CHAPTER 5

Discussion

Proteomic analysis allows us to study the levels and patterns of protein expression in organs, cells or sub cellular compartments. These such studies provide us with a better understanding of the cell's response to various external factors, such as viral and bacterial pathogens, under several different circumstances (Somboonwiwat *et al.*, 2010).

To date the understanding of immune responses at a molecular level of invertebrates especially prawn towards viral infection is very much limited (Chongsatja *et al.*, 2007). In this study, a gel-based proteomics approach was used to investigate the molecular responses of *M. rossenbergii* during IHHNV infection. Our results showed that 20 out of the average of 300 protein spots in each prawn hemocyte gel underwent considerable alterations in their expression levels upon IHHNV infection (Chongsatja *et al.*, 2007).

Two-dimensional gel electrophoresis showed that the complex proteome of prawn hemocyte expression was significantly up-regulated or down-regulated in 2 cohorts with the serum IHHNV infected and non-infected prawn from a wild population. Twenty differentially expressed protein spots (ten up-regulated and ten down-regulated) were annotated via mass spectrometry. One of the samples from the 20 selected samples was not identified from the annotated databases; however the othe 9 up- regulated and 10 down- regulated protein spots were successfully annotated. In view of the fact that the complete genome sequence of the prawn species is still unavailable, (Somboonwiwat *et al.*, 2010) the origins of all these unidentified protein spots are uncertain though it is likely that they are mostly prawns proteins.

Twelve of the total protein spots were annotated as isoforms of threeproteins, hemocyanine, AK and proPO2, which differ in either isoelectric points (pI) or mass (Table 1). Assuming that the different protein spots were correctly annotated (highly likely), these differences may be the result of portion posttranslational modifications of the same protein population, (Somboonwiwat *et al.*, 2010). This is discussed below.

These fluctuating proteins which are most of the up-regulated proteins (i.e., hemocyanin, prophenoloxidase) had significant functional roles in the defensive mechanisms of the prawn. The down-regulated proteins on the other hand had significant physiological effects in self defense. The up regulated proteins play important roles in energy production and catabolism (Arginin Kinase, Carbonic anhydrase 2, Sarcoplasmic calcium-binding protein). After database searches, these 20 detected proteins were categorized into 8 groups according to their cellular function (Fig 4). The divided groups are immune system related protein (33%). cell function and physiology (14%), energy production and catabolism (14%), antioxidants (5%), ATP-buffering and environmental stress (5%), calcium homeostasis (5%), cell structure (5 %) and oxygen transportation (19%) (Figure 4.5)

The innate immune response is a conserved trait shared by invertebrates and vertebrates. It defends the organism against pathogens and parasites through detection, signaling pathways and the initiation of defense mechanisms (Hoffmann, 2003);(Leulier *et al.*, 2003) In crustaceans, circulating hemocytes play a significant role in the innate immune response. These include the release of nonself-recognition proteins, clotting proteins, antimicrobial peptides, and prophenoloxidase. Activated phenoloxidase (tyrosinase) catalyzes the hydroxylation of monophenols to diphenols such as dopamine and dopa. In a second step (catecholoxidase reaction), the enzyme oxidizes the diphenol to an ortho-quinone, a highly reactive molecule that is involved in encapsulation and melanization of foreign organisms. Phenoloxidase is also important in sclerotization

(hardening) of the new exoskeleton after wound-repair and molting (Söderhäll and Cerenius, 1998); (Terwilliger, 1999);(Terwilliger, 2011).

The oxygen-transport protein hemocyanin functions as a phenoloxidase under certain conditions (Decker H *et al.*, 2001);(Jaenicke and Decker, 2004)**.** Hemocyanin belongs to the same family of copper proteins as phenoloxidase. Therefore, hemocyanin, hemocytes and phenoloxidase are related players in the crustacean immune response. The basic mechanisms for activation of arthropod hemocyanins and phenoloxidases and non-arthropod tyrosinases have been discussed recently (Decker *et al.*, 2006); (Matoba *et al.*, 2006; Terwilliger, 2011).

In this study 2 different types of hemocyanin subunits were observed in non-infected samples. They were hemocyanin subunit 1(Mass: 76304) and L (Mass: 77184). Another type of hemocyanin (Mass: 77538) was observed in infected samples at the same pH with pro-phenoloxidase. We suggest that this difference might be the result of a response to the virus. These different subunits may be the activated form of hemocyanin which is the main substance in the prophenoloxidase activating system. It may also show the relative contributions of hemocyte phenoloxidase and hemocyanin in the physiological ratio.

The proPO activating system is an important immune response that produces melanin and a reactive oxygen species in order to kill, trap and eliminate invading microorganisms. For invertebrates, this system is integral in the immune response towards viral and bacterial infections. Two different proPOs, proPO-1 and proPO-2, which individually play crucial roles in the proPO activating system, have been identified in several prawn species *including P. monodon, Fenneropenaeus chinensis* and *L. vannamei* (Somboonwiwat *et al.*, 2010). These proPO's have now been identified in *M. rosenbergii*.

Seven spots (Spots Number 10, 11, 12,13,14,15 and18) were presumed to be the same protein (proP0-2) judging by their partial amino acid sequences and molecular masses, however, these proteins may have different PI and posttranslational modifications. Expression of proPO-2 (Spots Number 11, 12, 13 and 14) were up-regulated in infected hemocyctes, while the other proPO-2 isoform (Spot Number 10) was down-regulated, suggesting a possible pI altering posttranslational modification of the proPO-2 of spots 10, 11, 12, 13 and 9 (Somboonwiwat *et al.*, 2010).

Because the up-regulated expression of proPO-2 was observed in non-infected samples, we hypothesize that the inactive form of proPO-2 is produced by the prawn to restore the level of proPO-2 in the hemocyte in order for the prawn to promptly fight against pathogenic microorganisms.

The up-regulation of hemocyanin may provide oxygen for an increase in energy production during viral replication. Massive damage of hemocyanin might be associated with IHHNV infections identical to infections of YHV (Rattanarojpong *et al.*, 2007). The ion uptake mechanisms in prawns has not been systematically studied, though in other groups of decapods crustaceans (e.g., crabs, lobsters and crayfish) specific ion transport proteins and transport-related enzymes which play a role in the active uptake of the major ions Na^+ and Cl^- have been identified (Mantel and Farmer, 1983) . One of the central proteins in this mechanism is believed to be the transport-related enzyme carbonic anhydrase (CA). CA is known to be present and active in high levels in the gills. The activity of CA is sensitive to changes in environmental salinity (Henry, 1984, 1988a).

Prawns are able to withstand a wide range of salinity fluctuations by maintaining their hemolymph osmotic and ionic concentrations within narrow levels. The enzyme carbonic anhydrase appears to be important in both hyper- and hypo-osmotic regulation

because it is induced in response to exposure to both low and high salinities (Roy *et al.*, 2007).

Enolase is a well known enzyme involved in cell metabolism. It is also associated with a multi functional role in disease. The enolases family is composed of 3 isozyme subunits alpha, beta and gamma which can form homodimers or heterodimers. Alpha-enolase performs many functions in both eukaryotes and prokaryotes, and plays an important role in various pathophysiological processes. Antibodies (Abs) against alpha-enolase have been detected in individuals affected by a large variety of infectious and autoimmune diseases. Still, the reasons why the spectrum of associated diseases is so wide and the exact pathogenic role of these auto- Abs remain unclear. Alpha-enolase may have a direct or indirect role in the immune response of prawns to viral infection (Terrier *et al.*, 2007) . Further study is still needed to improve our knowledge on the pathogenic role of alpha-enolase in viral diseases.

Arginine kinase is the most widely distributed phosphagen kinase, being found in all invertebrates (Suzuki *et al.*, 1997). The phosphagen kinases have been studied primarily in muscle tissues, in which their function is to buffer the ATP supply during periods of high energy demand by regenerating depleted ATP supplies (Walliman *et al.*, 1992).

In some tissues, phosphagen kinases may also function as energy shuttles between the mitochondria and the cytosol. This process is facilitated by the presence of separate isoforms in different parts of the cell (Bessman and Carpenter, 1985)**.**

The insignificant contribution of mitochondrial arginine kinase to the total activity in the posterior gills suggests that arginine kinase serves mainly as an ATP buffer rather than as part of a high energy phosphate shuttle system. The immunolocalization of arginine kinase in the mitochondria of heart muscle cells in the blue crab *C. sapidus* is

of particular interest to the present study (Pineda and Ellington, 1998), because it supports an earlier indication of a mitochondrial arginine kinase in the hepatopancreas of the same species (Chen and Lehnisger, 1973). In the present study, AK was a significantly down regulated protein in *M. rosenbergii* serum after IHHNV infection and it may show the impact of viruses on the cell energy system. Again further study is required to identify the exact cellular response.

The most significant up-regulation was found to be for AK Spot Number 9. These two protein spots had identical peptide fragment sequences. However, AK Spot Number 8 which was constantly expressed later on was of a slightly higher MW than AK Spot Number 9. These two AK's differ from each other in pI. Therefore, it is likely that these may represent separate alleles or isoforms under separate regulation and not simple interchanges of posttranslational modifications.

Sarcoplasmic Calcium-binding Protein (SCP) is believed to function as the invertebrate equivalent of vertebrate parvalbumin, which is used to "buffer" cytosolic Ca^2. Its expression works as a function of molting stage in non-epithelial and epithelial tissues (Gao *et al.*, 2006). SCPs from other crustaceans, including prawn and lobster, similarly exist as dimers of two different polypeptide chains (Wnuk and Jauregai-Adell, 1983) (Takagi and Konishi, 1984a, 1984b). SCPs exhibit a high degree of polymorphism and evolutionary drift between phyla (Cox, 1990) having evolved to satisfy cell-specific needs.

The transcriptional level of Arginine kinase 1 and some of the proteins involved in the glycolytic pathway and immune response are correlated with the results from the 2-DE (Table 3). Although changes in the expression level of arginine kinase in an IHHNV challenge suggest that arginine kinase expression correlates closely with the prawn's immune response (Arockiaraja *et al.*, 2011).

In conclusion, the results presented here provide preliminary data on the interaction between IHHNV and prawns. Although this study was not able to assign a specific IHHNV-response status to any of the nine up-regulated or ten down-regulated genes identified after the IHHNV challenge, it opens the way for more focused studies on their functions and possible interactions with prawn or viral proteins.