CHAPTER I
INTRODUCTION

1.1 General introduction

Thailand has been the world's leader for exporting shrimp products since 1993. The shrimp species which is mainly produced in Thailand and, of course, dominating the major global share of shrimp products and the total world shrimp production are *Penaeus monodon*, the black tiger shrimp. The frozen and value–added products have been exported to several countries, e.g. Japan, USA and the European Union. The industry is worth approximately 200,000-250,000 metric tons annually, providing an income of nearly 60,000 million bath yearly export earnings for the country (Figure 1.1) (Source: Thai Customs Department, cited in Shrimp Culture Newsletter).

In 2002, Thailand's shrimp production fell about 40% from 2001 to approximately 160,000 tons. This was due to many detrimental factors, which included the outbreaks of infectious diseases, the unfavorable weather, the high salt concentrations in water, the slow shrimp growth rates, the low yields per area, the switching to other shrimp species, such as the fresh water shrimp or white shrimp, and the concerning over the antibiotic residues. The shrinkage in the Thailand's shrimp export, traded to especially the United States, had an impact on the Thailand's share in the world markets, meaning that the other major shrimp exporting countries were able to increase their output in that year. The continual disease problem also caused the great impact on *P. monodon* production yield in the coming years. In 2004, *P. monodon* production of Thailand fell down to 75,000 tons, whereas those of the other countries were increased (Table 1.1). Nevertheless, the total shrimp production in Thailand was not significantly affected due to the increase production of the white shrimp *Litopenaeus vannamei*. According to the Thai Customs Department, the export value of Thai shrimp in 2001 to 2005 was not significantly decreased.

Since the outbreaks of shrimp diseases, the shrimp farming has been switched to the other species, namely, the Pacific white shrimp (*Litopenaeus vannamei*).

Because it is a genetically–improved strain, *L. vannamei* possesses many great advantages over the *P. monodon*, including the rapid growth rate, the high stocking density tolerance, the low salinity and temperature tolerance, the lower protein requirement (and hence the production costs), the certain disease resistance (in specific–pathogen–free shrimp), and the high survival rate during larval rearing (50-60% compared to 20-30% for *P. monodon*). Since it is an alien species for Thailand, *L. vannamei* possibly acts as a carrier of *Baculovirus penaei* (BP), Infectious hypodermal and hematopoietic necrosis virus (IHHNV), Reo-like virus (REO), and Taura Syndrome virus (TSV). These viruses can be transmitted to the native wild penaeid shrimp populations, and thus increase the concern over the spreading of the diseases by the releases of infected shrimps from the culture facilities (Overstreet *et al.*, 1997). The first signs of Taura Syndrome Virus (TSV) in Thai *L. vannamei* and *Macrobrachium rosengii* has been observed (Briggs *et al.*, 2004), and the possibility of virus outbreak on cultured *L. vannamei* as in Latin America is of great concern.

The world has learned that the intensive farming of only one shrimp species leads to the outbreak of diseases. Changing the species by the importing the alien species, *L. vannamei,* believed to be pathogen-free (resistant) and give better yield, along with the use of intensive framing system certainly follows the same cause as in the past. In addition, Thailand has to pay for the post larvae, the cost that reduces the profit, if any, from the farming. Although the farms can survive the crisis by the cultivation of a new shrimp species, it does not mean the sustainability of shrimp production in the long run since the actual causes of the infectious disease are still there in the farm. From the above reasons, the shrimp farming of the native rather than the alien shrimp species should be considered as essential for this area. Although overall biological systems of *P. monodon* are progressively studied at the molecular level, the knowledge of the immune system is also considered to be essential and the intensive study is needed in order to characterize the responses of the shrimp to infectious pathogens. The information would be applied for the selective breeding of the healthy shrimp in the near future for the sustainability of shrimp production industry in Thailand.

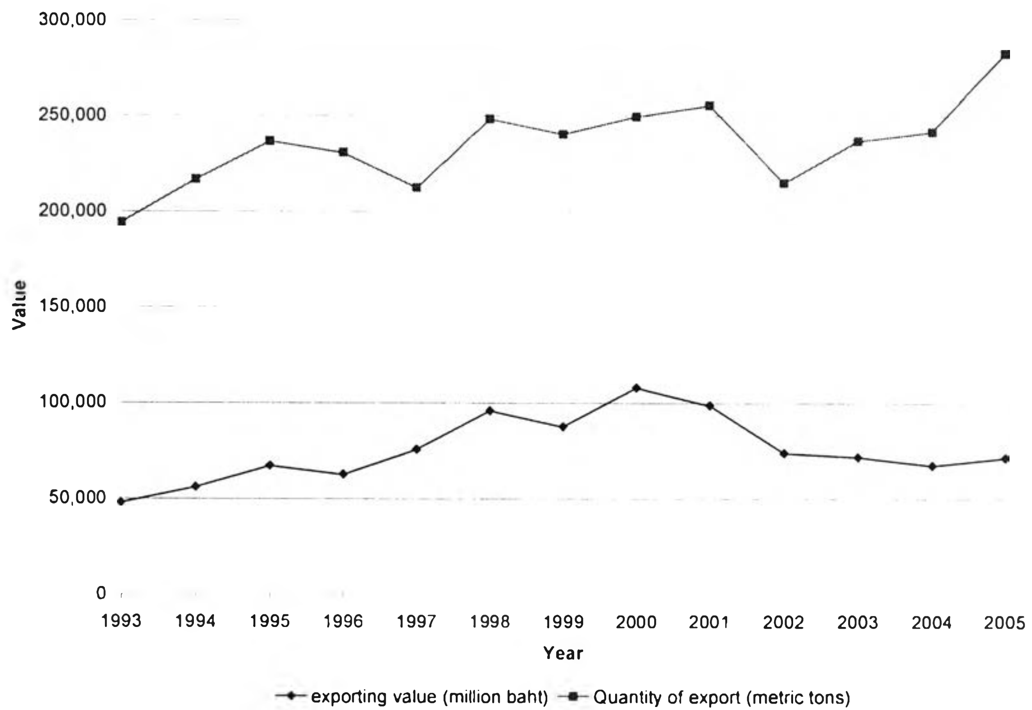

Figure 1.1 Cultured shrimp production in Thailand from 1993–2005 (Source: Thai Customs Department cited in Shrimp Culture Newsletter)

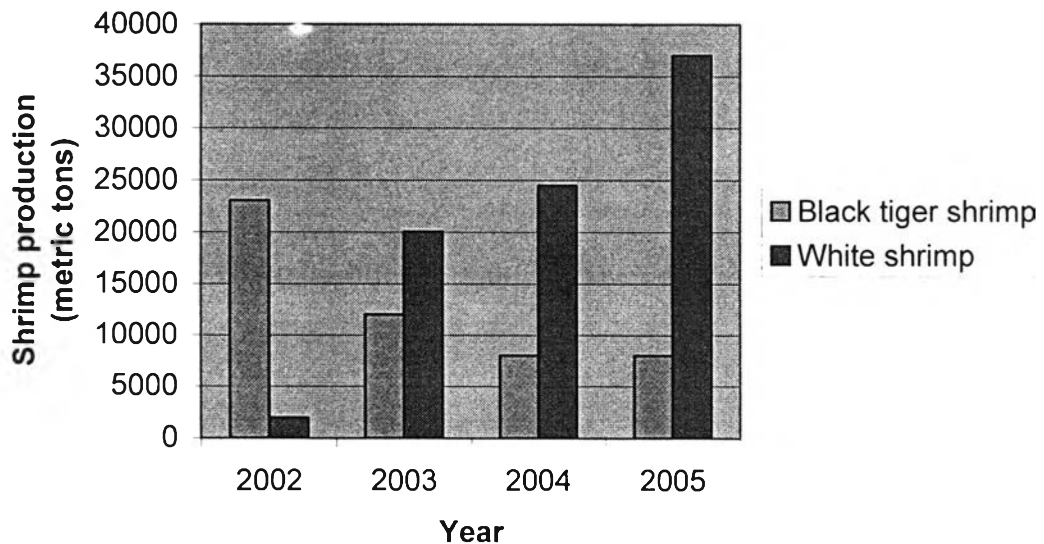

Figure 1.2 The values of black tiger shrimp and white shrimp production in Thailand from 2002 to 2005 (Source: http://www.shrimpcenter.com)

Table 1.1 *Penaeus monodon* production in various countries in 2004

Country	*P. monodon* production (tons)
India	120,000
Vietnam	100,000
Indonesia	60,000
Philippines	35,000
Malaysia	25,000
Thailand	75,000

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 tiger, Camaron tigre gigante.

1.3 Morphology

The shrimp body includes three regions: head, thorax, and abdomen (Figure 1.3). Each body region possesses appendages specialized for different functions. The head (five somites) and thorax (eight somites) are fused into a cephalothorax, which is completely covered by the carapace. Many internal organs, such as gills, digestive system, reproductive system and heart, located in thorax, are protected by the carapace. Shrimp muscles concentrate in the abdomen. The pleura of the cephalothorax form the branchiostegite or gill cover. The carapace has characteristic ridges (carinae) and grooves (sulci). The rostrum is always prominent, with a high median blade bearing dorsal teeth and, in some genera, ventral teeth as well. The compound eyes are stalked and laterally mobile and the somites of the head bear, in order, pairs of antennules, antennae, mandibles, maxillae 1 and maxillae 2 (not visible in Figure1.3). The thorax has three pairs of maxillipeds and five pairs of pereopods (legs), the first three being chelate and used for feeding, and last two simple (non-chelate) and used for walking. The mouth is situated ventrally and the cephalic appendages surrounding it, plus the first and second maxillipeds and sometimes the third as well may be referred to collectively as the "mouth parts". The abdomen has the obvious segmentation of invertebrates. The abdomen consists of six somites, the first five with paired pleopods (walking legs) (Baily-Brook & Moss, 1992; Bell & Lightner, 1988) and the sixth with uropods. A tail fan comprises of a telson, which bears the anus. 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 anus is on the ventral surface of the telson towards its base (Dall *et al.*, 1990).

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,

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while the old cuticle is moulted. After moulting the new cuticle is soft and is stretched to accommodate the increased sized of the shrimp.

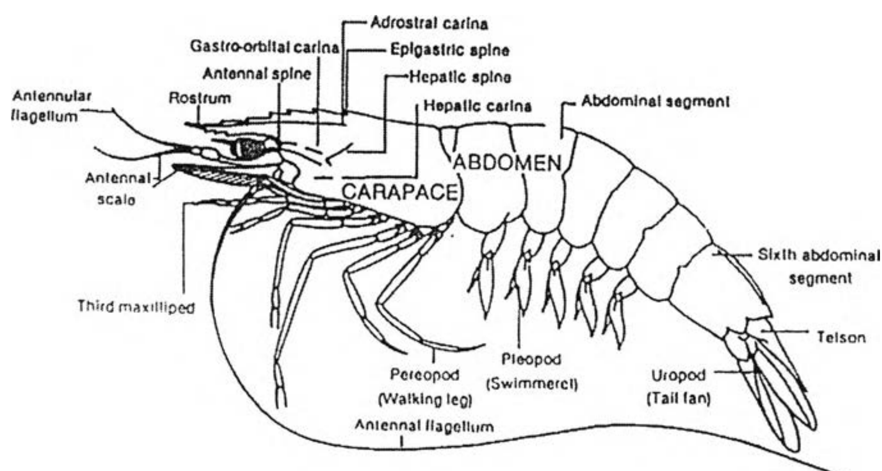

Figure 1.3 Lateral view of the external morphology of *Penaeus monodon* (Anderson, 1993)

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).

The internal morphology of penaeid shrimp is very well developed (Figure 1.4). Muscular, digestive, circulatory, respiratory, nervous, and reproductive systems are all present. The movements of the body such as walking, crawling, burrowing, swimming, feeding, and breathing are controlled by the muscles. The digestive system is complex, in which parts of the tract are differentiated into a foregut, a midgut, and a hindgut. The circulatory system consists of a heart, dorsally located in the cephalothorax, with branching arteries conducting blood to the various organs. Gills

are responsible for respiration process. The nervous system consists of two ventral nerve cords, a dorsal brain, and a pair of ganglia for each somite.

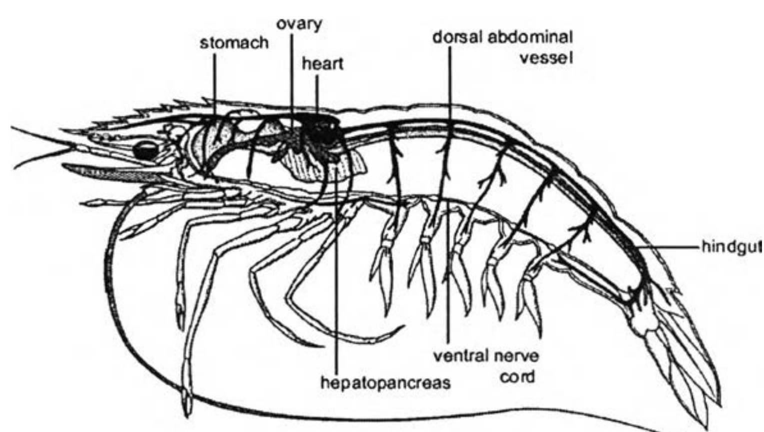

Figure 1.4 Lateral view of the internal anatomy of a female *Penaeus monodon* (Primavera, 1990)

Hepatopancreas connects to the gastrointestinal tract via the primary duct. It occupies a large portion of the cephalothorax in penaeid shrimp and functions on absorption of nutrients, storage of lipids and production of digestive enzymes (Johnson, 1980). One of the haemolymph vessels that leave the heart ends in the lymphoid organ where the haemolymph is filtered. This organ consists of two distinct lobes, each located ventro-lateral to the junction of the anterior and posterior stomach chambers. The lymphoid lobes are apposed slightly dorso-anterior to the ventral hepatopancreatic lobe. The haemocytes are produced in haematopoietic tissue. The hematopoietic tissue consists of densely packed lobules located at different parts of the shrimp anterior region. The first one is hematopoietic tissue surrounding the lateral arterial vessel, which joins the anterior recurrent artery at the base of the rostrum. The second one located within the 1st maxillliped. The third one is 2nd maxilliped hematopoietic tissue. The last one is epigastric hematopoietic tissue located dorsal to the anterior stomach chamber and just ventral to the dorsal cuticle.

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 1.5). 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).

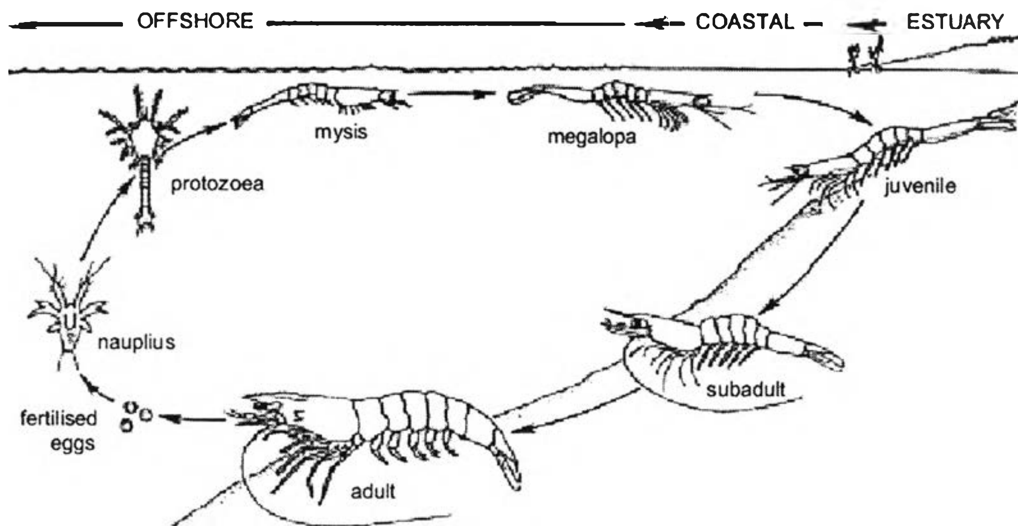

Figure 1.5 The life cycle of *Penaeus monodon* shrimp (Baily-Brook & Moss, 1992).

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. 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). This species is one of the most important aquaculture shrimp species in Asia (Rosenberry, 1997). The development of penaeid shrimps include several distinct stages in various habitats are shown in Figure 1.5.

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. As the shrimp farming grows, several

detrimental problems are developed. These include the environmental degradation, the outbreak of diseases, and the depletion of wild broodstock used to stock commercial hatcheries (Browdy, 1998).

The outbreaks of infectious diseases have become more and more serious, causing a great loss to the productions (Roch, 1999). The main causes of infectious diseases 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 the 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 and controllable. Therefore, more knowledge on shrimp immune system and genetics are required.

The farming of *P. monodon* relies entirely on wild caught broodstock for the supply of juveniles because breeding of this species in captivity is extremely difficult. The high demand on broodstock leads to over-fishing in the sea. The broodstock is gradually declining in the wild population (Browdy, 1998). For this reason, the research concerning domestication of this species has been initiated to overcome this problem by producing the 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 diseases

The outbreaks of infectious diseases become serious in the shrimp industry because of the increasing shrimp farming and the lack of proper knowledge involving shrimp biology, farm management and diseases. 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; Sung, *et al.*, 2001).

Outbreaks of yellow head disease were the most serious problem in Central and Southern Thailand during 1993-1994, while white spot disease was the most serious problem from 1994 to 1996. Also, from mid 1996 until now, luminescent bacterial disease has increasingly been considered to be the cause for unsuccessful shrimp culture. These pathogens particularly hamper the 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

Viral diseases cause a serious economic problem for shrimp farming in many countries of the world. Due to virus infection, disastrous failure of the shrimp farming industry in Thailand has continued for over a decade. White spot syndrome virus (WSSV) and Yellow-head virus (YHV) are the two important virus species, reported as hazardous agents in *P. monodon* farming. 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 viruses causes great losses in the shrimp industry in Thailand as well as in several countries.

White spot syndrome disease

White spot syndrome (WSS) first appeared in Northeast Asia in 1992-1993, and rapidly spread to several other shrimp farming countries. WSS affects 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) had named this virus, the white spot syndrome baculovirus (WSSV). This virus is an enveloped DNA virus of bacilliform to cylindrical morphology with an average size of $120 \times 275 \pm 22$ nm and has a tail-like projection at one end of the particle (Kasornchandra *et al.*, 1995; Wongteerasupaya *et al.*, 1995). The viral genome is a double-stranded DNA of ~292 to 305 kb in length (van Hulten *et al.*, 2001; Yang *et al.*, 2001). WSSV is morphologically similar to the insect baculovirus. However, phylogenetic analysis of ribonucleotide reductase and protein kinase genes

reveals that the WSSV does not share a common ancestor 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 that 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.

Disease signs observed in infected shrimp include shell spotting from abnormal deposits of calcium salts. Affected animals display a pink to reddish-brown coloration due to the expansion of their cuticular chromatophores, lethargy, a rapid reduction in food consumption. The infected shrimp swims slowly near the pond surface, eventually sinks to the bottom and dies. High mortality rate occurs 3 to 10 days after the first signs of this disease. Characterization of this disease is based on the histological observation, electron microscopy and molecular studies.

The causative agent of WSSV is extremely virulent and has a wide host range (Lo *et al.*, 1996). It 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. This notion still remains true nowadays.

Detection of the virus at an early stage is necessary to minimize damage from WSSV infection. Several diagnostic methods have been described such as PCR (Yoganandhan *et al.*, 2003), *in situ* hybridization (Wang *et al.*, 2003), miniarray (Quere *et al.*, 2002), observation of tissues subjected to fixation or negative staining (Inouye *et al.*, 1994), immunological methods using monoclonal or polyclonal antibodies to WSSV or their protein components, and a recently developed method, a reverse passive latex agglutination (RPLA) method (Adams *et al.*, 1991; Yi *et al.*, 2005).

To protect the shrimp or other crustaceans against WSSV, the vaccine against the WSSV subunits, the envelope proteins VP19 and VP28, was evaluated. The vaccine against the VP19 and VP28 fused to MBP gave significantly better surviving rate than the control group (Witteveldt *et al.*, 2005). The other research area, concerning the control of WSSV infection, is to identify and produce the antiviral proteins. A *PmAV* cDNA of WSSV-resistant *P. monodon* encoding for a 170 amino acid peptide with a C-type lectin-like domain were shown to possess a strong antiviral activity in virus infected fish cell line (Luo *et al.*, 2003). In 2004, Zhang and co-workers showed that hemocyanin, isolated from the *P. monodon* had non-specific antiviral property. In addition, interferon-like protein from haemocytes of the virus-resistant *Marsupenaeus japonicus* was found to have also the non-specific antiviral ability by inhibiting SGIV (iridovirus) in GP cells (embryo cells) (He *et al.*, 2005).

Yellow-head disease

In Thailand, the Yellow-head (YH) disease is called 'hua leung' (Chantanachookin *et al.*, 1993; Lightner, 1996). YHV is a pleomorphic, enveloped virus with a single stranded RNA of positive polarity, primarily localized in the cytoplasm of infected cells (Cowley *et al.*, 1999). It has recently been classified in the family Roniviridae, genus Okavirus within the order Nidovirales (Sittidilokratna *et al.*, 2006). It is known to infect and cause mass mortality in shrimp farming operations throughout South East Asian countries. The disease was first reported in Thailand in 1990. 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, rod-shape form, is present in the cytoplasm of the infected cells. Viral envelope is acquired by the passage of the provirions through the endoplasmic recticulum of the host cells. Enveloped virions then cluster in the cytoplasmic vesicles, sometimes densely packed, and resembling paracrystalline arrays, where they appear to divide into the smaller rod-shaped unit (Chantanachookin *et al.*, 1993).

Shrimp infected with YHV often shows light yellow coloration of the dorsal cephalothorax area and has a pale or bleached appearance (Limsuwan, 1991) At the onset of YHD, the shrimps consume the feed at an abnormally high rate for several days. Feeding abruptly ceases and within 1 day, a few moribund shrimps appear swimming slowly near the surface at the pond edges. The moribund shrimps generally appear pallid in color, with a yellowish, often swollen cephalothorax and die within a few hours. Infected shrimps frequently exhibit whitish or pale yellowish to brown gills, and often a pale yellow hepatopancreas (Lightner, 1996, Chantanachookin *et al.*, 1993). For the black tiger shrimp, typical signs of the YH disease are the characteristic yellowing of the hepatopancreas and gill. YHV may occur as latent, asymptomatic infections in broodstock shrimps, 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.

1.7.2 Bacterial disease

Vibrio species are a normal part of the bacterial flora in aquatic environment and formerly considered to be mostly opportunistic pathogens (Lightner, 1998). They exist in a high numbers in both the water and sediment of shrimp ponds, especially in an intensive culture system (Direkbusarakom *et al.*, 1998). However, 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). 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). Disease outbreaks attributed to *V. harveyi*, *V. alginolyticus*, *V. vulnificus*, *V. parahaemolyticus* and *V. penaeicida* have been observed in nursery or grow-out ponds of *P. monodon*, *L. vannamei*, *P. japonicus* and *P. stylirostris* (Saulnier *et al.*, 2000).

V. harveyi is a Gram-negative bacterium. It is a rod shape, 0.5-0.8 μm in width and 1.4-2.6 μm in length. It is able to emit light of a blue-green color. This

bacterium 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 results in mortality up to 100% for nauplius to Zoea stages of *P. merguiensis*. Living and dead shrimp larvae and even the seawater in the disease outbreak areas are luminescent in dim light.

Gross observation, wet mount, histology and bacteria cultivation are the main diagnostic methods of vibriosis. In gross observations, the infection is evidence as black or brown cuticular lesions, necrosis, opacity of musculature, black lymphoid organ, and melanization of appendages. Presumptive diagnosis is made on the basis of clinical signs and cultivation of the suspensions of hepatopancreas or blood on tryptic plate with 2% (w/v) NaCl. After incubation at 30 °C overnight, colonies of *V. harveyi* should show strong luminescence in dim light.

Many studies have been carried out for the control and management of vibriosis in shrimp (Vinod *et al.*, In press; Roque *et al.*, 2005; Saulnier *et al.*, 2000). The continuing use of antibiotics to control the pathogen might cause problems of drug resistance. The use of probiotics, a marine bacterial strain *Pseudomonas* I-2, which can produce a compound with inhibitory property against *Vibrio* is an interested strategy for controlling the shrimp pathogenic *Vibrio* in cultured system (Chythanya *et al.*, 2002).

1.8 Invertebrate defense system

Immune system has been developed to protect multicellular organisms from the foreign substances. Evolutionarily, two types of immune systems have been developed to detect the foreign substances, namely adaptive (acquired) immunity and innate (natural) immunity. Vertebrates possess both adaptive and innate immune system, whereas invertebrates have only the innate immunity. The adaptive immune system functions by producing the highly specific recognition molecules, namely antibodies, and memorizes the foreign molecules after the first time of exposure. The innate immune system involves a large number of generalized effector molecules.

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 the microorganisms (Janeway, 1998). The adaptive immune system has developed more sophisticated and complicated mechanisms (Lee and Söderhall, 2001).

In crustaceans, the innate immune system is based on cellular and humoral components of the circulatory system. The hard cuticle, which covers all external surfaces of crustaceans, is the first line of defense between them and the environment. However, the innate immunity responses rapidly if the microorganisms can invade the animals. The major defense systems of crustaceans are carried in the hemolymph, which contains a group of cells, called haemocytes. The recognition of conserved molecular patterns characteristic of pathogen is the ability of the innate immune system, which is instrumental in initiating and regulating the adaptive immnue response. The target recognition of innate immunity is 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)'.

1.8.1 Blood cells, the haemocytes

Crustaceans have an open circulatory system. The circulating haemocytes of crustacean are essential in immunity, performing functions such as phagocytosis, encapsulation, and lysis of foreign cells (Johansson and Söderhall, 1989; Söderhall and Cerenius, 1992). In general, crustacean circulating haemocytes are morphologically divided into three types: hyaline cells, semigranular cells (contain a variable number of small granules), and granular cells (contain a large number of large granules) (van de Braak *et al.*, 2000). Indeed, they are present in penaeid shrimp and freshwater crayfish (Rodriguez *et al.*, 1995).

Hyaline cells, which lack the cytoplasmic granules, are the smallest group. It was found only 1% of the total haemocytes (Iwanaga and Kawabata, 1998). The hyaline haemocyte is reportedly involved in phagocytosis (Söderhall *et al.*, 1986). Granular and semigranular haemocytes were oval, plate-shaped structure with 15-20 μm in their longest dimension. The semigranular cell is the most abundant type of

haemocyte. This haemocyte responses by phagocytosis and encapsulation (Persson *et al.*, 1987). The granular cells are involved in phagocytosis, encapsulation and storage of the prophenoloxidase (proPO) system (Hose *et al.*, 1990; Gargioni & Barracco, 1998; Burgents *et al.*, 2005).

1.8.2 Lymphoid organ

The lymphoid organ consists of two distinct lobes; each located just ventro-lateral to the junction of the anterior and posterior stomach chambers. The lymphoid lobes are apposed slightly dorso-anterior the ventral hepatopancreatic lobe. They are identical and consist of a generally basophilic mass of anastomosing tubules, all of which are connected via a single vessel, the subgastric artery, to the anterior aorta. The lymphoid organ in penaeid shrimp is responsible for the removal of foreign material from the haemolymph, before it goes from the arterial system into the open circulatory system (Van de Braak *et al.*, 2002). The shrimp lymphoid organ appears to be the main site of bacterial accumulation as well as the main site of bacteriostasis (Burgents *et al.*, 2005).

Since the information concerning the function of this tissue in shrimp is scanty. The basic knowledge of the lymphoid organ is required to further unveil its function in shrimp pathogen responses. The shrimp lymphoid organ is shown in Figure 1.6.

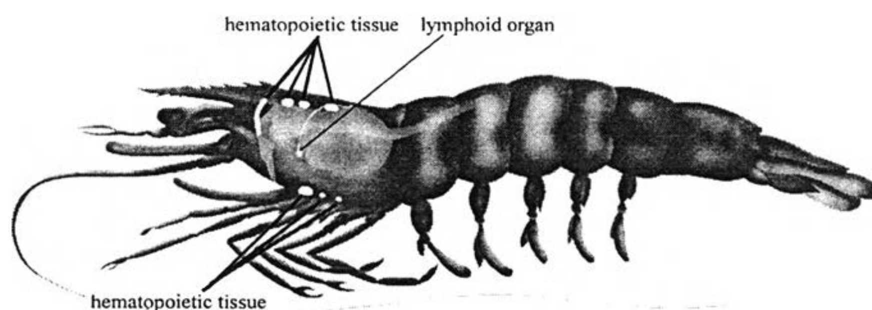

Figure 1.6 Location of the hematopoetic tissue and lymphoid organ of the penaeid shrimp.

1.9 Immune defense mechanisms

The major defense systems of crustaceans are carried in the hemolymph, which contains the haemocytes. Haemocytes and plasma protein recognizes a large group of pathogens by means of the common molecular patterns of particular microbes. The recognized molecules are the lipopolysaccharides (LPS) of Gram-negative bacteria, the glycolipids of mycobacteria, the lipoteichoic acids of Gram-positive bacteria, the mannans of yeasts, the β–1,3-glucan of fungi and the double-stranded RNA of viruses (Hoffmann *et al.*, 1999). These molecules may interact with the defense molecules and activate the hemocytes. Several types of pattern recognition proteins (PRPs) have been isolated and characterized in several invertebrates (Kim *et al.*, 2004; Lee & Soderhall, 2001). Some PRPs are lectins which can promote the agglutination. Therefore, they are likely to have a potential role in invertebrate non-self-recognition reactions. In crustaceans, examples of PRPs are lectins (Liu *et al.*, In press; Alpuche *et al.*, 2005; Okuno *et al.*, 2001; Marques & Barracco, 2000), and β–1,3-glucan binding protein (BGBP) in many crustaceans. The crustacean BGBPs were reported in freshwater crayfish, *Pacifastacus leniusculus* (Duvic & Soderhall, 1992), and three marine shrimp species, *P. californiensis* (Vargas-Albores *et al.*, 1996), *L. vannamei* (Jimenez-Vega *et al.*, 2002; Vargas-Albores *et al.*, 1997) and *P. monodon* (Sritunyalucksana *et al.*, 2002). Haemocyte activation results in the release of various immune effectors, such as antimicrobial peptides, coagulation factors, proteinase, and proteinase inhibitors, which function on the elimination of invading pathogens. The current knowledge of arthopod immunity was summarized in Figure 1.7.

1.9.1 The prophenoloxidase (proPO) system

Shrimp proPO system have been elucidated in *Penaeus californiensis* (Gollas-Galvan *et al.*, 1999; Vargas-Albores *et al.*, 1996), *P. panlensis* (Perazzolo and Barracco, 1997), *P. stylirostris* and *P. monodon* (Sritunyalucksana *et al.*, 1999). Shrimp proPO is synthesized in the haemocytes. Shrimp proPO is more closely related to the crayfish proPO than to the insect proPO according to the amino acid comparison.

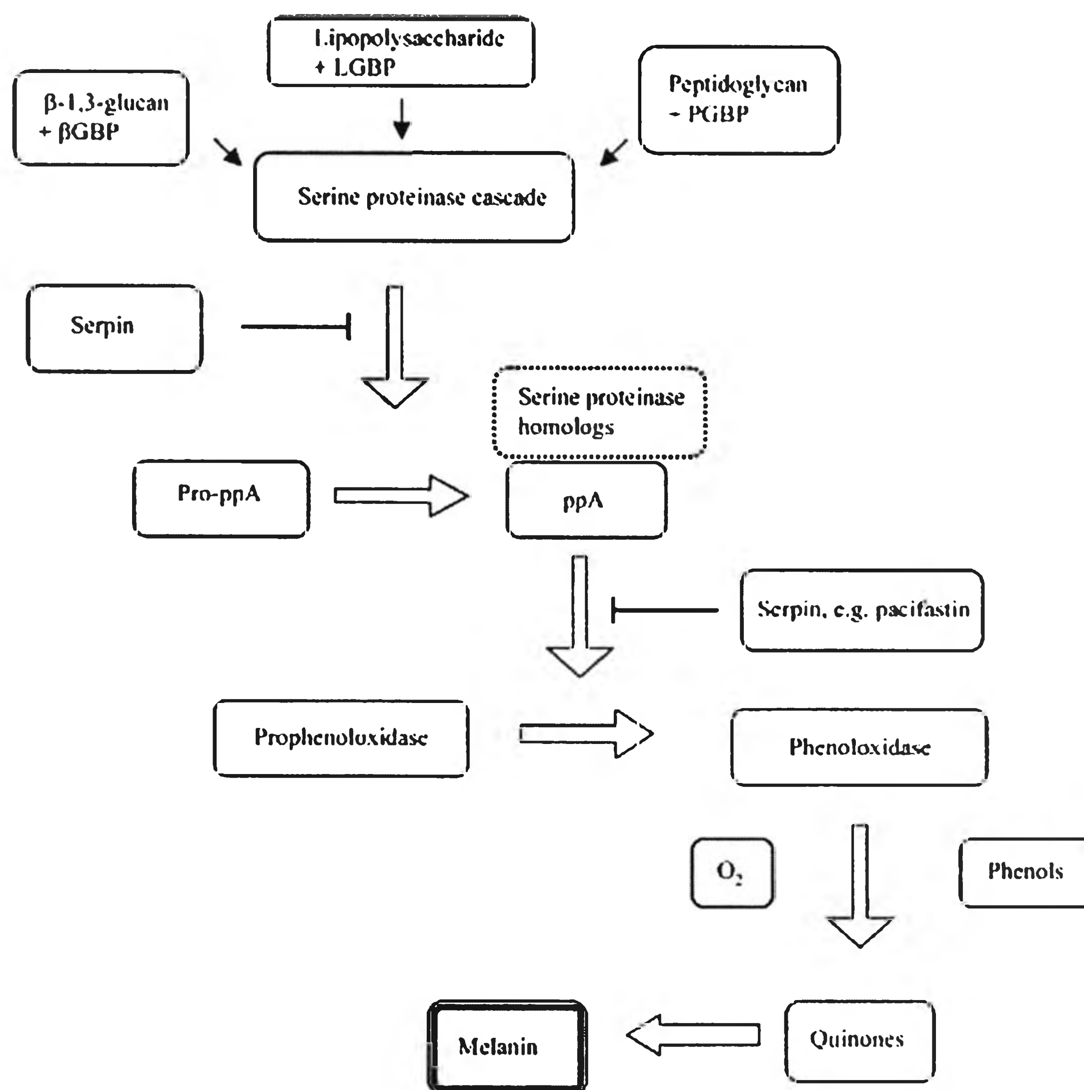

Figure 1.7 Overview of the arthropod prophenoloxidase (proPO)- activating system (Cerenius & Soderhall, 2004).

The proPO activating system comprises several proteins involving in the immune defense in invertebrates leading to melanin production, cell adhesion, encapsulation, and phagocytosis (Sritunyalucksana & Söderhäll, 2000). It is activated by extremely low quantities of lipopolysaccharides or peptideoglycans from bacteria and β–1,3-glucans from fungi, through pattern-recognition proteins (Ariki *et al.*,

2004). The proPO system is a proteinase cascade involving several zymogenic proteinases, and proPO (Cerenius & Soderhall, 2004). Upon stimulation, proprophenoloxidase-activating enzyme (proppA) is converted to an active ppA, a serine proteinase, which then converts the proPO to the active phenoloxidase (PO) by proteolysis. PO catalyses the two successive reactions: the hydroxylation of a monophenol to *o*-diphenol (monophenoloxidase activity) and the oxidation of the *o*-diphenol to *o*-quinone (diphenoloxidase activity) (Cerenius & Soderhall. 2004; Decker *et al.*, 2000; Decker & Tuczek, 2000). Production of the *o*-quinones by PO leads to melanization. The production of melanin pigment can often be seen as dark spots in the cuticle of arthropods in the process of sclerotisation, wound healing and encapsulation of foreign materials (Theopold *et al.*, 2004). Several components and associated factors of the proPO system have been also found to initiate several molecules in the defense reaction of the freshwater crayfish (Cerenius & Soderhall, 2004).

1.9.2 The coagulation/clotting system

A coagulation system entraps foreign molecules and prevents the excessive loss of haemolymh from a wound. Coagulation system in invertebrates, chelicerates and crustaceans, has been characterized in molecular detail. In horseshoe crab, *Tachypleus tridentatus*, clotting formation is activated by either lipopolysaccharide or β–1,3-glucans through proteinase cascade in the large granules of haemocytes (Iwanaga *et al.*, 1998; Kawabata & Tsuda, 2002). In this process, coagulogen is converted by proteinase into coagulin, which spontaneously aggregates to form insoluble clots. In crustacean, e.g. lobster (Fuller & Doolittle, 1971), crayfish (Kopacek *et al.*, 1993; Komatsu & Ando, 1998; Cerenius & Soderhall, 2004) and shrimp (Yeh *et al.*, 1998; Xu *et al.*, In press), transglutaminases (TGases) are identified. This enzyme is involved in the clotting process. TGases are Ca^{2+}-dependent enzymes capable of forming covalent bonds between the side chains of free lysine and glutamine residues on certain proteins resulting in polymerization. Another coagulation system, the type C coagulation, which is involved in the lysis of explosive

corpuscles or hyaline cells, is present in the spiny lobster and shrimp (Yeh *et al.*, 1999).

So far, the clotting reaction is only fully characterized in crayfish (Cerenius & Soderhall, 2004), the expected similar mechanism in other crustaceans remains to be elucidated in more detail for comparative studies of the clotting reaction in crustaceans.

1.9.3 Antimicrobial peptide or proteins

Antimicrobial peptides (AMPs) are effector molecules that play an important role in innate immune system. In all kingdoms, from bacteria to human, a variety of AMPs have been identified and characterized (Tew *et al.*, In press; Zhou *et al.*, 2006; Xu *et al.*, 2005; Buhimschi *et al.*, 2004; Hiemstra, 2001; Douglas *et al.*, 2001; Dathe & Wieprecht, 1999). All AMPs share common biochemical features such as small size, generally less than 150-200 amino acid residues, amphipathic structure and cationic property though the anionic peptides also exist. AMPs have a wide variety and diversity in amino acid sequences, structure, and range of activity. AMPs are active against a large spectrum of microorganisms; bacterial and filamentous fungi. In addition, some AMPs have antiviral or antiparasitic activities (Hancock & Diamond, 2000; Tang *et al.*, 2005; Murakami *et al.*, 1991) and may also exhibit an anti-tumor property (Cruciani *et al.*, 1991). In artropoda, a broad activity of AMPs against bacteria and filamentous fungi has been reported in insects, horseshoe crab, and shrimp (Bulet *et al.*, 1999; Vizioli & Salzet, 2002; Munoz *et al.*, 2002). 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. Some constitutively expressed within the secretory cells, others are induced upon microbial stimulation (Hancock & Diamond, 2000). In 2004, Bulet *et al.* assessed that over 1000 AMPs, mostly from insectd, have been isolated and characterized from multicellular organisms at the level of their primary structure.

In general, the net positive charge together with the amphipathic structure accounts for the preferential binding to the negatively charged membrane

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interface of microorganisms, which is different from the predominantly zwitterionic surface of normal mammalian cells. Thus, they are toxic to the microbes and not to the mammals. The features required for AMPs are the selective toxicity on microbial cells, short bacterial killing time, broad antimicrobial spectra, and no bacterial resistance development (Matsuzaki, 2001). AMPs can interact and insert into the lipid bilayer, causing the collapse of electrochemical gradients across the cell membrane. Consequently, the microorganisms lose their cellular components and source of energy for surviving.

Several antimicrobial peptides are isolated and characterized in arthropods, mainly in insects, especially *Drosophila* and chelicerates (horseshoe crabs) (Iwanaga *et al.*, 1998). In horseshoe crabs, these proteins are mainly synthesized in the haemocyte and are stored within the cytoplasmic granules. The cells are highly sensitive to LPS, a major outer membrane component of Gram-negative bacteria, and respond by degranulating the granules after stimulation by LPS. This antimicrobial system in insect differs from those of the crustaceans in that the fat body of the insects is the main site for the antimicrobial peptide synthesis (Engström, 1999; Hoffmann & Reichart, 1997). Upon injury, the transcription of the antimicrobial peptide gene is induced, resulting in their immediate synthesis and subsequent secretion into the blood.

There are only a few reports on antimicrobial peptides in crustaceans. Tachyplesin family and anti-LPS factors which act against Gram-negative bacteria were observed in horseshoe crab (Muta *et al.*, 1990; Nakamura *et al.*, 1988; Yamakawa *et al.*, 2001; Aketagawa *et al.*, 1986). In 1997, a small peptide, named callinectin, was reported to be responsible for the majority of antimicrobial activity observed in the haemolymph of blue crab, *Callinectes sapidus* (Khoo *et al.*, 1999). Recently, a new family of antimicrobial peptide which acting against Gram-positive bacteria and fungi, penaeidins, was reported in penaeid shrimp, *L. vannamei* (Destoumieux *et al.*, 1997). These peptides contain both a proline rich domain at the N-terminus and a C-terminal domain containing 6 cysteines forming three disulfide linkages. The cDNA clones of penaeidin isoforms were also isolated from the haemocytes of *L. vannamei* and *L. setiferus* (Gross *et al.*, 2001) and *P. monodon* (Supungul *et al.*, 2004). A cysteine-rich 11.5 kDa antibacterial protein was purified

and characterized from the haemolymph of shore crab, *Carcinus maenas* (Relf *et al.*, 1999). Crustins, an antimicrobial peptide homologues of the 11.5 kDa antibacterial peptide, were identified from 2 species of *Penaeid* shrimp, *L. vannamei* and *L. setiferus* (Bartlett *et al.*, 2002; (Vargas-Albores *et al.*, 2004). Peptides with antiviral activity, derived from the hemocyanin of *L. vannamei and P. stylirostris* and hemocyanin of *P. monodon*, have been identified (Destoumieux-Garzon *et al.*, 2001; Patat *et al.*, 2004; Altschul *et al.*, 1997). Recently, the histones and histone derived peptides of *L. vannamei* have been reported as an innate immune effectors because they can inhibit the growth of Gram-positive bacteria (Patat *et al.*, 2004).

1.10 Expressed sequence tags

The Expressed Sequence Tags (EST) analysis, first developed by Adams *et al.* (1991), has been widely employed as a means of discovering the novel and uniquely expressed genes, and characterizing the gene expression profiles of various tissues (Adams *et al.*, 1993; Affara *et al.*, 1994; Pawlak *et al.*, 1995; (Iida *et al.*, 2006; Thomas *et al.*, 2001; Sharma *et al.*, 1999). The ESTs are short, usually about 300-500 bp. The 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 the ESTs is a convenient and rapid way for the identification of new genes in various taxa, where knowledge about the genome under investigation is not available or limited. It is initiates with the mRNAs from an interesting tissue being used to construct the cDNA library in a λ vector. Upon *in vivo* excision, the λ clones are converted into the plasmid clones. Then, the plasmids are randomly selected for the determination of the insert size 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, and analyzed for the coding region, since the coding sequence, being more conserved than the untranslated region, should provide a better chance to identify a gene. This sequencing result is used as a tag for homology search through the sequence database in the NCBI GenBank by using Blastx program (Altschl *et al.*,

1997). The computerized translated protein sequences 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 a match length is >10 amino acid residues according to Anderson and Brass (1998). The basic procedure of this approach is summarized in Figure 1.8.

In the GenBank database, the number of public entries is 32,889,225 EST as of January 20, 2006. The top 10 organisms represented in the dbEST are summarized in Table 1.2. The fruit fly, *Drosophilla melanogaster*, is the 16th that has 383,407 ESTs. For Penaeid shrimps, there are 2,144 and 7,408 EST sequences deposited in the GenBank for *P. monodon* and *Litopenaeus vannamei*, respectively (dbEST, related by NCBI; January 20, 2006).

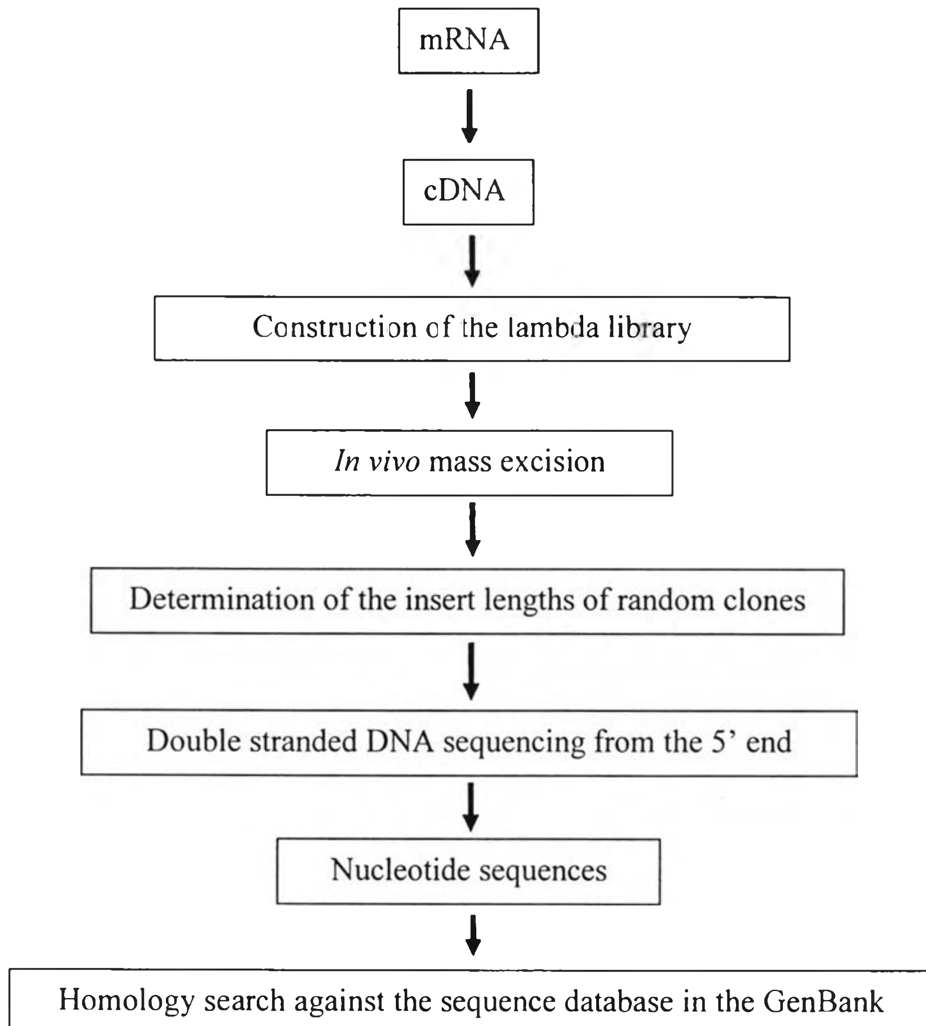

Figure 1.8 Basic procedure of the cDNA clones tagging

Table 1.2 The top 10 organisms presented in the dbEST. The number of EST sequence entries is as of January 20, 2006.

Organism	No. of ESTs
Homo sapiens	7,596,977
Mus musculus	4,690,536
Xenopus laevis	1,038,272
Bos taurus	837,648
Ratus sp.	812,662
Danio rerio	689,613
Ciona intestinalis	686,395
Zea mays	662,88
Triticum aestivum	600,205
Gallus gallus	588,739

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 had not previously identified in crustaceans. They found that the putative identities of these genes reflected the expected tissue specificity. Gross *et al.* (2001) constructed the haemocyte and hepatopancreas cDNA libraries from *L. vannamei* and *L. sertiferus*. These libraries showed high redundancy of penaeidin, the antimicrobial peptide, in the haemocyte libraries and lectin in the hepatopancreas. The haemocyte cDNA libraries of healthy and WSSV-infected *P. japonicus* were constructed by Rojtinnakorn *et al.* (2002). They found that the ESTs representing proteinase inhibitor and tumor-related protein were only identified in the WSSV-infected library, and the apototic peptides were expressed at high level in the same library. For *P. monodon*, Supungul *et al.* (2002) constructed the haemocyte cDNA library from the normal shrimp. They had isolated genes coding for enzymes and proteins in the clotting system and the prophenoloxidase-activating system, antioxidative enzymes, antimicrobial peptides, and serine proteinase inhibitors. Nevertheless, the above finding covers only a small number of immune genes in shrimp. More immune-related genes need to be identified and functionally characterized for better understanding of shrimp immunity.

1.11 Microarray analysis

Microarray (also commonly known as gene chip, DNA chip, or biochip) is simply the ordered sets of DNA molecules (probe) of known sequences as microscopic DNA spots, attached to a solid surface (substrate), such as glass, plastic or silicon chip, forming an array. The substrate, usually rectangular, can accommodate a few hundred to hundreds of thousands of sets. Each individual 'spot' or commonly known as 'feature' is on the array at precisely defined location on the substrate.

1.11.1 Probe

The type of molecule placed on the array units also varies according to circumstances. The most commonly used molecule is cDNA, or complementary DNA, which is derived from messenger RNA and cloned. Since they are derived from a distinct messenger RNA, each feature represents an expressed gene. Nevertheless, oligonucleotides, generally 20-70 bases in length, can be used in microarray slide.

1.11.2 Slide surface chemistry

Most microarrays are printed onto glass microscope slides. Glass is rigid, flat, and has low fluorescence. Nucleic acids will not bind to untreated glass, so a variety of chemical coatings have been developed to facilitate this interaction. These different chemistries determine not only how nucleic acids bind to the slide surface, but also at what density. Whatever the chemistry, the coating must be uniform, and the surface must be as smooth as possible for consistent spot density and even background. The most common surface coatings are aldehyde, amino, or poly-L-lysine groups.

Aldehyde

Aldehyde coatings consist primarily of aldehyde groups (H-C=O). Unmodified dsDNA binds to these aldehydes at an efficiency of 10% via aromatic amines on guanine, cytosine and adenosine. After printing, slides are post-processed with sodium borohydride ($NaBH_4$), which converts unreacted aldehyde groups to non-reactive primary alcohols (H_2-C=OH).

Amine

Aminated slides are treated with aminosilane, which yields a positively charged coating (NH^{3+}). This positive charge will bind to unmodified DNA, which carries a negative charge due to the presence of phosphate groups. Binding occurs along the length of the DNA backbone, resulting in highly efficient ionic bond formation. In addition to natural electrostatic binding (H_3N+/-*DNA*), UV light or heat are used after spotting to induce covalent attachments (H-N-*DNA*, where N is also bound to the array). Although the binding efficiency is high, both strands are equally bound and normally require denaturation prior to hybridization.

Poly-L-Lysine

Poly-L-lysine creates a positively charged surface, via a charged amino group, to which unmodified DNA can bond ionically. The binding properties are similar to aminated slides. Like aminated slides, electrostatic binding is supplemented by covalent attachment induced by heat or UV light.

1.11.3 Spotting (arraying or printing)

There are two main methods of robotic printing microarrays, direct contact and non-contact transfer, and also a specialized method, called photolithography, which does not involve the transfer of materials. All three will be discussed.

Direct contact printing

Contact printing is the most widely used method in today's research community. A motion control robot is fitted with a printhead which holds a number of small spotting pins. The tip of each pin contains a small channel which holds a precise volume (0.2 – 0.6 μl, depending on the pin type) via capillary action. The end of each tip is horizontally level against the surface of the slide. This causes a small amount of liquid to form a thin layer at the surface of the pin when it loads. Slides are positioned and secured on a tray, and the print plates are queued in a hotel which allows the robot to select them one by one. First the pinhead dips into the print plate, and acquires its volume of probe. The pins are then guided to each slide in sequence, and touched down onto the surface. When the pin comes in contact with each slide, that thin layer of liquid is left behind on the slide surface. This pull comes from surface tension between pin and slide. The deposit is therefore passive, in the sense that no liquid is

ejected from the pin. There is very little contact between pin and slide, eliminating wear and tear on very fragile pins. The diameter of the spot left behind is normally 100-300 μm. This spot size is determined by the size of the pin tip, and is highly reproducible.

Non-contact printing (Inkjet Printing)

Non-contact printing refers to any technology that enables microarray printing without direct contact between slide and printer. The most common type is piezoelectric dispensing, or inkjet printing, a technique employed by many computer printers. Slides and plates are arranged in a fashion similar to that for contact printing. Liquid is collected in a capillary tube and ejected at specific volumes when an electric pulse causes temporary deformation on the walls of the tube. Whereas contact printing requires a stop and start movement of the printheads, piezo jet print heads can maintain a constant, smooth motion along the x and y axes, reducing wear and tear and overall print time immensely. Spot sizes are generally larger than with contact printing (250-300 μm), due to the distance between the pin and the slide.

Photolithography (Semiconductor printing)

Photolithography array manufacture uses strategies culled from the computer chip industry to create extremely small and dense microarrays containing short oligonucleotides. A Californiabased company, Affymetrix, employs this light-based approach to build 25mer oligonucleotides directly on the surface of their array. The substrate (Chip) is glass coated with aminosilane to create reactive amino groups. A second chemical serves as a photoprotective group, inactivating the Chip surface in the absence of UV light. Each feature on the array corresponds to a region on a mask. Masks contain holes corresponding to specific features for each step of synthesis. A nucleotide is washed over the entire array surface, but only features which correspond to holes in the mask are exposed to concurrent UV light. Only features which are exposed to light accept the incoming nucleotide for coupling. For each step of base addition, four nucleotide washes/mask exposures are required in succession.

1.11.4 Experimental samples (Target)

Most gene expression studies compare a baseline, control or wild type sample to an experimental sample. Both samples are prepared in parallel, using different

colored fluorescent dyes, which can be incorporated directly as a nucleotide conjugate, or indirectly through the incorporation of a nucleotide conjugated to a molecule which is later conjugated to a dye. The target can be DNA, derived from RNA by reverse transcription or from DNA by PCR, or cRNA derived from cDNA. It is important to match target strand to probe strand. For example, if DNA probes on an array are sense strand, then labeled target must be antisense. In this case, first strand cDNA could be prepared for use as a target. If spotted DNA is antisense, then cRNA derived from cDNA should be used as target.

1.11.5 Target labeling strategies

The most commonly used target labeling dyes are cyanine fluorophores Cy3 and Cy5. Under scanning conditions, Cy3 appears green, while Cy5 appears red. By using fluorophores with different emission wavelengths, it is possible to hybridize both the control and experimental samples onto the same array simultaneously. It is critical to optimize the amount of label incorporated. Incorporation of too few labeled nucleotides into a sample can lead to reduced signal. Too many fluors in close proximity can also reduce signal, because much of the absorbed light energy can go into fluor-fluor interactions, which causes energy to be dissipated as heat. Optimization can be achieved by striking the best balance between concentrations of modified and unmodified nucleotides going into a reaction. Labeling mixtures are usually comprised of four unmodified nucleotides, and one modified nucleotide. The modified and unmodified nucleotides compete for incorporation, and based on its availability, the modified nucleotide will incorporate on a regular basis.

Direct Incorporation

Direct incorporation into DNA (by oligonucleotide synthesis, reverse transcription or PCR) is usually done with a cynanine fluor-conjugated dUTP (Cy3-dUTP, Cy5-dUTP) (Figure 1.9A). Nothing else need to be done with such samples to make them ready for hybridization, except for rigorous purification to wash away unincorporated Cy-dyes, which can spuriously bind to the surface of the slide, causing background fluorescence, which hinders hybridization signal.

Indirect Incorporation

Indirect dye incorporation is a two step process (Figure 1.9B). The first step is the incorporation of an aminoallyl-modified nucleotide (aa-dUTP). The second step, after purification to remove unincorporated aa-dUTP, is to "post-label" by conjugating the aminoallyl group to a Cy-dye NHS ester in a secondary step. A second purification removes unconjugated esters (which are present in great excess).

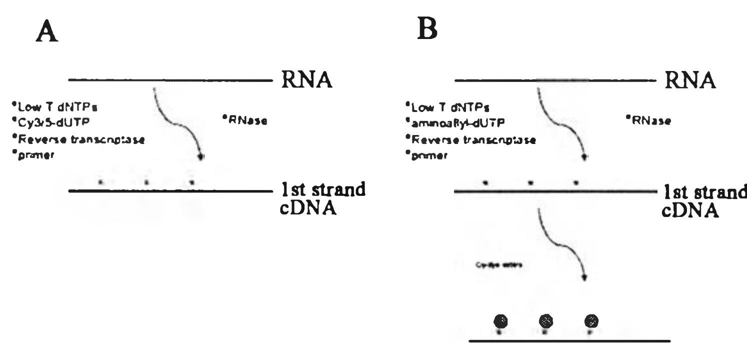

Figure 1.9 The 1st strand cDNA Labeling Strategies; Direct incorporation (A) and Indirect incorporation (B)

1.11.6 Hybridization and washing

With respect to nucleic acids, "hybridization" refers to the formation of double-stranded DNA, RNA, or DNA/RNA hybrids by complementary base pairing. In a typical microarray experiment, labeled target is applied to a pre-printed microarray surface. Concentration and volume are pre-optimized. Hybridization cocktails should be protected against evaporation and overspreading with a sealed, humidified hybridization chamber and coverslip. The slide is incubated in a temperature controlled, humid environment for a number of hours, normally between three and forty-eight. The coverslip or chamber is then removed, and the slide is washed in a series of buffers which is optimized to remove any nonspecific binding of target and probe. Once washed, the slide surface is dried using a stream of air (often nitrogen), or centrifugation. The stringency of the hybridization and washes are

critical to the optimization process. Insufficient stringency can result in a high degree of nonspecific binding. Excessive stringency can lead to a failure of appropriate binding, which results in diminished signal to noise ratios.

1.11.7 Image capture and data extraction

Microarray scanners, which employ lasers to capture fluorescent images, are routinely used for microarray data capture. Lasers deliver a beam of light on a microarray positioned in a fixed holder. Filters are used to selectively narrow the light to within a small wavelength range, enabling optimal excitation and emission wavelengths for each dye to be used. The emitted fluorescence from a fluorophore will radiate in all directions, although it is desirable to collect light from only off of the printed surface of the array. Additional filters and focusing are therefore needed to eliminate scattered light. Filters which focus on the wavelengths of interest are also narrowing the light data being generated. A photomultiplier tube (PMT) is used to convert photon energy into electrical energy. This electrical energy is measurable, and is proportional to the photon energy, The amplified light generates an analog signal which is subsequently converted to a digital signal. This digital signal translates to a number representing the intensity of fluorescence from a given pixel. Each feature of the array is made up of several such pixels. The final result of a scan is an image of the array surface greatly magnified, where each pixel's intensity is colorized according to a continuum of color density spanning absent to saturated, and the corresponding numerical data for each pixel can then be acquired. Scanner software is used to identify individual features, associate them with spot identifiers (such as gene names), and delineate their boundaries. Once each feature's borders have been established, the software generates numerical data. Each pixel is individually measured, and the mean, median and standard deviation are reported for each feature. In addition, the local background fluorescence for each feature is measured. The output is usually a spreadsheet detailing each spot's identification and pixel data. Normally the ratio of expression between two biological samples is evaluated, so the binding ratio is also reported, indicating an up- or down-regulation of gene expression.

Microarrays are quickly adaptable to many different types of experimental design, and a lot of uses have been found to take advantage of the miniaturization

microarrays afford. In 2004, Kuninger *et al.* used microarray to identify novel genes induced during growth factor mediated muscle cell. In addition, this technology has been used to study expressed genes in various organism (Mu *et al.*, 2006; Roberts *et al.*, 2006; Taleb *et al.*, 2006; Wang *et al.*, 2003). The cDNA microarray analysis has been used to differentiate gene expression pattern of human salivary gland, gastric cancer cell and Atlantic salmon (Ewart *et al.*, 2005; Kainuma *et al.*, 2004; Park *et al.*, 2004).

In shrimp, Dhar *et al.* (2003) used a small microarray composed of 100 cDNAs to compare gene expression patterns in the hepatopancreas of healthy and white spot syndrome virus (WSSV)-infected shrimp (*Penaeus stylirostris*). This approach was also used to study viral gene expressed in WSSV-infected and specific-pathogen free shrimp (Khadijah *et al.*, 2003). However, oligonucleotide microarray was widely used in many organism (Walker *et al.*, 2006; Moertel *et al.*, In press; Xu *et al.*, In press; Seo *et al.*, In press).

Basic procedure for cDNA microarray analysis can be divided into 3 parts consisting of preparation of the microarray slide, sample labeling, hybridization and scanning and analysis. The graphical summery of the cDNA microarray procedure are shown in Figure 1.10.

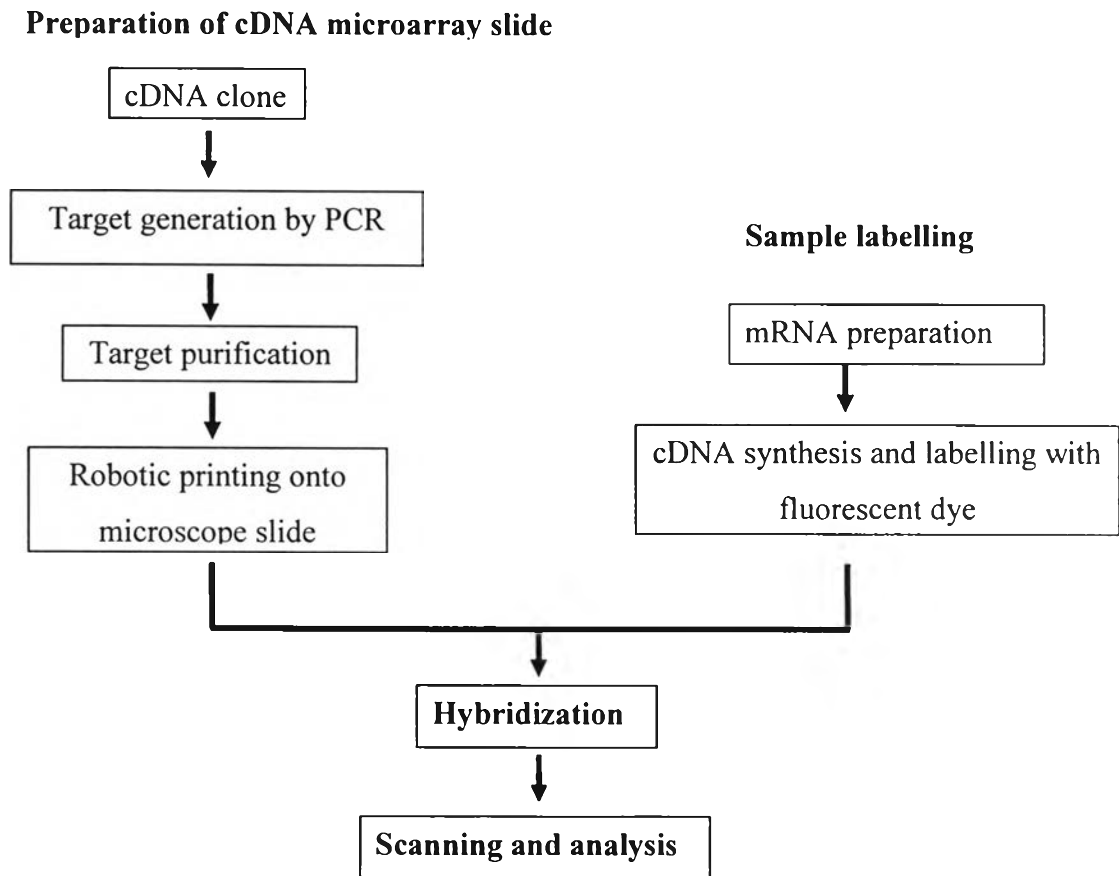

Figure 1.10 Basic procedure of cDNA microarray analysis

1.12 Real-Time RT-PCR analysis

As the name suggests, real time RT-PCR is a technique used to monitor the progress of a PCR reaction in real time. At the same time, a relatively small amount of the PCR product (DNA, cDNA or RNA) can be quantified. Real-time RT-PCR is currently the most sensitive method for the detection of low-abundance transcripts. Limitation of the classical endpoint RT-PCR could be overcome by the kinetic or real-time RT-PCR assay. The truly linear part of the amplification can be easily observed with the developed fluorescence based PCR technique together with the specialized detector and software. Real-time RT-PCR can be used to compare and quantify the expression of selected genes in different biological samples. Moreover, there is no need for the post PCR processing which saves the resources and the time. It is remarkably useful for confirming differential expression of candidate genes identified by other techniques, for example; DD-PCR, cDNA microarray, suppression-substractive hybridization (Astrofsky *et al.*, 2002; de Lorgeril *et al.*, 2005).

The amplified products can be detected by using fluorescent dyes that are specific for double-stranded DNA (dsDNA) or by sequence-specific fluorescent oligonucleotide probes. The first dye used for this purposes was ethidium bromide. A dsDNA-specific dye frequently used in real-time PCR today is SYBR Green I (Figure 1.11). It is an asymmetric cyanine dye which binds sequence independently to the minor groove of dsDNA. The binding affinity is more than 100 times higher than that of ethidium bromide. It is suitable for monitoring the accumulation of the product during PCR, without a separate assay to detect this product, because the fluorescence of the bound dye is more than 1,000 fold higher than that of the free dye. However, the major drawback of SYBR Green is that it binds to nonspecific products like primer dimers and the desired PCR product with equal affinity. The fluorescence emitted from the total dsDNA, both the desired amplicons as well as the primer dimers, thus constitutes the interference of primer dimers to the quantitation of the target gene.

Quantitation of mRNA transcriptions by real-time RT-PCR can be either absolute using the standard curve method or relative using the comparative threshold

method. In the standard curve method, a sample of known concentration is used to construct a standard curve. Since the cycle at which PCR enters log linear amplification is directly proportional to the amount of starting template, one determines the concentration of an unknown sample by comparing it to such standard curve. Absolute quantification should be performed when determination of the absolute transcript copy number is required.

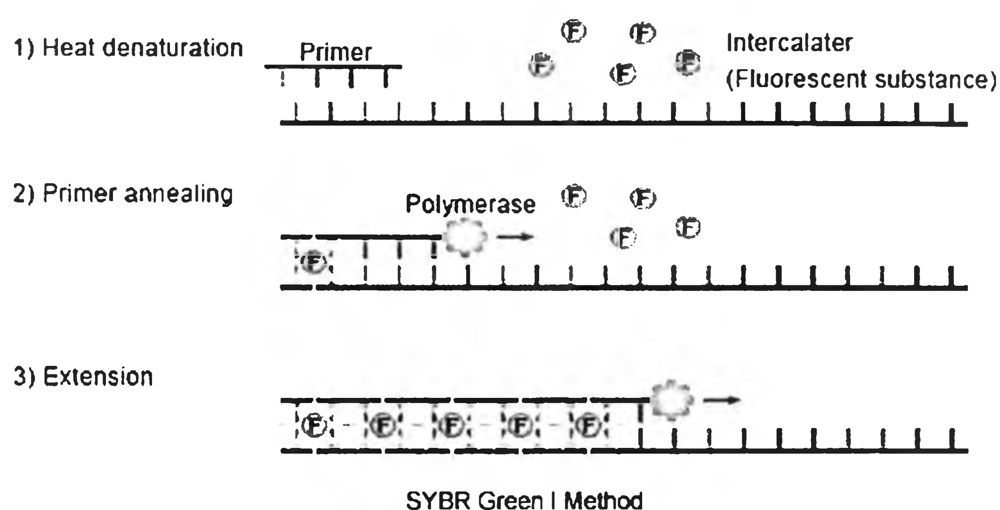

Figure 1.11 SYBR green in real time RT-PCR analysis

The comparative threshold method was used to report the changes in the expression of interested genes relative to a reference gene in a given treatment. Pfaffl (2001) reported a mathematical model for relative quantification determined from real-time PCR experiments without a calibration curve. The expression of target genes was normalized with a reference of housekeeping gene, either glyceraldehyde-3-phosphate dehydrogenase (G3PDH or GAPDH), actins, cyclophilin, 18S rRNA or 28S rRNA. In shrimp, elongation factor 1-alpha gene (EF 1α) is identified as constitutively expressed gene and has been used as reference gene for real-time RT-PCR analysis (Astrofsky *et al.*, 2002). The relative expression ratio (R) of a target gene is calculated as in an equation below based on the PCR efficiency (E) and the

deviation of crossing points (CP) of an unknown sample versus a control, and expressed in comparison to a reference gene:

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta CP_{\text{target}}(\text{control-sample})}}{(E_{\text{ref}})^{\Delta CP_{\text{ref}}(\text{control-sample})}}$$

where the E_{target} is the real-time PCR efficiency of target gene trancript; the E_{ref} is the real-time PCR efficiency of reference gene trancript; the $\Delta CP_{\text{target}}$ is the CP deviation of control-sample of the target gene transcript; and the ΔCP_{ref} is the CP deviation of control-sample of the reference gene transcript.

In theory, the amount of amplified product is a doubling amount of DNA at each cycle during exponential amplification when the PCR efficiency is equal to 100%. However, the actual PCR efficiency of each amplified DNA may be slightly different, and can make a lot of difference in the amount of the final product. To determine the PCR efficiency, CP cycles versus cDNA input are plotted to calculate the slope. The corresponding real-time PCR efficiencies are then calculated, according to $E = 10^{[-1/\text{slope}]}$ (Pfaffl, 2001).

1.13 *In situ* hybridization

In situ hybridization technique is very useful in detecting nucleic acid in morphologically preserved chromosomes, cells or tissue sections. Both radioactive and nonradioactive hybridization are available. However, working with nonradioactive system is preferable, probably because it is safer and easier to perform, and has a longer probe-shelf life. The nonradioactive hybridizaiton methods can be divided into two types: direct and indirect. Direct method uses fluorochromes directly coupled to a nucleic acid probe so that the probe-target hybrids can be visualized under microscope immediately after hybridizaiton. Indirect procedure requires the probe to contain a detectable molecule, such as digoxigenin (DIG) and biotin, introduced chemically or enzymatically, that can be detected by a specific antibody. For the above methods, the presence of the label should not interfere with the hybridization reaction and the stability of the resulting hybrid.

The DIG is naturally extracted from the digitalis plants (*Digitalis purpurea* and *Digitalis lanata*). It is linked to the C-5 position of uridine nucleotides. The DIG-labeled uridine nucleotides are incorporated into the nucleic acid probes enzymatically with the DNA polymerases, RNA polymerases, and terminal transferase. Hybridized DIG-labeled probes may be detected with the high affinity anti-digoxigenin (anti-DIG) antibodies, conjugated to the alkaline phosphatase, peroxidase, fluorescein, rhodamine, AMCA, or colloidal gold. For unconjugated anti-DIG antibodies, detection by the conjugated secondary antibodies may be used instead. Detection sensitivity depends on the method used to visualize the conjugate. In this study, an anti-DIG antibody, conjugated to the alkaline phosphatase, is visualized with the colorimetric (NBT and BCIP) substrates.

In order to detect the interested transcripts in the cells, RNA probes are generated by *in vitro* transcription from the linearized plasmids, The reaction contains a template and a promoter for RNA polymerase. The SP6, T3, or T7 RNA polymerases are commonly used for the synthesis of the run-off transcripts which complementary to the DNA template. The sequence of probe begins from the promoter site to the restriction site used for linearization. The RNA probes are single-stranded, and about 10 µg of probe are produced from about 1 µg of DNA template. *In situ* hybridization is widely applied for the localization of many genes in shrimp research (Yi *et al.*, 2005; Tang *et al.*, 2005; Phromjai *et al.*, 2002; Lee *et al.*, 2000).

In situ hybridization technique effectively combines histochemistry with molecular biology and enables the rapid analysis of the distribution of RNA, or DNA, in the tissues. The information gained from this has caused something of a revolution in our understanding of developmental biology, since a fundamental aspect of development is the spatial and temporal expression of genes. In addition the technique has found application in the field of medicine, where it is giving new insights into the functioning of healthy tissues, the diagnosis, and the study of diseases.

1.14 Immunohistochemistry or immunocytochemistry

Immunohistochemistry is the localization of antigens in tissue sections by the use of labeled antibodies as specific reagents through antigen-antibody interactions that are visualized by a marker such as fluorescent dye, enzyme, radioactive element or colloidal gold. In 1941, Coons *et al.* were the first to label antibodies with a fluorescent dye, and used it to identify antigens in the tissue sections. With the expansion and development of immunohistochemical technique, enzyme labels have been introduced such as peroxidase (Nakane and Pierce, 1966; Avrameas and Uriel, 1966) and alkaline phosphatase (Mason and Sammons 1978). Colloidal gold label (Faulk and Taylor, 1971) has also been discovered and used to identify immunohistochemical reactions at both light and electron microscopy level. Other labels include radioactive elements, in which the immunoreaction can be visualized by autoradiography. Since immunohistochemistry involves specific antigen-antibody reaction, it has apparent advantage over traditionally used special and enzyme staining techniques that identify only a limited number of proteins, enzymes and tissue structures. Therefore, immunohistochemistry has become a crucial technique, and are widely used in many medical research laboratories as well as clinical diagnostics. The diagram of immunohistochemistry techniques is shown in Figure 1.12.

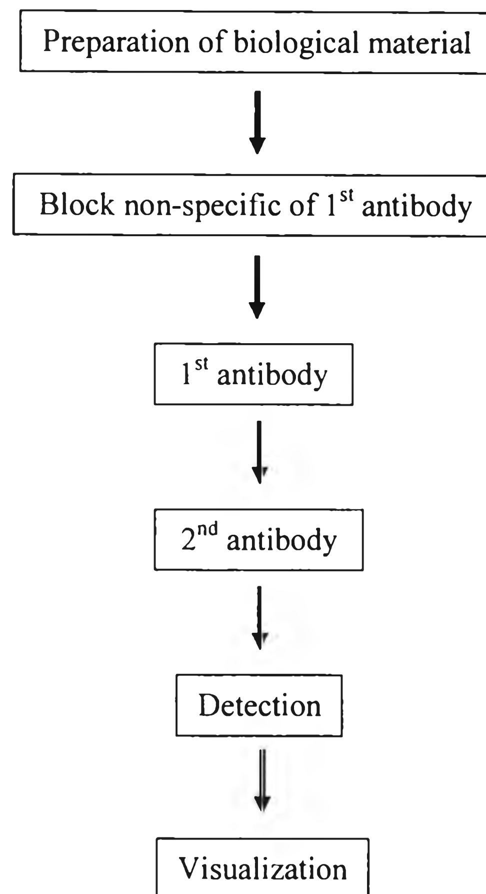

Figure 1.12 Graphical summary of the immunohistochemical technique

1.15 Objectives of the thesis

Basic knowledge on shrimp immunity is needed for prevention and management of shrimp diseases. So, the aim of this thesis is to identify the cDNAs of immune-related protein in the lymphoid organ of the unchallenged and *V. harveyi* challenged *P. monodon* using the expressed sequence tag (EST) analysis. Additionally, microarray analysis is used to study the gene expression in the haemocyte of the white spot syndrome virus (WSSV) and Gram-negative bacteria *V. harveyi* challenged shrimp. Furthermore, some of the up-regulated genes are assessed for their expression using the real time PCR analysis and *in situ* hybridization. Immunohistochemistry is performed to detect the expression of the up-regulated protein in shrimp cephalothorax.