CHAPTER IV
DISCUSSIONS

Disease is the major problem in shrimp aquaculture worldwide. The outbreaks of viral and bacterial diseases cause a great loss of shrimp production. A better understanding of the shrimp immune response will help in the design of more efficient strategies for shrimp disease control and management. The shrimp defense mechanisms are based mainly on the innate immune response comprising cellular and humoral reactions. Shrimp haemocytes are the main site of shrimp immune response. The involvement of shrimp haemocytes in immunity has been investigated (Jiang *et al.*, In press; Lai *et al.*, 2005; Liu *et al.*, In press). Identification of genes, involved in the shrimp immunity, has been reported using an expressed sequence tag (EST) approach (Gross *et al.*, 2001; Rojtinnakorn *et al.*, 2002; Supungul *et al.*, 2002). In addition, the *Vibrio harveyi* responsive genes in shrimp haemocytes were also identified by differential display PCR (Somboonwiwat *et al.*, 2006). In the present study, lymphoid organs from the unchallenged and *V. harveyi* challenged shrimps were subjected to EST analysis to identify novel genes in the black tiger shrimp. The detection of the biodefense-related genes was expected because the lymphoid organs function as a target for phagocytosis. Moreover, the lymphoid organs have been speculated to be responsible for the removal of foreign materials from the hemolymph before it goes from the arterial system into the open circulatory system (Van de Braak *et al.*, 2002).

Similar to those found in other shrimp libraries, the titers of normal and *V. harveyi* challenged libraries were estimated to be 3.2×10^5 and 3.1×10^6 plaque forming units (pfu), respectively (Gross *et al.*, 2001; Supungul *et al.*, 2002). These numbers indicated sufficient clones that covered the predominantly expressed mRNA (Adams *et al.*, 1991). EST clones were randomly selected, sequenced, homology searched against the sequence databases in the Genbank, and data analysis. It was found that the normal cDNA library contained 40.4% of the sequenced clones representing the known genes while the *V. harveyi* challenged cDNA library contained 44.5%. The

remaining clones of these two libraries (59.6% and 55.5% of normal and *V. harveyi* challenged cDNA library, respectively) were classified as unknown sequences. The high proportion of unknown genes was due to the limited sequences of known functions in shrimp and related species deposited in the GenBank database.

The libraries derived from the lymphoid organs of unchallenged and *V. harveyi* challenged shrimps showed differences in general patterns of gene expression reflecting the different physiological conditions in the sacrificed shrimps. For the normal library, the dominant functional class of messages was the 'defense and homeostasis' while that of *V. harveyi* challenged library was the 'ribosomal protein and rRNA'. The high representation of immune related genes in the lymphoid organ library indicates the possible role of this tissue in defense response. The high percentages of the immune function category was also observed in shrimp haemocyte libraries (Gross *et al.*, 2001; Rojtinnakorn *et al.*, 2002; Supungul *et al.*, 2002). Although the percentage of the defense and homeostasis category in normal library was higher than that of *V. harveyi* injected library (Table 3.2), the number of different genes in the normal library is smaller (Table 3.6). This result implies that several immune-related genes were expressed in lymphoid organ after bacterial infection. Among the genes in this category, the high redundancy of genes in the cathepsin family, especially cathepsin B and L, was observed in the normal library. The cathepsin family, the lysosomal cysteine endopeptidases, has been reported to be involved in an apoptosis mechanism (Chwieralski *et al.*, 2006; Liu *et al.*, 2006; Pacheco *et al.*, 2005). In 2003, Wongprasert *et al.* reported that apoptosis occurred after WSSV infection in *P. monodon*. Nevertheless, many reports had shown that apoptosis was not the main immune response mechanism after *V. harveyi* infection (Somboonwiwat *et al.*, 2005; Supungul *et al.*, 2002). In this study, we found that the expression of genes involved in apoptosis was apparently not increased after bacterial stimulation which supported the previous reports by Somboonwiwat *et al.* (2005) and Supungul *et al.* (2002). In contrast, Rojtinnakorn, *et al* (2002) found the high levels of apoptotic peptides in WSSV injected shrimp haemocyte library. In addition, in the *V. harveyi* challenged library, the antimicrobial molecules (anti-lipopolysaccharide factor (ALF) and lysozyme), the proPO system and oxidative enzymes (haem peroxidase, prophenoloxidase activating factor-III and transglutaminase), and some

other immune molecules (peptidyl-prolyl cis-trans isomerase5, profilin, survivin, techylectin-5B, thymosin beta-4 and translationally controlled tumor protein) that had been reported in shrimp in response to bacterial infection (Liu *et al.*, 2005; Supungul *et al.*, 2004) were also identified. This implies that these genes are possibly involved in the defense mechanism in *P. monodon* against bacterial invasion. The bacterial infection led to the up regulation of the translational mechanism as well, judging from the ribosomal protein and rRNA content (Table 3.2), which indicated more protein synthesis in shrimp lymphoid organ in response to pathogen invasion. The increase in the expression of ribosomal protein genes was also found in WSSV-infected shrimp (Dhar *et al.*, 2003).

Genes in other functional classes comprising gene expression, regulation and protein synthesis (protein synthesis factor), metabolism, cell division/DNA synthesis, repair and replication, mitochondrial protein, and transport were nearly equally represented in both libraries. Interestingly, many molecules such as calcium binding protein, cartilage oligomeric matrix protein, integrin beta subunit, polyglutamine tract binding protein-1, receptor for activated protein kinase C and thrombospondin classified in signaling and communication category were only found in the *V. harveyi* injected library. The increased expression of many molecules involved in signal transduction was also observed in *Marsupeneaus japonicus* injected with a heat-killed microorganism (He *et al.*, 2004). The detection of these genes in the bacterial challenged shrimp indicates that some signal transduction pathways were possibly involved in the shrimp defense mechanism. However, more study is needed as to how they function and which pathway is most likely involved in the response. From the above gene expression analysis, the detection of the immune related genes in shrimp lymphoid organ was similar to that of Burgents *et al.* (2005), whose results suggested that the lymphoid organ played a major role in bacterial uptake and bacteriostasis.

The EST approach provides not only the gene discovery in shrimp, but also the tool for the investigation of the mechanisms of regulating shrimp immune response. Therefore, we chose to take a transcript profiling approach, cDNA microarray analysis, to characterize the response of known and unknown genes in haemocytes of pathogen invaded shrimp. The microarray approach provides an attractive solution for effectively identifying candidate genes involved in the

pathogenesis of diseases. This technique could generate new hypotheses for the mechanisms underlying the development of damage and might enhance our understanding in pathogenesis of diseases in the penaeid shrimp. Because of the time limitation, the EST clones from the two shrimp haemocytes (Rojtinnakorn *et al.*, 2002; Supungul *et al.*, 2002) were used to develop the cDNA microarray instead of the EST clones described above. The shrimp cDNA microarrays consist of 1,026 distinct genes: 718 genes from *P. monodon* and 308 genes from *M. japonicus*. The functions of 489 genes were unknown. The full data set can be accessed at http://www.ebi.ac.uk/arrayexpress/ with the accession number of A-MEXP-306.

The results from cDNA microarray showed that the highest number of up regulated genes was observed at 72 hpi whereas a largest number of down regulated genes were found at 24 hpi in the WSSV challenged shrimp. In the *V. harveyi* injected shrimp, the highest numbers of up and down regulated genes were observed at 6 hpi, and much fewer genes were changed at 24 and 48 hpi. These results suggested that the host response was more rapid for a bacterial challenge than a viral challenge.

The microarray data were further processed to explore the relationship among the expressed genes in the haemocytes of the WSSV and *V. harveyi* challenged shrimp by Gene Cluster 3 analysis. For WSSV challenged shrimp, ninety-six genes were filtered and grouped into two clusters. The genes with elevated expression at a late time (72 hpi) fell into group A, and mainly encoded ribosomal proteins and several unknown genes. Increased expression of the transcripts for ribosomal proteins had also been observed in fish challenged with bacteria (Ewart *et al.*, 2005), human challenged with bacteria (Wang *et al.*, 2003) and shrimp challenged with WSSV (Rojtinnakorn *et al.*, 2002; Dhar *et al.*, 2003). The other cluster was classified into group B by an increase in expression at 24 to 48 hpi. In this cluster, some ribosomal proteins as well as the asialoglycoprotein receptor (ASGPR) showed transient expression. The hierarchical clustering of the *V. harveyi* challenged shrimp microarray data failed to identify gene clusters because of the inconsistent hybridization results across three different time points (data not shown).

Immune related genes showed different responses to pathogens. The WSSV infection caused up regulation of transcripts for GeneMAC25 protein and heat shock protein70 at the late phase (72 hpi) whereas transcripts for the antimicrobial

molecules, penaeidin and 11.5 kDa protein (crustin) were down regulated after 24 hpi. Interestingly, penaeidin transcripts were, in turn, up regulated in *V. harveyi* challenged shrimp at 48 hpi, suggesting that the immune responses of the penaeidin genes to the two pathogens were different. Jiravanichpaisal (2005) had demonstrated that WSSV interacts with different haemocyte types in freshwater crayfish *Pacifastacus leniusculus*. Semigranular cells were more susceptible than the granular cells, and the WSSV infection might affect the protein kinase C pathway. Other immune-related genes, prophenoloxidase activating factor (PPAF) and anti-lipopolysaccharide factor (ALF) also did not respond to WSSV infection. The results were corresponding to those of Van de Braak *et al.* (2002b) that they could not observed the massive haemocytic aggregation, a possible result from the interaction of ALF with the bacteria, and melanisation, the PPAF-related activity, in WSSV infected shrimp. These proteins are possibly involved in the bacterial response. On the contrary, proteins involved in apoptosis might be implicated in shrimp death caused by the WSSV (Sahtout *et al.*, 2001).

In previous studies, the up regulation of ALF (Somboonwiwat *et al.*, 2005) and PPAF (Jitwaropat, unpublished data) were observed in *V. harveyi* infected shrimp. However, up regulation of these two genes in *V. harveyi* challenged shrimp could not be detected by the microarray technique due to the inconsistent hybridization results. Besides the changes in expression of the immune genes in shrimp haemocytes, several clones with unknown function were up regulated in the pathogen infections. These clones should be subjected to further investigation for their roles and functions in the shrimp immune response.

From the cDNA microarray results, the three up regulated genes in response to pathogen challenge, calmodulin (CaM), ASGPR and β-tubulin, were subjected to real-time PCR to accurately measure and verify their up regulated expression. The real-time PCR analysis showed that the genes encoding for CaM, tubulin and ASGPR were up regulated in response to the pathogen challenge, in accordance with the results of the microarray analysis. These three selected genes could be actually involved in shrimp immune response.

CaM is a protein of primary importance in the regulation of cellular Ca^{2+}-dependent processes. In plant, CaM is implicated in host-pathogen interactions (Ali *et*

al., 2003). Bergey *et al.* (1999) suggested that CaM gene expression was associated with the signaling cascade that activated the defensive genes in response to wounding. In 1998, Sung *et al.* reported that Ca^{2+} was involved in the activation of proPO system. However, the activation mechanism of Ca^{2+} in the proPO system was not well understood. The up-regulated expression of CaM in response to pathogen in this study indicated that the CaM might be involved in the Ca^{2+}-mediated activation of the proPO system.

For the second selected gene, tubulin is the building block of microtubules, which are involved in many cellular processes such as cell division, cytoplasmic streaming, organelle positioning and signal transduction (Ebel *et al.*, 2001). However, how tubulin is involved in the shrimp defense is not known. For the ASGPR, it is a calcium-dependent (C-type) animal lectin. It mediates endocytosis and degradation of serum proteins in mammalian hepatocytes (Shimada *et al.*, 2003), and is involved in the clearance of apoptotic bodies from the liver (Dalto *et al.*, 2003). Our real time PCR results could corroborate the involvement of CaM, ASGPR and tubulin in the shrimp defense.

Interestingly, the functions of CaM, ASGPR and tubulin are affected by the calcium ion (Baricevic *et al.*, 2002; Yamakawa *et al.*, 2001). In fact, the calcium ion (Ca^{2+}) is a fundamental intracellular messenger that is involved in many cellular pathways. It is an element vital for copious biological functions. In resting phase, the concentration of intracellular free Ca^{2+} is considerably lower than that outside the cells. This provides a potential across the membrane for the import of Ca^{2+} into the cells, at which the Ca^{2+} can act as a second messenger. Cell survival and apoptosis have been reported to be induced by cellular calcium ions (Hajnoczky *et al.*, 2003). Ashisa, *et al.* (1990) suggested that mosquito prophenoloxidase required Ca^{2+} for its activation. The mechanisms underlying the ability of a majority of the Ca^{2+}-sensor proteins to integrate Ca^{2+} signals into specific cellular responses are not clearly understood. Additional studies are necessary to elucidate the mechanism responsible for their cellular pathways. Moreover, the information on molecular target of this Ca^{2+} signal and how it regulates the downstream events in the defense-signaling pathway is required. Much of what we do know about the mechanisms that the sensor proteins use to transduce Ca^{2+} signals is based on the information gained from the CaM.

Because CaM is a sensor protein of intracellular calcium fluxes that functions by binding to and regulating the CaM binding proteins (CaMBPs) (Myre and O'Day, 2004), identification of the target proteins that bind CaM may help to elucidate the signaling pathways that link CaM to the defense response in shrimp. In this study, we elucidated the expression of CaM binding protein and the candidate-CaM binding genes; calcineurin (CN), CDC like kinase 2 and protein phosphatase 1 in shrimp haemocytes after pathogen challenge using real time PCR analysis.

Calcineurin (CN) is a Ca^{2+}/calmodulin (CaM)-dependent protein phosphatase that belongs to the family of protein phosphatase 2B. Kim *et al.* (2004) reported that this protein phosphatase was thought to play the important roles in the immune system and in the cell death mechanism. They also suggested that the CN mediated the inflammatory response to LPS in macrophages. Moreover, Ca^{2+}/calcineurin signaling pathway has profound effects on the gene expression programs in immune cells (Feske *et al.*, 2003), but the mechanisms of gene repression by calcineurin remain poorly understood. Real time PCR analysis revealed differentially expression of this gene in haemocytes from viral and bacterial challenged shrimps. Considering the CN expression profiles upon viral and bacterial challenge, we found that the expression was decreased at 24 hours after viral challenge, followed by the up regulation at 48 hours For bacterial challenge, this transcript was increased at the early phase of injection and showed slightly decrease in expression at the late phase. The expression profile of CN was relatively similar to that of CaM. The signal transduction via Ca^{2+}, CaM and CN may be a possibly one of the shrimp pathogen-responsive pathway. Nevertheless, the exact relation between them and the immune response should be further investigated by techniques such as yeast two hybrid (Bruno *et al.*, 2005; Daly *et al.*, 2001; Tonganunt *et al.*, 2005) or phage display (Fang *et al.*, 2005; Chen *et al.*, 2003).

CDC like kinase 2, clk-2, belongs to the Ser/Thr protein kinase family. CDC like kinase 2 activity is required for DNA damage and S phase replication checkpoints, for embryonic development, and for normal biological rhythms and life span. Real time PCR analysis revealed the change in expression of this gene after pathogen challenge. We found that the expression pattern was very similar to that of CaM. That is, the gene expression is relatively high at the early and late phases of pathogen

injection, not the intermediate phase at 24 hpi. This result implied that the CDC like kinase 2 might be involved in signal transduction via CaM. To date, little is known about the function of the clk2. Nevertheless, the involvement of clk2 in phosphorylation and activation of tyrosine phosphatase was reported by Moeslein, *et al.* (1999).

Protein phosphatase 1 (PP1) is a Mn^{2+}-dependent protein phosphatase with activity towards phosphoserine/phosphothreonine residues. Protein phosphatases catalyze the dephosphorylation of proteins. Modification of the proteins by phosphorylation is a rapid and reversible mechanism to control their function, and is central to many signal transduction pathways. The serine/threonine phosphatase appears to be critical molecules in the control of apoptosis and proliferation (Ayllon *et al.*, 2000). The expression analysis by real time PCR revealed that upon viral challenge, mRNA level significantly was increased from 24 to 48 hpi. While that of bacterial challenge slightly was increased at 6 hpi and decreased at 24 and 48 hpi. We found that the expression pattern of PP1 was very similar to that of CN after pathogen challenge. The results implied that these genes might be involved in signal response to pathogens, especially the viruses. However, more investigation was needed to clarify its function and regulation.

In addition to cDNA microarray and real time PCR analysis, the localization of CaM transcript and protein was carried out using *in situ* hybridization and immunohistochemistry, respectively. According to the results of *in situ* hybridization, purple positive staining of control shrimp haemocytes indicated that the expression of CaM was localized in the haemocytes. Among the positive haemocytes, the differential staining was found and referred to as the difference in CaM expression. Only the relatively strong enough stained cells were counted as the positive haemocytes. It was found that the CaM producing cells were observed the most at 6 hpi and 24 hpi after challenged with WSSV and *V. harveyi*, respectively. This result suggested that signal transduction triggered via the CaM from viral challenge was relatively faster than that of bacterial challenge. Moreover, the results showed that the percentage of the positive cells in the hemolymph of the shrimp challenged with either of the pathogens was higher than that of the control shrimp, thus suggesting that the CaM might play an important role in the response to pathogen infection.

The present immunohistochemistry study provides the first evidence of the present of calcium binding protein (CaM) in shrimp tissues. The immunolocalization experiment revealed that the CaM protein was detected in the epithelium of shrimp lymphoid organ, like that found in the supporting cells of *Lumbricus terrestris* (Licata *et al.*, 2000). The similar results were found in gills and hepatopancreas. In addition, there were many infiltrated CaM-positive haemocytes in these tissues, especially at 48 hpi. The present of positive haemocytes within the tissues suggesting that the CaM-producing haemocytes infiltrated the tissues after the bacterial invasion. Munoz *et al* (2002) showed that distribution of penaeidin-positive cells was found in the hepatopancreas of *Penaeus vanamei* at 48 hours after bacterial challenge. It was possibly then to hypothesize that the CaM might be a first-line trigger for this response mechanism in the haemocytes. However, questions about the function of this intracellular CaM protein and their potential involvement in the elimination of internalized microbes in shrimp remained.

In summary, the CaM, ASGPR, tubulin, CN, CDC like kinase and PP1 were suspected to be responsible for the mechanism(s) involved in shrimp immunity. Further investigation of their roles in shrimp immune response would allow a better understanding of pathogen defenses in shrimp and other related species. This present study thus provided the basic clues for further investigation on shrimp immune mechanisms.