CHAPTER V

DISCUSSION

5.1 Non-lethal temperature treatment in *P. monodon*

Prior to the investigation on thermal responses, the assessment of the thermal effect was conducted in order to obtain the optimal temperature which gained maximum induction but no harm to the shrimps. The determination on the effect of thermal exposure in *P. monodon* has revealed the non-lethal temperature ranging between 15 and 35°C for at least 14 h. Complete mortality of the shrimps was obtained when exposed to lower or higher than that range within an hour of exposure. Therefore, the temperature at 15°C recognized as cold shock treatment and the temperatures at 30, 33, and 35°C recognized as heat shock treatments were used for thermal shock conditions in most experiments in this study.

5.2 Protein induction in the haemolymph of thermal-shock shrimps

Haemolymph contains proteins that are important for the transportation of ions and molecules. Quantitative and qualitative variations of the haemolymph proteins have been recognized as an important information supply on physiological and pathological state of shrimps. Several types of protein are found in crustacean haemolymph. The major component is haemocyanin, which accounts for 60% of haemolymph total protein. Other proteins are coagulation, apohaemocyanin, hormones and antibodies. The level of haemolymph protein is one of the physiological parameter that related to the stress response in shrimps (Chen and Cheng, 1996).

In this study, total protein concentrations of the haemolymph from the survived shrimps exposed to cold and heat shock were analyzed. The results revealed that protein level of the haemolymph could be raised within the first hour and corresponded to the increase levels of temperature which the shrimps were exposed to. Shrimps exposed to cold shock have lower plasma protein level than normal shrimps. Plasma protein concentration tended to increase according to time after heat shock while it tended to decrease in the shrimps exposed to cold shock. This indicates that plasma protein level of *P. monodon* can be induced by heat and this certain level

remains high for more than 72 h. There was no significant difference among the shrimps exposed to 30, 33, and 35°C, indicating the maximum plasma protein induction. There was no evidence on induction of *Vibrio* exposure on plasma protein level, however, the infection seemed to suppress protein level in the shrimps pretreated with heat shock. The lowest level (15.7 mg/ml) of plasma proteins was obtained from the shrimps at ambient temperature and the highest level (75.0 mg/ml) of plasma proteins was obtained from the shrimps maintained at 35°C. The difference between the lowest and the highest levels of plasma protein was almost 60 mg/ml (nearly 4 folds) and about 29.2 mg/ml (1.3 folds) higher than that of the shrimps at normal condition. Therefore, it can be concluded that the plasma protein level in *P. monodon* depends on the raise of surrounding temperature and it tends to return to normal level after the thermal condition has passed.

It is interesting to note that the haemolymph protein level of the shrimps detected in this study was considerably low even from the induced shrimps when compared to those reported by Supamattaya *et al* (2000a) who found that the average levels of haemolymph protein in the shrimps from the culture farms located in Eastern region was 118.5±55.1 mg/ml and those from the shrimps in captured or laboratory tank was 115.3±38.1 mg/ml. However, the difference was probably caused by a number of factors. Haemplymph proteins have been known to vary according to physiological states (e.g. molting), nutritional stage, sex and season.

A trend of the season variation of total protein concentration in portunid crab, *C. maenas*, depended on the seawater temperature. Other factors, e. g. infection, hypoxia and salinity fluctuation have been found to influent the release of an immunological reactive protein into the haemolymph when infected with the rhizocephalan parasite (Bell, 1993). In *C. sapiuis* rathbun, the males had a lower protein level in haemolymph than that of female (Robert, 1969). Haemolymph protein level in *C. maenas* was associated with molting (Busselen, 1970). Protein concentration reached the lowest level shortly after molting and then progressively increased during the intermolt period. The pattern of total protein was similar to the pattern of ecdysteroid hormone or molting hormone in *P. vannamei* (Adriana and Fernando, 2002). The increase in haemolymph ecdysteroids was correlaed with an increase in haemolymph protein content. The increase in haemolymph protein

concentration might result from an increased protein synthesis, reduced degradation of protein and/or resorbtion of cuticular protein in premolt stage.

5.3 Glucose induction in the haemolymph of thermal-shock shrimps

In crustaceans, glucose, mannose, fructose, maltose oligosaccharides and trehalose have been found in haemolymph. Glucose was a major reducing agent in crustaceans and was also a major component of circulating carbohydrate. Haemolymph glucose was used as an indicator to stress in aquatic animals. Glucose content represented an average of approximated one-fourth of the total reducing substance in the haemolymph of fresh-water crayfish, *O. vivilis* (McWhinnie and Connor, 1967). While the ratios of the total reducing sugar to blood glucose did not vary, the level of total reducing sugar depended upon the amount of blood glucose.

In this experiment, glucose concentrations in haemolymph of *P. monodon* exposed to the temperature at cold shock (15°C), normal (27°C) and heat shock (30, 33, and 35°C) for 6 h were measured. High level of plasma glucose was found in shrimps exposed to heat shock while low level was found in shrimps exposed to cold shock. Plasma glucose in normal shrimps was in between that of the cold and heat shocks. The increase of glucose level was found in heat shock shrimps 3 h after the thermal treatment. Low glucose level was found in cold shock shrimps and the level increased in corresponding to post exposure time. Plasma glucose detected in control shrimps (maintained at 27°C) was in the level between that of the cold and heat shocks. Glucose levels from cold shock shrimps tended to decrease within 12 h and increased afterward. On the other hand, glucose levels from heat shock shrimps significantly increased within 12 h of post thermal exposure and decreased afterward.

It can be concluded that the level of plasma glucose responds according to the degree of temperature exposure. Glucose level can reach its lowest (22.52 mg%) and highest (101.77 mg%) concentrations within 12 h after thermal induction. Glucose concentration from the haemolymph of the shrimps was not induced by *Vibrio* exposure. The highest levels of plasma glucose was 71.31 mg% (more than 5 folds) higher than the lowest level and about 45.63 mg% (2 folds) higher than that of the shrimps at normal condition (32.0 mg%).

It has been reported that a baseline of haemolymph glucose concentrations in black tiger shrimp, *P. monodon* was 32.91±27.95 mg% from the shrimps in culture farms and 53.87±55.84 mg% from the shrimps in laboratory conditions. (Supamattaya

et al, 2000a and 2000b). Glucose level in the shrimps maintained in laboratory tank was clearly higher than that of the shrimps in culture pond. Similar result of glucose level was obtained from the shrimps in this experiment. However, glucose levels obtained in this study were much higher, roughly a hundred times higher than earlier report, indicating a very high response to on *P. monodon*. Haemolymph glucose of the river crab, *Potamonaytes warreni* increased from 8.52±7.53 mg% to 173.80±77.29 mg% after 6 h of anoxia conditions (Van Aardt, 1988). It was also suggested that the glucose concentration did not depend on sex and size of shrimps (Supamattaya *et al*, 2000a and 2000b).

The study in Gilthead sea bream, *Sparus aurata* showed that the concentration of plasma glucose in this fish increased when fishes were kept at high density (Barton *et al*, 2004). Potential molecular and biochemical indicators are suitable for evaluating stress, because stress classically leads to a rapid onset and a cascade of molecular and physiological responses.

Recently, there has been a report on the effect of a variety of putative stressors on blood glucose concentrations in an attempt to obtain a simple and reliable biological index of stress for shrimp. A significant elevation of blood glucose in *P. monodon* after a depletion of dissolved oxygen, and increased dissolved CO_2 levels were detected. However, the blood glucose levels were not useful as an indicator of the detrimental effects of crowding or water pH values between 8.3 and 5.9 (Hall and Van-Ham, 1998). Racotta and Palacios (1998) showed that blood glucose is strongly and rapidly increased in response to repeated blood-sampling stress, with lactate levels more slowly affected, in *L. vannamei* juveniles.

5.4 Protein analysis of stress induced shrimps

Despite the enormous amount of literature available on the HSP response in a variety of organisms, very little is known about the HSP response in aquatic invertebrates and the data available has been obtained largely from *in vitro* studies. An increase in HSP70 and HSP90 was observed following thermal stress in crayfish (*Procambarus clarkii*) (Rochelle *et al*, 1991, Sheller *et al*, 1998), encysted brine shrimp (*Artemia*) (Clegg *et al*, 2000b, Frankenberg *et al*, 2000), and *Homarus americanus* (Chang *et al*, 1999). In oyster haemocytes (*Crassostrea virginica*), three different isoforms of HSP30 (HSP32, HSP34, and HSP37), HSP45 and HSP85 were detected *in vitro* by autoradiograph of radioactive proteins after hyperthermal shock

from 20 to 41°C (Tirard *et al*, 1995). In most organisms studied so far, HSP70 proteins were among the most prominent proteins induced by heat, and these proteins played a central role in tolerance to high temperatures, as they allowed cell survival during and after thermal stress (reviewed by Parsell and Lindquist, 1993). A number of investigations have focused on the HSP70 family as the majority of HSPs in Crustaceans. Many studies have reported that members of the HSP70 family commonly showed up-regulation during times of stress (Rochelle *et al*, 1991, Dunlap and Matsumura, 1997, Frankenberg *et al*, 2000).

In this study, attempts to analyze the protein profiles obtained from the tissue extracts of thermal and *Vibrio* induced shrimps using SDS-PAGE were not achieved to differentiate the induced proteins from other constitutive proteins. Coomassie Brilliant blue stained SDS-PAGE gels revealed several different protein bands in the unstressed and stressed samples. However, the un-consistency results were detected among samples from both control and treatments. Furthermore, most tissue extracts, especially from haemocytes, were interfered by the haemocyanin, the major proteins commonly found in the haemolymph of invertebrates. Therefore, the difference between proteins stressed and unstressed shrimps was not conclusive with this method.

The results from Western blotting analysis of the haemocyte lysates from the shrimps from control, cold and heat shock experiments showed a considerably clear signal of cross reaction of anti-HSP70 monoclonal antibody and the proteins at 76 kDa. The presence of HSP70 was detected in both control and induced shrimps because the antibody used in the experiment recognized both inducible and constitutive HSP70. The increase of HSP70 accumulation after thermal shock due to time and temperature level indicated that HSP70 was present in normal shrimps and could be induced by both cold and heat shock. The detection of HSP60 and HSP90 in the haemocyte lysate of the shrimps delivered no positive results which were presumably caused by low cross reactivity and low sensitivity of the antibodies. Although, HSP70 in *P. monodon* was possibly be determined by the cross reactivity of monoclonal antibody against human HSP70, the successful dilution used in this study was considerably low (1:1,000). Therefore, it was not practically rational to perform quantitative analysis (ELISA) using this antibody with a large number of samples. In addition, to precisely determine the levels of HSP70 in the samples, a homologous antibody and a calibration curve from a pure HSP70 are required. To

date, no homologous antibodies and pure HSP standards have been produced for *P. monodon*.

This result was in agreement with other reports (Rochelle *et al*, 1991, Sheller *et al*, 1998, and Cimino *et al*, 2002). The result in Western blotting described by Cimino *et al*, (2002) showing the presence of an immuno-reactive protein to mouse anti-human HSP70 IgG1 monoclonal antibody at a mass of 86 kDa (HSP86) in pleopod samples of *P. monodon* but it was not sensitive enough to detect differences in response to stress. However, an ELISA was reported to detect the significantly higher levels of HSP86 in hyperthermally stressed and hypoosmotically stressed of *P. monodon*.

5.5 Bacterial tolerance of the shrimps pre-treated with thermal shock

In order to challenge the shrimps with *V. harveyi*, the appropriate concentration of *V. harveyi* for challenge test was determined. It was performed by exposing the shrimps to *V. harveyi* by suspension administration ranging from 10^6 to 10^9 CFU/ml. Fifty percent mortality (LC_{50}) of the shrimps exposed to *V. harveyi* at the concentrations of 10^6, 10^7, 10^8, and 10^9 CFU/ml were detected at 5, 4, 3, and 2 days, respectively. These results were in agreement with a number of other reports (Table 5.1).

Table 5.1 Results of pathogenicity experiments with *Vibrio harveyi* bacteria isolated from diseased animals.

***Vibrio* species, strain**	**Shrimp species**	**LD_{50}**	**Route of infection and survey duration**	**Reference**
V. harveyi, 770527	*P. monodon* (5-6 g.)	$8.2x10^4$ CFU/shrimp	In muscle (7 days)	Liu *et al*, 1996b
V. harveyi, 820514	*P. monodon* (5-6 g.)	$8.7x10^4$ CFU/shrimp	In muscle (7 days)	Liu *et al*, 1996b
V. harveyi, In I strain	*P. monodon* (5-6 g.)	$7.7x10^4$ CFU/shrimp	In muscle (7 days)	Liu *et al*, 1996b
V. harveyi, A1 strain	*P. monodon* (5-6 g.)	$5.9x10^4$ CFU/shrimp	In muscle (7 days)	Liu *et al*, 1996b
V. harveyi	*P. monodon* (5-6 g.)	10^6-10^7 CFU/shrimp	In muscle (7 days)	Liu *et al*, 1996b
V. harveyi	*P. monodon* Larvae	10^2-10^3 CFU/ml	Bath (40-48 hours)	Lavilla-pitogo *et al*, 1990
V. harveyi STD3-101	*P.vananmei*	10^6-10^7 CFU/ml	Bath of 2 hours	Robertson *et al*, 1998
V. harveyi BL1	*P. monodon* larvae	$<10^2$ CFU/ml	Bath (2 days)	Le Groumellec *et al*, 1995
V. harveyi from larvel tank	*P. monodon* PL	$2.6x10^3$ CFU/ml	Bath	Lavilla-pitogo *et al*, 1990
V. harveyi from seawater	*P. monodon* PL	$1.52.6x10^5$ CFU/ml	Bath	Lavilla-pitogo *et al*, 1990
V. harveyi BP04	*P. monodon* larvae, Z	$<10^5$ CFU/ml	Bath (48 hours)	Prayitno and latchford, 1995
V. harveyi BP04	*P. monodon* M and PL	$>10^5$ CFU/ml	Bath (48 hours)	Prayitno and latchford, 1995

Abbreviations; N: Nauplius, Z: Protozoa, M: Mysis, PL: post-larvae

V. harveyi concentration at 10^8 CFU/ml was applied by suspension administration to 2 groups of shrimps; normal shrimps and the shrimps pre-heated at 35°C for 6 h. LC_{50} of the pre-heated shrimps exposed to *V. harveyi* was detected at 7 days while the LC_{50} of non-heated shrimps was detected at 5 days of exposure. The result indicated that pre-heat at 35°C for 6 h can enhance the *V. harveyi* tolerance in *P. monodon*. It was in agreement with the study of Granja *et al*, (2003) on the enhancement of pathogenic tolerance by hyperthermia. It has been suggested that hyperthermia could facilitate apoptosis in WSSV-infected *L. vannamei* and might be one of the mechanisms responsible for increased survival of infected shrimp maintained at relatively high (32°C) temperature. However, another report has suggested differently that apoptosis may not be a principal protective factor in immune shrimp (Wu, 2002). Apoptosis or programmed cell death, is a highly regulated process involved in injury and disease. Synthesis of heat stress proteins is thought to increase resistance to apoptosis. Overexpression of human hsp70 gene protects against heat-induced apoptosis.

5.6 Differential expressed genes in *P. monodon* responded to heat shock

RNA arbitrarily primed PCR technique is based upon the use of reverse transcribed RNA as a template to identify differentially expressed genes in a manner analogous to that of arbitrarily primed PCR (AP-PCR, Welsh and McClelland, 1992), which used genomic DNA as a template. RAP-PCR has proven to be a powerful method for the detection and isolation of differentially expressed genes in several systems including tumor cells (Wong *et al*, 1993, Nelson *et al*, 1996), human brain cells (Dalal *et al*, 1996), and rat glial cells.

The determination of heat-induced genes expressed in the haemocytes of the shrimps in response to heat shock was performed by RAP-PCR using 10 primer combinations. The results revealed that 7 DNA fragments were displayed differentially between control and heat shock shrimps, 10 DNA fragments were up-regulated and 3 DNA fragments were down-regulated in corresponding to the heat shock temperature. It should be noted that almost all of the RAP-PCR results that displayed the difference of gene expression between treatments were analyzed within the first hour of post exposure. Only a small number of markers were detected from the samples collected at 6 and 12 h of post exposure. It was possible that the difference in expression of these genes returned to normal level within 6 h of post

exposure. However, judging by the small numbers and less intensities of the bands present on the gels, it was presumably indicated that this result was probably caused by the un-consistency of the RNA extraction, first stranded cDNA preparation and the PCR amplification from different time, rather than the decline of protein expression level from the haemocytes at post exposure time.

The results from sequence comparison showed no significant similarity (E<-4) to any known genes in the GenBank excepted clones RAP4, RAP9, and RAP21, which were indicated as *P. monodon* clone TUZX4-6:86 microsatellite sequence, *P. (Litopenaeus) vannamei* microsatellite TUMXLv10.221 sequence, and ENSANGP00000010415 (*Anopheles gambiae* str. PEST) and Niemann-Pick type C1 disease protein (*Oryctolagus cuniculus*), respectively.

The expression levels of some unknown markers (RAP12, RAP16, RAP21, and RAP58) were quantitatively analyzed using semi-quantitative RT-PCR.

Since most of the markers were unknown genes and the ones that have been identified were not related to stress condition. These markers were, therefore, selected based on the appropriate sizes (longer than 200 bp) and the complete reading frame of the sequences (no interrupt ORF). The expression of RAP12 gene from both heated and un-heated shrimps were slightly induced in the same level and decreased slowly in corresponding to exposure time. It appeared that RAP12 gene was not induced by exposing the shrimps with *vibrio*. Similar expression strategies were also obtained from RAP16, RAP21, and RAP58 genes. The result has confirmed heat related characteristics of these 4 genes. However, the involvement of these genes to pathogenic stress in *P. monodon* is still un-conclusive.

Daniel *et al*, (2004) used differential display PCR to identify hepatic genes responsive to handling stress and genes that differ in expression between populations of a fish, *Fundulus heteroclitus*, from different thermal environments. Despite substantial inter-individual variation, twenty putatively stress-regulated bands were cloned from Northern fish, 10 of which had high similarity to gene of known function. Five of these genes were selected for further analysis based in their known roles in the stress response. Three of these genes (glucokinase, serin-threonine kinase 10 and cRAF) were confirmed as stress-responsive using real-time PCR. These genes increased in expression in response to a 7-day chronic stress protocol in fish from the Southern population of *F. heteroclitus*, but did not change significantly in fish from the Northern population. These three genes also differed in expression between

populations in control fish, suggesting a link between the response to chronic stress and inter-population differences in gene expression laboratory-acclimated fish. Two genes that did not respond to stress (glycogen synthase kinase and warm acclimation-related protein (WAP)) also differed between populations. Expression of WAP was eight-fold higher in Southern than in Northern fish, consistent with a previously suggested role for this gene in thermal acclimation or adaptation in fish.

5.7 Expression level of PO and HSP genes

PO is an enzyme commonly known to play an important role in immune response in invertebrates. From this finding, the expression level of PO gene in *P. monodon* was obviously induced by *vibrio* exposure but it was not induced or suppressed by the heat treatment, indicating no correlation between the expression of PO gene and other heat stressed genes. This result supports the idea of using PO as biomarker for the assessment of shrimp immune condition.

The study revealed no induction in expression level of HSP60 gene from the heated and unheated shrimps and also no correlation to the *vibrio* exposure. On the other hand, the expression of HSP70 and HSP90 genes was induced by heat shock but it was not induced by *vibrio* exposure. The expression of HSP90 gene seemed to response to heat shock faster that of HSP70 in heat shock shrimps while HSP70 gene remained in high level for longer period of time when the shrimps were exposed to *vibrio*. Although, there was no obvious evidence from this study that the expressions of HSPs were related to *vibrio* infection or responsible for the *vibrio* tolerance of the preheated shrimps reported in this study, a large number of studies on the involvement of heat stress proteins to the defense system of many organisms.

Expression of heat stress proteins is a cellular response to stress, and subsequently leads to an acquired protected state. This state of protection confers resistance to further episodes of exposure to the same stress or a different harmful stimulus. Heat stress protein accumulation improves functional recovery and is associated with preservation of high energy phosphates (Jayakumar *et al,* 1998) and reduction in infarct size (Currie *et al,* 1993), see comments), activation of ATP-sensitive potassium channels (Sadd and Hahn, 1992) and anti-apoptotic effects (Mehlen *et al,* 1996). However, the precise details of cytoprotective mechanisms of these proteins have yet to be fully elucidated. Heat stress proteins are a small family of gene products which are expressed as an intracellular response to stress (Lindquist

and Craig, 1988). Many agents and conditions are known to initiate the heat stress response. These stimulants include elevated temperature (Slakey *et al,* 1993), ischaemia (Richard *et al,* 1996), hypoxia (Petel *et al,* 1995), depletion of ATP (Kabakov and Gabai, 1997), free radicals (Kukreja *et al,* 1994) and hypothermia (Yang *et al,* 1996). Cells and tissues which accumulate heat shock proteins develop a transient resistance to subsequent episodes of that particular stress or a cross-tolerance to a different stress (Heads *et al,* 1994).