CHAPTER I

INTRODUCTION

1.1 General introduction

The black tiger shrimp (Penaeus monodon), cultured throughout Asia, has always represented more than fifty percent of world production of farmed shrimp. 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 of shrimp production while in the East (Chanthaburi) and Central regions (Samut Sakhon and Samut Songkhram) 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 was 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, respectively.

Table 1.1 The world 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
Vietnam	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
Colombia	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

Whereas, Thailand had a severe outbreak during 1995-1997 causing the decrease in the shrimp production at that period (Figure 1.1). Nevertheless, Thailand is still the largest *P. monodon* producer (Figure 1.2).

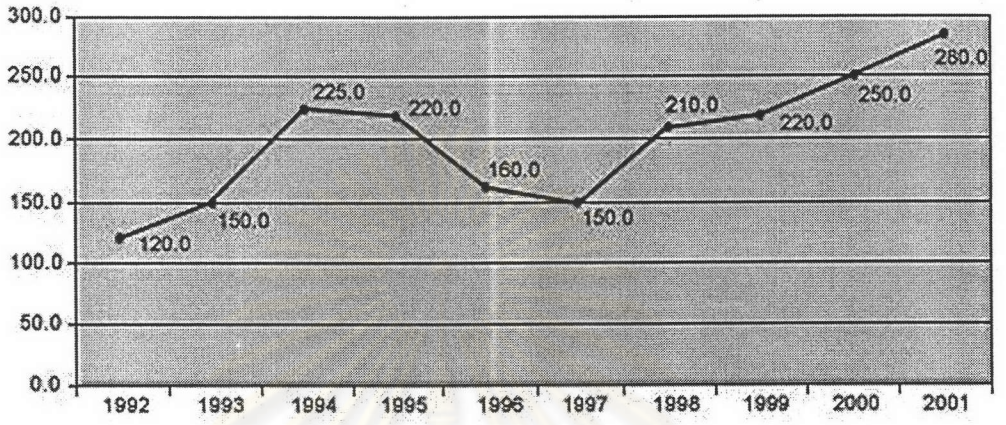

Figure 1.1 Thai shrimp aquaculture production during1992-2001(in 1000 metric tons)

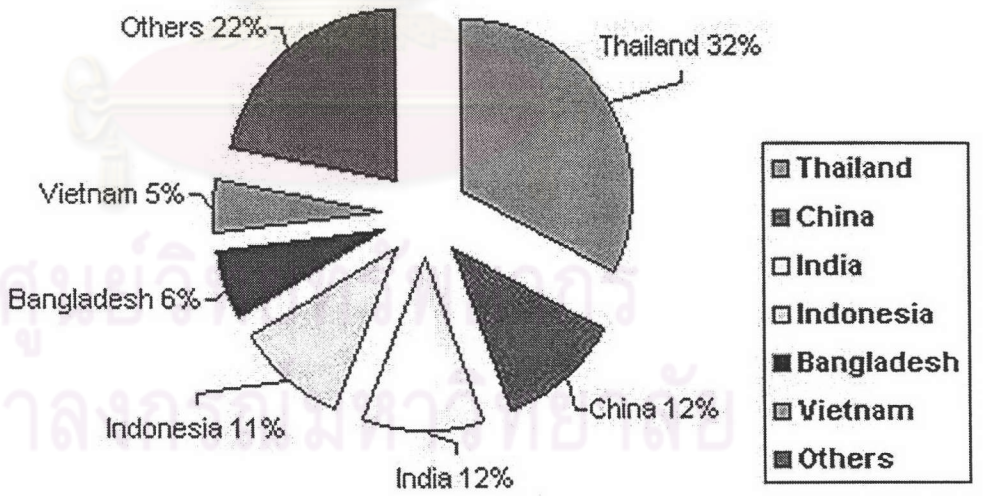

Figure 1.2 Major producers of aquaculture shrimp in the world (year 2001)

Source: Globefish

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, worth for 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)
U.S.A.	66,998.08	27,207.52
JAPAN	24,854.61	11,663.23
CANADA	5,757.72	2,245.39
SINGAPORE	6,610.47	2,128.58
TAIWAN	6,307.84	1,762.31
AUSTRALIA	3,638.26	1,405.53
KOREA	4,120.70	1,270.28
CHINA, PEOPLE'S REPUBLIC	3,411.50	1,051.16
HONG KONG	2,621.07	975.37
United KINGDOM	1,587.22	597.55
OTHER COUNTRY	9,039.26	2,927.61
Total	134,946.73	53,234.53

Source: Department of Fisheries

The farming activity of *P. monodon* in Thailand has rapidly increased the large annual production. In year 2003 from January to September, export of *P. monodon* 120,451,243 kg account for 39,795.2 million baht. 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 : Black tiger shrimp or Giant tiger shrimp
FAO Names : Giant tiger shrimp, Crevette giante tigre, Camaron tigre gigante.

1.3 Morphology

Externally the prawn can be divided basically into the thorax and abdomen (Figure 1.3). The thorax (or head) is covered by a single, immobile carapace, which protects internal organs and supports muscle origins. The rostrum, extending beyond the tip of the antennular peduncle, is sigmoidal in shape. 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 pair of swimming legs pleopods arises from each of the six 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 quick 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 molted. After molting 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).

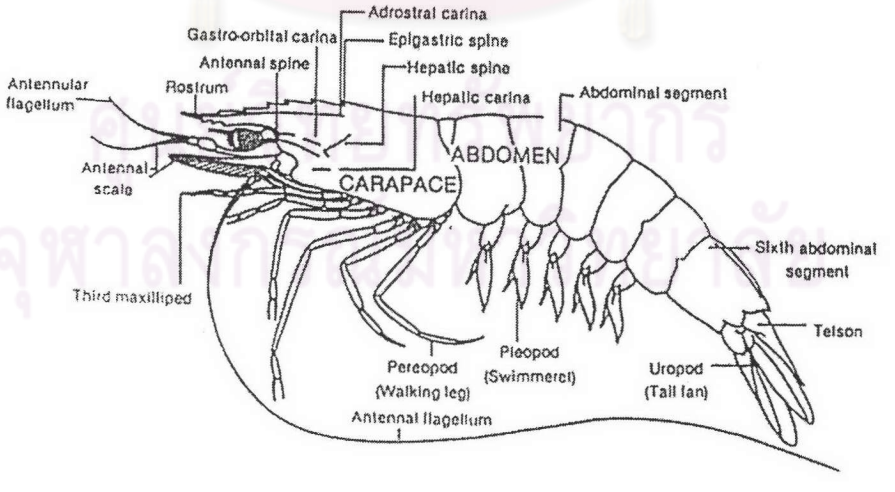

Figure 1.3 Lateral view of *P. monodon* showing important parts

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 1.4). 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 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).

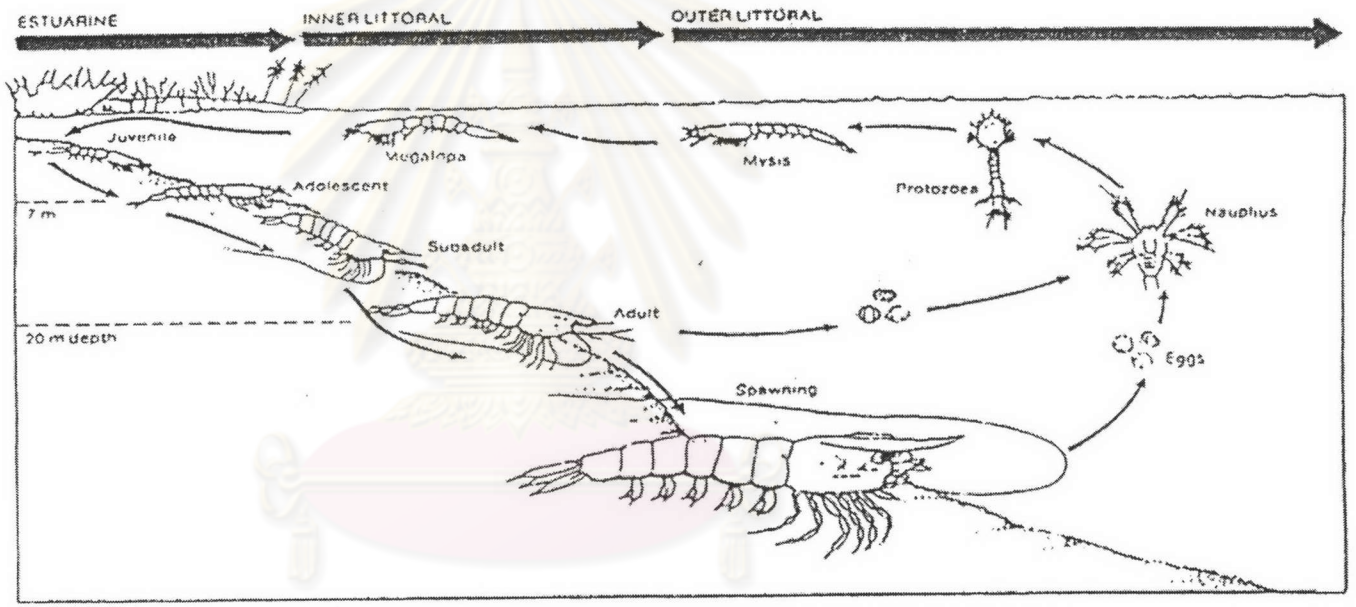

Figure 1.4 Life cycle of penaeid shrimp (Bailey-Brock and Mass, 1992)

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1.5 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). 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.

Consequently, the control of disease became a priority at the world level, if shrimp production is to be ecologically and economically sustainable. To a greater extent, the durability of the production is dependent on the equilibrium between the environment quality, the prevention of diseases by diagnosis and epidemiological surveys of the pathogens, and the health status of the shrimp. Finally, shrimp aquaculture is also dependent on the selection of the disease resistant animals. Therefore, the prevention and the control of shrimp diseases need an integrated approach in which basic knowledge of shrimp immunity must be improved. Consideration, should also be given to other research areas related to shrimp pathology and physiology, in close connection to research in genetics.

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

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 is the clinical sign of this disease. 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. However, 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., 1996).

Yellow-head (YH) disease

YHV, called Hua leung in Thailand (Chantanachookin et al., 1993; Lightner, 1996), 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).

Shrimp infected with YHV often show light yellow coloration of the dorsal cephalothorax area and have a pale or bleached appearance (Limsuwan, 1991. 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. After infection, mortality may reach as high as 100% of affected populations within 3-5 days from on set of disease. 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).

1.5.2 Bacterial disease

Vibriosis is a major bacterial 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, 1998). However, some 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 (Figure 1.5). 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:

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

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 (Figure 1.6).

The diseased shrimp has milking white body and appendages, weakness, disoriented swimming, lethargy, eventually leading to death.

Vibriosis is the main cause of production loss in Thailand. The major vibrio species isolates from diseased shrimp are *Vibrio parahaemolyticus, V. harveyi, V. alginolyticus,* and *V. vulnificus,* while *V. damsela, V. anguillarum,* and *V. fluvia* have been reported less frequently (Leangphibul et al., 1985; Lightner, 1988; Ruangpan and Kitao, 1991; Nash et al., 1992; Jiravanichpaisal et al., 1994). Vibriosis caused mortality in larvae, post-larvae, juveniles, sub-adults and adults. At times, outbreaks cause mortality up to nearly 100% of affected populations (Lightner, 1983). The gross signs of localized infection in the cuticle or sub-cuticle are called shell disease or black or brown spot disease and these superficial infections can develop into systemic infections under some circumstances. It is the systemic infections that cause mortality.

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 from increasing number of bacterial resistance to antibiotics. Tjahjadi et al. (1994) reported that most luminious vibrios isolated from a shrimp hatcheries in Kalianget, East Java were resistant to a number of antibiotics tested, except 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 antibiotics residuals.

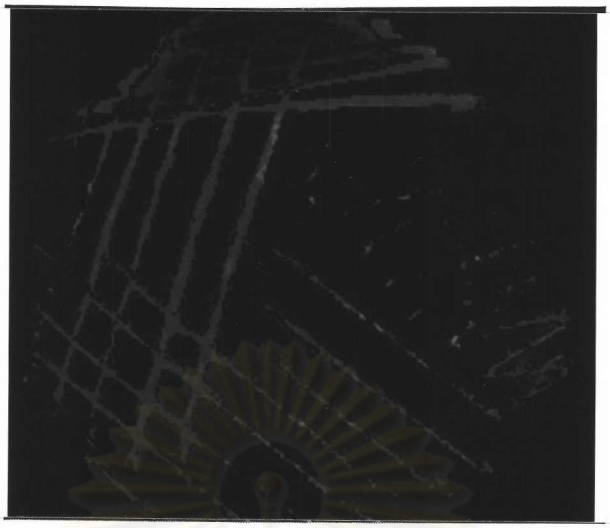

Figure 1.5 The blue-green color of light emission form *Vibrio harveyi* (Photograph by Premruethai Supungul)

Figure 1.6 Shrimp with luminous disease (Photograph by Premruethai Supungul)

1.6 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 recognize conserved molecular patterns present on microorganisms (Janeway, 1998). 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 whereas invertebrates have a rapid and efficient innate system to recognize 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.

Invertebrates have characteristic host defense systems different to mammalian immune systems. Invertebrates lack of antibodies, they do not possess proteins with domains belonging to the immunoglobulins, nor memory following the first encounter with a pathogen. Instead, they have developed innate immune systems to defend themselves against invading foreign materials.

In crustaceans, this innate defense system includes hemolymph coagulation, melanization, cytolysis, cell agglutination, antimicrobial actions, phagocytosis, and encapsulation against pathogens. Their defense systems are activated by the recognition of common epitopes on the surface of pathogens. 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 lipopolysaccharides (LPS) of Gram negative bacteria, glycolipids of mycobacteria, lipoteichoic acids of Gram positive bacteria, mannans of yeasts, the β–1,3-glucan of fungi and double-stranded RNA of viruses (Hoffmann et al., 1999).

Crustacean immune response to pathogens is based on both cellular and humoral component of the circulatory system, which cooperates to eliminate potentially infectious pathogens. The humoral factors comprise molecules that act in the defense without direct involvement of cells, humoral response, although many of the factors are originally synthesized and stored in the blood cells. Consequently, the actions with direct participation of blood cells are understood by the term cellular response.

1.6.1 Blood cells

Crustaceans have open circulatory system. The major defense systems of crustaceans are carried in the hemolymph, which contains a cells called hemocyte. The recognition molecules may interact with and activate the hemocytes, which play an important and central role in host defense. Hemocytes are the effectors of the cellular immune response and they are also involved in the synthesis of the majority of humoral effectors.

In crustaceans, according to numerous works dealing with the identification of hemocyte cell types, a classification scheme has been commonly adopted with three types of circulating hemocytes: hyaline cells, semigranular cells, and granular cells (Bauchau, 1981; Martin and Graves, 1985; Tsing et al., 1989; Rodriguez et al., 1995; Van de Braak et al., 1996).

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 contains 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). Semigranular cells and granular cells were participating in prophenoloxidase (proPO) system, which is an important component of the cellular defense reaction (Johansson and Soderhall, 1985).

1.6.2 Mechanism of defense reaction

1.6.2.1 Pattern recognition proteins

Pattern recognition proteins have been isolated and characterized in several invertebrates. These PRPs recognize and 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; 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 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 agglutinins from *P. monodon* plasma have been purified by affinity chromatography. This lectin, named monodins, which induced the agglutination of the highly pathogenic bacteria *V. vulnificus,* a major infective bacterium for prawns (Ratanapo and Chulavatnatol, 1992).

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. The first crustacean BGBP was reported in freshwater crayfish, *Pacifastacus leniusculus* (Davic and Soderhall, 1990), and two marine shrimp species, *P. californiensis* (Vargas-Albores et al., 1996) and *Penaeus vannamei* (Vargas-Albores et al., 1997). 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 haemolymph are engulfed and immobilized by the clot, and subsequently killed by antimicrobial substances that are also released from the two types of granules.

1.6.2.2 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). The activation 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 involving in the process of sclerotisation, wound healing and encapsulation of foreign materials (Lai-Fook, 1996; Sugumaran, 1991). Several components and associated factors 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* 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 isolated in several insects (Jiang 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.

The proposed proPO activation model for crustaceans (Johansson and Soderhall, 1989; Soderhall, 1992; Soderhall et al., 1994) involves a proteolytic

cleavage mediated by a serine proteinase (Aspan et al., 1990a, Aspan et al., 1990b), namely proPO activating enzyme (PPAE)(Figure 1.7). In shrimp, there are two steps involved in the activation of proPO. The first one is the degranulation that occurs when hemocytes are stimulated with bacteria, LPS or beta glucans, and inactive forms of both proPO and PPAE are released. The second one requires the participation of Ca2+ for the conversion of inactive PPAE to an active proteinase that, in turn, transforms proPO to active PO (Aspan and Soderhall, 1991; Gallas-Galvan et al., 1997). PO has the ability to adhere to surfaces, and this leads to the formation of melanin on the surface of the pathogen.

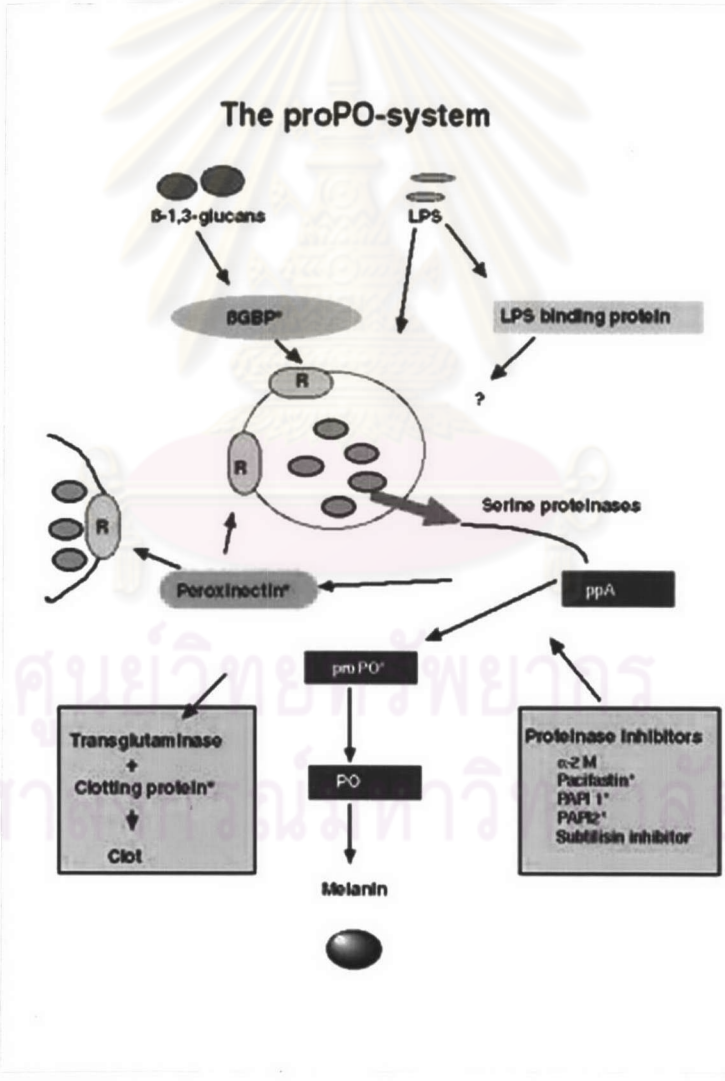

Figure 1.7 proPO activation model (Soderhall K.)

1.6.2.3 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) (Figure 1.8) (Iwanaga, 1993; Kawabata et al., 1996).

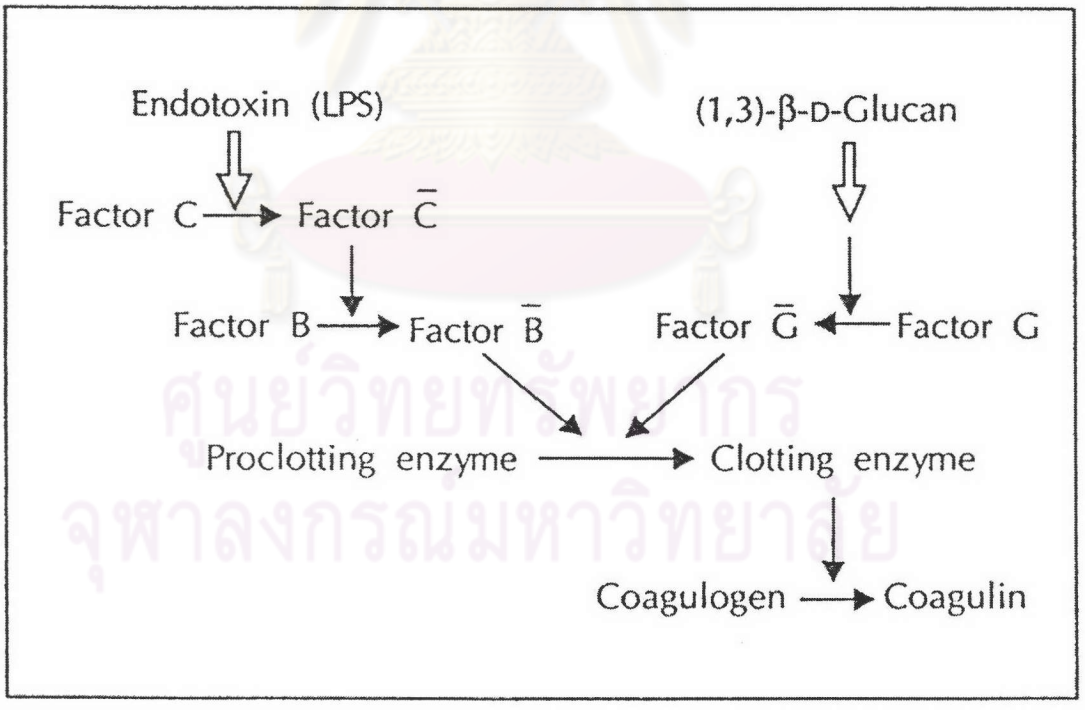

Figure 1.8 Haemolymph coagulation cascade in *Tachypleus tridentatus* (Iwanaga, 1993)

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.

1.6.2.4 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.

There are few reports on antimicrobial peptides in crustaceans. Tachyplesin family and anti-LPS factors which acting against Gram negative bacteria were reported in horseshoe crab (Nakamura et al., 1988; Muta et al., 1990; Ohashi et al., 1984; Aketagawa et al., 1985). 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 cysteine-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).

In arthropods, several of antimicrobial peptides were isolated and characterized, mainly in insects and chelicerates (horseshoe crabs) (Iwanaga et al., 1998). In horseshoe crabs, these proteins 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. 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.

1.7 Expression system

1.7.1 Baculovirus expression system

Baculovirus is a group of large double-stranded DNA virus that infect many different species of insect. In this system, baculovirus nonessential gene in the tissue culture life cycle was replaced by heterologous gene.

The baculovirus expression system offer a number of advantages, including high expression level, limitless size of the expressed protein, efficient cleavage of signal peptides, post-translational modifications and corrected protein folding. The host of baculovirus was insect, which was an arthropod as same as shrimp consequently the expression and modification of gene products are probably more similar than other eukaryotic expression system (Baculovirus expression manual, Pharmingen).

1.7.2 *Pichia pastoris* expression system

Pichia pastoris is a methylotrophic yeast, which metabolized methanol as its sole carbon source. The first step in metabolizing methanol is oxidation of methanol by enzyme alcohol oxidase. The promoter regulating the production of alcohol oxidase was used to drive recombinant protein expression in *Pichia*, so methanol can be used to induce the expression of recombinant protein.

Yeast (*Pichia*) expression system is selected because of, *Pichia pastoris* has many of the advantages of higher eukaryotic expression systems such as protein processing, protein folding, and posttranslational modification, while being as easy to manipulate as *E. coli* or *Saccharomyces cerevisiae*. It is faster, easier, and less expensive to use than other eukaryotic expression systems and generally gives higher expression levels (Pichia expression manual, invitrogen).

Anti-lipopolysaccharide (anti-LPS) factor

Anti-LPS factor is a small basic protein, which was found during the purification of proteins in coagulation system initially characterized in horseshoe crab. This protein, which has been identified to be an anticoagulant towards the LPS-mediated coagulation system, was found in the hemocyte lysate from Japanese *(Tachypleus tridentatus)* and American *(Limulus polyphemus)* horseshoe crabs (Iwanaga et al., 1985).

Anti-LPS factor had an activity to binds and neutralizes bacterial endotoxin (LPS) and has a strong antibacterial effect especially on the growth of Gram-negative R-type bacteria (Morita et al., 1985) and a hemolytic activity on the red blood cells sensitized with LPS. In hemocytes, this protein is located in L-granules with several clotting factors (Iwanaga et al., 1998).

The primary structure of anti-LPS factor isolated from the Japanese horseshoe crab was first determined in 1986 (Aketagawa et al., 1986) and the next year the American horseshoe crab was established (Muta et al., 1987). The NH_2-terminus of the Japanese horseshoe crab anti-LPS factor was masked with pyroglutamic acid whereas; the American horseshoe crab anti-LPS factor has its NH_2-terminus masked with an aspartyl residue. American horseshoe crab and Japanese horseshoe crab anti-LPS factors are single chain polypeptides composed of 101 or 102 amino acid residues respectively, which contain two cysteine residues linked with an intramolecular disulfide bridge, and have a relative molecular mass of 12000. No glycosylation was found. The sequence of *L. polyphemus* anti-LPS factor obtained here shows 83% sequence identity with that of *T. tridentatus.* The hydropathic profile indicates that the NH_2-terminal region of anti-LPS factor is highly hydrophobic and the remaining region contains positively charged residues, mainly found within the disulfide loop. The region from Arg-41 to Lys-49 has basic amino acids at every second residues, and the region from Arg-61 to Arg-76 is also rich in basic residues. In this region (Arg-61 to Arg-76), there are positive charges at almost every third residues. The, clusterings of charged groups and high hydrophobicity of the

NH_2-terminal region suggest that the molecule of anti-LPS factor is amphipathic.

The three-dimensional structure of *Limulus* anti-LPS factor (LALF) was reported in 1993. The crystal structure of anti-LPS factor reveals a simple tertiary fold with a striking charge distribution and amphipathicity. The molecule is wedge shaped, about 40 Å tall and 28 Å wide at its base (Figure 1.9) (Hoess et al., 1993). The molecule has a single domain consisting of three a-helices packed against a four-stranded β-sheet. The binding site for LPS probably involves the extend amphipathic loop of anti-LPS factor, which binds the phosphoglucosamine portion of lipid A. An analogous LPS-binding loop also exists for two human proteins that bind LPS, LPS-binding protein (LBP) (Schumann et al., 1990) and bactericidal/permeability-increasing protein (BPI) (Marra et al., 1992).

As mentioned earlier, anti-LPS factor contains two positively charged regions and an NH2-terminal hydrophobic region. These two positively charged clusters might provide interaction site with phosphate groups in the lipid A portion of LPS. Once anti-LPS factor interacts with LPS on the cell membrane through the LPS-binding loop, the membrane structure seems to be perturbed by insertion of the hydrophobic NH_2-terminal region, including up to about the 27^{th} residue. The region appears to be long enough to cross a lipid bilayer-like transmembrane α-helix of the bacteriorhodopsin molecule.

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Figure 1.9 Schematic ribbons representation of Limulus anti-LPS factor with the ends of secondary structure elements numbered (Hoess et al., 1993)

In our laboratory previous study, expressed sequence tags (ESTs) from the hemocyte of the black tiger shrimp, *P. monodon,* were generated in order to identify gene associated with shrimp immunity. A hemocyte cDNA library was constructed. Randomly selected cDNA clones with insert over 500 bp in length were sequenced. The partial nucleotide sequences of cDNA clones were compared with sequences in the GenBank database using the BLAST program. From these ESTs, 9% matched with genes involved in defense reaction molecules, such as the components of the proPO system including PO, proPO activating enzymes; the components of the clotting system including glutamine gamma-glutamyl transferase, hemocyte protease; the antioxidative enzymes, peroxidase and catalase; antimicrobialpeptides including anti-LPS factor, penaeidins, 11.5 kDa antibacterial peptides, lysozyme, and serine proteinase inhibitors (Supangul et. al., 2002).

Three full-length cDNA clones were found to encode a homologue of anti-LPS factor (Sh13, Sh20 and Sh71). The insert fragment of the cDNA clone was 517 bp in length, which contains an open reading frame (ORF) of 372 bp encoding 123 amino acids. The deduced amino acid shows 57% homology to the anti-LPS factor from Atlantic horseshoe crab.

The amino acid sequences alignment of anti-LPS factor purified from the horseshoe crabs, *L. polyphemus, T. tridentatus,* and the black tiger shrimp *P. monodon* shows the two conserved cysteine residues, and highly positive charged residues within the disulfide loop, which are necessary for disulfide bridge formation in three dimensional structure and the binding to the lipid A moiety of LPS, respectively. The NH_2-terminal region is highly hydrophobic and anti-LPS factor purified from horseshoe crabs were found to lack the NH2-terminal portion. The extra 26 amino acid residues at the NH_2-terminus of the *P. monodon* anti-LPS factor was proposed to be a signal peptide for protein transport.

Gene encodes a homologue of anti-LPS factor clone Sh71 was selected to cloning and expression in the baculovirus expression system. Two versions of the gene expressing full-length and NH_2-terminal truncated derivative of the

protein were constructed ptr13 and pΔNAL7 respectively. A full-length version of anti-LPS factor with a signal peptide was constructed to direct the expressed protein out of the cells for the ease of protein purification, while NH2-terminal deletion version was constructed to eliminate the signal peptide to obtain a mature protein (Anurakolan, 2001). Whereas two types of constructed virus were expressed intracellular. There are many species of protein in the host cells thus it was difficult to purified anti-LPS factor proteins. The solution is constructed two new version of virus by added histidine tag to the N-terminal of full-length and NH_2-terminal truncated anti-LPS factor proteins to help the purification step so easily.

The aim of this study is to characterize the anti-LPS factor of *P. monodon* by cloning, expression and purification of the cDNA clone (Sh71) in the Eucaryotes expression system, baculovirus and yeast. The gene product will be investigated for the activity of the expressed proteins.

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