CHAPTER IV

DISCUSSION

4.1 Tissue distribution and polymorphisms of Mn-SOD and AK

Very high expression level of Mn-SOD was found in all 6 examined tissues of *P.monodon.* This included gill, heart, haemocyte, hepatopancreas, muscle and digestive tract. Four variants of Mn-SOD sequences were obtained and they differed from other Mn-SOD sequences of *P.monodon* reported in other studies. This result is supported by other reports on polymorphism of Mn-SOD. High variant numbers of Mn-SOD are common among animals. A polymorphism [valine (V) → alanine (A)] at codon -9 of mitochondrial targeting sequence of Mn-SOD has been recently associated with prostate cancer (Mitrunen *et al.*, 2001). The relationship between prostate cancer and the Mn-SOD polymorphism and its interactions with baseline plasma antioxidant levels (selenium, lycopene, and a-tocopherol) and h-carotene treatment was examined. It was found little overall association between Mn-SOD polymorphism and prostate cancer risk; however, this polymorphism significantly modified risk of prostate cancer associated with prediagnostic plasma antioxidants (Li *et al.*, 2005).

Mn-SOD as an endogenous antioxidant in mitochondria may play a role in preventing prostate cancer. Malignant prostate epithelium has lower Mn-SOD expression than benign prostatic epithelium (Baker *et al.*, 1997) and overexpression of Mn-SOD in the prostate inhibits cancer cell growth in vivo (Li *et al.*, 1998). Although the function of the Mn-SOD polymorphism is not fully understood, a recent study showed that the A-containing Mn-SOD is transported more efficiently through the mitochondrial membrane (Sutton *et al.*, 2003), suggesting that, compared with those with the VV/VA genotype, the individuals with the homozygous AA genotype may have higher Mn-SOD activity.

In mitochondria, the superoxide anion is dismutated by Mn-SOD into oxygen and hydrogen peroxide (H_2O_2), which is further detoxified by mitochondrial glutathione peroxidase, an enzyme requiring selenium, or catalase into water. Thus, high levels of Mn-SOD expression may lead to enzyme imbalance and induce toxicity if glutathione peroxidase activity is low due to inadequate selenium intake or if antioxidants are in high demand due to physiologic conditions. In pregnant women, Mn-SOD variant AA was significantly related to increased formation of urinary 8-hydroxy-2V-deoxyguanosine, a biomarker of oxidative DNA damage (Hong *et al.*, 2002).

AK can also be found in all tissues examined in this study. High amount of AK was observed in gill, followed by heart, haemocyte, hepatopamcreas, muscle, and digestive tract, respectively. No polymorphism of AK was observed in this study. The rate of evolution and extant levels of polymorphism for arginine kinase were examined in species of the genus *Drosophila*. Surveys of 11 species for electrophoretic variation revealed an average heterozygosity of 0.003. Using antisera prepared against arginine kinase purified from a representative of each of three major subdivisions of the genus, immunological distances were measured by microcomplement fixation (MC'F) assay for 20 species of *Drosophila*. It is suggested that this difference in evolutionary rate arises because a single gene encodes *Drosophila* arginine kinase, whereas at least three different genes encode the various forms of vertebrate creatine kinase (CK) (Collier, 1990). Both CK and AK belong to a family that is so highly conserved that functional hybrid enzymes can be formed (Reddy and Watts, 1994). Vertebrates have four CK genes that encode tissue-specific isozymes (Muhlebach *et al.*, 1996). Although echinoderms and annelids can express both CK and AK (Moreland *et al.*, 1967), only AK has been reported in arthropods (Wallimann and Eppenberger, 1973).

AK is the most widely distributed phosphagen kinase, being found throughout the invertebrates and in hemichordates and tunicates as well, and is usually considered to be most closely related to the ancestral phosphagen kinases (Suzuki *et al.*, 1997). AK is represented by a single gene, and the sequence or partial sequence is available from *Drosophila*, *Limulus*, lobster (*Homarus*), shrimp (*Panuliris*), and abalone. It is abundant

in muscle, where they maintain ATP homeostasis during muscle contraction (van Deursen *et al.*, 1993). AK can be controlled by at least three mechanisms: selective expression, subcellular positioning, and phosphorylation (Hemmer *et al.*, 1995).

In general, high expression of AK was found in muscle that experience a high energy demand may also benefit from an ATP-buffering or energy shuttle system. Gills of euryhaline crustaceans, which achieve high rates of osmoregulatory ion uptake from reduced-salinity environments, driven by the hydrolysis of ATP are also found to have high expression level of AK. AK is expressed in crustacean gills, at the level of both protein and mRNA. Although the enzyme activity levels in gills are much lower than those measured in heart and claw muscle, both anterior (respiratory) and posterior (ion-transporting) gills exhibit substantial levels of arginine kinase activity. Amplification and sequencing of arginine kinase cDNA from the gills of *C. sapidus* and the shore crab *C. maenas* revealed close sequence homologies with other arthropod arginine kinases and, indeed, with phosphagen kinases in general (Kotlyar *et al.*, 2000).

4.2 Expression of stress responsive genes in *P.monodon*

During oxidative, osmotic, and handling stresses, genes of interest including Mn-SOD, AK, HSP70, HSP90, DAD-I, and TPx were monitored on their expressions.

Superoxide dismutase

Extreme environment condition such as osmotic stress could enhance the generation of reactive oxygen species (ROS), such as superoxide radical ($O2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$). Incompletely reduced ROS can be extremely reactive and oxidize biological molecules (DNA, RNA, proteins and lipids), inactivate enzymes, decrease the rate of protein synthesis (Jolán, 2005). The enzymatic protection is partly performed by SOD that eliminates $O_2^{\cdot-}$ radicals and by catalase and peroxidases that degrade H_2O_2. Mn-SOD functions primarily to protect mitochondrial components from superoxide liberated as a normal byproduct of respiration. Complex I

and Complex III release superoxide radical as a consequence of normal respiration, with estimates of 1-5% of the oxygen consumed being liberated as superoxide anion. Mn-SOD is therefore the cells primary defense against free radical mediated damage. In addition, stimulated levels of the enzyme have evolved to address increased free radical production during an inflammatory episode showed increase of catalase, peroxidase and SOD activities. In *Drosophila*, overexpression of manganese superoxide dismutase, which is confined to mitochondria, extends lifespan. In Crabs (estuarine-resident species) exposed to chronic hypoxia showed increased in gene expression of cytosolic Mn-SOD (cyt-Mn-SOD).

As obtained from this study, the results are in agreement with studies previously reported. It can be concluded that the expression of Mn-SOD gene in both haemocytes and gills of *P. monodon* clearly responses to oxidative, osmotic, and handling stresses. The induction of Mn-SOD in haemocytes appeared to be slightly more sensitive to the stress than in gills especially during handling stress. In osmotic stress, the expression level of Mn-SOD gene in the gill was raised by the salinity change.

Heat shock proteins

HSPs have been studied in various numbers of organisms from bacteria to mammals. There are obvious evidences indicating the high potential of HSPs, especially HSP70 and HSP90, as biomarkers to determine the stress conditions in many organisms (Köhler *et al.*, 1992; Sharp *et al.*, 1994; De Pomerai 1996; Eckwert and Köhler 1997; Lewis *et al.*, 1999).

HSP70 families in *Drosophila* have been described as chaperones with several functions. First, HSP70 is regarded as protein that induces stress tolerance by the benefits of massive expression of Hsp70. The other function includes roles in the regulation of cell cycle, apoptosis, transcription factors, cell signaling, and membrane conductance. These roles necessitate stringent regulation of Hsp70 levels, and unregulated, massive, or prolonged expression of Hsp70 can be harmful.

HSP90 is the most abundant protein in cytosols of eucaryotic cells. It is a major molecular chaperone, which is distributed ubiquitously among all living organisms and

which is indispensable for yeast cells to survive even under nonstressful conditions (Borkovich *et al.*, 1989). *In vitro*, HSP90 prevents the aggregation of chemically denatured or heat-denatured proteins (Jakob *et al.*, 1995; Wiech *et al.*, 1992; Yonehara *et al.*, 1996) and inherently unstable proteins such as casein kinase II (Miyata, and. Yahara.,1992). Nonnative proteins associated with HSP90 were released from the complexes and then refolded with the aid of reticulocyte lysates (Yonehara *et al.*, 1996) or a combination of HSP70 and HSP40 (Freeman and Morimoto, 1996; Minami *et al.*, 2000). Consistent with the results of these in vitro studies, overexpression of HSP90 confers a tolerance to stress in mammalian and yeast cells (Borkovich *et al.*, 1989; Yahara *et al.*, 1986). On the other hand, in vivo studies have revealed that HSP90 functions as a molecular chaperone for specific substrate proteins, most of which are involved in signal transduction (Nathan *et al.*, 1997; Rutherford and Zuker, 1994). The role of HSP90 in stress tolerance of *Saccharomyces cerevisiae* show 20-fold overexpression of Hsc82, an HSP90 homologue in yeast, was hypersensitive to high-NaCl or H-LiCl stresses. Hsc82-overexpressing cells appeared reduced levels of gene expression.

There were several studies reporting the conflicting results regarding the responses of animals to different kinds of stresses. In the studies of Buethong (2004) and Doungpanta (2004), the expressions of HSP70 and HSP90 in the haemocyes of *P.monodon* were induced by heat stress but not by vibrio infection. The expression of HSPs are widely regarded and used as an indicator of cellular stress. However, recent data suggest that insect diapause may inhibit the increased expression of Hsp70 typical of the heat stress response (Luís and Sridhar, 2005). Transcriptional levels of HSP70 and HSP90 were determined in muscle and hepatopancreas of American lobster, *Homarus americanus,* encountering osmotic stress. Lobsters in seawater at salinity of 32 ppt were moved into seawater at salinity of 16 ppt and 48 ppt to create hypo- and hypo-osmotic stress conditions to the lobster, respectively. Hypo- and hyper-osmotic stress significantly increased the levels of the mRNAs encoding HSP70 and HSP90 in abdominal muscle. Hyperosmotic stress increased HSP90 mRNA levels in hepatopancreas, but hypo-osmotic stress did not (Spees *et al.*, 2002)

In this study, the expression levels of HSP70 and HSP90 genes were repressed by stresses, which contradict to studies previously reported. Several reasons are needed to be considered. These include the condition of shrimps prior to the stress experiment and the number of sample replications. During acclimation, it is necessary for the shrimps to adjust themselves to the condition of the laboratory tanks. If the acclimation period is not long enough or the condition of the tanks is too different from what the shrimp previously had, the shrimps therefore longer time is needed to allow the shrimp to adjust themselves to the new environment. If this is the case, it is not possible to detect the increasing level of stress from the shrimps, which are already in the stressful condition. Therefore, the basal levels of the gene expression from the shrimps without any disturbances would be very important.

Arginine kinase

AK is a ATP/guanidino phosphotransferases that provide ATP by catalyzing the conversion of ADP and phosphorylarginine to ATP and arginine (Eppenberger *et al.*, 1967; Blethen and Kaplan, 1968; Morrison, 1973; Bessman, 1985; Wallimann, 1994; Wyss *et al.*, 1995). Phosphotransferases belong to a family that is so highly conserved that functional hybrid enzymes can be formed (Reddy and Watts, 1994). AK has been reported in arthropods (Wallimann and Eppenberger, 1973). AK is represented by a single gene (James and Collier, 1988; Munneke and Collier, 1988), and the sequence or partial sequence is available from *Drosophila* (Hecht *et al.*, 1995), *Limulus* (Strong and Ellington, 1995), lobster (Dumas and Camonis, 1993), shrimp (*Panuliris*), and abalone (Suzuki and Furukohri, 1994).

The hyperosmotic treatments led to a reduction in arginine kinase flux, while the hypo-osmotic treatments led to an enhanced arginine kinase flux (Stewart, 2002). The activity of arginine kinase, which is important in the temporal, and possibly spatial, buffering of cellular ATP concentrations (Ellington, 2001) and which has recently been shown to be induced by low salinity in posterior gills of adult blue crabs (Kotlyar *et al.* 2000).

In this study, the level of AK gene expression in gill reduced during osmotic stress. This result is in agreement with the result of Stewart (2002), which was conducted with blue crab (*Callinectes sapidus*) muscle.

Defender against apoptotic cell death I

DAD-I was initially identified as a negative regulator of programmed cell death in the BHK21-derived tsBN7 cell line. Of interest, the 12.5-kDa DAD1 protein is 40% identical in sequence to Ost2p, the 16-kDa subunit of the yeast oligosaccharyltransferase (OST). (Daniel *et al.*, 1997). In multicellular organisms, programmed cell death or apoptosis is an obligatory, exquisitely regulated event during normal cell differentiation, development, and tissue homeostasis of the mature organism (Vaux, 1993). During the past several years, it has been established that regulatory proteins (e.g., *ced9* and *bcl-2*) and enzymatic activities [e.g., *ced3* and interleukin 1-*b*converting enzyme (ICE)-like proteases] that control commitment to, and mediate progression of, the programmed cell death pathway are conserved between invertebrates and vertebrates (Hengartner and Horvitz, 1994; Chinnaiyan *et al.*, 1996).

DAD1 protein was identified as a mammalian cell death suppressor that may act downstream of the *bcl-2* protein (Nakashima, *et al.*, 1993; Sugimoto, *et al.*, 1995). It is a novel protein conserved through evolution, suggesting that the DADI protein plays an essential role in cell growth. Its loss of function induces apoptosis, since the DADI protein disappeared in tsBN7 cells following a temperature shift. An interesting and important question, which currently investigating, is how loss of the DADI protein induces apoptosis. The hydrophobicity of the DADI protein suggests that it is located in a membrane. Oxidative stress is an important component of the stress response in marine organisms exposed to a variety of insults as a result of changes in environmental conditions such as thermal stress, exposure to ultraviolet radiation, or exposure to pollution. As in the clinical setting, reactive oxygen species are also important signal transduction molecules and mediators of damage in cellular processes, such as apoptosis and cell necrosis, for marine organisms. Organized dismantling of cells, also called programmed cell death (PCD), is observed in both animal and plants (Pennell and Lamb,

1997). In animal systems, several genes that induce or repress PCD have been identified (Kop *et al.*,2003). Most of previous studies have reported that animals facing osmotic pressure can also be suffered from oxidative stress. This is because the imbalance of ions can create free radicals, which are the main cause of oxidative stress in cells. DAD-1 is one of potential proteins that contain the property to prevent programmed cell death in animal cells (Halliwell and Gutteridge, 1999). Endogenous Dad1 levels are modulated during T cell development to reach maximal expression in mature thymocytes. Transgenic mice that overexpress Dad1 in both the thymus and peripheral immune system have been generated.

Apoptosis of thymocytes from such mice is largely unaffected, but peripheral T cells display hyperproliferation in response to stimuli. Therefore, the linkage between the TCR and Dad1 genes may have important consequences for T cell function (Hong *et al.*, 1999). A *P.monodon* cDNA encoding a novel protein designated as DAD1 was cloned by heat induced haemocyte EST cDNA library (Bauthong, 2004).

In this study, the expression of DAD-I genes detected in gill of *P.monodon* was not induced by osmotic stress. On the contrary, it was repressed by oxidative and handling stresses. This is quite different from the result of the studies reported in other species. DAD-I, which is involved in apoptosis, does not clearly show responses to oxidative stress conducting by vibrio exposure in this study. Only slightly down regulation was detected in oxidative stressed gill. This is probably because *vibrio*, which is bacteria, does not stimulate the production of ROS in high levels as other stressors do. Although, direct induction of oxidative stress to the shrimps is difficult, stronger stressors like virus or the combination of stressors are needed for further study regarding oxidative stress.

Thioredoxin peroxidase

TPx is a small soluble protein capable of catalyzing thiol-disulfide redox reactions. The two cysteines of the conserved active site form a disulfide bond after reduction of exposed disulfides in a variety of substrate proteins. The reduced form is regenerated by the flavoenzyme thioredoxin reductase, which in turn is kept reduced by

NADPH (Holmgren, 1985). In *Escherichia coli*, thioredoxin is an electron donor for ribonucleotide reductase, for methionine sulfoxide reductase (Russel, and Model, 1986), and for 39-phosphoadenylylsulfate (PAPS) 1- reductase in the sulfate assimilation pathway (Lillig *et al.*, 1999, Russel *et al.*, 1990). The redox activities of the protein are not required for these latter functions (Huber *et al.*, 1986). Many additional roles for Thioredoxin have been proposed in eukaryotic organisms (Schulze-Osthoff *et al.*, 1995, Iwata *et al.*, 1997). TPx can help in the cellular defense against oxidative stresses, such as exposure to hydrogen peroxide or hydroxyl radicals (Spector *et al.*, 1988, Takemoto *et al.*,1998). Therefore, TPxs are believed to be present in insects for the protection against the toxicity of ROS. The TPx genes in some insect species including *D. melanogaster*, *Aedes aegypti* and *Apis mellifera* have been isolated (Fang and Li, 2001; Radyuk *et al.*, 2001; Whitfield *et al.*, 2002). Especially, five distinct genes of the Prx family were reported in *D. melanogaster* (Radyuk *et al.*, 2001). A recent work of Radyuk *et al.* (2003) confirmed that TPxs can foster cytoprotection or cell death in response to different stressors.

In this study, the expression level of Thioredoxin peroxidase gene was not induced by osmotic stress. This is in contrast with many early reports stating that osmotic stress could enhance the generation of active oxygen species.

4.3 Potential genes as biomarkers for stress conditions in *P.monodon*

The responses of all 6 candidate genes were summarized in table 4.1. Mn-SOD has potential as biomarker for the determination of oxidative, osmotic, and handling stresses and also both haemocyte and gill can be used as target tissues. The disadvantage of such a broad biomarker is that it needs more than one indicator to identify the specific stressor.

HSPs do not provide qualified properties to apply as biomarker for any kind of stresses in this study. Since, there are some contradicting results among experiments, more investigation is needed before conclusion on the responses of HSPs in *P.monodon* is made.

DAD-I gene responds to oxidative and handling stresses by showing down regulation instead of up regulation. This interesting result is quite helpful for further study on its inhibiting mechanism from both kinds of stresses before applying as biomarker.

TPx is effective in gill but not in haemocye. Therefore, it can also be used as biomarker for oxidative stress but care must be taken with the choices of target tissues.

The result of AK induction reveals a response, which is in agreement with most of earlier studies. Therefore, it can be used as biomarker for osmotic stress. The concern of using AK as biomarker is that there are 2 forms of AK and each form response differently.

Table 4.1 Summary of the gene responses in *P.monodon* during stresses

Genes	**Expression levels of the genes during stress**					
	Oxidative stress		**Osmotic stress**		**Handling stress**	
	Haemocyte	Gill	Haemocyte	Gill	Haemocyte	Gill
Mn-SOD	Up (++)	Up (++)	ND	Up (++)	Up (++)	Up (+)
DAD I	No change	Down (-)	ND	No change	Down (-)	Down (-)
HSP70	Down (--)	Down (-)	ND	No change	Down (-)	Down (-)
HSP90	Down (--)	Down (--)	ND	No change	+/-	+/-
AK	+/-	+/-	ND	Down (--)	Up (+)	No change
TP	Down (--)	Up (++)	ND	No change	Down (-)	No change

Up - up regulation of the gene

Down - down regulation of the gene

No change- no detectable changes of the gene expression

+/- - un-consistent change of the gene during stress