CHAPTER III

MATERIALS AND METHODS

3.1 Samples

Adult black tiger shrimps, *P. monodon*, approximately 3 month-old or 20 g of body weight were used in the experiments. They were purchased from local shrimp farms (Pathumthani and Nonthaburi) and maintained in rectangular concrete tanks (0.75x0.75x0.75 m^3) containing approximately 200 L. of brackish water (the salinity at 10 ppt) with ambient temperature (27°C). Shrimps were fed twice daily with commercial shrimp pellet diet. Ten to fifteen percent of seawater was changed every morning. Shrimps were acclimated at least 1 week before the experiment. All experiments were carried out at Marine Biotechnology Reserch Unit (MBRU), Chulalongkorn University.

3.2 Determination of the appropriate thermal treatment in shrimp

After separating into 8 groups (20 shrimps in each group) and acclimating as described in 3.2, the shrimps were subjected to thermal treatment by placing the shrimps from each group into seawater at the temperatures of 10, 15, 27 (ambient), 30, 33, 35, 40, and 45°C, for 6 h. The survival rates of the shrimps from each treatment were recorded.

3.3 Thermal shock conditions for the shrimp

The thermal shock conditions used in this experiment were chosen from the conditions in the study of thermal treatment (3.3), which revealed no mortality of the shrimp. The experiment was conducted as follow.

Acclimated shrimps were separated into 5 groups (35 shrimps in each group). The shrimps from each group were placed into the tank containing brackish water at the temperature of 15, 27 (ambient), 30, 33, and 35°C, respectively, and maintained condition for 6 h. The survival rate of the shrimps from each treatment was monitored. Following the thermal shock, all shrimps were moved back to the water at ambient temperature and 5 shrimps from each treatment were collected at 0, 3, 6, 12, 24, and 72 h. of post exposure. Haemolymph from each shrimp was withdrawn and a

number of organs (gill, muscle hepatopancreas, digestive tract, and heart) were also dissected. All collected samples were subjected to further analyses.

3.3.1 Haemocyte and tissue preparation

The haemolymph was withdrawn from the ventral sinus of the shrimp using a 26 G 1/2 inch needle fitted onto a 1.0 ml syringe pre-loaded with 500 µl of anti-coagulant (10% sodium citrate, w/v). Haemolymph was immediately centrifuged at 3,000 rpm for 10 min at 4°C to separate haemocytes from the plasma. Haemocyte lysate was prepared by re-suspending the haemocytes in 150 µl of 1xPBS (appendix A). Cell debris was separated by centrifugation. Haemocytes and haemocyte lysate were used immediately or kept at -80°C for further analysis.

Following haemolymph collection, a number of tissues including gill, muscle, hepatopancreas, digestive tract, and heart were immediately dissected, dropped into liquid nitrogen and ground to fine powder using mortar and pestle. Protein extract from each tissue was prepared separately by homogenizing in homogenize buffer (0.5 mM PMSF in 1xPBS). After centrifugation at 12,000 rpm at 4°C for 25 min, the supernatant was stored at -20°C.

3.4 Preparation of *Vibrio harveyi*

The method used in this experiment was modified from the method described by Roque *et al*, (1998).

Culture stock of *V. harveyi* stain 1526 was streak on marine agar plate to obtain colonies. A single colony was then inoculated in marine broth and incubated at room temperature for 16 h. The titer of the culture was monitored by plate count method in marine agar plate (modified from Austin, 1988). The culture was diluted to the experiment condition at the concentration of 10^6, 10^7, 10^8, and 10^9 CFU/ml for challenge test.

3.4.1 Acute virulence test of *V. harveyi* in *P. monodon*

The acute virulence test of *V. harveyi* to shrimp, estimated by median lethal dose (LD_{50}) at 96 h, was conducted on *P. monodon* with batches of 30 shrimps/dose by bath of a bacterial suspension (10^6, 10^7, 10^8, and 10^9 CFU/ml) with aeration. Mortalities were recorded daily for 7 days after infection and the survival rate from

each treatment was recorded. Bacteria were re-isolated from all moribund shrimps by culturing haemolymph samples on TCBS plates and incubated at room temperature overnight. Colonies of *V. harveyi* strain 1526 from infected shrimps showed strong luminescence in the dark.

3.5 Determination of pathogenic tolerance of the shrimp pre-treated with thermal shock

Acclimated shrimps were randomly selected and separated into 3 treatments. Each group contained 50 shrimps. Shrimps in first group were moved into a tank containing seawater at the temperature of 35°C for 6 h and moved back to seawater at ambient temperature, followed by the suspension challenge of *V. harveyi* at 10^8 CFU/ml. For the second group, shrimps were also moved into a tank containing seawater at ambient temperature for 6 h, followed by the suspension challenge of *V. harveyi* at 10^8 CFU/ml. For the last group, which was a control group, shrimps were moved into a tank containing seawater at ambient temperature. The mortality of the shrimps in each treatment was monitored. Six shrimps from each treatment were collected at 0, 3, 6, 12, 24, 72, 120, and 168 h after all shrimps were back to ambient temperature. The haemolymph of the collected shrimps from each tank were then withdrawn. Haemocytes were separated as described in 3.4.1. The tissues including gill, muscle, hepatopancreas, digestive tract, and heart were also dissected from the collected shrimps as described in 3.4.1. Haemocytes and tissues were subjected to protein preparation and RNA extraction (in 3.12).

3.6 Haemolymph protein determination

Protein concentration of tissues extract, haemolymph and haemocyte lysate was determined by dye-binding method described by Bradford (1976). The assay was performed by mixing sample (10 µl) and dye reagent (200 µl) in the well of microtiter plate. The mixture was left at room temperature for 5 min. The absorbance of the mixture was measured at wavelength of 595 nm using microplate reader model 450 (Bio-Rad, U. S. A.). Bovine serum albumin (BSA) was used as standard protein at the concentrations of 20, 40, 60, 80, and 100 mg/ml. The protein contents were estimated from standard curve (appendix B).

3.7 Determination of glucose concentration in shrimp haemolymph

The glucose concentration in the haemolymph of *P. monodon* was determined by dye-binding method described by Halfman (1962). Haemolymph sample (50 µl) was added to 50 µl of 30% TCA for protein precipitation, the mixture was stored at room temperature for 2-5 min before centrifugation at 8,000 rpm for 10 min. The colorless upper aqueous phase was transferred to a 1.5 ml microcentrifuge tube and mixed with 1.0 ml of colour reagent (appendix A). The mixture tube was placed in boiling water for 8 min then cooled on ice. The absorbance of the blue-green colour was spectrophotometrically measured at 630 nm. The glucose contents were calculated according to standard curve (appendix B). Glucose standard was prepared at the concentrations of 20, 40, 60, 80, and 100 mg%.

3.8 Determination of proteins from haemocyte lysate and tissue extracts from shrimps treated with thermal stress

Accumulated proteins from haemocyte lysates and tissue extracts from the normal shrimps and the shrimps treated with different levels of thermal stress (15, 27, 30, 33, and 35°C) were determined using gel electrophoresis. Heat shock protein, HSP60, HSP70, and HSP90, were detected by Western blot analysis using antibodies against human-HSP60, bovine-HSP70, and water mold-HSP90, respectively.

3.9 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

An electrophoresis of denaturing gels was performed using a discontinuous buffer system as described by Laemmli (1970).

Twelve percent discontinuous SDS-PAGE was prepared on a mini-PROTEAN II electrophoresis apparatus (9.5 cm x 10 cm x 1 mm) (Bio-Rad, U. S. A.). Separating gel was prepared by mixing 3.4 ml of deionized water, 4.0 ml of 30% acrylamide mix solution (appendix A), 3.5 ml of 1.5 M Tris-HCl, pH 8.8, 100 µl of 10% (w/v) SDS, 50 µl of 10% (w/v) ammonium persulfate, and 8 µl of TEMED, respectively. Without delay, the mixture was swirled rapidly and poured into the gap between the glass plates. The gel was placed in a vertical position and deionized water was carefully overlaid on the top of the separating gel solution. After allowing the gel to solidify at room temperature for 60 min, the overlaid water was poured off and washed again with deionized water to remove any unpolymerized acrylamide.

The stacking gel (4%) was prepared by mixing 3.05 ml of deionized water, 0.65 ml of 30% acrylamide mix solution, 1.25 ml of 0.5 M Tris-HCl, pH 8.8, 50 µl of 10% (w/v) SDS, 50 µl of 10% (w/v) ammonium persulfate, and 8 µl of TEMED, respectively. The mixture was swirled rapidly and poured onto the top of solidified separating gel and the comb was immediately inserted into the stacking gel solution and was carefully avoided trapping air bubbles. The gel was placed in a vertical position at room temperature. While the stacking gel was polymerizing, the samples were mixed with 5x loading buffer (appendix A) and heated in boiling water for 10 min to denature proteins. After polymerization was completed (30-60 min), the comb was removed carefully and washed the wells immediately with deionized water to remove any unpolymerized acrylamide. The gel was placed into the electrophoresis chamber and added electrophoresis buffer (appendix A) to the top and bottom reservoirs. The samples and low molecular weight marker were loaded into the bottom of the well and the gel was run at 200 V until the bromophenol blue reached the bottom of the separating gel (about 50 min). The gels were coomassie stained and silver stained as described in 3.10.1 and 3.10.3.

3.9.1 Coomassie staining

After electrophoresis, the gel was rinsed briefly with deionized water, followed by the addition of coomassie staining solution (appendix A). The gel was left at room temperature for at least 1 h with gently agitation. After staining, the gel was then destained in the de-staining solution (appendix A) until the protein bands were seen and the background was clear.

3.9.2 Silver staining

Following electrophoresis, the gel was rinsed briefly with deionized water and then placed in fix solution (50% (v/v) methanol) for 30 min at room temperature. The gel was transferred into 5% (v/v) methanol for 10 min and 160 mM DTT for 10 min. The gel was washed with dd-H_2O and soaked with 0.1% (w/v) silver nitrate for 10 min with gentle agitation. The gel was briefly rinsed by milli-Q H_2O for 2-3 s before it was developed in a cool developing solution (3% (w/v) sodium carbonate and 50 µl of 37% (v/v) formaldehyde) until protein bands were visible. The developing reaction

was stopped by soaking the gel with 1 M citric acid for 5 min. Finally, the gel was rinsed with dd-H_2O and dried by a cellophane paper at room temperature.

3.10 Western blot Analysis

Samples (15 μg) were loaded and separated on 12% denaturing polyacrylamide gel electrophoresis. The gel was then subjected to the protein transfer using mini-Trans blot apparatus (Bio-Rad, U. S. A). The process was as follow.

After equilibrating in Towbin buffer (25 mM Tris, 192 mM glycine, 0.1% SDS and 20% methanol) for 15 min, the gel was aligned next to a pre-equilibrated nitrocellulose membrane and assembled to a mini-Transblot electrophoretic transfer cell (Bio-Rad, U. S. A.). The system was run at 25 V for 90 min.

After the transfer was finished, the gel was removed and blocked the non-specific binding sites on the membrane by incubating in blocking solution (1%BSA/1xPBS) for 1 h at 4°C. The membrane was washed 3 times (5 min each) with 1xPBS before incubating with the appropriate dilution of primary antibody in 1xPBS containing 1%BSA for 60 min at 4°C. The membrane was then washed 3 times (5 min each) with 1xPBS followed by the incubation of the membrane with peroxidase-conjugated secondary antibody at the dilution of 1:1000 in 1xPBS ontaining 1%BSA for 1 h at 4°C. The membrane was washed 2 times (10 min each) with 0.05% Tween20 in 1xPBS, followed by 3 washed (5 min each) with 1xPBS. The membrane was developed by the addition of diaminobenzidine (DAB) solution (0.054 g DAB/30% H_2O_2 in 1 M Tris, pH 7.6) until the brown bands appeared, indicating the sites of antibody binding. The development reaction was stopped by washing the membrane with large volumes of distilled-H_2O.

3.11 Total RNA preparation

Total RNA was extracted from *P. monodon* tissues and haemocytes by using Tri reagent® (Molecular Research Center, Inc.). The process was carried out as described by the manufacture's protocol. Haemocyte pellet or tissues (approximatedly 50-100 mg) were homogenized in 1 ml of Tri reagent and incubated at room temperature for 5 min to permit complete dissociation of nucleoprotein complexes. After that, 200 μl of chloroform was added and the mixture was stirred vigorously for 15 s. The resulting mixture was left at room temperature for 2-5 min before it was

centrifuged at 12,000 rpm for 15 min at 4°C. The colorless upper aqueous phase was transferred to a 1.5 ml microcentrifuge tube. RNA was precipitated by the addition of 500 µl of isopropanol. The mixture was left at -20°C for 30-60 min and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was discarded. The RNA pellet was washed with 1.0 ml of 75% ethanol. The RNA pellet was kept under ethanol at -80°C until used. When required, the samples were centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was removed. The RNA pellet was briefly air-dried at room temperature. The total RNA was dissolved with an appropriate amount of diethyl pyrocarbonate (DEPC)-treated water. After completely dissolving, it was stored at -80°C for a short period of time.

3.11.1 Determination of RNA concentration

The amount of RNA was measured by determination of the optical density at 260 nm. The OD value at 260 nm allows calculation of total nucleic acids whereas the value reading at 280 nm determines the amount of contaminated protein in the sample.

The concentration of total RNA can be determined by measuring the OD at 260 nm. An OD_{260} of 1.0 corresponds to approximately 40 µg/ml of RNA (Sambrook *et al*, 1989) and estimated in µg/ml using the following equation,

$$[RNA] = OD_{260} \times \text{dilution factor} \times 40$$

3.12 Agarose gel electrophoresis

One percentage (w/v) of agarose gel was prepared by mixing agarose with 1xTBE buffer (appendix A). The gel slurry was heated until complete solubilization, the gel was then poured into a chamber set. A comb was inserted. After the gel was solidified, the comb was carefully withdrawn and sufficient 1xTBE buffer was added to cover the gel for approximately 0.5 cm. Each sample was mixed with ¼ volume of the gel-loading dye (appendix A) and loaded into the well. Lambda-*Hind* III fragments or a DNA ladder (100 bp marker) were used as standard DNA markers. Electrophoresis was carried out in 1xTBE buffer at 100 V until the bromophenol blue dye marker migrated about ¾ of the gel length. After electrophoresis, the gel was stained in a 3.5 µg/ml ethidium bromide (EtBr) solution for 5 min and de-stained to

remove unbound EtBr by submerging the gel in distilled water for 15 min. Fractionated DNA was visualized under a UV transilluminator (UVP, U. S. A.).

3.13 First stranded cDNA synthesis

The first stranded cDNA was generated using an ImProm-II™ Reverse Transcription system kit (Promega, Co., U. S. A.). Combine the experimental RNA (up to 1 μg) and olido $(dT)_{15}$ (0.5 μg/reaction) nuclease-free water for a final volume of 5 μl per reaction. The mixture was preheated at 70°C for 5 min and immediately placed on ice for 5 min. The reverse transcription reaction mix (15 μl), containing 4 μl of ImProm-II™ 5x reaction buffer, 1.8 μl of 25 mM $MgCl_2$, 1 μl of dNTP Mix (10 mM each), 20 units of Recombinant Rnasin® Ribonuclease inhibitor and 1 μl of ImProm-II™ reverse transcriptase, was added and mixed gently. The reaction was incubated at 25°C for 5 min and at 42°C for 60 min. The reaction was finally incubated at 70°C for 15 min to terminate the activity of reverse transcriptase.

The concentration of cDNA can be determined by measuring the OD at 260 nm. An OD_{260} of 1.0 corresponds to approximately 33 μg/ml of single-stranded DNA and estimated in μg/ml using the following equation,

$$[\text{cDNA}] = OD_{260} \times \text{dilution factor} \times 33$$

3.14 Determination of thermal-induced genes in *P. monodon* using RNA arbitrarily primed polymerase chain reaction (RAP-PCR)

The expression level of thermal-induced genes in the haemocytes of *P. monodon* was differentially displayed using RAP-PCR. The technique used in this experiment was modified from the method described by Welsh *et al* (1992). The shrimps were shocked in different levels of temperature (27, 30, 33, and 35°C for 6 h) then returned to the ambient condition. Total RNA was extracted from the haemocytes of the shrimps at 0, 6, and 12 h. First stranded cDNAs were synthesized as described in 3.14 and were subjected to RAP-PCR.

Arbitrarily primers consisted of 11 primers, UBC119, UBC122, UBC128, UBC135, UBC158, UBC174, UBC228, UBC268, UBC299, UBC457 and UBC459 were used. Sequences of these 10-base primers were purchased from Biosynthesis using the sequence from the Biotechnology Laboratory, University of British

Columbia, Canada. Details of the primers were shown in Table 3.1. UBC119 was used as forward primer and the other 10 primers were used as reverse primers.

Table 3.1 Sequence nucleotide in this study technique RAP-PCR.

Primer	Sequence
UBC119	ATTGGGCGAT
UBC122	GTAGACGAGC
UBC128	GCATATTCCG
UBC135	AAGCTGCGAG
UBC158	TAGCCGTGGC
UBC174	AACGGGCAGC
UBC228	GCTGGGCCGA
UBC268	AGGCCGCTTA
UBC299	TGTCAGCGGT
UBC457	CGACGCCCTG
UBC459	GCGTCGAGGG

PCR was carried out in a final volume of 50 µl, containing 2 µM of primers (combination of UBC119 and either one of reverse primers), 10 mM Tris-HCl, pH 8.3, 50 mM of KCl, 2 mM of $MgCl_2$, 0.2 µM each of dNTP, 1 unit of DyNAzyme II DNA polymerase and 1.5 µg template single stranded cDNA. The amplification was performed for 40 cycles. Each cycle consisted of denaturation step at 94°C for 30 s, annealing step at 36°C for 60 s, and extension step at 72°C for 90 s. PCR reaction was operated by Hybaid thermo-cycler (Hybaid Limited, England). After amplification, PCR product was precipitated by the addition of one-tenth volume of 3 M sodium acetate pH 5.5 and two volume of ice-cold absolute ethanol. The mixture was incubated at -80°C for 30 min prior to centrifugation at 12,000 rpm for 15 min at room temperature. The pellet containing DNA was washed twice with 70% ethanol. The DNA pellet was air-dried and resuspended in 13.5 µl of TE buffer (10 mM Tris-HCl, pH 7.4 and 1 mM EDTA). The DNA solution was incubated at 37°C for 1-2 hours for complete re-dissolving and kept at -20°C until further use.

3.14.1 Analysis of RAP-PCR products using polyacrylamide gel electrophoresis

The products of RAP-PCR were analyzed using polyacrylamide gel electrophoresis. The method was performed following the method of Welsh *et al*, 1993. Before the assembly of the apparatus and electrophoresis, the glass plates were prepared as follow. The long glass plate was treated with binding solution (3 µl of bind silane, 955 µl of 95% ethanol, and 0.5% glacial acetic acid) for 4-5 min. Ethanol (95%) was then applied to the plate and wiped out by tissue paper in one direction and then perpendicular to the first direction using gentle pressure. For the short glass plate preparation, tissue paper saturated with repel solution (Amersham Life Science, England) was applied to the plate and left horizontally for 5-10 min. The excess repel solution was removed by wiping the plate with tissue paper, followed by the application of 95% ethanol. The plate was dried by wiping out with a tissue paper in one direction and then perpendicular to the first direction using gentle pressure. A pre-run of the gel was carried out for 20 min at 40 W before loading the sample. 3 µl of formamide dye, was added to 8 µl of each PCR sample. The samples were heated to 95°C for 5 min and 10 µl of each was loaded on a 4.5% denaturing polyacrylamide gel. The gel was run in 1xTBE buffer at a constant power of 40 watts until the xylene cyanole dye reached the bottom.

3.14.2 Silver staining

After electrophoresis, the plates were carefully separated using a plastic wedge. The gel, which was attached to the small glass plate was placed in a shallow tray containing Fix/Stop solution (10% acetic acid). The gel was agitated vigorously for 30 min or until the tracking dyes were no longer visible. The gel was rinsed 3 times using ultrapure water with agitation. The gel was lifted out of the wash and allowed to drain for 20 s before transferring to the next wash. The gel was then transferred to staining solution (appendix A) and agitated well for 30 min. The gel was rinsed briefly with ultrapure water and placed into a tray containing a liter of developing solution (1 L of 30 g Sodium carbonate/0.75 ml formaldehyde/30 µl sodium thiosulfate). The time taken to dip the gel in the water and transferred it to developing solution should be no longer than 5-10 s. The gel was agitated well by rocking until the template band started to develop or until the first bands were visible. The gel was transferred to a liter of fresh developing solution and the reaction was

continued for an additional 2-3 min or until all bands became visible. The developing reaction was terminated and fixed by adding the equal volume of Fix / Stop solution directly to the developing solution and the gel was incubated for 2-3 min with agitation. The gel was rinsed for 2 min with ultrapure water. The bands of DNA were observed and photographed under visible light.

3.15 Cloning and sequencing of RAP-PCR fragments

3.15.1 Isolation and re-amplification of RAP-PCR products

Isolation of PCR products was conducted as previously described by Welsh, *et al*, 1993. The candidate bands were excised from the gel using a razor blade. The piece of acrylamide was placed in a microcentrifuge tube containing dd-H_2O and the DNA was allowed to diffuse from the gel overnight at room temperature. The diffused DNA was amplified using primer complementary to the adapter sequence and the PCR amplification was carried out in the presence of the template with the conditions described in 3.15.

3.15.2 Elution of DNA fragments from agarose gel

After electrophoresis, the DNA fragments were excised from a 1.5% agarose gel using a scalpel and placed in a preweighed microcentrifuge tube. Three volumes of the buffer QG (supplied by the manufacturer by QIAquick Gel Extraction Kits (QIAGEN)) were added. The mixture was incubated at 50°C for 10 min or until the gel slice was completely dissolved. The gel mixture was vortexed every 2 to 3 min during the incubation period. The mixture turned into yellow after the gel was completely dissolved. The mixture was transferred into a QIAquick column, inserted in a 3.0 ml collection tube, and centrifuged at 12,000 rpm for 90 s. The flow-through solution was discarded. Another 0.5 ml of buffer QG was added to the QIA quick column and recentrifuged for 90 s. Then, a 0.75 ml of buffer PE (supplied by the manufacturer) was added to the QIAquick column and centrifuged. The flow through solution was discarded. The QIAquick column was centrifuged to remove a trace amount of the washing solution. The QIAquick column was placed into a sterile 1.5 ml microcentrifuge tube. DNA was eluted by an addition of 30 µl buffer EB (10 mM Tris-HCl, pH 8.5) or H_2O to the center of the QIAquick membrane and let the column

stood for 5 min, before centrifugation at 12,000 rpm for 90 s. The eluted DNA was kept at -20°C.

3.15.3 Ligation of PCR products to plasmids

Each of the reamplified DNA fragments was ligated to pGEM®-T Easy Vectors (Promega, Co., U. S. A.). In a 10 µl reaction, it contained 50 ng of pGEM®-T Easy Vectors, 10 ng of DNA insert, 5 µl of 2x rapid ligation buffer (60 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP, 10% polyethylene glycol; MW 8000), and 3 unit of T4 DNA ligase. The reaction mixture was incubated overnight at 4°C before the transformation.

3.15.4 Transformation of ligated products to *E. coli* host cells by heat shock

3.15.4.1 Preparation of competent cells (Ausubel *et al*, 1995)

A single colony of *E. coli* JM-109 was inoculated in 3 ml of LB-broth (1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5% NaCl) with vigorous shaking at 37°C overnight. The starting culture was inoculated into 1 L of LB-broth and continued culture at 37°C with vigorous shaking until the OD_{600} reached 0.4-0.5. The cells were chilled briefly on ice for 10 to 15 min, and harvested by centrifugation in a pre-chilled rotor at 2,700 rpm for 10 min at 4°C. The pellets were resuspended in 30 ml of ice-cold $MgCl_2/CaCl_2$ solution. After incubation on ice for 10 min, the cells were sedimented by centrifugation at 2,700 rpm for 10 min at 4°C. The cell pellets were gently resuspended in 3.0 ml of ice-cold 0.1 M $CaCl_2$ and 15% glycerol. This concentrated cell suspension was divided into 200 µl of aliquots. These cells could be used immediately or stored at -80°C for later use.

3.15.4.2 Transformation

The competent cells were thawed on ice for 5 min. One or two microlitres of the ligation mixture was added and gently mixed by pipetting The mixture was left on ice for approximately 1 min. The mixture was heat shock at 42°C for 45-60 s before the mixture were immediately removed from the tube and added to a new tube containing 1.0 ml of SOC medium (appendix A). The cell suspension was incubated at 37°C for 1 to 2 h with agitation. Approximately 10-30 µl of the culture was spreaded on a selective LB agar plate containing 50 µg/ml of ampicillin, 25 µg/ml of

IPTG and 20 μg/ml of X-GAL. The plate was incubated at 37°C overnight. The recombinant clones containing inserted DNA appeared in white whereas those without inserted DNA were blue.

3.15.4.3 Colony PCR

Colony PCR was performed in a 25 μl PCR reaction containing 1.25 mM of each dNTP (dATP, dCTP, dGTP, and dTTP), 1xPCR buffer, 1.2 mM $MgCl_2$, 2 pmole of each primer (pUC1 and pUC2) and 1 unit of DyNAzyme™ II DNA polymerase. A recombinant colony was scraped by the micropipette tip and mixed well with the PCR reaction. The amplification was performed for 35 cycles. In the first cycle, the reaction was heated to denature DNA at 94°C for 3 min. The following cycles was consisted of the denaturation step at 94°C for 30 s, annealing step at 50°C for 1 min and extension step at 72°C for 5 min. The last cycle of the reaction was finally extended at 72°C for 7 min. The resulting PCR products were eletrophoretically analyzed in 1xTBE buffer at 100 V. DNA ladder (100 bp) was used as DNA marker.

3.15.4.4 Isolation of recombinant plasmid DNA

An *E.coli* consisted of recombinant plasmid was inoculated into 3 ml of LB broth containing 50 μg/ml of ampicillin. The culture was incubated at 37°C with constant shaking at 250 rpm overnight. The culture was transferred into 1.5 ml microcentrifuge tube and centrifuged at 12,000 rpm for 1 min. The cell pellet was collected and resuspended with 250 μl of buffer P1 supplied by the manufacturer by QIAprep Spin Miniprep Kits, (QIAGEN). The mixture was completely dispersed by vortexing and the mixture was then treated with 250 μl of buffer P2 (sodium hydroxide). The tube was inverted gently 4-6 times before and after adding 350 μl of buffer N3 (guanidine hydrochloride and acetic acid). Cell debris was separated by centrifuging the mixture at 12,000 rpm for 15 min. Supernatant was transferred into the QIAprep column inserted in a 3.0 ml collection tube and centrifuged at 12,000 rpm for 90 s. The flow-through solution was discarded. Another 0.5 ml of buffer PB was added to the QIAprep column and recentrifuged for 90 s. After this step, 0.75 ml of buffer PE (guanidine hydrochloride and isopropanol) was added to the QIAprep column and centrifuged. The flow through solution was discarded. The QIAprep column was centrifuged to remove a trace amount of the washing solution. The

QIAprep column was placed into a sterile 1.5 ml microfuge tube. DNA was eluted by an addition of 50 µl of buffer EB (10 mM Tris-HCl, pH 8.5) or H_2O to the center of the QIAprep membrane. The column was allowed to stand for 5 min and centrifuged at 12,000 rpm for 90 s. The eluant product was kept at -20°C.

3.15.4.5 DNA sequencing and data analysis

Recombinant clones were sequenced using an automated DNA sequencer (Shrimp Molecular Biology and Genomic Laboratory, Department of biochemistry, Chulalongkorn University)

Sequences of cDNAs were edited and compared with sequences in the nucleotide sequence database of the GenBank (the National Center for Biotechnology Information; NCBI) using the BLAST*N* and BLAST*X* programs (Altschul *et al*, 1997). Significant probabilities and numbers of matched nucleotide/proteins were considered when E-values < the 10^{-4} and a match >100 nucleotides for the BLAST*N* and a match >10 amino acid residues for the BLAST*X*, respectively.

3.16 Semi-quantitative Reverse Transcription–Polymerase Chain Reaction

A number of DNA sequences obtained from RAP-PCR was subjected to quantitative analysis. Semi-quantitative RT-PCR used in this study was modified from the method described by Maria *et al*, 2001.

Single stranded cDNAs were synthesized from the reverse transcription of RNA extracted from thermal-treated shrimps. These cDNAs were used as templates. Specific primers were designed from the sequences of DNA fragments, which were differentially displayed in RAP-PCR result. The sequences of these primers were shown in Table 3.2.

Table 3.2 List of oligonucleotide primers used in semi-quantitative RT-PCR. Primers were designed from DNA sequences obtained from the result of RAP-PCR, PO and HSP gene in heat-stressed shrimps.

Primer name	Sequence (5'-3')	Size (bp)	Tm (^{0}C)
RAP12F	TCT CTG GAA CCA CAC ACA CC	234	62
RAP12R	GAG GCG AAG ATG TAA TTC CA		60
RAP16F	GAA TTA TTC ATC CCA GGG TA	253	58
RAP16R	AGG CTA GAT CAT AAT ACG GT		58
RAP21F	TCG CGC ATT GCC AGA CCA GT	255	64
RAP21R	GTC ACA GTC CAC AGT CCC AC		64
RAP58F	GGG ATC CGA CTG TAG CAA AC	242	62
RAP58R	ACA TCC CAC TCA ATT TCA GG		58
POF	CAC GGC AAA GTG AAC GAG	417	56
POR	CTT CGG GAG ACC CAG ACA		58
HSP60F	AGG TTG GTC GTG AGG GTG TC	843	64
HSP60R	GAG TCT GGA TAG CCT TGC GG		64
HSP70F	CCT CTA TCA CTC GTG CTC GC	719	64
HSP70R	GTC CCT CTG CTT CTC ATC GT		62
HSP90F	TCC ACG AGG ATT CCA CCA ACC	612	66
HSP90R	TCG GCA TCC GCC TTT GTC TCA		66
ActinF	GGT ATC CTC ACC CTC AAG TA	327	60
ActinR	AAG AGC GAA ACC TTC ATA GA		56

The amplification was performed in a PCR thermal cycler (Hybraid Limited, England) with 25 µl reaction volume containing 100 µM of each dNTPs, 1xPCR buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl and 0.1% Triton x-100), 1 unit of DyNAzyme^{TM} II DNA polymerase. The optimum concentration of $MgCl_2$, specific primers, and templates applied separately to each reaction was dependent on the optimized condition for each gene.

Prior to the quantitative analysis, the appropriate PCR conditions such as template concentration, number of cycles, and $MgCl_2$ concentration for each of candidate genes and β-actin gene which was used as reference were verified. First, the

concentration of DNA templates various from 50 to 200 μg were examined, for PO and HSP gene. Then, different numbers of PCR cycles (20, 25, 30, 35, 40, and 45 cycles) were carried out. The condition that amplified the PCR product in the exponential range and did not reach a plateau level was chosen. Also, the application of $MgCl_2$ concentration ranged from 1.5, 2, 3, and 5 mM was determined. The concentration that gave the highest yield and specificity was chosen. After amplifications, PCR products were run on 1.5% agarose gel electrophoresis as described in 3.13. Six microliters of PCR product from each sample was combined with ¼ volume of the gel-loading dye before loaded to the agarose gel. A DNA ladder (100 bp marker) was used as a standard DNA marker. After electrophoresis at 100 V, the gel was stained with 3.5 μg/ml of EtBr for 5 min and destained in distilled water for 15 min. The intensity of DNA bands from each sample was analyzed.

The ratio between the candidate gene and the internal control products (β-actin gene) was determined.

3.17 Data analysis

The expression level of each transcript at a particular time was normalized with the internal control (β-actin). Significant difference of expression levels were treated using One Way Analysis of Variance (ANOVA) following by a post hoc test (Duncan's new multiple range test).