CHAPTER I
INTRODUCTION

1.1 Shrimp culture industry

Shrimp farming is a multi-billion dollar industry contributing a major income to several countries in Asia and South America. Thailand has been the world's leading exporter of cultured shrimp since 1992, and used to be the largest producer of the black tiger shrimp supplying 20 percent of the world trade in shrimp and prawn (Wyban 2007). The rapid growth of shrimp farming led to an economic boom and also enhanced employment distribution in the country.

In Asia, Thailand is the one of top ten countries in shrimp production. The black tiger shrimp hatcheries and farms are dispersed along the coastal area of the country. In the past, the black tiger shrimp *Penaeus monodon* farming is one of the most important aquaculture in Thailand. The black tiger shrimp farms and hatcheries are dispersed along the coastal areas. Southern provinces such as Nakon Sri Thammarat and Surat Thani yield the majority of harvests, whereas eastern and central provinces such as Samut Sakhon and Samut Songkhram yield the minority in terms of number. The location in Thailand has several advantages for shrimp farming, including no typhoon or cyclone seasons, small variable of sea water during season and ideal soy and terrain for pond construction. Unfortunately, during the past decades, the production of the black tiger shrimp has rapidly been decreased due to the outbreaks of bacterial and viral diseases as well as the lack of high-quality shrimp broodstock (Mohan et al. 1998)

1.2 Shrimp aquaculture in Thailand

Previously, the main cultured species shrimp in Thailand was the black tiger shrimp *P. monodon*. This made *P. monodon* shrimp an economically important species in Thailand (Source : FAO Fishstat 2006). Since the year 2002, the production of the black tiger shrimp in Thailand was decreased continuously due to infectious disease. Until now, the export value of the black tiger shrimp in Thailand fell down over 20 times from 28,300.20 million baht in 2002 to 1,538.34 million baht in 2013 (Table 1.1).

Table 1.1 Aquaculture production of black tiger shrimp in Thailand (Source: office of Agricultural Economics, (www.oae.go.th/oae_report/export_import/export.php)

Year	Total	
	Quantity (tons)	Value (million baht)
2002	83,040	28,300.20
2003	77,922	24,201.44
2004	53,043	15,023.23
2005	33,053	8,570.54
2006	29,045	6,971.55
2007	19,314	4,450.28
2008	14,283	2,680.86
2009	11,238	2,355.00
2010	14,099	2,680.86
2011	5,108	2,749.11
2012	6,080	1,383.47
2013	4,661	1,538.34

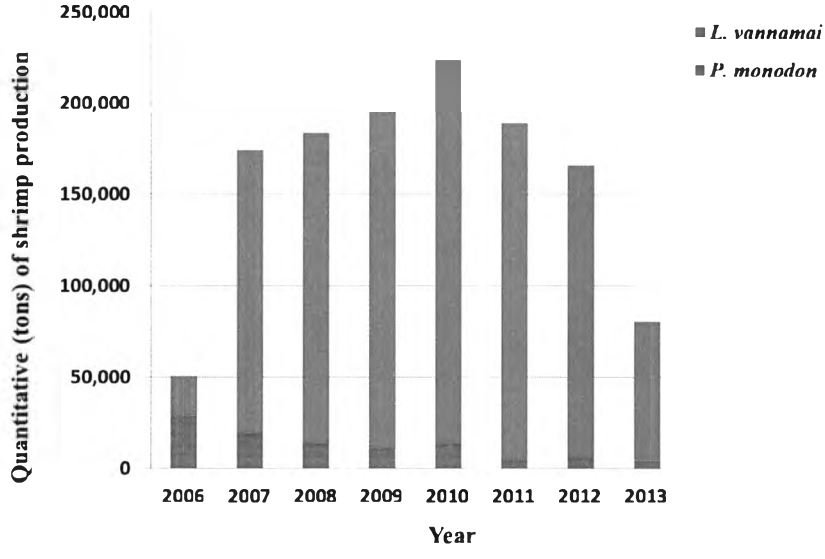

Figure 1.1 The black tiger shrimp (*Penaeus monodon*) and white shrimp (*Litopenaeus vannamei*) export of Thailand during 2006 to 2013 (Source: office of Agricultural Economics, www.oae.go.th/oae_report/export_import/export.php).

The shrimp aquaculture in Thailand has been shift from the native black tiger shrimp (*Penaeus monodon*) to the white shrimp (*Litopenaeus vannamei*) as the white shrimp has higher growth rate, higher survival rate during larval rearing, more availability of specific pathogen free stocks and lower production cost than the black tiger shrimp. In addition, white shrimp aquaculture required less resource and produced less waste than black tiger shrimp (Louis 2010).

However, the black tiger shrimp has some advantages as they are native species in Thailand. They are fast growing and tolerant to a wide range of salinity. Moreover, the white shrimp act as a carriers of various viral pathogens such as *Taura Syndrome virus* (TSV) and *Baculovirus penaei* (BP). These viruses can be transmitted to native penaied shrimp and caused massive losses in *P. monodon* production. The white shrimp *L. vannamei* has lower export price than the black tiger shrimp, and this affects the country's shrimp export in term of value. Therefore, the improvement of *P. monodon* are essential to maintain the shrimp farming industry of black tiger shrimp and important to study the shrimp immune-related genes and their function to understand the immune system in the black tiger shrimp and consequently conserve the black tiger shrimp farming.

1.3 Major viral diseases in shrimp

The cultivation of penaeid shrimp is an important economic activity in the world. This industry, however, has been suffering serious problems brought by viral and bacterial diseases. The infectious diseases, especially viral diseases, become serious problems in shrimp industry worldwide. In Thailand, the outbreaks of infectious disease have become the most serious problem since 1993. White spot syndrome virus (WSSV) and yellow head virus (YHV) are two of the major viral pathogens of *P. monodon* which cause white spot syndrome and yellow head disease, respectively (Boonyaratpalin 1993, Wongteerasupaya 1995) The outbreak of these virus diseases causes great losses in the shrimp industry.

1.3.1 White spot syndrome (WSS) disease

White spot syndrome is a viral disease resulting from WSSV infection. WSS disease affects most of the commercially cultivated marine shrimp species worldwide. The origin of white spot syndrome outbreak began in Taiwan shrimp farms in 1992 and rapidly spread throughout Asia; and subsequently, the disease crossed over the Pacific Ocean and spread in North, Central and South America, creating by far the greatest economic loss (Chou 1995, Lightner 1996, Flegel 1997, Lotz 1997, Spän 1997). The reports of WSSV outbreaks in various shrimp farming countries are presented in Table 1.2 (Escobedo-Bonilla *et al.* 2008).

Table 1.2 Chronology of white spot syndrome virus outbreaks in shrimp farming countries in Asia and America.

Year first reported	Country	Reference
1992	Taiwan	Chou et al., 1995
1993	China, Japan, Korea	Zhan et al., 1998; Inouye et al. 1994; Park et al., 1998
1994	Thailand, India, Bangladesh	Lo et al., 1996; Karunasagar et al., 1997; Mazid & Banu 2002
1995	USA	Lightner 1996; Wang et al., 1999
1996	Indonesia, Malaysia, Sri Lanka	Durand et al., 1996; Kasornchandra et al., 1998; Rajan et al., 2000
1997	Vietnam	Bondad-Reantaso et al., 2001
1998	Peru	Rosenberry 2001
1999	Philippines, Ecuador, Colombia, Panama, Honduras, Nicaragua, Guatemala, Belice	Magbanua et al., 2000; Bondad-Reantaso et al., 2001; Hossain et al., 2001; Wu et al., 2001
1999-2000	Mexico	Bondad-Reantaso et al., 2001
2002	France, Iran	Dieu et al., 2004; Marks 2005
2005	Brazil	APHIS-USDA 2005

WSS disease is spread by contaminated water, decomposing fecal matter or tissue, cannibalism and fluid from infected females. Shrimp may be indirectly infected by exposure to previous hatchery or pond growing cycles, contaminated water supplies, contaminated food, equipment surfaces and clothing, or animals which have ingested diseased shrimp.

WSSV causes the onset of a rapid and mass mortality within 2-7 days post-infection. A clinical sign of white spot syndrome typically is the development of many white spots on the carapace of the infected shrimp which results from white calcium accumulated in shell (Chou 1995). The disease can occur without the presence of white spots. Other signs of the WSSV infection in shrimp also include the body surface and appendages turning to red or pink, loosing shell, lower food consumption and show lethargic behavior. This virus has a wide host range of decapod crustaceans and more than 93 species of arthropods have been reported as hosts or carriers of WSSV (Lo 1996, Flegel 1997, Flegel 1998). WSSV can infect various tissues including antennal grand, cuticular epidermis, gill, muscle, lymphoid organ, nervous tissue, hematopoietic tissue, connective tissues of some organs (Chang 1996, Wang 1997).

WSSV is the type specie of the genus *Whispovirus* in the viral family *Nimaviridae*, containing a circular double-stranded DNA of about 305 kb, and is an enveloped rod-shaped particle with a single filamentous appendage-like tail at one end of the nucleocapsid (Yang *et al.* 2001, Vlak 2004). The average size of the virus is about 298 ± 21 nm long and 107 ± 8 nm in diameter (Wang et al., 1997). A total of 531 putative open reading frames (ORFs) have been identified of which 181 ORFs are likely to encode the functional proteins ((OIE). 2003).

White spot virus (WSV) is a double-stranded DNA (dsDNA) virus. The intact virion is a large (80-120 × 250-380 nm) enveloped rod-shaped particle (Inouye 1994, Wang 1995, Durand 1997, Van Hulten *et al.* 2001) (Figure 1.2). WSSV is a lytic virus that replicates and assembles in the nucleus. At the late stage of infection, the infected nuclei/cells are lysed, causing the infected tissues to become severely damaged and necrotic.

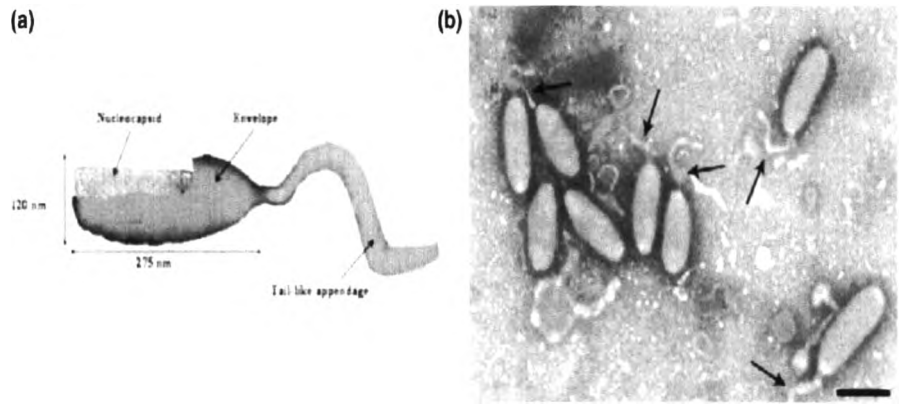

Figure 1.2 (a) Morphology of the white spot syndrome virus (WSSV) virion. (b) Electron micrograph showing WSSV virions with the tail-like appendage (black arrows) (bar = 250 nm) (Durand 1996).

The morphogenesis of WSSV have also reviewed (Escobedo-Bonilla et al., 2008). The morphogenesis is directly related to the development of cellular lesions (Figure 1.3).

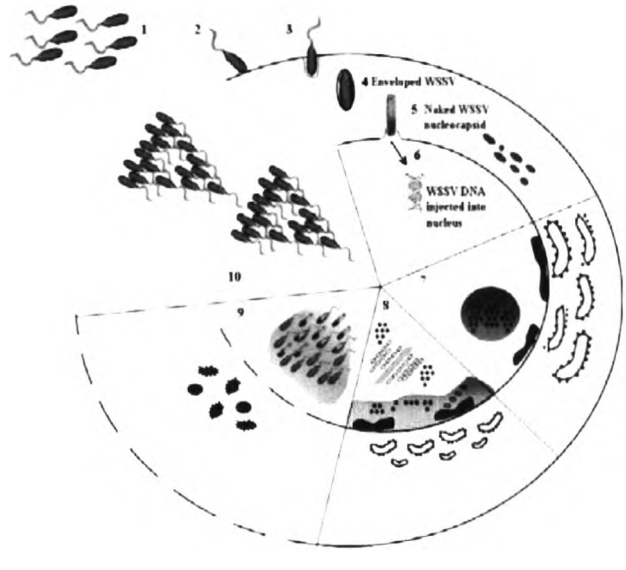

Figure 1.3 A proposed model of the morphogenesis of white spot syndrome virus (WSSV) (Escobedo-Bonilla *et al.* 2008).

(1) Infectious WSSV particles.

(2) An infectious WSSV virion attaches to a susceptible cell using envelope proteins with a cell attachment motif.

(3) WSSV enters the cell.

(4) The envelope of the WSSV virion probably fuses with the endosome and the naked nucleocapsid is transported to the nucleus, in a similar way as in baculoviruses (Lua *et al.* 2000).

(5) The naked WSSV nucleocapsid attaches to the nuclear membrane and the WSSV genome is released into the nucleus.

(6) The WSSV genome replication starts. In the cytoplasm, the mitochondria start degenerating (Wang *et al.* 1999).

(7) In the nucleus the early virogenic stroma appears composed of loose granular material. Cellular chromatin accumulates near the nuclear membrane and the rough endoplasmic reticulum (RER) becomes enlarged and active.

(8) The marginated chromatin is transformed in a dense ring zone (shaded area). The virogenic stroma is less dense and starts forming vesicles that will form the viral envelope. The vesicles are probably formed with membranous material found in the ring zone, as in baculoviruses (Lua *et al.* 2000). A viral nucleosome is also observed as a filamentous structure in the virogenic stroma. This structure contains proteins that will form the nucleocapsid.

(9) New WSSV particles are assembled in the nucleus within an electron-dense inclusion. The empty envelopes are filled with a nucleocapsid. In cytoplasm, organelles become disintegrated and the cellular and nuclear membranes are disrupted (Wang *et al.* 1999).

(10) WSSV virions are completely formed and ready to be released from the disrupted cell to begin the cycle in other susceptible cells.

Apoptosis is a host cell defense to eliminate invading virus. ALG-2 gene is an apoptotic responsive gene that plays a role in apoptotic pathway in response to WSSV infection (Liu *et al.* 2011). Recently, it was shown that the target of ALG-2 is AIP1/Alix, which is an adaptor protein proved to bind with many growth-related signaling molecules (Odorizzi 2006). Moreover, AIP1/Alix involved in endocytosis processes like virus budding and multivesicular endosome formation (Strack *et al.* 2003). However, the mechanism of apoptosis induced by WSSV infection remains unknown.

Host-cell provides biosynthetic machinery of internalization process for virus entry such as receptor mediated endocytosis, a complex array of vesicles and proteins (clathrin- and caveolar-dependent pathways). The entry of enveloped viruses typically reach the endosomal compartment by trafficking in clathrin-coated vesicles. Examples of enveloped viruses with entry mechanisms have been well-defined for HCV, influenza A and HIV (Thorley et al., 2010). In response, some viruses have developed strategies to hijack the cell's endocytic machinery to enter the cell cytoplasm or the nucleus for replication of the particular virus. Transcripts clathrin, dynein related genes and actins/tublins, were also found to be enhanced upon WSSV challenge in crayfish haematopoietic cells. Besides, WSSV infection leads to the activation of cytoskeletal genes in crayfish haematopoietic cells. Cytoskeletal proteins like actins and tublins support diverse cellular processes (Lundin *et al.* 2010).

1.3.2 Yellow head (YHV) disease

Yellow Head Virus was the first major viral disease problem, affecting Asian shrimp farms when it was diagnosed as causing extensive losses in Thailand starting in 1990. The first records of this virus were from *P. monodon* ponds in Eastern Thailand in 1990. By 1992, it had moved to Southern Thailand and was causing substantial mortality. YHV is prevalent wherever *P. monodon* are cultured, including Thailand, Taiwan Province of China, Indonesia, Malaysia, Mainland China, the Philippines and Vietnam. It responsible for the first major industrial crashes in Taiwan Province of China in 1987 (Flegel 1997, Lightner 1998). Losses due to YHV continued, although the severity and frequency of outbreaks declined sharply by 1994 when WSSV became the prime cause of mortality in cultured *P. monodon*. Although research has shown that YHV is still present in culture ponds, the shrimp now rarely show gross symptoms and are latently infected. There appears to be a currently

unknown mechanism for rapid tolerance or resistance to RNA-type viruses (such as YHV in Asia, and TSV in Latin America) in Penaeid shrimp. The primary mechanism of YHV spread in pond culture appears to be from water and mechanical means or from infected crustacean carriers (Flegel 1997). This virus can be spread either by ingestion or cohabitation. In addition, infected broodstock can pass on the virus to larvae in the maturation/hatchery facilities if thorough disinfection protocols are not strictly adhered to (Flegel 1997). YHV principally affects *P. monodon* at juvenile stages from 5-15 g (Lightner 1996).

YHV infections can cause swollen and light yellow coloured hepatopancreas in infected shrimp, and a general pale appearance, before dying within a few hours. Total mortality of the crop is then typically seen within three days. Experimentally infected shrimp develop the same signs as those naturally infected, indications of the disease are noted after two days and 100 percent mortality results after three to nine days (Lu 1995). YHV is a pleomorphic, enveloped virus with single stranded RNA of positive polarity primarily localized in the cytoplasm of infected cells (Cowley *et al.* 1999). Infected shrimp often exhibits light yellow coloration of the dorsal cephalothorax area and have a generally pale or bleached appearance (Limsuwan 1991).

YHV is an invertebrate nidovirus containing single-stranded RNA of about 22 kb, and is an enveloped rod-shaped particle of approximately 195 ± 5 nm long and 55 ± 5 nm in diameter (Sittidilokratna *et al.* 2002). The target tissues of YHV are diverse such as the lymphoid organs, gills, nerve cord, heart, midgut, hepatopancreas, head soft tissues, abdominal muscle, eyestalks and hematopoietic tissue (Boonyaratpalin 1993, Chantanachookin 1993, Lu 1995). YHV may occur as latent and asymptomatic infections in broodstock shrimp and possibly transfer from the parental shrimp to their offspring (Chantanachookin 1993). The YHV infection diagnosis could be performed by using several methods such as RT-PCR, *in situ* hybridization, and immunological methods using specific antibodies to a surface glycoprotein and the nucleocapsid proteins of the virus (Wongteerasupaya 1995, Tang *et al.* 1999, Sánchez-Barajas 2009).

1.4 The innate immune responses in crustaceans

The innate defense system of invertebrates including crustaceans is grouped into cellular and humoral responses. Hemocytes are the main effective tissue, which plays the important role to both responses against the invading pathogens. In

crustacean, the hemocytes are classified into three groups; hyaline cell (agranular), semigranular cell (small granular) and granular cell (large granular) (Bauchau 1980, Tsing 1989). Hemocytes are the major immune cells of shrimps and play an crucial role in both the cellular and humoral immunity. Different types of hemocytes have different defense functions. Hyaline cells have small size and contain no or few granules involved in phagocytosis. Semigranular cells have many eosinophilic granules and active in encapsulation. Moreover, these cells have a limited function in phagocytosis and contain the prophenoloxidase activating system (proPO system). Granular cells have numerous large eosinophilic granules participating in storage and release of the proPO system and cytotoxicity (Smith *et al.* 1983, Kobayashi 1990, Bachère 2000).

1.5 Cell-mediated defense reactions

Cellular defense reactions include various biological processes. Three main reactions of cell-mediated defense mechanisms are known, namely phagocytosis, encapsulation and nodule formation (Millar 1994).

Phagocytosis, a common phenomenon in all organisms, includes foreign particle attachment, ingestion and destruction (Jeon *et al.* 2010).

Encapsulation, a process wherein layers of cells surround the foreign material to prevent spreading of the pathogen, occurs when a parasite is too large to be ingested by phagocytosis (Gillespie *et al.* 1997).

Nodule formation, which appears to be similar to capsule formation, occurs when the number of invading bacteria is high. These mechanism is an entrapment of invading pathogen. The entrapped foreign matter is in the center of the forming nodules. Subsequently, the invading pathogens are destroyed by secretory humoral defense molecules from the cell such as oxygen species produced by the prophenoloxidase activating system (Jiravanichpaisal *et al.* 2006).

1.6 Humoral defense system

1.6.1 Pattern recognition proteins

Unlike vertebrates, invertebrates lack adaptive immune system and rely on innate immune response against invading pathogens (Hoffmann *et al.* 1999). When

pathogenic microorganisms attack animals, the initial immune process is recognition of cell wall components that present on the surface of invading microorganisms. This process is mediated by hemocytes and plasma proteins. In crustaceans, several types of modulator proteins recognizing cell surface components of pathogens have been identified. The target recognition of innate immunity, so-called "pattern recognition molecules (PRMs)", is 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 patterns include the lipopolysaccharides (LPS) of gram-negative bacteria, the lipoteichoic acids (LTA) of gram-positive bacteria, the glycolipids of mycobacteria, the mannans of yeasts, the peptidoglycan of microbial cell wall, the ß-1,3-glucan of fungi, and double-stranded RNA of viruses (Hoffmann *et al.* 1999). Most current researches have emphasized the possible roles of non-self recognition molecules in the vertebrate and the invertebrate immune system.

Carbohydrate recognition is a common constituents of microbial cell wall, and microbial carbohydrates have distinct structures from those of carbohydrates of eukaryotic cells. Therefore, LPS or/and ß-1,3-glucan-binding proteins (LBP, ß-GBP, or LGBP), peptidoglycan recognition protein (PGBP), several kinds of lectins, and hemolin have been identified in a variety of invertebrates with different biological functions proposed following their binding to their targets (Lee and Söderhäll, 2002). In shrimp, the LPS-binding protein has been reported as a multivalent carbohydrate-binding agglutinin, besides its bacterial agglutination ability and phagocytic induction (Vargas-Albores 1995).

Lectins are sugar-binding proteins that results in cell agglutination and/or glycoconjugate precipitation with a carbohydrate portion such as polysaccharide, glycoproteins, glycolipids, and the others. There are many different lectins, including tachylectins from hemolymph plasma of the horseshoe crab, *Tachypleus tridentatus* (Gokudan *et al.* 1999). They are involved in a variety of processes, including the innate immune response, and are critical for the detection and elimination of infectious microorganisms (Weis *et al.* 1998, Kilpatrick 2000).

In animals, lectins regulate the cell adhesion to glycoprotein synthesis, control protein levels in blood, and bind soluble extracellular and intracellular glycoproteins. Also, in the immune system, lectins recognize sugar or carbohydrates structures found specifically on the surfaces of pathogens that do not present on host cells. Generally, they recognize sugar or carbohydrate. It has been reported that

lectins have an LPS-binding property (Jomori *et al.* 1992, Koizumi *et al.* 1999). These LPS-binding proteins have biological functions of a bacterial clearance activity and an opsonic effect. Lectins are responsible for promoting phagocytosis and stimulating the proPO system (Yu *et al.* 1999, Yu *et al.* 2000). Tachylectins were found to have hemagglutinating and antibacterial activities which are important in the immune system (Kawabata *et al.* 1999). So far, C Type lectins carry a wide range of functions such as cell to cell adhesion, immune response to foreign bodies and self-cell destruction. C-type lectins from shrimp have been reported to be involved in antibacterial, antifungal and antiviral processes. For example, *Fc*-hsl and *Lv*Lec displayed immune activities against some bacteria and fungi (Sun *et al.* 2008, Zhang *et al.* 2009), while *Fc*lectin showed up-regulated expression levels after challenge with bacteria, lipopolysaccharide or WSSV (Liu *et al.* 2007). Likewise, *Lv*CTL1 and *Pm*AV exhibited antiviral activity against infection with WSSV and grouper iridovirus, respectively (Luo *et al.* 2003, Zhao *et al.* 2009) whilst *Pm*LT played a role in the pattern recognition receptor for initial recognition of WSSV, and could also activate the cellular defense mechanism of shrimp hemocytes (Ma *et al.* 2008).

1.6.2 The prophenoloxidase (proPO) system

The proPO activating system is composed of several proteins involved in melanin production, cell adhesion, encapsulation, and phagocytosis (Söderhäll *et al.* 1998). One of the important innate immune response in crustaceans is the prophenoloxidase (proPO) activating system, which is activated by a stepwise process involving serine proteases activated by microbial cell wall components such as lipopolysaccharides or peptidoglycans from bacteria, and ß-1,3-glucans from fungi through pattern-recognition proteins (PRPs) (Ariki *et al.* 2004).

An enzyme, capable of activating the proPO *in vivo,* is called prophenoloxidase-activating enzyme (factor) (ppA, PPAE, PPAF). Proteinases stored as an inactive form into active phenoloxidase-activating factor (PAEs, PAFs, PAPs), proteinases in an active form. This activation leads to a cascade of stepwise activation of proteinases in the proPO system, and eventually produces prophenoloxidase (PO) (Ariki *et al.* 2004).

The proPO system is controlled by serine protease inhibitors. In crayfish, ppA is a trypsin-like proteinase presenting as an inactive form in the hemocyte granules. After degranulation, the enzyme is released together with proPO and becomes an

active form in the presence of microbial elicitors. The active ppA can convert proPO to an active form, phenoloxidase (PO) (monophenyl L-dopa: oxygen oxidoreductase; EC 1.14.18.1) (Aspán 1991, Aspan *et al.* 1995).

PO is a copper-containing protein and a key enzyme in melanin synthesis (Söderhäll *et al.* 1998, Shiao *et al.* 2001). It catalyses o-hydroxylation of monophenols to diphenols and oxidises diphenols to quinones, which can polymerize nonenzymatically to melanin as a toxic to micro-organisms (Söderhäll 1996). PO is a sticky protein and can adhere to the surface of parasites leading to melanization of the pathogens.

Melanization is usually observed by blackening of the parasite in the hemolymph or black spots on the cuticle. The melanin and intermediates in the melanin formation can inhibit growth of microbial parasites such as crayfish plague fungus, *Aphanomyces astaci* (Söderhäll 1982). In addition, the production of insoluble melanin is important for the process of sclerotisation, wound healing, and encapsulation of foreign materials (Theopold *et al.* 2004).

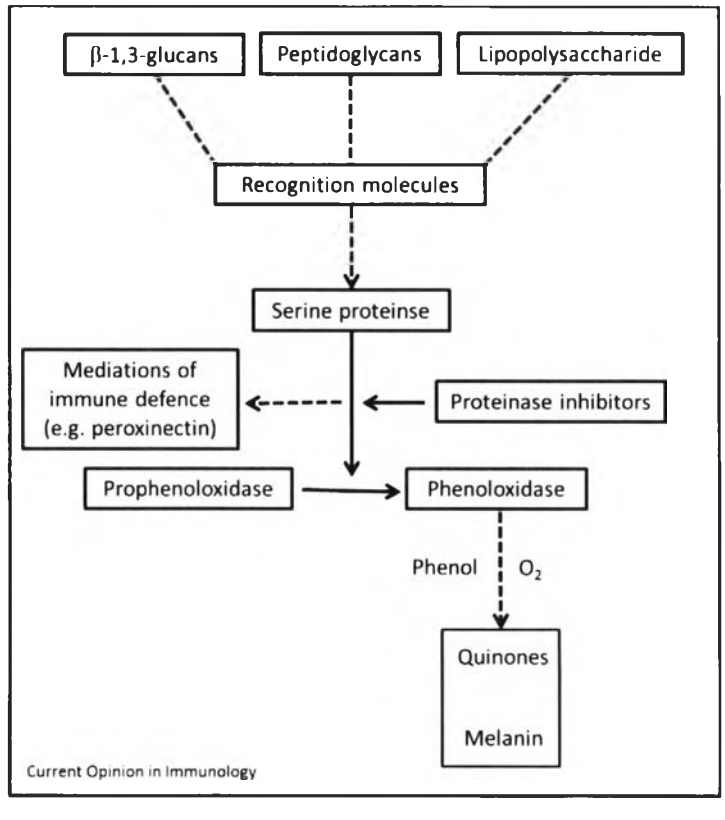

Figure 1.4 A scheme for prophenoloxidase-activation in arthropods. (Söderhäll *et al.* 1998).

Crustacean proPO is synthesised in the blood cells (Aspán et al., 1995), whereas in crayfish haemocyanin is known to be synthesised in the hepatopancreas. In insects, the proPO also seems to be synthesised in the blood cells (Kawabata *et al.* 1995). Recently, proPO has been shown to be transported and deposited in the cuticle of the silkworm, *B. mori* (Ashida 1994).

In the crayfish, *P. leniusculus*, RNA interference-mediated depletion of crayfish proPO led to lower PO activity, increased bacterial growth, lower phagocytosis, lower nodule formation, and higher mortality when infected with a highly pathogenic bacterium, *Aeromonas hydrophila* (Liu *et al.* 2007). In contrast, RNA interference of pacifastin, an inhibitor of the crayfish proPO activation cascade, resulted in higher phenoloxidase activity, lower bacterial growth, increased phagocytosis, increased nodule formation, and delayed mortality of the infected crayfish (Liu *et al.* 2007).

Moreover, in penaeid shrimp, enzymes in the proPO system are localized in the semigranular and granular cells (Perazzolo *et al.* 1997). This is in accordance with the former studies showing that *P. monodon* proPO and PPAE mRNAs as well as a *L. vannamei* proPO mRNA are expressed only in hemocytes (Sritunyalucksana 2000, Ai *et al.* 2009, Amparyup *et al.* 2009, Charoensapsri *et al.* 2009, Charoensapsri *et al.* 2011). These results indicate that proPO and PPAE are important in proPO system as well as the innate immune response in crustaceans.

In the black tiger shrimp, RNAi-mediated silencing of two *P. monodon* proPO genes (*Pm*proPO1 and *Pm*proPO2) and two *P. monodon* PPAE genes (*Pm*PPAE1 and *Pm*PPAE2) show significantly decreased the total PO activity, leading to an increase in the bacterial number in *Vibrio harveyi*-infected shrimp and also enhanced their the mortality rate after the infection (Amparyup *et al.* 2009, Charoensapsri *et al.* 2009, Charoensapsri *et al.* 2011).

1.6.3 Antimicrobial peptides (AMPs)

Animal and plant organisms possess an innate immune system for immediate protection from invading foreign microorganisms. Shrimps have evolved and use a diverse array of antimicrobial peptides (AMPs) as part of an important first-line response of the host defense system. Besides many other peptides and proteins in the system, AMPs are one of the key elements (Bulet *et al.* 1999). The AMPs have broad spectra of antimicrobial activity, an ability to kill or neutralize Gram-negative and Gram-positive bacteria, fungi, parasites and viruses (Hancock *et al.* 2000). Lacking

the adaptive immunity, crustaceans including shrimps rely on only the innate immunity. Therefore, the AMPs are crucial for them to fight the pathogenic invasion.

Antimicrobial peptides (AMPs) are an integral component of the innate immune system. These peptides share certain common characteristics, such as small size (generally less than 200 amino acid residues), with amphipathic structure, cationic character and similarities in structural patterns or motifs. Similar to those found in other animals, AMPs are also key factors in the innate immunity of shrimp (Bachère *et al.* 2004, Jenssen *et al.* 2006). They have a broad range of antimicrobial activity against Gram-positive and Gram-negative bacteria, filamentous fungi and, in some cases, viruses and protozoa (Bachère 2003).

For many of these peptides, there is evidence that one of the targets for the peptides is the lipid bilayer of the membrane because these peptides can often increase the rate of leakage of the internal aqueous contents of liposomes. Furthermore, most of the antimicrobial peptides are cationic and their interaction with anionic phospholipids would provide a ready explanation for their specificity for bacterial membranes. With regard to the mechanism by which the peptide breaks down the membrane permeability barrier, it is possible that the peptide induces complete lysis of the microorganisms by rupturing the membrane or perturbing the membrane lipid bilayer, allowing for leakage of certain cellular components as well as dissipating the electrical potential of the membrane.

AMPs are active against a large spectrum of microorganisms: bacteria and filamentous fungi. In addition, some AMPs have antiviral or antiparasitic activities (Murakami *et al.* 1991, Hancock *et al.* 2000, Pan *et al.* 2000) and may also exhibit an anti-tumor property (Cruciani et al., 1991). AMPs are primarily expressed in circulating hemocytes, which is the main site of the immune response, and hemocytes expressing AMPs probably migrate to infection sites to fight against pathogen invasion.

In penaeid shrimp, several putative antimicrobial peptides have been identified. These are the penaeidins (Destoumieux *et al.* 1997, Destoumieux *et al.* 1999, Destoumieux *et al.* 2000, Cuthbertson *et al.* 2004, Kang *et al.* 2004, Munoz *et al.* 2004, Kang *et al.* 2007), whey acidic protein (WAP) domain containing proteins [crustins and single WAP containing peptides (SWD)] (Gross *et al.* 2001, Amparyup *et al.* 2008, Jia 2008), antilipopolysaccharide factors (ALFs) (Gross *et al.* 2001, Liu *et al.* 2005, Somboonwiwat *et al.* 2005, Liu *et al.* 2006, Tharntada *et al.* 2008), histones (Patat *et al.* 2004), hemocyanin (Destoumieux-Garzon *et al.* 2001, Zhang *et al.* 2004),

lysozymes (Hikima *et al.* 2003, Sotelo-Mundo *et al.* 2003, de-la-Re-Vega *et al.* 2006, Burge *et al.* 2007, Bu *et al.* 2008, Ye *et al.* 2009), a C-type lectin (Sun *et al.* 2008), and peritrophins (Loongyai *et al.* 2007).

Penaeidins is a family of antimicrobial peptides acting against gram- positive bacteria and fungi. It had been reported in the white shrimp, *L. vannamei* (Destoumieux *et al.* 1997). cDNA clones of penaeidins were also isolated from the hemocytes of *P. setferus* and *P. monodon* (Gross *et al.* 2001, Supungul *et al.* 2004). Recently, a new function of penaeidin5 from the *P. monodon* has been reported to play a possible role in protection against viral infection (Woramongkolchai *et al.* 2011).

Crustins, the crustacean antimicrobial peptides, were identified from several species of *Penaeid* shrimp, *L. vannamei*, *L. setiferus*. Several isoforms of crustins were found in Penaeid species. The 11.5 kDa antibacterial protein from *Carcinus maenas*a and crustins from shrimp showed no homology with other known antibacterial peptides, but possesses sequence identity with a family of proteinase inhibitory proteins, the whey acidic protein (WAP). Crustin possesses antimicrobial activity and antiviral activity (Destoumieux-Garzon *et al.* 2001, Patat *et al.* 2004, Supungul *et al.* 2004, Zhang *et al.* 2004).

ALFs is a small basic protein which was initially isolated and characterized from hemocytes of the horseshoe crab *Limulus polyphemus* (Aketagawa *et al.* 1986, Muta *et al.* 1987). L. polyphemus ALF binds lipopolysaccharide (LPS) and has a strong antibacterial activity, particularly on the growth of Gram-negative bacteria. In shrimp, cDNA clones homologous to the horseshoe crab ALFs were initially identified in hemocytes of *P. monodon* and *L. setiferus* by expressed sequence tag (EST) analysis (Gross *et al.* 2001, Supungul *et al.* 2004).

ALFs have broad antimicrobial activities towards gram-positive and gram-negative bacteria, filamentous fungi, and viruses (Somboonwiwat *et al.* 2005, Liu *et al.* 2006, Li *et al.* 2008). ALF3 from *P. monodon* showed the anti-viral activity against WSSV (Tharntada *et al.* 2009) and anti-bacteria activity against vibriosis (Ponprateep 2009). ALF transcript level in the crayfish, *P. leniusculus,* was enhanced after WSSV injection, and ALF knockdown resulted in an increase of WSSV propagation in cell cultures (Liu *et al.* 2006). In addition, peptides derived from the hemocyanin of *L. vannamei, P. stylirostris*, and *P. monodon* possess antiviral activity (Destoumieux-Garzon *et al.* 2001, Patat *et al.* 2004, Zhang *et al.* 2004). Histones and histone-derived peptides of *L. vannamei* have also been reported as innate immune

effectors because they could inhibit growth of gram-positive bacteria (Patat *et al.* 2004).

1.6.4 The coagulation system/ the clotting system

A coagulation system is essential in both invertebrate and vertebrate animals for prevention of excess blood loss from a wound and obstructing microorganisms from invading the wound. Coagulation has also been studied in detail in two invertebrate groups: chelicerates and crustaceans. The coagulation system of a chelicerate, the horseshoe crab, is composed of a clot-forming protein coagulagen, and a proteinase cascade in the large granules of hemocytes, which can be activated by either microbial lipopolysaccharides or β-1,3-glucans (Kawabata *et al.* 1995, Iwanaga *et al.* 1998). Upon activation of this system, coagulogen can be converted by proteinases into coagulin and the latter spontaneously aggregates to form insoluble clots.

In crustacean coagulation systems, a TGase was found to be one of the essential components, e.g. in lobster (Fuller *et al.* 1971), crayfish (Kopacek *et al.* 1993, Komatsu *et al.* 1998, Hall *et al.* 1999) and shrimp (Yeh *et al.* 1998). In the coagulation system of the freshwater crayfish, *Pacifastacus leniusculus*, a plasma clotting protein (CP) was found to form stable clots via polymerisation during wound healing or defence reactions (Kopacek *et al.* 1993, Hall *et al.* 1999). This blood clotting process is catalysed by an endogenous Ca^{2+}-dependent TGase (Kopacek *et al.* 1993), which is released from the hemocytes under foreign particle stimulus or tissue damage (Hall *et al.* 1999).

The TGase, under this process, forms the ε-(γ-glutamyl)- lysine crosslinks between glutamine and lysine of the clottable protein (CP) (Kopacek *et al.* 1993). This polymerization step has been conserved through evolution (Wang et al., 2001). TGase and CP have been documented to be involved in the blood coagulation system of shrimp.

In the black tiger shrimp *P. monodon*, there are three isoforms of Tranglutaminase in the black tiger shrimp, the *Pm*STG I (Huang 2000) and two members of *Pm*STG II (Chen *et al.* 2005, Yeh *et al.* 2006). These *Pm*STG IIs exert the polymerization activity using clottable proteins as a substrate; whilst *Pm*STG I lacks the blood coagulation activity.

1.6.5 Apoptotic and tumor proteins

Apoptosis, a kind of programmed cell death, is an important cellular defense mechanism that inhibits viral multiplication and eliminates infected cells in multicellular organisms (Everett *et al.* 1999, Koyama *et al.* 2000).

Apoptosis occurs through energy-dependent, distinct biochemical reactions, and it is accompanied by characteristic morphological features, including chromatin condensation, cell shrinkage, blebbing of the plasma membrane and fragmentation of the cell body (Kerr *et al.* 1972, Wyllie *et al.* 1980). Apoptosis is used to remove surplus, damaged or diseased cells in multicellular organisms, and is controlled by complicated molecular networks that are highly conserved from nematodes to mammals.

Apoptosis, which occurs after viral infections, plays an important role in the antiviral mechanism of crustaceans (Liu *et al.* 2009). However, this also leads to a significant reduction in the number of circulating hemocytes, probably resulting in a decline of antiviral immunity as well as mortality of crustaceans (van de Braak *et al.* 2002, Granja *et al.* 2003, Wongprasert *et al.* 2003, Wang *et al.* 2008). Therefore, maintenance of the hemocyte level in the blood-circulating system, including the rapid production of new hemocytes from hematopoietic tissue, is essential for the survival of the animals as is the capacity to protect against pathogenic invaders.

1.6.6 Proteinase inhibitors

Proteinase inhibitors are necessary to protect host from microbial proteinases and regulate the proteinase cascades (the proPO and coagulation system). They were produced by the hemocytes. Proteinases function in many pathogenic fungi to aid in penetrating the cuticle of their arthropod hosts. Proteinases can also contribute to the virulence of bacterial pathogens. Some of the proteinase inhibitors in hemolymph may defend the host against such microbial proteinases. For example, the silk worm (*Bombyx mori*) serine proteinase inhibitor is active against proteinases from fungal pathogens (Eguchi 1993) and several of the *Manduca sexta* serpin gene-1 variants inhibit bacterial or fungal serine proteinases (Jiang *et al.* 1997). Inducible proteinase inhibitors may also play a role in antifungal defense (Vilcinskas 1997). Proteinase inhibitors in the cuticle or at the surface of the integument might also function in protection against fungal infection. An external secretion from

grasshoppers has been shown to contain proteinase inhibitors with a wide range of specificity (Polanowski 1997). An external secretion from grasshoppers has been shown to contain proteinase inhibitors with a wide range of specificity.

In vertebrates, injury and microbial infection lead to activation of the blood coagulation and proPO systems. Both of these systems for maintaining homeostasis employ cascades of serine proteinases to amplify an initial signal (wounded tissue or the presence of microbial polysaccharides), resulting in rapid and efficient responses to threats to health (O'Brien 1993, Whaley 1993). Blood clotting and phenoloxidase activation can be harmful to the host if they are not limited as local and transient reactions, and for this reason the proteinases in these systems are tightly regulated by serpins that exist in plasma (Polanowski 1997).

In arthropods, serine proteinases are supposed to be one of the most vital elements in the immune system. There is relatively high concentration of their inhibitors in the hemolymph (Kanost 1996). Several serine proteinases have been characterized. A 39 kDa serine proteinase purified from plasma of *B. mori* exists as a zymogen that is proteolytically activated upon exposure of plasma to β-(1-3) glucan (Katsumi *et al.* 1995) and then is rapidly inactivated by a plasma serpin (Ashida 1994). However, this enzyme (which cleaves specifically after Arg residues) does not appear to be part of the prophenoloxidase activation pathway (Katsumi *et al.* 1995). Serpin-1J of *M. sexta* from hemolymph inhibits the activity of a serine proteinase linked to prophenoloxidase activation (Jiang *et al.* 1997). The *M. sexta* serpin-6 was isolated from hemolymph of the bacteria-challenged larvae, which selectively inhibited proPO-activating proteinase-3 (PAP-3) (Wang *et al.* 2004). Moreover, the function of serpin-6 was further characterized by cloning and expression in *E. coli* expression system (Zou *et al.* 2005). The results indicated that serpin-6 played important roles in the regulation of immune proteinases in the hemolymph. It seems that each proteinase in the proPO cascade is regulated by one or more specific inhibitors present in plasma or in hemocyte granules.

Moreover, the recently reported function of kazal-type proteinase inhibitors (KPIs) includes the reproductive process in the fresh water prawn *Macrobrachium rosenbergii* required KPI to inhibit the sperm gelatinolytic activity (Li et al., 2009) and bacteriostatic activity of KPI in *P. monodon* (Donpudsa *et al.* 2009). A 23 kDa inhibitor from the Kazal family was isolated from hemocytes of the crayfish *Pacifastacus leniusculus*, and a cDNA encoding the inhibitor was cloned (Johansson et al., 1994).

This protein contains four Kazal family domains and inhibits chymotrypsin and subtilisin.

In addition, pacifastin and α-macroglobulin (A2M) inhibited crayfish proPO activation (Aspan 1990). Among the low molecular weight inhibitors from insect hemolymph, Kunitz family inhibitors from *M. sexta, Sarcophaga bullata*, and *B. mori* (Sugumaran *et al.* 1985, Saul *et al.* 1986, Aso *et al.* 1994) and the 4 kDa locust inhibitors (Boigegrain *et al.* 1992) can also interfere in proPO activation.

1.7 Other immune molecules

Eicosanoids including prostaglandins (PGs) are fatty acid metabolites that in mammals are produced by many cell types, including epithelial and immune cells, and play key roles in hemostasis and immune regulation, among many other functions (Harris *et al.* 2002). Prostaglandins (PGs) are able to regulate the expressions of genes in various mechanisms such as cell protection, lipid metabolism, energy metabolism, signal transduction and proPO-activating cascade in insect cells (Downer *et al.* 1997, Stanley *et al.* 2008). PGs mediate the expression of genes encoding the antibacterial peptide cecropine and lysozyme in the silkworm *Bombyx mori* (Morishima *et al.* 1997) and regulate the activation of prophenoloxidase (PPO) in two insect species, *Galleria mellonella* (Downer *et al.* 1997) and *Rhodnius prolixus* (Garcia *et al.* 2004). However, they do not activate PPO in other insects (Lord *et al.* 2002, Goldsworthy 2003). In addition, *Anopheles albimanus* prostaglandin E_2 (PGE2) modulated the expression of three antimicrobial peptides: *Aa*-Attacin, *Aa*-Cecropin and *Aa*-Gambicin (Garcia Gil de Munoz *et al.* 2008). Although, until now, there is no information on the functions of PGs in crustaceans, the results from previous studies strongly suggested that PGs acted as mediators in the immune response pathways of various invertebrates.

Rab GTPases belong to the *Ras* superfamily and are known to play key roles in phagocytotic development, and are probably involved in virus evasion. Certainly, Rab7 is a small GTPase protein mediates early to late endosome and from late endosome to lysosome trafficking in mammalian cells (Feng *et al.* 1995, Press *et al.* 1998, Mohrmann *et al.* 1999, Ohashi *et al.* 1999, Zuk *et al.* 1999). In *Penaeus monodon*, *Pm*Rab7 was previously identified as a WSSV- binding protein. It binds directly to the VP28 envelope protein of WSSV and is probably involved in the transport of viruses into the cells (Sritunyalucksana *et al.* 2006). Moreover, Rab7 from

P. monodon has previously been implicated in WSSV and YHV infections (Ongvarrasopone *et al.* 2008). In addition, Rab7 is involved in sorting of virus and in the formation of transport vesicles (Vidricaire *et al.* 2005, Vonderheit *et al.* 2005). Additionally, the Rab6 protein homologue of the kuruma shrimp, *P. japonicus*, was documented to be essential in the regulation of hemocytic phagocytosis against WSSV and the bacterium *Vibrio parahemolyticus* (Wu *et al.* 2008, Zong *et al.* 2008).

Individual genes or proteins has been described for partial functional work but for which a clear role in the viral response network has not yet been determined. For example, a small heat shock protein was identified with chaperone activity (HSP21) that is down-regulated in *P. monodon* after WSSV challenge (Huang 2000). In *P. chinensis*, the expression of hematopoietic proliferating cell nuclear antigen gene (PCNA) was down-regulated compared with that of the control group at the early phase of WSSV infection, suggesting that might inhibit the proliferation activity of hematopoietic tissue during that disease phase (Xie *et al.* 2008). In *P. (Marsupenaeus) japonicus*, a ras-related nuclear protein (Ran protein) with GTP-binding activity was up-regulated in WSSV resistant shrimp and in WSSV-challenged shrimp at 4 h post-challenge (Han *et al.* 2007), suggesting a role in defense against WSSV. Also in *P. japonicus*, a double-WAP domain protein (Mj-DWD protein) (Chen *et al.* 2005) was found that exhibited high expression levels in WSSV-resistant shrimp and up-regulation at the early stage of WSSV infection. It was suggested that it played a role in defense against WSSV via protease inhibitory activity.

1.8 Viral Responsive Protein 15 (*Pm*VRP15)

Many genes and proteins with unknown or putative functions have been reported to change in expression level after viral challenge; however, roles in the shrimp viral response network are not well clarified (Flegel *et al.* 2011).

Viral Responsive Protein 15 was a novel gene. It was found to be up-regulated in the hemocytes of WSSV-infected *P. monodon* upon WSSV infection using suppression subtractive hybridization approach (Vatanavicharn *et al.* 2014). This gene was found to be greatly abundant in both early (24I) and late (48/72I) WSSV infection SSH libraries. Table 1.3 shows that 20/110 clones and 104/228 clones of *Pm*VRP15 were found in 24I and 48/72I in SSH libraries, respectively (Table 1.3).

Table 1.3 Up-regulated genes in the hemocytes of WSSV-infected *P. monodon* identified from suppression subtractive hybridization

Genes and putative functions	The closest species	GenBank accession no.	No. of clone in libraries 24I	48/72I	*E*-Value (% Identity)
Apoptosis					
Translationally controlled tumor protein	*Penaeus monodon*	EU492535	1	-	8e-55 (100%)
Cell defense and homeostasis					
Alpha-2-macroglobulin	*Penaeus monodon*	AAX24130	-	3	1e-12 (100%)
Heat shock cognate 70	*Litopenaeus vannamei*	ABP01681	1	-	4e-04 (100%)
Hemocyte homeostasis-associated protein (*Pm*HHAP)	*Penaeus monodon*	HQ130431	13	32	5e-100 (100%)
Histone H1	*Venerupis philippinarum*	ABV25905	-	18	4e-15 (65%)
Nazal-type proteinase inhibitor, isoform 2	*Penaeus monodon*	AY267200	17	-	1e-57 (100%)
Transglutaminase	*Penaeus monodon*	AAV49005	-	2	5e-29 (100%)
Sodium/potassium-transporting ATPase subunit beta	*Penaeus monodon*	EF672698	2	-	1e-64 (98%)
Cytoskeletal component					
Non-muscle myosin heavy chain	*Hordegnathos saltator*	EFN89532	33	-	1e-25 (60%)
Energy metabolism					
ATP synthase beta subunit	*Tethya actinia*	ACV88000	1	-	1e-13 (100%)
Cytochrome c oxidase subunit I	*Penaeus monodon*	EU139434	-	1	3e-112 (100%)
Protein synthesis and processing					
16S ribosomal RNA gene	*Penaeus monodon*	AF217843	-	6	6e-31 (100%)
Eukaryotic translation initiation factor 5A	*Penaeus monodon*	ABI30653	4	-	6e-10 (100%)

Genes and putative functions	The closest species	GenBank accession no.	No. of clone in libraries 241	48/721	*E*-Value (% Identity)
Signal transduction					
G protein, alpha subunit	*Aplysia californica*	ABD62078	-	2	8e-33 (40%)
Transcription and RNA processing					
X-box-binding protein 1	*Lycosa singoriensis*	ABX75460	8	-	8e-10 (85%)
Viral infection mechanism					
Viral responsive protein 15 kDa (VRP15)	*Penaeus monodon*	EE661626	20	104	5e-58 (100%)
WSSV protein-coding genes					
ICP11	White spot syndrome virus	HM778020	-	11	1e-46 (100%)
Thymidine kinase	White spot syndrome virus	AF132668	-	2	3e-34 (100%)
VP15	White spot syndrome virus	DQ681072	4	37	3e-81 (100%)
VP68	White spot syndrome virus	EF534252	-	1	1e-67 (100%)
Unknown sequences			6	9	
		Total clones	110	228	

1.8.1 The full-length cDNA of *Pm*VRP15 and sequence analysis

The partial sequence of the *Pm*VRP15 cDNA was obtained from SSH library of WSSV-challenged *P. monodon* hemocytes. Thus, the full-length cDNA of *Pm*VRP15 was then identified using 5′ RACE and *Pm*VRP15 contained 722 base pairs with a complete open reading frame encoding 137 amino acids. The size of *Pm*VRP15 mRNA was confirmed by Northern blot analysis. It was about 722 base pairs (Figure 1.5).

By BLAST homology search, the putative predicted protein sequence encoded by *Pm*VRP15 had no significant amino acid sequence similarity to any proteins in the database (E-value > 10^{-4}). From a protein-structural analysis (TMHMM Server v. 2.0) (Krogh *et al.* 2001), this protein contained a transmembrane helix of 23 amino acids and it has no predicted signaling domain (Simple Modular Architecture Research Tool (SMART)) (Schultz *et al.* 1998).


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1   AGCGGCCGCGACCGAGCCAAGAGAACGTTCACTCGATCACCACTCTCGTTCTTTGATCTA   60
61  CGCAATGTTAACAGAGGACTTAGTAAACCTGGTGTACGAGGTGTGTCAAGAGAAGAAGCT  120
        M  L  T  E  D  L  V  N  L  V  Y  E  V  C  Q  E  K  K  L
121 GCGAGCGGCGGTGAAATGCATCCTTCAGTGCGCTTCCATACCATTCGTCTCAACAATAGC  180
    R  A  A  V  K  C  I  L  Q  C  A  S  I  P  F  V  S  T  I  A
181 CGTAGCTCTGTATATGGGCCCCTTGGGCGTCTTGCTGGGTGGCGCTGTAGGTACTGGGAT  240
    V  A  L  Y  M  G  P  L  G  V  L  L  G  G  A  V  G  T  G  I
241 CTCCTACGTCTATGCTAGGGGGAAGTTCAAAAGCGTCGTTAGCATTATCAGGGACGACTT  300
    S  Y  V  Y  A  R  G  K  F  K  S  V  V  S  I  I  R  D  D  L
301 GACTCCACAGGAAAGGGAGAGGCTCATGATGAGGGTGCGGGCCGCTCTCGTAGACCTTGG  360
    T  P  Q  E  R  E  R  L  M  M  R  V  R  A  A  L  V  D  L  G
361 AGTCGCTGTCGGGGCCTCTGTGGCCTTCCGTCAGCTCACCGAGCCCATGAAGTCGGAGAT  420
    V  A  V  G  A  S  V  A  F  R  Q  L  T  E  P  M  K  S  E  I
421 CGCTGCTACTGTCAAGAAGTACTTGGAGTATGACCACAACATGTCAGTAGAGCATTAAAT  480
    A  A  T  V  K  K  Y  L  E  Y  D  H  N  M  S  V  E  H  *
481 GCCTAAAAGACTGTTCAGGTGAATGGCGAGAACGACGGTTTCTTTTCTGTTTGCATTTGT  540
641 TAGCGAAGATGGGTTCGCTGTTAGTACTACTTTTGGAAATTGGATTTGTTTTATGTTCGA  600
601 GGCAAAAATGTGAAAGAGACAGTTCCAAAATAAACAAATAAAACTATCAAAAAAAAAAAA  660
661 AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  720
721 AA  722
```

Figure 1.5 Nucleotide and deduced amino acid sequences of *Pm*VRP15. An asterisk indicates the stop codon. A transmembrane domain is boxed. The polyadenylation site is underlined (Vatanavicharn *et al.* 2014).

*Pm*VRP15 transcript was mainly expressed in the hemocytes in normal shrimp (Figure 1.6). Moreover, *Pm*VRP15 transcript was also found in heart, hepatopancreas, lymphoid, gill and intestine (Vatanavicharn et al., 2014). *Pm*VRP15 mRNA expression was highly up-regulated in *P. momodon* hemocytes at 24, 48 and 72 h after WSSV challenge (Figure 1.7). Interestingly, *Pm*VRP15 transcript was greatly up-regulated about 3.6, 9,410.1 and 1,351.2 fold at 24, 48 and 72 h post-infection, compared to the non-infected hemocytes (Figure 1.8) (Vatanavicharn et al., 2014). Furthermore, *P. monodon* injected with VRP15 dsRNA prior WSSV challenge reduced WSSV replication in shrimp (Figure 1.9).

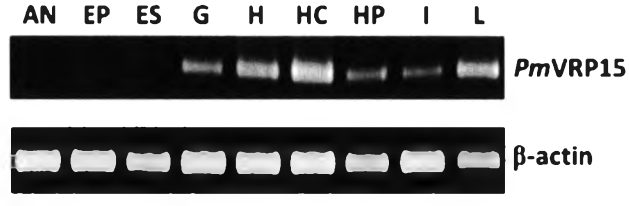

Figure 1.6 *Pm*VRP15 expression analysis in various shrimp tissues by RT-PCR. The figure shows antennal gland (AN), epipodite (EP), eye stalk (ES), gill (G), heart (H), hemocyte (HC), hepatopancreas (HP), intestine (I), lymphoid (L). β-actin was used as an internal reference (Vatanavicharn *et al.* 2014).

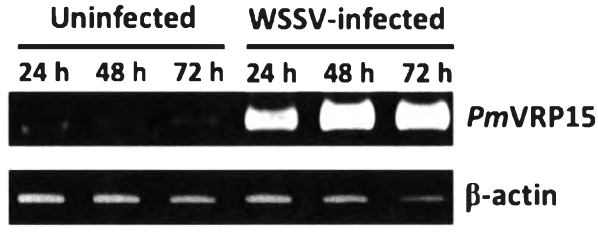

Figure 1.7 *Pm*VRP15 expressions in response to WSSV at 24, 48 and 72 h post-infection in *P. monodon* hemocytes compared to uninfected hemocytes by RT-PCR. β-actin was used as an internal reference (Prapavorarat 2010).

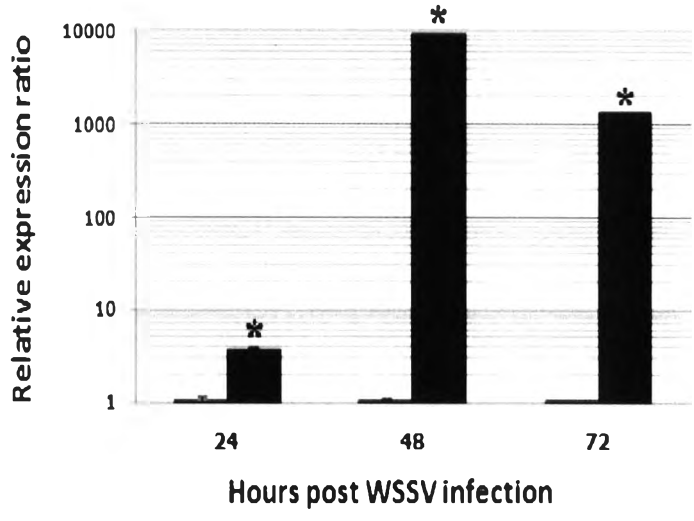

Figure 1.8 Up-regulation of *Pm*VRP15 mRNA in response to WSSV infection. A relative expression ratios of *Pm*VRP15 transcript levels in the hemocytes of WSSV-infected *P. monodon* at 24, 48 and 72 h post-WSSV infection compared to the control (non-infected) shrimp and standardized against β-actin as the internal reference as determined by Real-time PCR. Means with an asterisk are significantly different ($P < 0.05$, analyzed by paired samples *t*-test). Relative expression ratio < 1, 1, and > 1 mean that the target gene expression is down-regulated, the same or up-regulated, respectively, in the hemocytes of WSSV-infected shrimp compared with the control (Vatanavicharn *et al.* 2014).

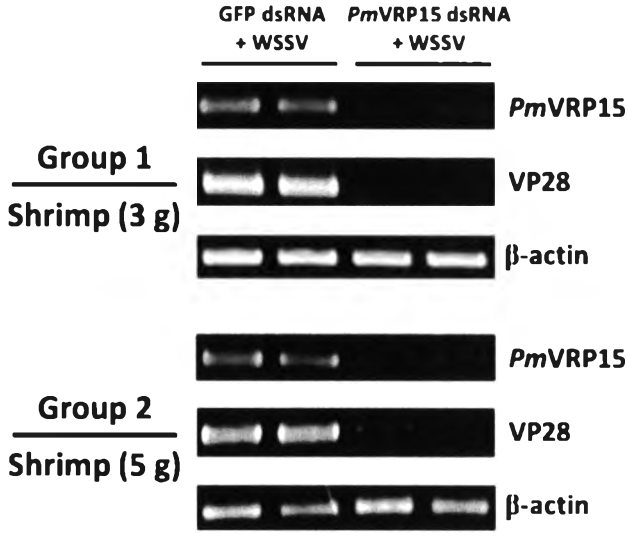

Figure 1.9 Knockdown of *Pm*VRP15 gene expression leads to a reduction of WSSV replication in *P. monodon*. The shrimp hemocytes from two groups (~3 and ~5 g body weight) injected with 150 mM sodium chloride solution containing GFP dsRNA (3 μg/g shrimp) or *Pm*VRP15 dsRNA (3 μg/g shrimp) for 24 h followed by being injected with WSSV for 24 h, were collected and used to analyze VRP15 and VP28 gene expression levels by RT-PCR with β-actin as an internal reference (Prapavorarat 2010).

Localization of *Pm*VRP15 and VP28 in uninfected and WSSV-infected *P. monodon* hemocytes was examined by confocal laser scanning microscopy (CLSM) using the antibodies specific to *Pm*VRP15 and the WSSV late protein VP28 coupled with different fluorescence-conjugated secondary antibodies. *Pm*VRP15 and VP28 were detected as green and red fluorescence, respectively, the accepted fraction of the emission spectra of TO-PRO-3, used to stain the nuclear DNA, was adjusted to show in blue.

Bright field image shows three types of hemocytes (hyaline, semigranular and granular cells (Figure. 1.10A). *Pm*VRP15 showed weakly positive in all three types of hemocytes in uninfected shrimp hemocytes and *Pm*VRP15 was localized in the cytoplasm near to the nuclear membrane (Figure. 1.10B). Interestingly, *Pm*VRP15 and VP28 protein expression were found in the same hemocytes at the late infection phase (48 hpi) of viral infection (Figure. 1.10). Thus, the expression of *Pm*VRP15 in *P. monodon* hemocytes appears to be linked to a response to the acute phase of WSSV infection (Vatanavicharn *et al.* 2014).

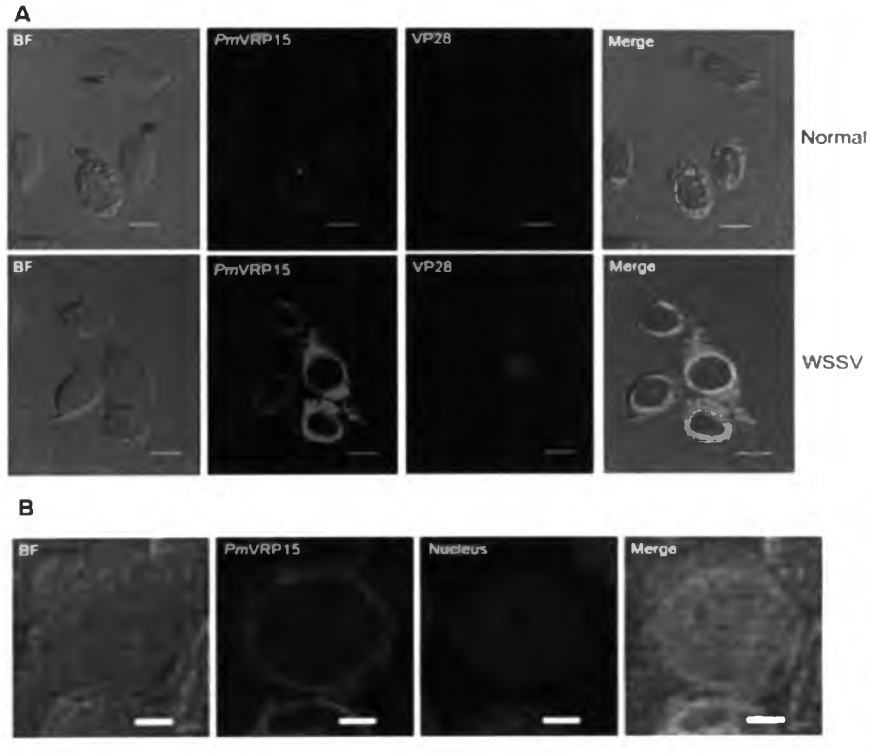

Figure 1.10 CFLM-derived images of the uninfected (control) and WSSV-infected hemocytes at 48 hpi with WSSV. Rabbit anti-r*Pm*VRP15 and mouse anti-VP28 primary antibodies were detected with corresponding Alexa488 and Alexa568 secondary antibodies revealing *Pm*VRP15 (green color) and VP28 (red color), respectively. Scale bars represent (A) 5 μm and (B) 2 μm. Nucleus was stained with TO-PRO-3 iodide and color was adjusted to blue. The bright field image showed hyaline cell (HC), semigranular cell (SGC) and granular cell (GC) (Vatanavicharn *et al.* 2014).

1.9 Purposes of the thesis

In this study, the function of *Pm*VRP15 in response to WSSV has been studied *in vitro*. The expression of *Pm*VRP15 gene upon WSSV infection has been examined in primary hemocyte cell culture using Real-time PCR. Then, effects of *Pm*VRP15 on WSSV propagation have been studied by RNA interference. After WSSV infection, VP28 transcripts of normal and *Pm*VRP15-silenced hemocytes have been compared by Real-time PCR. To confirm *Pm*VRP15 localization, WSSV-infected hemocyles have been fractionated into five fractions (cytoplasm, cell membrane, soluble nuclear, chromatin-bound and cytoskeletal fractions) and subjected to Western blot analysis. A possible role of *Pm*VRP15 in nuclear import/export has been further investigated. In addition, recombinant *Pm*VRP15 has been produced for crystallization purpose. Full-length and truncated forms of *Pm*VRP15 gene have been cloned and expressed in either *Saccharomyces cerevisiae* or *Escherichia coli* expression system. Then, the recombinant *Pm*VRP15 have been analyzed by MALDI-TOF mass spectrometry and circular dichroism spectroscopy. Furthermore, screening of crystallization conditions of *Pm*VRP15 has been performed in order to obtain a single crystal for protein X-ray crystallography study.