CHAPTER IV

DISCUSSION

Construction of cDNA library

Haemocytes were prepared from haemolymph of normal and infected shrimps and were used to prepare total RNA. Approximately 19 μg of total RNA was obtained from each normal shrimp whereas the average total RNA obtained from *V. harveyi*-infected shrimp was 11 μg per individual. The result indirectly indicated that haemocytes of infected shrimps may be lower than those of normal shrimps. It has been reported that exposure of haemocytes with LPS of Gram-negative bacteria like *V. harveyi* results in rapid degranulation releasing granular components of granular and semigranular haemocytes (Iwanaga et al., 1998).

Two cDNA libraries were established from haemocytes of normal shrimps (primary library titer of 4 x 10^6 pfu) and *V. harveyi*-infected shrimps (2.5 x 10^5 pfu). The titer of the normal library was higher than of the infected library because the former was non-directional whereas the latter was directional cloned at *Not* I and *Sal* I sites (Sambrook et al., 1989). Theoretically, numbers of clones in both libraries were sufficient for determination of predominantly expressed mRNAs in haemocytes of normal and infected *P. monodon* (Adams et al., 1991).

Basically, construction of cDNA libraries is rather expensive and time consuming. As a result, λ phage-based libraries are preferred to plasmid libraries due to stability and wider applications (Gong, 1999). After *in vivo* excision λ libraries can be converted to plasmid libraries (Sambrook et al., 1989). Directional cloning of the infected library also provided the possibility to screen positive recombinant with antibody. Moreover, sequencing at 5' end

of directional clones ensure the ability to determined part of ORF whereas up to 50% of clones the non-directional library (e.g. the normal library in this study) may represented 3' sequences of genes which generally contained different length of poly A (Gong, 1999). Practically, 75% (231 clones) of match EST clones from the non-directional normal library had the same orientation of mRNA when sequencing with the M13 forward primer.

EST analysis and homology search

Sizes of cDNA inserts were verified by either colony PCR or plasmid digestion before subjected to DNA sequencing. Clones were randomly selected and unidirectional sequenced. In a total, 615 clones and 447 clones for the normal and the infected libraries were examined, respectively. The average insert length of the EST clones in the former library was 964 bp whereas that of the latter was 858 bp.

Among overall investigated sequences, 30% and 38% of characterized ESTs from normal and infected libraries were identified at both nucleotide (BlastN) and deduced amino acid (BlastX). Additional 65% and 62% of ESTs from respective libraries were only identified by deduced amino acid. Results indicated that identification of gene homologues using amino acid sequences was more efficient than using nucleotide sequences (Aoki et al., 1999).

Unknown gene transcripts found in both libraries were nearly identical (49.9% and 51.9%, respectively), but the percentage of unknown transcripts containing ORFs from the infected library were approximately 2 times greater than of those from the normal library. These unknown (50.8% of total clones from both libraries) represented a novel gene or a functionally unidentified gene (Nam et al., 2000).

The percentage of unknown genes found in this study was as similar as ESTs from haemocyte of normal and WSSV-infected *P. japonicus* (55.3% and 53%), coelomocyte of sea urchin (Smith et al., 1996) and *Schistosoma mansoni*

(57.5%) (Santos et al., 1999), but higher than unknown ESTs found in haemocyte of *P. vannamei* and *P. setferus* which were were 16.7% and 29.5% of overall investigated genes, respectively (Gross et al., 2001). Notably, a threshold to consider whether sequenced ESTs was unknown transcript was the E-value < 10^{-4} differentiate which were lesser than 10^{-2} in *P. vannamei* and *P. setiferus* (Gross et al., 2001).

A number of homologous mitochondrial transcripts (44 clones from the normal library and 22 clones from the infected library) were also found. ESTs corresponding to mitochrondrial sequences are often found in cDNA libraries (Adams et al., 1991; Adam et al., 1993; Howe, 2001; Paraoan et al., 2000). The presence of A+T rich mitochondrial sequences in *P. monodon* mitochondrial genome (> 70% A+T; Wilson K., unpublish data) may lead to binding of mitochondrial RNA to oligo (dT) columns used to isolate poly (A)-contained mRNA (Lehnert et al., 1999).

Homologous ESTs in both libraries showed significant similarity with genes previously identified in several organisms. Mammalian gene homologues was the largest EST group (81 and 64 clones from normal and infected libraries, respectively). Seventy-nine ESTs represented penaeid gene homologues (49 and 30 clones from the normal and infected libraries, respectively). Of these, 45 clones (30 clones and 15 clones) were mitochondrial origin (cytochrome C subunits, ATP synthase subunit and NADH dehydrogenase). ESTs matched with those of normal and WSSV-infected *P. japonicus* were also found for 28.7% and 9.5%, respectively (Rojtinnakorn et al., 2002).

The matched ESTs from both libraries were classified into 6 broad functional categories based on significant sequence homology according to the criteria proposed by Adam et al. (1999).

Identification of ESTs from various functional categories

High abundant transcripts (94 different genes from 171 clones) were assigned into gene expression and regulation of protein synthesis. Ribosomal (23 types of small subunit and 28 types of large subunit of ribosomal protein) A remarkable increase of ribosomal proteins was observed the *V. harveyi*-infected cDNA library suggesting that *V. harveyi* strong affected gene expression in *P. monodon* haemocytes. Only 14 and 19 types of small and large subunits of ribosomal proteins were noticed in *P. japonicus* EST libraries (Rojtinnakorn et al., 2002).

Large numbers of housekeeping ribosomal RNAs (75 clones of 16S, five clones of 18S and eight clones of 28S rRNA) were found in a *P. japonicus* EST library. Only 3 clones representing 16S rRNA were found in this study. Likewise, the same number of cDNA containing 16S rRNA insert was reported in a coelomocyte cDNA library of the sea urchin. The rRNA was not found in *P. monodon* cDNA libraries established from cephalothorax, eyestalk and pleopod mRNAs from *P. monodon* (Rojtinnakorn et al., 2002; Lehert et al; 1991).

A total of 58 clones representing 29 gene transcripts were homologous to genes functionally involved with structure and mobility. The normal library contained relatively high percentage (7.6%) and various types of these genes (24 different genes). The percentage of gene homologues in this category of the *V. harveyi*-infected library was only 2.5% and contained 9 different, transcriptional types. Infection of *V. harveyi* may have suppressed expression of genes in this category.

The number of structure and mobility related cloned and types of normal and WSSV-infected libraries in *P. japonicus* were roughly identical (Rojtinnnakorn, 2002). Several types of gene in this group were found in cDNA librares constructed from leukocytes of rhabdovirus-infected Japanese flounder, coelomocytes of sea urchin library and *T. brucei* cDNA (Nam et al.,

2000; Smith et al., 1996; Djikeng et al., 1998). The cDNA libraries of different larval stages of *Haemonchus contortus* were analyzed and found that the numbers and types of structure/mobility-related cDNA after the 1st larval L_1 (Hoekstra et al., 2000).

A total of 104 clones, representing 55 different genes were classified as putative metabolism-related genes. Although the percentage of gene involving with metabolism was greater in the normal library, mitochondrial transcripts were predominated in both libraries (41 and 22 clones). This is concordant to Rojtinnakorn et al.(2002) who reported high abundance of mtDNA transcripts in the normal library (45 clones) and WSSV infected library (17 clones) of *P. japonicus*. Interestingly, metabolism-related nuclear ESTs in *P. monodon* were higher than those in *P. japoninus* in both normal and infected conditions.

Isolation of genes involving defense and homeostasis

One hundred and fifteen clones composed of 65 clones from the normal library and 50 clones form the infected library accounting for 10.8% of the total clones sequenced representing 34 different putative immune genes (antimicrobial peptides, ProPO-related enzymes, proteinase and proteinae inhibitors, heat shock protein and other immune genes)

Antimicrobial molecules

Antimicrobial peptide was the largest subgroup of gene classified as the defense and homeostasis category. ESTs representing antimicrobial peptide homologues were found for 29.2% of defense and homeostasis genes in the normal library. They were, however, increased to 64% in the *V. harveyi*-infected library.

Results also showed highly significant increasing of ALF in the infected library (16 clones; 32 % of the defense genes). This was resulted from stimulation of LPS which is the major component of the cell surface of *V. harveyi* to genes encoding ALF (Morita et al., 1985; Hoess et al., 1993). In the

horseshoe crab, it was shown that granular cells with contained granular component including ALF are degranulated upon recognizing the cell surface of bacteria and release the granular components. ALF binds and then neutralizes LPS of the bacteria (Morita et al., 1985; Hoess et al., 1993).

Crustin is the second most common ESTs in immune genes. This protein was found in both libraries (5 clones from the normal library; 8 clones from the infected library). Crustin is a homologue of 11.5 kDa antibacterial protein in the shore crab, *C. maenas* and acts against Gram positive bacteria (Relf et al., 1999). In addition, the other antimicrobial peptide; penaeidins were found (7 clones from the normal library and 6 clones from the infected library). Lysozyme (2 clones from each library) was also found.

Only two (lysozyme and penaeidin-2) and one EST (bactinectin11) representing immune-related genes were found in the normal and WSSV-infected *P. japonicus* cDNA libraries. Gross et al., 2001, found that penaeidins were highly abundant transcripts in haemocytes of *P. vannamei* (53 clones) and *P. setferus* (106 clones). Penaeidins exhibit antimicrobial activity against marine Gram negative bacteria and fungi (Destoumieux et al., 1997; Destoumieux et al., 1999) and transcription of penaidins was not related with microbial infection (Destoumieux et al., 2000). Results of those and this study indicated that the findings of newly identified antimicrobial molecules vary greatly among different species.

ProPO systems and oxidase enzyme

One would expect to find components of the ProPO cascade and the enzyme involved in producing reactive oxygen intermediates in crustacean cDNA libraries established from haemocyte mRNA. Granular and semigranular haemocytes were involved in phagocytosis and encapsulation and contain ProPO and peroxinectin (Johansson et al., 1999; Perazzolo and Barracco, 1997).

In this study, ProPOs were only found in the normal library as previously reported in *P. japonicus* and *P. vannamie* (Rojtinnakorn et al., 2002; Gross et al., 2001). Nevertheless, ProPO activating enzymes which regulate conversion of ProPO to PO, was only found in *P. monodon* haemocyte libraries but not in other shrimp species (Gollas-Gulvan et al., 1997; Aspán and Söderhäll, 1991 ; Aspán et al., 1995).

Clottable proteins localized in the haemocytes, especially in hyaline and semigranular cells are recognized and covalently linked to one another by the promotion of transglutaminase (Hall et al., 1999). Clottable protein were found in both libraries and exhibited highly significant matching (97-98% identity and E-value < 1×10^{-130}) to that previously reported in *P. monodon.* Transglutaminase, a homologue of haemocyte protein-glutamine gamma-glutamyltransferase of *T. tridentatus*, was only found in the normal library (4 clones). In contrast, Rojtinnakorn et al. (2002) found transglutaminase in both normal (1 clone) and WSSV-infected (2 clones) libraries of *P. japonicus*.

Superoxide dismutase glutathione peroxidase and catalase play an essential role against reactive oxygen species produced as a by-product of aerobic metabolism (Docampo and Moreno, 1984). These enzymes reduce the superoxide radical O_2^- into hydrogen peroxide and molecular oxygen. A homologue to cytosolic manganese superoxide dismutase (MnSOD) precursor was identified in the infected libraries (78% identity and E-value < 1×10^{-129} to that of *C. sapidus*).

Proteinase and inhibitor

Among arthropods, serine proteases act as the potential activator of diverse immune cascades. A serine protease is autocatalytically activated in the presence of LPS of gram negative bacteria and activates the 2nd serine protease (factor B) which initiates the clotting cascade in the horseshoe crab, *P. polyphemus*. This same clotting cascade is also activated by catalytic activity of the 3rd serine protease (factor G) in the presence of β1-3 glucan (Muta and

Iwanaga, 1996). Two types of serine proteinase, factor D and masquerade-like protease were found in haemocytes of *P. japonicus* (Rojtinnakorn et al., 2001). In this study, a single clone from each library represented a homologue of *P. leniusculus* proteiase (79% identity) while additional 9 clones from the normal library were identified as a *Manduca sexta.* proteinase homologue. Moreover, a clone represented a cathepsin B-like cystein proteinase of *P. vannamei* was also found (Gross et al., 2001).

Nine members of the Kazal family clones (8 and 1 clones from the normal and the infected libraries) were homologues to proteinase inhibitor originally identified in *P. leniusculus* (Lu et al., 2001). Three additional clones from the infected library were homologues of gene MAC25 (Kazal proteinase inhibitor homology). Whey acidic proteins, one of the putative proteinase inhibitor, were found in both libraries.

Homologues of serine proteinase inhibitor recognized as the Kazal family (2 clones) and the Kunitz family (1 clone) were found in the WSSV infected library of *P. japonicus* (Rojtinnakorn et al., 2002). A large number of The Kazal type (12 clones) and secretory leukocyte proteinase inhibitors (4 clones) were found in the haemocyte cDNA library of *P. sertiferus* but not in *P. vannamei* (Gross et al., 2001). A homologue of proteinase inhibitor (Kazal family) was found in sea urchin cDNA library (Smith et al., 1996). In the liver cDNA library of rainbow trout a clone homologous to cathepsin (Kazal family) was identified. Surprisingly, proteinase inhibitor could not be isolated the leukocyte cDNA library of the Japanese flounder (Bayne et al., 2001; Nam et al., 2000). The above results indicated that the Kazal family of serine proteinase was abundantly expressed in shrimp haemocytes.

Heat Shock Protein

HSPs prevent denaturation and facilitate refolding of denatured proteins. HSP 70 is a prominent among this protein family. It acts as a ligand for Toll-like receptor 4 in mouse macrophages. The HSP 90 family binds ATP and has

autophosphorylating activity. It was reported that HSP 90 and a 50 kDa phophoprotein are essential for the function of the other receptor; tyrosine kinase (Inanobe et al., 1994).

Four types of HSPs (HSP10, HSP70, heat shock cognate 70 and HSP 90) were found. Of these, HSP 10 and HSP 90 were only found in the normal library. Gross et al. (2001) found several types of HSP (HSP 3, HSP 5, HSP 10, HSP 28, HSP 29, HSP 70 and HSP 82) in haemocytes and hepatopancrease of *P. vannamei* and *P. setiferus* whereas HSPs were not found in haemocytes of *P. japonicus* (Rojtinnakorn et al., 2002).

Other immune molecules

Other immune and potential immune transcripts were also identified in haemocytes cDNA libraries of *P. monodon.* Among these, homologues of peptide-propyl cis-trans isomer 5 were equally found in both libraries (7 clones). This protein family comprises enzymes that increase the rate of protein folding. Interestingly, lectins were not found in haemocyte cDNA libraries of *P. japonicus*, *P. vannamei* and *P. sertiferus* (Gross et al., 2001; Rojtinnakorn et al., 2002), but 2 clones were homologous to perlucin, a C type lectin were identified in *P. monodon.*

Signaling and communication

Fifteen clones (28%) were identified as putative genes for signaling and communication and were regarded as homologues of several receptors and signaling protein.

Cell division/DNA synthesis, repair and replication

This group was the smallest group comprising 4 different genes. Typically, low abundant expression of genes exhibiting synthesis, repairing and replication activity was found in haemocyte cDNA libraries of penaeid

shrimps. Nine clones representing histone were identified in *P. monodon* whereas only one clone was found in *P. japonicus*.

Full-length of immune related genes

Complete ORFs were found in several ESTs that homologous with defense and homeostasis genes. These results showed 6 different genes which contained complete ORF.

Anti-lipopolysaccharide factor

ALF is a small basic protein with binds and neutralizes LPS (Wainwright et al., 1990) and has a strong antibacterial effect on the growth of Gram negative R-type bacteria (Morita et al., 1985). Sixteen out of 21 ALF found in this study contained complete ORFs. At least 4 types of ALF were found in haemocytes of *P. monodon.* Multiple alignment of nucleotide and deduce amino acids sequences and phylogenetic analysis of all ALF of *P. monodon* with ALF of *T. tridentatus*, illustrated that ALF*Pm*1 was closely related to ALF*Pm*2 where ALF*Pm*3 and ALF*Pm*4 were more distantly related. ALF*Pm*3 was the most abundant transcript (13 clones) whereas a single EST clone represented the remaining family (ALFPm1, -2 and –4).

ALF consisted of 2 cysteine residues linked with an intramolecular disulfide bridge. ALF is highly hydrophobic and the positive charged resides were clustered mainly with the disulfide loop. The clustering of charged groups and high hydrophobicity of the NH_2-terminal region suggest that ALF is amphipathic molecule (Aketagawa et al., 1986).

ALF has a single domain consisting of three α–helix packed against a four-stranded β–sheet. The binding site for LPS probably involves an extended amphipathic loop (Hoess et al., 1993). Ried et al.(1996) designed synthetic peptide comprising different part of the exposed amphipathic loop in the proposed of endotoxin-binding domain of *Limumus* ALF. They observed that

only a linear peptide, corresponding to amino acids 36-45 of *Limulus* ALF, was able to bind lipid A and endotoxin above the background levels.

Several studies concerning interaction between cationic antimicrobial peptides and LPS had been reported and likely suggested that the binding of cationic antimicrobial peptides to the outer membrane could play a major role in their toxicity towards microorganisms. The cationic peptides could displace the divalent cations, hence leading to destabilization of the outer membrane of gram negative bacteria.

Penaeidins

Penaeidins are family of antimicrobial peptides, first isolated from the *P. vannamei* (Destoumieux et al., 1997). Their antibacterial activities essentially acted against Gram negative bacteria, with a strain-specific inhibition mechanism (Destoumieux et al., 1999). In this study, deduced amino acids indicated the existence of 3 penaeidin types in *P. monodon* haemocyte.

This protein combines a proline-rich amino-terminal domain and a carboxyl-domain containing six cysteines engaged in three disulfide bridges (Bachère et al., 2000). Although penaeidins of *P. monodon* were homologous with Penaeidin2, Penaeidin3C and Penaeidin3K, multiple alignment of nucleotide and deduced amino acid of *P. monodon* penaeidins revealed high significant similarity to penaeidin-2 of *P. vannamei* (Bachère et al., 2000). Thus, they were named penaeidin2a, -2b and –2c. Like previously reported penaeidins, two organized doublets of cysteine residues are separated by 5 amino acid residues in *P. monodon* (RQL(G/R)). The conserved PRP motif which is present in the proline-rich antimicrobial peptide family from insect (Hetru et al., 1998) and penaeidin in *P. vannamei* (Destoumieus et al., 1997) was also found. The expression and localization of penaeidins were analyzed in shrimps and found that mRNA levels of penaeidins in circulating haemocytes decreased in the first 3 hours following stimulation but the plasma penaeidin concentration increased (Destoumieux et al., 2000).

Serine proteinase inhibitor

Nine clones in this study, were homologous of SPI, an inhibitor of serine protease (such as trypsin and elaster) (Williamson et al., 1984; Laskowski et al., 1987). Three ESTs contained complete ORF and all of then (SPI*Pm*1-3) were classified as members of the kazal family.

The Kazal-type motif characterized by six cysteine residues distributed over a region of about 40-60 amino acids with conserved residues adjacent to C_3 and a tyrosine located between C_3 and C_4 (Bode and Huber, 1992). Six cysteine residuals engaged in 3 disulfide bonds, which in the former case is C_1/C_5, C_2/C_4 and C_3/C_6 (Niimi et al., 1999). SPI*Pm*1 and SPI*Pm*2 of *P. monodon* contained 5 and 4 complete Kazal domains, respectively. SPI*Pm*3 had 2 complete and 2 incomplete Kazal domains. Besides conserved cysteines, PVCB and TYXN were consensus in each Kazal domain of SPI from *P. monodon.*

Crustins

Crustins are antimicrobial peptide/proteins with inhibited the growth of Gram positive bacteria, previously reported in *P. vannamei* and *P. setiferus*. Thirteen clones from both haemocytes libraries of *P. monodon* were identified as crustin homologues.

Two clones from the infected library contained complete ORF were named Crustin*Pm*1. The deduced amino acids alignment suggested 5 different types of crustins in *P. monodon* (Crustin*Pm*1 - *Pm*5). This protein contained glycine-rich region at NH_2-terminal and 2 domains of cysteine-rich residues. Cysteine-rich residues, WAP, contained 8 cysteine residues in conserved arrangement and engaged a 4 disulfide core (4-DSC) domain, comprising approximately 50 amino acids. Domain II of the WAP appeared more conserved than domain I and demonstrates surface electrostatic potentials

(Ranganathan et al., 1999). The rigidity of multiple disulfide bonds helps holding a reactive site in the correct conformation needed for inhibition (Kanost, 1999). WAP domain contains either confirmed or putative proteinase inhibitory activity (Couto et al., 1993) were aligned and show the characteristic conservation of cysteine residues arranged in a C_1-(Xn)-C_2-(X_n)-C_3-(X_5)-C_4-(X_5)-C_5-C_6-($X_{3\text{-}5}$)-C_7-($X_{3\text{-}4}$)-C_8 pattern where X is any amino acid residue, and X_n is a stretch of n residues (Rangariathan et al., 1999).

Moreover, other consensus sequences also appeared in the recognition of the 4-DSC domain: i) the consensus KXGKCP containing C_1; ii) a conserved aspartate (D) residue between C_3 and C_4 iii) KCC with C_5 and C_6; iv) CXXP with C_8 (Ranganathan et al., 1999). All of these remarks were also seen in *P. monodon* crustins with the exception of KXGXCP A substitution of leucine (L) for glycine (G) in the C_1 peptide was observed in crustins of *P. monodon P. vannamei* and *P. sertiferus*.

Heat Shock Protein 10

An EST possessing a complete HSP10 (also called cpn10) ORF was also found. The deduce amino acids of *P. monodon* HSP 10 showed 73 to 75% homology which those of vertebrates. These protein showed conserved DXX (D/N)(K/R) motif of cnp10 at the amino-terminal. Hartman et al. (1992) compared partial HSP10 sequence of *Rattus norvegicus* with HSP10 from seven bacterial species and indicated identical residues and conservative replacement of K/R, T/S, D/E, Q/E, D/N and I/L. All except T/S and D/N replacement were observed when compared the putative HSP10 of *P. monodon* with that of *Homo sapiens*, *Mus musculus* and *Gallus gallus.*

HSP play an important role during stress condition (depressed ATP levels, oxygen depletion, decreased intracellular glucose levels). Previous studies indicated that the mammalian HSP10, present in the mitochondria, is unclearly code. For full chaperonin function, HSP10 forms a complex with another distance family member, cpn60 (1989; Dickson et al., 1994). This

chaperonin complex is the primary site for folding of multimeric enzyme complexes and is necessary for folding of newly synthesized or translocated proteins. Besides these, mammalian cpn10 without post-translational modification can be used to reliably assess the link between its well established role in protein folding and its suspected role as a secreted growth factor with immunosuppressive characteristics.

Cytosolic manganese superoxide dimutase

MnSOD homologues were identified from the infected library of *P. monodon.* Both oxygen metabolism under respiration and naturally occurring chemical reaction in different kinds of cause toxic by-product responsible for damage to many cell components such as lipids, proteins, and nucleic acids (Cannio et al., 2000). MnSOD reduces the superoxide radical into hydrogen peroxide and molecular oxygen. The consensus sequence DXWEH was located between Asp 244 and His-248. Four residues known to be involved in metal binding in Fe or were found in this protein (His-112, His-159, Asp-244 and His 248) (Carlioz et al., 1988; Borgstahl et al., 1992)

From the analysis of full-length EST clones of putative immune genes in *P. monodon*, interestingly, we found several types of antimicrobial molecules existed in the haemocytes. Four types of *P. monodon* ALF whereas, ALF of the horseshoe crabs showed different between ALF of the Atlantic horseshoe crab (*Limulus polyphemus*) and the Japanese horseshoe crab (*Tachypleus tridentatus*). ALF of the Atlantic horseshoe crab shows 83% sequence identity with ALF of the Japanese horseshoe crab (Muta et al., 1987). Bachère et al. (2000) found penaeidin1, -2, -3a, -3b and –3c in haemocytes of *P. vannamei* and Cuthbertsom et al. (2002) found several new class 3 of penaeidins in *P. vannamei* and *P. setiferus*. All *P. monodon* penaeidins are similar to pen2 of *P. vannamei* but 3 variants were found. For crustins, Bartlett et al. (2002) observed each 3 types of these proteins in *P. vannamei* and *P. setiferus* whereas, our results suggested sveral types in *P. monodon*. Discovery of

several types of each antimicrobial molecules indicated that these molecules were contain broad spectrum of activity against various microorganism.

Determination of expression levels of immune related genes using semi-quantitative PCR

To determine expression levels of protein coding genes, several parameters must be taken into consideration. These include: 1) feasibility in the laboratory; 2) necessity to study different markers in the same sample; 3) availability of sample; 4) accuracy required for a specific application (i.e. need to measure the specific number of RNA molecules or rather rough variation in RNA levels) (Marone et al., 2001). Relatively accurate methods have been developed, such as competitive PCR, real-time PCR base on the use of fluorescent probes and/or RT-PCR.

Competitive PCR is carried out based on the competition of the target RNA and known amount of a synthetic homologous competitor template, usually engineered to share the primer recognition site with the target sequence but to differ either in length or by a short heterologous sequence stretch and the overall PCR efficiency is not affected by the modification (Orlando et al., 1998; Goerke et al., 2001). This technique is an "end-point" measuring method occurs when the PCR reaction is completed. An amplification curve with the internal competitor is then built for each sample. Quantification is rather accurate, but this technique requires a relatively large amount of cDNA and a large number of amplification reactions per sample. Moreover, intensive initial works are required when different RNAs have to be analyzed and hence different competitors need to be constructed.

The real-time PCR technique allows detection of the PCR product during the entire course of amplification by hybridization of two internal probes labeled with two different fluorescent dyes following the fluorescence resonance energy transfer principle. Thus, sequence-specific detection is ensured by the use of internal hybridization probes. The kinetics obtained

during the exponential phase of PCR are used for quantification (Goerke et al., 2001). Although this technique undoubtedly has a number of advantages, especially in terms of accuracy, it is not commonly available to the standard laboratories and may be cumbersome to set up when it is not used routinely.

RT-PCR is a highly sensitive and specific method which is useful for the detection of rare transcripts or for the analysis of samples available in limiting amounts (Carding and Bottomly, 1992). However, this technique is based on an "end point" measurement (e.g. reaching a plateau of amplification), therefore, the PCR product at this point does not correlate to the amount of mRNA at the initial stage.

Semi-quantitative RT-PCR for evaluation transcriptional levels of immune-related genes found in this study was developed. This method simultaneously amplifies interesting genes and the internal control (a housekeeping gene). The PCR product is quantitatively analyzed at the exponential phase of amplification. As a result, the PCR product at this point is surely related to the amount mRNA at the initiation. This method is a highly sensitive, specific and easy to set up (Marone et al., 2001). Semi-quantitative RT-PCR assumes that the experimental conditions do not alter the expression levels of the selected control RNA. The PCR product of the interesting genes was analyzed relatively to the internal control in the single tube eliminating varied amount of mRNA in each tube.

β-actin mRNA is expressed at moderately abundant levels in most cell types and encodes a ubiquitous cytoskeleton protein. It was one of the first RNAs used as an internal standard for evaluation of gene expression (Kreuzer et al., 1999).

rRNAs, which constitute 85-90% of total cellular RNA, are also useful internal controls (Paule and white, 2000). However, rRNA transcription may be affected by biological factors and drugs (Spanakis 1993) and the variations

in levels of transcription between samples taken from different individuals have not been quantified. In addition, there are two other drawbacks to its use: rRNA cannot be used for normalization when quantified targets have been enriched for mRNA because rRNA is lost during mRNA purification, and rRNA is expressed at much greater levels than target mRNAs. Therefore, β–actin was selected as an internal control in this experiment.

Expression levels of eight putative genes; ALF, penaeidins, crustin, ProPO, SPI, HSP 90, HSP 70 and lysozyme, in haemocytes of *P. monodon* were analyzed at different treatment period after shrimps were injected with the appropriate amount of *V. harveyi* using semi-quantitative RT-PCR. The PCR amplification conditions were optimized for the exponential phase of amplification. Two sets of primers used in each reaction did not compete among each other. After eletrophoresis and band intensity analysis, ALF, HSP 90 and lysozyme transcription levels were significantly increased ($p<0.05$) whereas crustin and penaeidin levels were significantly down-regulated ($p <0.05$). Constitutive expressions of SPI, proPO and HSP 70 were observed ($p> 0.05$).

V. harveyi, a Gram-negative bacteria , has the LPS which is the major component of the cell surface, therefore this LPS stimulates transcription of ALF to bind lipid A portion of LPS and neutralize toxin of the bacteria (Morita et al., 1985; Hoess et al., 1993). Lysozyme is defined as muraminidases that catalyze the hydrolysis of β(1-4) glycosidic bands. Such hydrolysis of bacteria peptidoglycan, a major component of the bacteria cell wall, cause lysis of bacteria (Jolles and Jolles, 1984; Hikima et al., 2000; Minagawa et al., 2001), thus *V. harveyi*-injection induced transcription of this protien. Although the expression level of HSP90 is increased after *V. harveyi*-injection, the highest expression level of this protein is lower than the expression level at all time points of HSP70.

The expression levels of crustin and penaeidin transcripts were decreased after injection with *V. harveyi* and the expression levels of both

protein transcripts were later increased after the lowest level. This may be because crustin and penaeidin inhibit the growth of Gram-positive bacteria (Ranganathan et al., 1999; Destoumieux et al., 1997) so they were down-regulated while ALF was up-regulated upon injection of the Gram Negative bacteria.

Several immune genes in invertebrates have been shown to constitutively expressed the same as those we found in the expression of SPI, proPO and HSP 70. Suggesting that some of the defense reactions are already activated.