacteria disease is increasingly considered to be the cause for unsuccessful shrimp culture. These pathogens particularly hampered larval production and lead to profitability problems due to stock mortality. They also lead to the over-fishing of wild shrimp larvae and an overexploitation of broodstock.

1.7.1 Viral disease

Disastrous failures have occurred in the shrimp farming industry in Thailand over a decade mostly due to virus infection. White spot syndrome virus (WSSV) and Yellow-head virus (YHV) are the important virus species that have been reported in *P. monodon*. They cause white spot syndrome disease (WSS) and yellow-head disease (YH), respectively (Boonyatatpalin et al., 1993;Wongteerasupaya et al., 1995). The outbreak of these virus causes a great losses in the shrimp industry in several countries including Thailand.

White spot syndrome (WSS) disease

White spot syndrome (WSS) is a viral disease which affect most of the commercially cultivated marine shrimp species, not just in Asia but globally (Chou et al., 1995; Lightner, 1996; Flegel, 1997; Lotz, 1997; Span and Lester, 1997). Lightner (1996) has called this virus white spot syndrome baculovirus (WSSV). This virus is an enveloped DNA virus of bacilliform to cylindrical morphology with an average size of 120 x 275 +/- 22 nm and has a tail-like projection at one end of the particle (Kasornchandra et al., 1995; Wongteerasupaya et al., 1995) (Fig 1.4). The viral genome contains double-stranded DNA of ~ 292 to 305 kb in length (van Hulten et al., 2001; Yang et al., 2001). WSSV is morphologically similar to insect baculovirus. However, phylogenetic analysis of ribonucleotide reductase and protein kinase genes revealed that WSSV does not share a common ancester with baculovirus (van Hulten et al., 2000; van Hulten and Valk, 2001).

The disease is thought to spread by means of contaminated water, decomposing fecal matter or tissue, cannibalism and fluid from infected females. Direct transmission can occur between unrelated crustacean species. Shrimp may be indirectly exposed to the disease through expose to previous hatchery or pond growing cycles, contaminated water supplies (new or previously utilized) contaminated food (through unlikely), equipment surfaces and clothing, or animals who have ingested diseased shrimp. Humans may also facilitate transmission of the disease by global transportation of viruses in

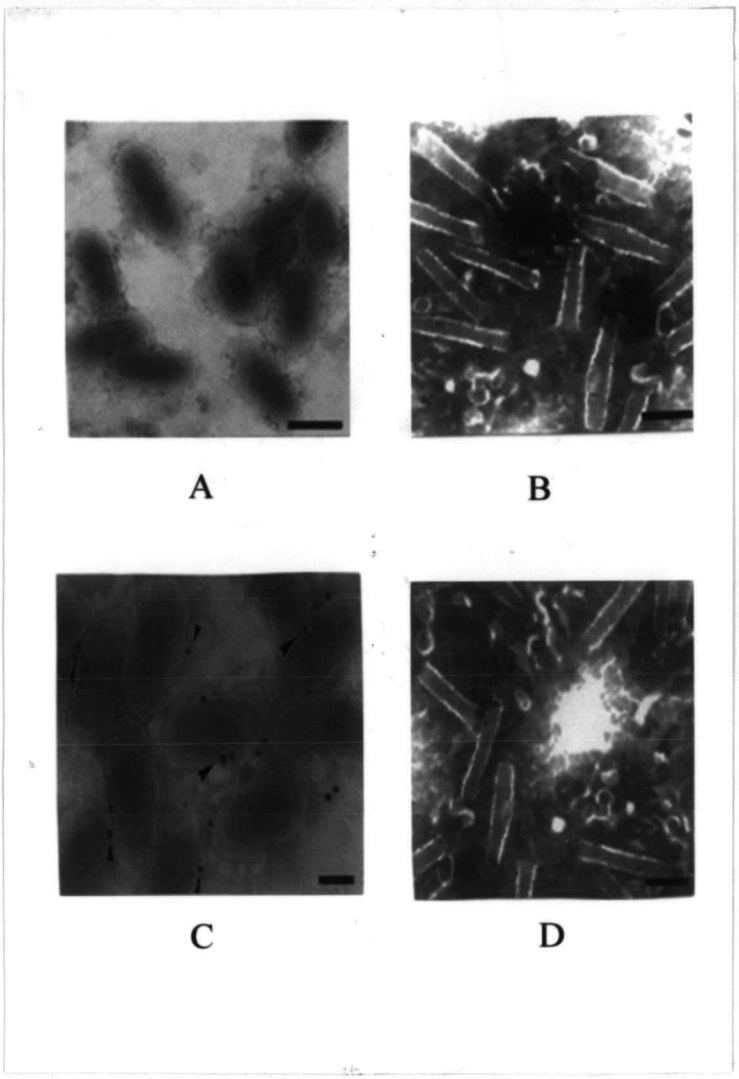


Figure 1.4 Immuno-electron microscopy of purified WSSV virions and nucleocapsids with anti-GST–P22 IgG followed by gold-labelled secondary antibody. (A) Intact WSSV virions (scale bar, 185 nm); (B) nucleocapsids of WSSV (scale bar, 185 nm); (C) WSSV virions labelled with gold (scale bar, 238 nm); and (D) WSSV nucleocapsids labelled with gold (scale bar, 185 nm). Arrows indicate gold particles.

infected frozen imported shrimps. Shrimp, which survive the infection, are suspected to be life-long carriers of WSS.

The clinical sign of this disease include white spots in the exoskeleton and epidermis, lethargy, a pink to reddish-brown coloration, the gathering of affected shrimp around the edges of ponds throughout the day and a rapid reduction in food consumption (Fig 1.5). Characterization of this virus is based on histological observation, electron microscopy and molecular studies. However, the presence of WSSV does not always mean that the condition is terminal. For instance, under non-stressful conditions, infected shrimp that have WSSV may survive indefinitely. Howevėr, if the shrimp also appear the clinical signs, then a very high mortality rate in the shrimp population can be expected within a few hours to a few days of the onset of the signs.

WSS can cause up to 100% mortality, with a correspondingly devastating economic impact. In 1996, Lightner pointed out that no significant resistance to this disease had been reported for any species of shrimp, and this still remains true today. The causative agent of WSS, WSSV is extremely virulent and has a wide host range (Lo et al., 1996b).

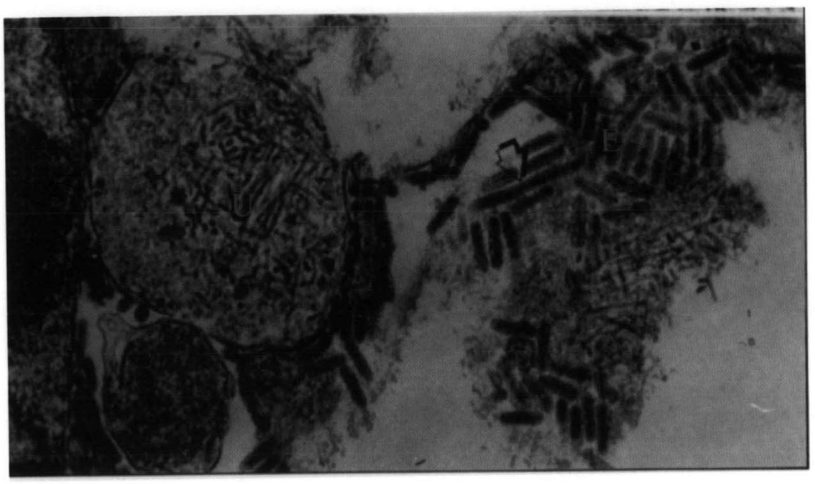
Yellow-head (YH) disease

In Thailand the disease is called Hua leung (Chan tanachookin et al., 1993; Lightner, 1996). YHV is a pleomorphic, enveloped virus with single stranded RNA of positive polarity primarily localized in the cytoplasm of infected cells (Cowley et al., 1999, Fig 1.6). It may belong in the family coronaviruses (Cowley et al., 1999). YH disease was first reported in Thailand in 1990, but is known to infect and cause mass mortality in shrimp farming operations throughout South East Asian countries. This syndrome occurs in the juvenile to sub-adult stages of shrimp, 5 to 15 grams in size, especially at 50-70 days of grow-out (Lightner, 1996).

Viral replication seems to occur only in the cytoplasm without any sign of replication in the intact nuclei of infected cells. A long filamentous form of the virus (some over 800 nm in length), perhaps a precursor to the enveloped,

Figure 1.5 Shrimp with White Spot Syndrome disease

a



b

Figure 1.6 Transmission eletron micrographs of hepatopancreatic interstitial cells from yellow-head specimens. (a) A viral infected cell. The viral material is sectioned transversely and longitudinally. It appears in two densities, probably indicating the presence (dense) or absence (less dense of capsid material (bar=200 nM). (b) Unenveloped virions (U) bell. The average length of the short virions was approximately 170 nM. Note the double length virion (arrow) (bar=200 nM).

rod-shape form is present in the cytoplasm of many cells. Viral envelope appear to be acquired by passage of these provirions through the endoplasmic recticulum of the host cells. Enveloped virions then cluster in cytoplasmic vesicles, sometimes densely packed, resembling paracrystalline arrays, where they appear to divide into the smaler rod-shaped unit (Chantanachookin et al., 1993).

Shrimp infected with YHV often show light yellow coloration of the dorsal cephalothorax area and have a pale or bleached appearance (Limsuwan, 1991) (Fig 1.7). At the onset of YHD, shrimp have been observed consuming feed at an abnormally high rate for several days. Feeding abruptly ceases and within 1 day, a few moribund shrimp appear swimming slowly near the surface at the pond edges. Moribund shrimp with YHV generally appear pallid in color, with a yellowish, often swollen cephalothorax and die with in a few hours. Infected shrimp frequently exhibit whitish or pale yellowish to brown gills, and often a pale yellow hepatopancrease (Lightner, 1996, Chantanachookin et al., 1993). In the black tiger shrimp, typical signs of YH disease include characteristic yellowing of the hepatopancrease and gill. YHV may occur as latent, asymptomatic infections in broodstock shrimp and may possibly transfer from these shrimps to their offspring in larval rearing facilities (Chantanachookin et al., 1993).

Presumptive diagnosis is made on the basic of pond history, clinical signs, gross changes and histopathology. Bioassay reinfection studies and transmission electron microscopy are used for definitive diagnosis.

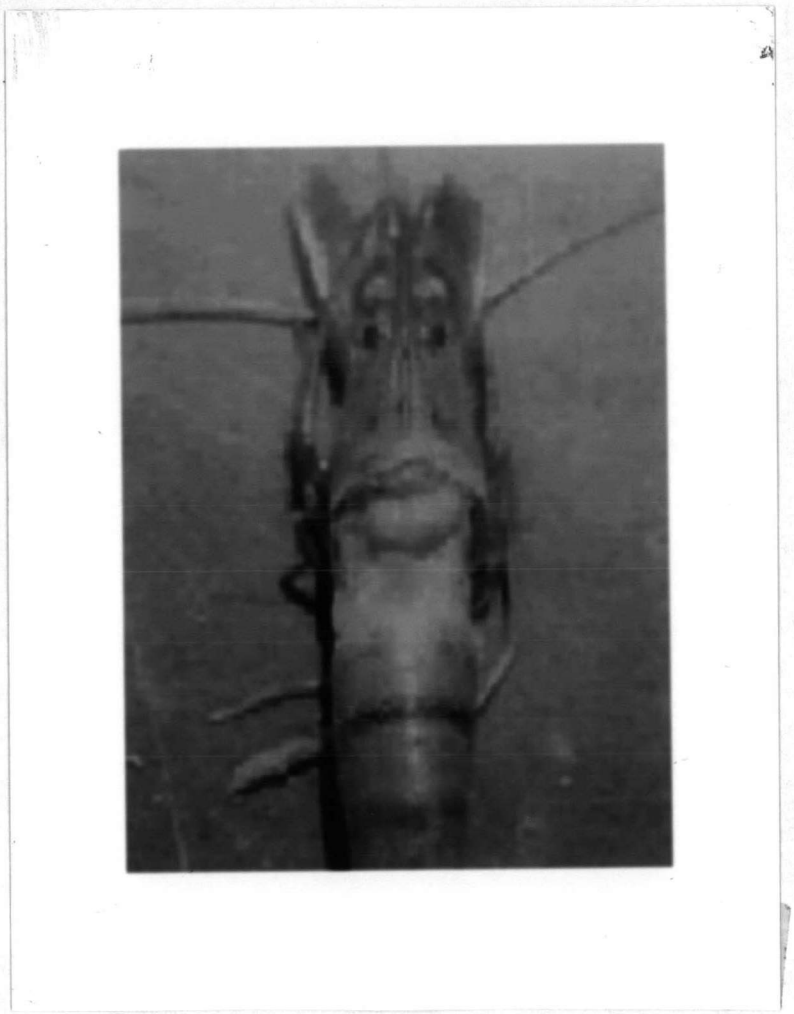


Figure 1.7 Shrimp with Yellow-head disease

1.7.2 Bacterial disease

Vibriosis is a major disease problem in shrimp aquaculture, causing high mortality and severe economic loss in all producing countries (Brock et al., 1992; Crosa et al., 1980; Mohney et al., 1994.) *Vibrio* species are a normal part of the bacterial flora in aquatic environment and formerly considered to be mostly opportunistic pathogens (Lightner, 1988). However, somemore recently occurring disease syndromes of penaeid shrimp have been caused by *Vibrio* species which behave more like true pathogens than opportunistic invader (Lightner et al., 1992).

The luminescent bacterium, *Vibrio harveyi*, has been described frequently in outbreaks of luminous vibriosis in cultured *P. monodon* in hatcheries or farms in Australia (Pizzutto et al., 1995), China (Vandenberghe et al., 1998), India (Karunasagar et al., 1994), Indonesia (Sunaryanto et al., 1986), Thailand (Jiravanichpaisal et al., 1994), the Philippines (Lavilla-Pitogo et al., 1990) and Taiwan (Liu et al., 1996; Song and Lee, 1993). *V. harveyi* is a Gram-negative bacterium. It is a rod shape, 0.5-0.8 μm width and 1.4-2.6 μm in length. It is able to emit light of a blue-green color (Fig 1.8). The reaction leading to light emission, catalyzed by the enzyme luciferase, has been shown to be similar in all prokaryotes. The substrates are reduced flavin mononucleotide ($FMNH_2$), a long chain aldehyde (RCHO; probably tetradecanal), and molecular oxygen which react according to the following overall stoichiometry:

$$FMNH_2 + O_2 + RCHO \xrightarrow{\text{Luciferase}} FMN + HO + RCOOH + light$$

Bacterial luciferase is a heterodimer having a molecular weight (MW) of about 80,000 and consisting of α and β subunits with MWs of about 42,000 and 38,000, respectively.

Figure 1.8 The blue-green color of light emission form *Vibrio harveyi*

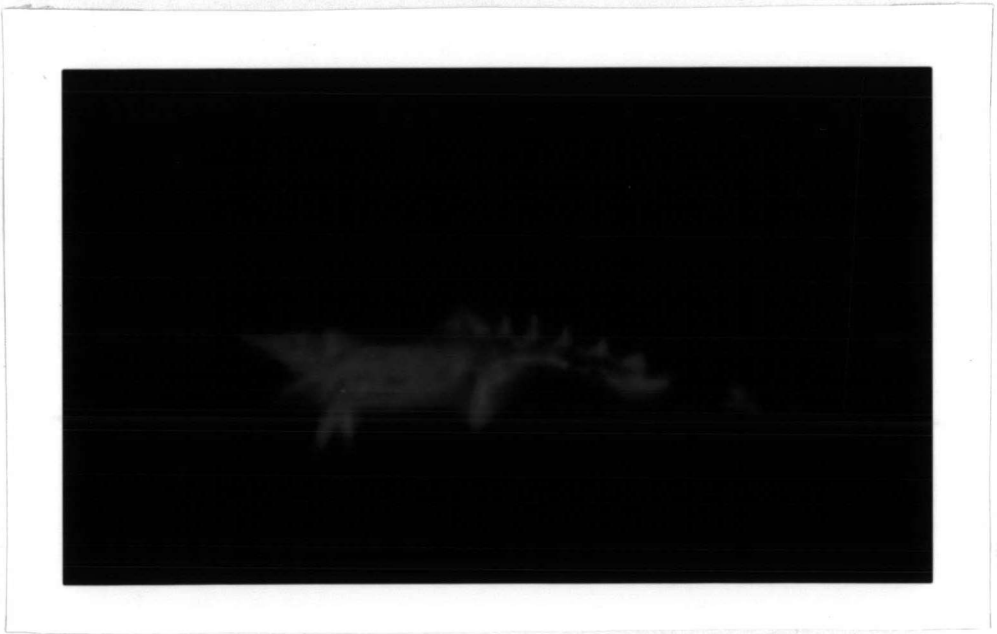


Figure 1.9 Shrimp with luminous disease

V. harveyi is claimed to be the most causative agent associated with shrimp mortality. The disease is widely known as luminous disease or Kung-rungsang in Thai. The bacterial pathogen is resulted in mortality up to 100% for nauplius to Zoea stages of *P. merguiensis*. Living and dead shrimp larvae and even the seawater in disease outbreak areas were luminescent in dim light (Fig 1.9). The diseased shrimp is milking white body and appendages, weakness, disoriented swimming, lethargy, eventually leading to death.

Presumptive diagnosis is made on the basis of clinical signs and culture of the suspensions of hepatopancreas or blood on trytic plate with 2% (w/v) NaCl. After incubation at 30 °C overnight, colonies of *V. harveyi* showed strong luminescence in dim light.

Control of luminous vibrios by supplementation of antibiotics has become less effective to a number of antibiotics occurrence of bacterial resistance to a number of antibiotics. Tjahjadi et al., (1994) reported that most luminious vibrios isolated from ahrimp hatcheries in Kalianget, East Java were resistant to a number of antibiotics tested except to rifampicin (50mg/ml). Use of excessive antibiotics has also been implicated in shrimp growth retardation, abnormal morphogenesis and rejection of the exported shrimp due to the residuals.

1.8 Invertebrate Defense system

Immune system has developed to protect multicellular organisms from foreign substances. During evolution, two types of immune systems have developed to detect foreign substances, namely innate (natural) immunity and adaptive (acquired) immunity.

The innate immune system is phylogenetically a more ancient defense mechanism and can be found in all multicellular organisms. This system is the first line of defense that helps to limit infection at an early stage, and relies on germ line encoded receptors that recognism conserved molecular patterns present on microorganisms (Fearon et al., 1996; Fearon, 1997; Janeway, 1997).

The adaptive immune system has developed more sophisticated and complicated mechanisms including an immunological memory with generation of a large receptoire of antigen recognition receptors (Lee and Soderhall, 2001).

The adaptive immune system is found only in vertebrates (Thompson, 1995) whereas invertebrates have a rapid and efficient innate system to recognism and destroy non-self material, including pathogens. Although this system generally lacks immunologic memory and the discrete specificity of the antigen:antibody response components of classical immunology.

In crustaceans, the innate immune system is based on cellular and humoral components of the circulatory system. The recognition of conserved molecular patterns characteristic of pathogen is a property of the innate immune system, which is instrumental in initiating and regulating the adaptive immnue response. The target recognition of innate immunity is the so-called "pattern recognition molecules (PRMs)" shared among groups of pathogens. Host organisms have developed the response to these PRMs by a set of receptors referred to as "pattern recognition proteins or receptors (PRPs or PRRs)". These pattern include the lipopolysaccharides (LPS) of Germ negative bacteria, the glycolipids of mycobacteria, the lipoteichoic acids of Gramp positive, bacteria, the mannans of yeasts, the β–1,3-glucan of fungi and double-stranded RNA of viruses (Hoffmann et al., 1999).

Proteolytic cascades triggered by nonself recognition molecules have major roles in innate immunity. Examples are the complement cascade in mammals, hemolymph coagulation in horseshoe crab and the phenoloxidase mediated melanization in crustaceans and insects (Söderhäll, 1982; Söderhäll et al., 1994). The complement cascade is activated directly (via alternative and lectin pathways) or indirectly (via classical pathways) by microorganisms and results in their opsonization for phagocytosis, chemotaxis by the assembly on their surface of a pore-forming membrane attack complex. The lectin pathway requires mannose-binding protein (MBP). MBP recognises sugar moeities on

microbe surfaces and results in the activation of MBL-associated serine proteases, MASP-1 and –2, which in turn activate the C3 convertase. Recent cloning of MASPs in lamprey (Matsushita et al., 1998) and tunicates, C3-like molecules from tunicates (Smith et al., 1999) and sea urchins (AL-Sharif et al., 1998), and related thioester-containing (TEP) in *Drosophhila melanogaster* and *Anopheles gambiae* leads to the prediction that the lectin pathway of mammalian complement system seems to be ancient. An earlier link between recognition of microbial molecular patterns, proteolytic cascades and activation of host defence came from the studies of the cloting cascade in the horseshoe crab, Limulus polyphemus (Iwanaga et al., 1998). The protein participating in the horseshore crab clotting system all reside in the hemocytes and, upon activation they are released from the cytoplasmic L-granules into the hemolymph through rapid exocytosis. Gram negative bacteria and fungi invading the horseshoe crab hemolymph activate factor C and factor G, respectively, which result in the formation of an insoluble coagulin gel that limits the infection (Iwanaga et al., 1998). Factor C in this cascade has five short consensuses reports (SCR, also called CCP or the sushi domain) (Muta et al., 1991) that are found in mammalian complement proteins, suggesting an early common origin of the complement and coagulation cascades. The prophenoloxidase activating system (the proPO system) is an enzymatic cascade reported in many invertebrates and large amount of information about this system has come from work done on crustacean; the freshwater crayfish, *Pacifastacus leniusculus* (for reviews see Söderhäll et al., 1994; Söderhäll and Cerenius, 1998). The activation of the proPO system is brought about by extremely low amount (pg/L) of microbial cell wall components such as LPS and β–1, 3-glucans. Activation of the proPO system not only leads to the synthesis of melanin, but also initiates several biological molecules responsible in the defense system of the crayfish. Recently, Nagai and Kawabata (2000) showed that *Tachypleus* clotting enzyme and activated factor B are capable to functionally transform hemocyanin to phenoloxidase without proteolytic

cleavage suggesting that the two host defense systems of blood coagulation and prophenoloxidase activation are evolutionary related protease cascades.

1.8.1 Blood cells

Crustaceans have open circulatory. The circulating haemocytes of crustacean are essential in immunity, performing functions such as phagocytosis, encapsulation, and lysis of foreign cells (Smith and Soderhall, 1998a; Soderhall and Smith, 1986; Johansson and Soderhall, 1989; Soderhall and Cerenius, 1992). The number of free haemocytes can vary and can, for instance, decrease dramatically during an infection (Persson et al., 1987b; Smith and Soderhall, 1983a; Smith et al., 1984). Crustaceans have three morphologically different haemocyte types: hyaline, semigranular, and granular cells (Bauchau, 1980). These different haemocyte types carry out different functions in immunity (Johansson et al., 2000).

Hyaline cell, which lacking cytoplasmic granules, is the smallest group. It found only 1% of the total haemocytes (Iwanaga and Kawabata, 1998). The previous report indicates that, this haemocyte is involved in phagocytosis (Soderhall et al., 1986). Granular and semigranular haemocytes were oval, plate-shaped structure, 15-20 μm in their longest dimension. The semigranular cell is the most abundant type of haemocyte and contain a variable number (1-40) of small (S) granules (0.4 μm diameter). This haemocyte response by some phagocytic and encapsulation (Persson et al., 1987). Granular cell contains a large number of secretory large (L) granules (0.8 μm diameter). On the other hand, the circulating haemocytes, which are filled with the two secretory L- and S-granules, contain many kinds of defense proteins and peptides (Iwanaga et al., 1994). L-granules contain at least 24 proteins, a majority of which are clotting factor serpins, and various lectins. In contrast, S-granules contain at least 6 proteins with molecular masses of less than 30 kDa, in addition to an antimicrobial peptide tachyplesin and its analogues (Shigenaga et al., 1993; Muta et al., 1990).

1.8.2 Mechanism of defense reaction

1.8.2.1 Pattern recognition proteins

Pattern recognition proteins (PRPs or PRRs) have been isolated and characterized in several invertebrates. These PRPs recognitione ans respond to microbial invaders by the presence of signature molecules on the surface of the intruders. Some of them contain common motifs for example, bacterial glucanase-like (Lee et al., 2001; Ochiai and Ashida, 2000; Cerenius et al., 1994; Ma and Kanost, 2000; Beschin et al., 1998; Lee et al., 2000; Kim et al., 2000), bacteriophage lysozyme-like (Yoshida et al., 1996; Ochiai and Ashida, 1999) and immunoglobulin-like (Sun et al., 1990) motif in their primary structures. Some of them are haemagglutinins or lectins that have the ability to bind to specific carbohydrates expressed on different cell surfaces. Due to the fact that they are, in general, at least bivalent, they can bind cells and an agglutination reaction occurs. 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. As with vertebrate immunoglobulins, they can agglutinate microorganisms and enhance their phagocytosis by mediating binding between the haemocyte surface and a foreign body (opsonic role), and are apparently synthesised by invertebrate immune cells. However, in contrast to immunoglobulins, the specificity of invertebrate agglutinin is restricted only to sugar residues.

The surface recognizing protein detected in arthropod plasma has the capability to react with β–1,3-glucan, and therefore, it is named beta glucan binding protein or BGBP. β–1,3-glucan is a major cell wall component of fungi. Although BGBPs have glucanase-like motif, none has been shown to contain glucanase activity suggesting that the BGBPs developed from a primitive glucanase and then evolved into proteins without glucanase activity, but instead bind glucans and after binding, operate as elicitors of defense responses. The activation of this zymogen triggers the clotting cascades

(clotting reaction, factor C and factor G), resulting finally in the conversion of coagulogen to an insoluble coagulin gel (Tokunaga et al., 1987; Muta et al., 1995; Seki et al., 1994). Thus, the invaders in the haemolymp are engulfed and immobilized by the clot, and subsequently killed by antimicrobial substances that are also released from the two types of granules.

1.8.2.2 The prophenoloxidase (proPO) system

The proPO activating system consists of several proteins involved in the immune defense in invertebrates leading to melanin production, cell adhesion, encapsulation, and phagocytosis (Söderhäll et al., 1998; Sritunyalucksana and Söderhäll, 2000). It is an efficient immune system for non-self recognition and is initiated by recognition of lipopolysaccharides or peptideoglycans from bacteria and β–1, 3-glucans from fungi. This system contains a proteinase cascade compose of pattern-recognition proteins (PRPs), several zymogenic proteinases, and proPO (Soderhall and Cerenius, 1998). Theactivation of the proPO cascade is exerted by extremely low quantities of microbial cell wall components, resulting in limited proteolysis of proPO to the active phonoloxidase(PO). PO is a bifunctional copper-containing, also known as tyrosinase, catalyses two successive reaction: hydroxylation of a monophenol to o-diphenol (monophenoloxidase activity) and the oxidation of the o-diphenol to o-quinone (diphenoloxidase activity) (Soderhall and Cerenius, 1998; Decker and Tuczek, 2000). Production of o-quinones by PO is an initial step in the biochemical cascade of melanin biosynthesis. The production of melanin pigment can often be seen as dark spots in the cuticle of arthropods involvingin the process of sclerotisation, wound healing and encapsulation of foreign materials (Lai-Fook, 1996; Sugumaran, 1991). Several components and associated facttors of the proPO system have been found to play several important roles in the defense reaction of the freshwater crayfish (Söderhäll and Cerenius, 1998).

Studies on shrimp proPO system have been carried out in Penaeid shrimps including *Penaeus californiensis* (Vargas-Albores et al., 1993, 1996;

Gallas-Galvan et al., 1999), *P. panlensis* (Perazzolo and Barracco, 1997), *P. stylirostris* (Le Moullac et al., 1997) and *P. monodon* (Sritunyalucksana et al., 1999). Shrimp proPO is synthesized in the haemocytes and not in the hepatopancreases. By comparison of amino acid sequences, shrimp proPO is more closely related to crayfish proPO than to the insect proPO. The conversion of inactive proPO to PO is by a serine protease named the prophenoloxidase activating enzyme (ppA). This enzyme has been isolared in several insects (Jiang et al., 1998; Lee et al., 1998; Satoh et al., 1999) and from a crayfish haemocyte lysate. It was shown in crayfish that only ppA enzyme is sufficient for the activation of proPO. However, the mechanism by which it converts proPO to active enzyme is still unclear.

1.8.2.3 The coagulation system/the clotting system

Two different coagulation mechanisms have been characterized in molecular detail in invertebrates, those were the haemocyte-derived clotting cascade in horseshoe crab, *Tachypleus tridentatus* (Kawabata et al., 1996) and the transglutaminase (TGase)-dependent clotting reaction in crayfish, *Pacifastacus leniusculus* (Hall et al., 1999). The proteins participating in the horseshoe crab clotting system all reside in the haemocytes and upon activation they are released from the cytoplasmic L-granules into the haemolymph through rapid exocytosis (Kawabata et al., 1996). The microbial cell wall components activate factor C and G, respectively, which results in subsequent activation of proclotting enzyme and the resulting clotting enzyme catalyses the conversion of a soluble protein (coagulogen) into an insoluble aggregate (coagulin) (Iwanaga, 1993; Kawabata et al., 1996).

The crayfish clotting protein, a dimeric protein consisting of 210 kDa subunits, is a VHDL (Hall et al., 1995a) and each of the 210 kDa subunits has both free lysine and glutamine, which are recognized and become covalently linked to each other by TGases. The crayfish clotting protein polymerizes, forms clot in the presence of Ca^{2+} and TGases released from haemocyte, and starts to crosslink the clotting protein, into large aggregates. TGases are Ca^{2+}-

dependent enzymes capable of forming covalent bonds between the side chains of free lysine and glutamine residues on certain proteins. This enzyme is localized in the haemocytes, especially in hyaline and semigranular cell, and is shown to be involved in the clotting process.

However, the clotting reaction has only been fully characterized in crayfish (Hall et al., 1999), the mechanism in other crustaceans have to be elucidated in more detail for comparative studies of the clotting reaction reaction in crustaceans.

1.8.2.4 Antimicrobial peptide or proteins

Antimicrobial peptides are major components of innate immunity that have been conserved in evolution and found in different phyla of the plant and animal kingdom. Although these immune effectors share common characteristics and similarities in structural patterns or motifs (Bulet et al., 1999), one striking feature is their great diversity in term of amino acid sequences, antimicrobial activities and modes of action. Moreover, depending on their distribution, antimicrobial peptide expression appears to be regulated by different tissue-specific pathways and these effectors may consequently participate in either a local or a systemic reaction.

For convenience, these antimicrobial peptides are tentatively classified into four distinct groups base on amino acid sequences, secondary structures and functional similarities: (I) linear basic peptides forming amphipathic α–helices which are devoid of cysteine residues including the cecropins, the first antimicrobial peptide isolated from insect haemolymph; (ii) peptides with one to six intramolecular disulfide bridges including the arthropod defensins, antifungal peptides from *Drosophila*, drosomycin and metchnikowin, thanatin from *Podisus*, anti-LPS factor, tachyplesin, big defensin and tachycitin from *Limulus*; (iii) the proline-rich peptides such as the apidaecins or drosocin; (iv) the glycine-rich peptides or polypeptides such as attacins, diptericin, and sarcotoxins.

In general, the mechanism of action of any of these agents is not very well established. For many of these peptides, there is evidence that one of the targets for the peptide is the lipid bilayer of the membrane. This is because these peptides can often increase the rate of leakage of the internal aqueous contents of liposomes. In addition, most of the antimicrobial peptides are cationic and their interaction with anionic phospholipids would provide a ready explanation for their specificity for bacterial menbranes. With regard to the mechanism by which the peptide breaks down the membrane permeabilty barrier, it is possible that the peptide induces complete lysis of the organism by rupture of the membrane or that it perturbs the membrane lipid bilayer, which allows for leakage of certain cellular components as well as dissipating the electrical potential of the membrane.

In arthropods, several of antimicrobial peptides were isolated and characterized, mainly in insects and chelicerates (horseshoe crabs) (1998; Iwanaga et al., 1998). In horseshoe crabs,these protein are mainly synthesized in haemocyte and are stored within the cytoplasmic granules (Iwanaga and Kawabata, 1998). The cells are highly sensitive to LPS, a major outer membrane component of Gram negative bacteria, and respond by degranulating these granules after stimulation by LPS (Iwanaga et al., 1997). This system differs from that described in insects, where the fat body is the main site for the antimicrobial peptide synthesis (Hoffman and Reichhart, 1997; Engstrom, 1998), and upon injury antimicrobial peptide gene transcription is induced, resulting in their immediate synthesis and subsequent secretion into the blood.

There are few reports on antimicrobial peptides in crustaceans. Tachyplesin family and anti-LPS factors which acting against Gram negative bacteria were observed in horseshoe crab (Nakamura et al., 1988; Muta et al., 1990; Ohashi et al., 1984; Aketagawa et al., 1985; Muta et al., 1990). In 1997, a small peptide named callinection was reported to be responsible for the majority of antimicrobial activity observed in the haemolymph of blue crab, *Callinectes sapidus* (Lester et al., 1997) and recently, penaeidins, a new family

of antimicrobial peptide which acting against Gram positive bacteria and fungi were reported in penaeid shrimp *P. vannamei* (Destoumieux et al., 1997). These peptides contain both a proline rich domain at the N-terminal and a carboxy-terminal domain containing 6 cysteines which from 3 disulfide linkages. cDNA clones of penaeidin isoform were also isolated from the haemocytes of *P. vannamei* and *P. setferus* (Gross et al., 2001). A cystein-rich 11.5 kDa antibacterial protein was purified and characterized from haemolymph of shore crab, *Carcinus maenas* in 1999 (Relf et al., 1999). And in 2002, crustins, an antimicrobial peptide homologues of an 11.5 kDa antibacterial peptide were identified from 2 species of *Penaeid* shrimp, *P. vannamei* and P. *setiferus.* Several isoforms of crustins were observed in both shrimp species. Like the 11.5 kDa antibacterial protein from *C. maenas*, crustins from shrimp show no homology with other known antibacterial peptides, but possess sequence identity with a family of proteinase inhibitory proteins, the whey acidic protein (WAP).

1.9 Expressed Sequence Tags

The Expressed Sequence Tags (EST) method of analysis, first developed by Adams et al. (1991), has been widely employed as a means of discover of novel and uniquely expressed genes, and for characterizing the gene expression profiles of several tissues (Adams et al., 1993; Affara et al., 1994; Pawlak et al., 1995; Liew et al., 1994; Takeda et al., 1993). ESTs are shot (usually about 300-500 bp), single-passed sequence reads from mRNA (cDNA) and relatively inaccurate (around 2% error) (Schuler, 1997). They represent a snapshot of genes expressed in a given tissue and/or at a given development stage. They are tags (some coding, other not) of expression for a given cDNA library. Characterization of ESTs is a convenient and rapid way for identification of new genes in various taxa where knowledge about the genome under investigation is not available or rather limited. The basic procedure of this approch is summarizied in Fig 1.10. It initials with the mRNAs from

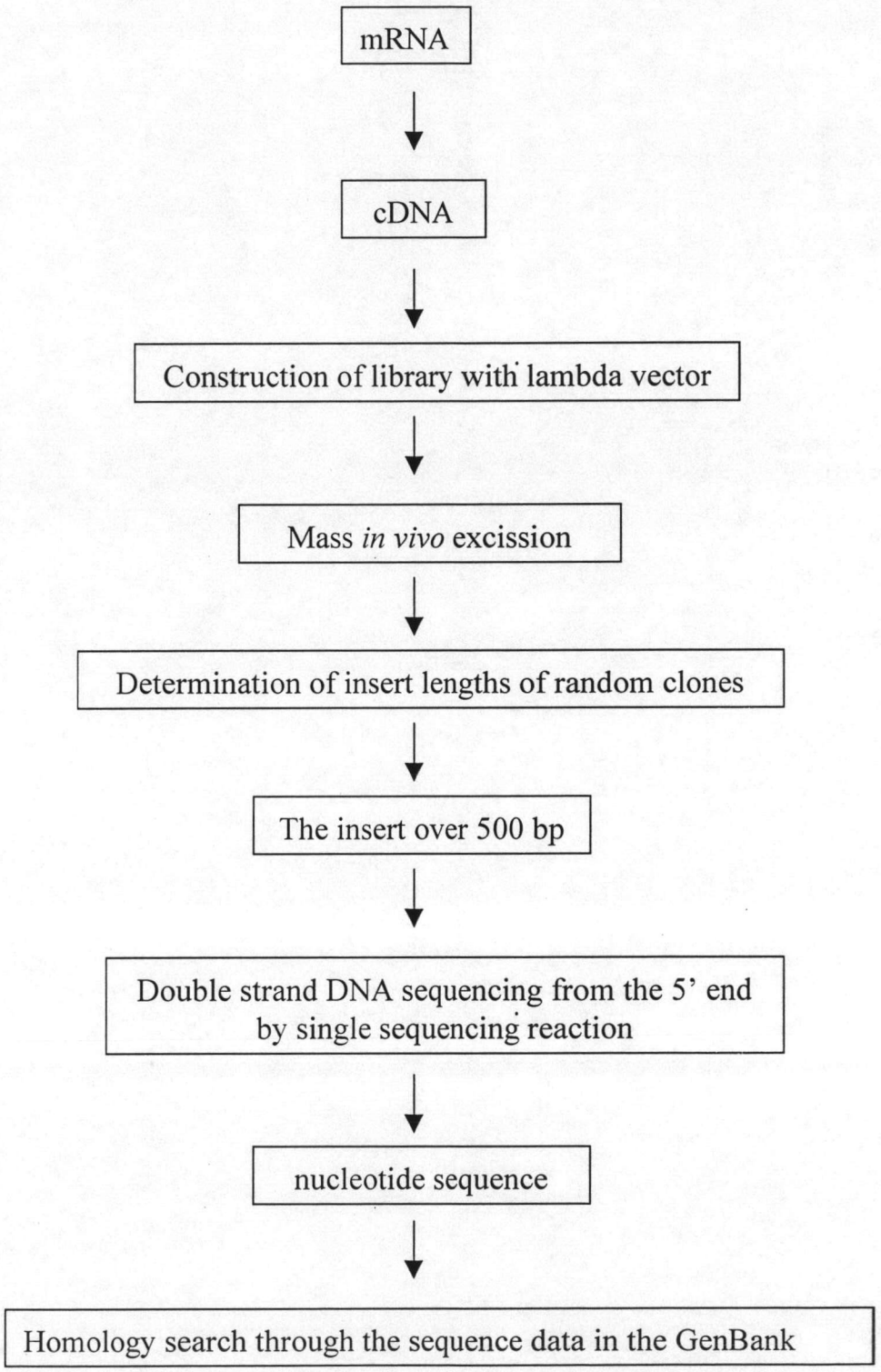


Figure 1.10 Flowchart of cDNA clone tagging

interesting tissue and these mRNAs are used to construct the cDNA library in λ vector. After in *vivo* excision, the λ converts to the plasmids. Then the plasmids are randomly selected and determined the insert length by PCR or enzymatic digestion to ensure that every clone that is sequenced contains at least part of the coding region. The inserts over 500 bp are partial 5' sequenced to access the coding region. Because the coding sequence is more conserved than the untranslated region and should provide a better chance to identify a gene. This sequence result was used as a tag to homology search through the sequence data in the GenBank by using the Blastn and Blastx program (NCBI) Advanced Blast search (Altschl et al., 1997). The Blast program is used the nucleotide sequence to compared against the NCBI nucleotide database in all 6 reading frames. The translated protein products are then compared against the NCBI protein database. The sequences are considered to be significantly matched when the possibility value (E-value) is less than 0.0001 and the match length is > 100 nucleotides for Blastn and a match length is > 10 amino acid residues for Blastx, respectively, according to Anderson and Brass (1998).

In the GenBank database, the number of public entries is 16,292,594 EST as of April 11, 2003. The top 10 organisms represented in dbEST were summarized in Table 1.3. The fruit fly, *Drosophilla melanogaster*, is the 11th that has 261,271 ESTs. For Penaeid shrimps, there are 1,042 and 703 EST sequences diposited in the GenBank for *P. setiferus* and *P. monodon*, respectively (dbEST related by NCBI April 11, 2003).

Table 1.3 Top 10 organisms represent in dbEST (number of EST sequence entries as of 11 April, 2003.

Organism	No. ofESTs
Homo sapiens	5,041573
Mus musculus	3,710,187
Ciona intestinalis	492,488
Ratus sp.	476,677
Gallus gallus	418,093
Triticum aestivum	415,728
Bos taurus	319,713
Danio rerio	309,461
Glycine max	308,583
Xenopus laevis	268,354

The cDNA libraries constructed from many tissues of Penaeid shrimps were reported. In 1999, Lehnert et al. constructed 3 cDNA libraries from cephalothorax, eyestalk and pleopod tissue of the black tiger shrimp, *P. monodon*. They found 60 newly isolated genes, 49 of which have not previously identified in crustaceans. They found the putative identities of these genes reflected the expected tissue specifity. For example, genes for digestive enzymes were identified from the cephalothorax library and genes involved in the visual and neuroendocrine system from the eyestalk library. Gross et al. (2001) constructed the haemocyte and hepatopancreas cDNA libraries from *P. vannamei* and *P. sertiferus*. These libraries showed high redundancy of penaeidin, the antimicrobial peptide in the haemocyte libraries and high redundancy of lectin in the hepatopancreas of both libraries. The haemocyte cDNA libraries of healthy shrimps and WSSV infected shrimps, *P.*

japonicus were constructed by Rojtinnakorn et al. (2002). They found that ESTs representing proteinase inhibitor and tumor-related protein only in the WSSV-infected library and the apototic peptides were expressed at high level in the infected library.

Application of tagged cDNA clones

Expression profile

Large-scale cDNA clone tagging has become a important approach to analyz gene expression. By this approach, two types of information can be obtained from an unbiased cDNA library: the profile of expressed genes in the source tissue and the relative abundance of these transcripts (Adams et al., 1995). Both types of information are important the undrestanding of the function of the source tissue or cell type.

Isolated of full-lenth cDNA clones

Many of the identified cDNAs, full-length clones remain to be isolated for other studies such as functional analysis and expression of recombinant proteins. To recover the full-length cDNA, the tagged partial clone can be determined the full nucleotide sequences by 5' or 3' Rapid amplification cDNA end (RACE).

Tissue and cell-type markers

For some of the clones, tissue specificity can be predicted from the gene name and confirmed by *in situ* hybridization and northern hybridization. These clones can also be used to express recombinant proteins to raise antibodies for expression analysis. Some clones can be used as molecular markers for various tissues and all types.

Genome mapping

EST clones should be useful for identification and mapping of single nucleotide polymorphism (SNP) markers. Moreover, EST clones will also be used to construct the genetic linkage map using type I markers (coding genes), comparative genetic maps between species and closely related species.

1.10 Objective of the thesis

The aim of this thesis is to identify and characterize cDNAs of immune-related protein in the haemocyte of normal and *V. harveyi* infected *P. monodon* by using the expressed sequence tags. The cDNA homologues of immune-related protein were analyzed the expression level after injection of Gram-negative bacteria, *V. harveyi*.