CHAPTER III

MATERIALS AND METHODS

3.1 Test organisms

Giant tiger shrimp, *P. monodon* (weight, 18-20 g) were obtained from local shrimp farm and acclimated to laboratory condition in rectangular tanks (0.75x0.75x0.75 m^3) for 1 week prior to use. The tank contained approximately 500 liters of seawater (salinity: 10 ppt.; temperature: 28 °C). Shrimp were fed twice daily with commercial shrimp diet before the experiment. Intermoult shrimp were selected and used in the experiments.

3.2 Chlorpyrifos

Commercial grade chlorpyrifos (40% W/V) used in the experiment was obtained from local supplier while laboratory grade chlorpyrifos was purchased from CHEM SERVICE, Inc (USA). The stock solutions were prepared by diluting chlorpyrifos in methanol to give stock concentration. The dosing volume for all of the experiment was not over 1%, of which the solvent did not affect survival of tested organisms or cells.

3.3 Quantification of commercial chlorpyrifos

Gas chromatography (GC) analysis was performed to quantify 40% W/V chlorpyrifos stock solution. The stock solution was diluted to 10 mg/l and subjected to an Agilent 6890 GC equipped with an AG6890 Series autosampler and split/splitless injector. An Agilent US1653687H HP-5 fused-silica capillary column, 30 m x 0.32 mm i.d., 0.25 μm film thickness was used as analytical column. The GC conditions were: injector temperature, 200 °C; detector temperature 325 °C; carrier flow (helium) 29 cm/sec, oven temperature, 180 °C (0 min), rate 5 °C /min to 280 °C, 280 °C (3 min); nitrogen flow, 30 ml/min. The detector was Agilent micro-ECD detector. Calibration standard were prepared using 99.5% purity chlorpyrifos (CHEM SERVICE). The dilutions, for both sample and standards, were carried out using pesticide grade 95% n-hexane (Lab-Scan).

3.4 Acute toxicity test of chlorpyrifos

Static acute-toxicity tests were conducted in basic accordance with procedures described by the standard method for the examination of water and wastewater (APHA, AWWA, WEF 1992). Exposures were conducted in 200-L plastic tanks containing 100 L test solution.

For the range finding test, shrimps at intermoult stage were randomly assigned to the test by separating into 4 test tanks. Each tank contained 20 shrimp. Serial concentrations of chlorpyrifos, 0, 6.81, 68.1, and 681 µg/l, respectively, were applied to shrimp in test tanks. The mortality of the shrimp was recorded during 120 h of experiment. The shrimp were not fed during the experimental period. The result of range finding test was then used as criteria for the definitive test.

The definitive test was conducted by exposing the shrimp to water containing 5 concentrations of chlorpyrifos (0, 6.81, 13.62, 27.24, 54.48, and 68.1 µg/l) (N=20). Mortality was recorded in all treatments at 24 h intervals until 96 h. LC_{50} was estimated by the probit method according to Finney (1971) using EPA Probit Analysis Program, Version 1.5.

3.5 Assay for AChE activity

Tissues, including haemolymph, gill, and muscle from tested shrimps (N=5) were subjected to AChE activity measurement base on the Ellman method (Ellman et al.1961) adapted to microplate operation (Lundebye et al., 1997; Scaps et al., 1997). The tests were conducted for both lethal (0, 0.681, 6.81, 68.1, and 681 µg/l) and sub-lethal concentrations of chlorpyrifos (0, 0.00681, 0.0681, and 0.681 µg/l).

For the lethal concentration test, the AChE activity was determined after 30 min of exposure. For the sub-lethal concentration test, AChE activity was determined at the interval times of 24, 48, 72, and 96 h, respectively.

Haemolymph was withdrawn from ventral sinus of the shrimp using 26G ½ inch needle fitted onto a 1.0 ml syringe and centrifuged at 3600 g for 2 min to separate haemocytes from the haemolymph. Gills and muscles were dissected,

homogenized in 4 volumes of ice-cold 0.01 M Tris/HCl, pH 8.0, and centrifuged at 10,000 g for 10 min. Extracted samples (10 µl each) was added to the wells of microtiter plate containing 300 µl of 0.1-M phosphate buffer, pH 8.0, 20 µl of 0.01 M DTNB (5, 5'- dithio-(2 nitrobenzoic acid)), and 20 µl of 0.075 M acetylthiocholine iodide. The kinetic of the enzyme activity was monitored with the use of a spectrophotometer in the visible range at 415 nm. The enzymatic activity was corrected for spontaneous hydrolysis of the substrate and was expressed as µmole/min/mg of protein. Protein content was measured based on the method of Bradford (1976) in which a volume of 160 µl of a diluted tissue extract (1/1000 for haemolymph, 1/500 for gill and muscle) is reacted with 40 µl of Bio-Rad protein reagent. The absorbance was read at 595 nm, and the protein content of the sample was determined with bovine serum albumin calibration curve. The inhibition of AChE activity was determined by the comparison of AChE activities between normal shrimp and the shrimp exposed to different concentrations of chlorpyrifos.

Normality and homogeneity of variances was tested using Shapiro-Wilk and Levene's test. Significant difference among group of treatments was examined using post hoc Duncan's new multiple range test in SPSS (version 11.5). Statistically significant difference was considered at $P<0.05$.

3.6 Single cell gel electrophoresis analysis (Comet assay)

In vitro testing was conducted to examine the genotoxic effect of chlorpyrifos on shrimp haemocytes using single-cell gel electrophoresis assay. The method was performed according to Giovanelli et al. (2002) with modification.

Haemolyph was withdrawn from ventral sinus of cephalothorax of the shrimp using 24 G needle and 1 ml syringe containing 10% sodium citrate as anticoagulant (1:1 dilution). Haemolymph was collected from 5 or more shrimp, pooled, and diluted to 10^6 cell/ml. This stock of haemocytes was then resuspended in M199 media (1.1% W/V M199 media, 10% fetal bovine serum, 0.02 µg/l penicillin, 0.02 µg/l streptomycin, pH 7.6) to a required density for each experiment.

Prior to the assay, cell viability was examined on the normal and chlorpyrifos-exposed haemocytes after 1, 6, 12, and 24 h of exposure using trypan blue exclusion. The method was carried out by mixing 30 µl of M199 media containing haemocyte with 10 µl of 0.4% trypan blue dye. The mixture was left at room temperature for 5 min before layering 20 µl of the mixture onto hematocytometer. The number of dead cells was recorded. Dead cells were identified by the blue color inside the cells due to the losses of the ability to exclude the dye out of their cells.

Haemocytes obtained from healthy shrimps were separated into 4 treatments with 3 replications. The test was conducted by adding 10 µl of 10% sodium citrate containing approximately 10^4 haemocytes with methanol-diluted chlorpyrifos stock solution to make the final concentrations of 0, 0.007, 0.034, and 0.170 µg/l, respectively. M199 media was added to make up the total volume of 100µl. The mixture was then kept at room temperature for 1 and 6 h.

The exposed haemocytes were then mixed with melted 1% low-melting-point agarose, subsequently layered on 1% agarose-precoated microscope slide, covered with a cover slip, and allowed to solidify at 4 °C. The coverslip was removed and a second layer of low-melting-point agarose was placed on the top of the solidified layer. The gel was then covered with the cover slip and stored at 4 °C for solidification. After the removal of cover slip, the slide was subjected to a lysis step by placing the slide into chilled lysis solution (2.5 M NaCl, 10 mM Tris-HCl, 0.1 M Na_2EDTA, 1X Triton X, 10% Dimethylsulfoxide, pH 10) at 4 °C for 1 h. At the end of lysis step, the slide was placed in an electrophoresis chamber containing alkaline electrophoresis buffer (0.3 M NaOH, 1 mM Na_2EDTA, 0.2% Dimethylsulfoxide) for 30 min to allow DNA to unwind. The electrophoresis was conducted for 30 min at 26 V and 300 mA. After completion of the electrophoresis, the DNA was neutralized by placing the slide into neutralization buffer (400 mM Tris-HCl, pH 7.5), stained with ethidium bromide, and dried by immersing in absolute ethanol. For DNA damage visualization and analysis, microscopic analysis was conducted using Olympus BX 50 microscope. Randomly chosen nuclei images ($N \geq 50$) from each slide were taken using Olympus DP11-P and categorized into ghost cells or cells damaged by cytotoxicity and comet cells or cells whose DNA was damaged by

genotoxicity. The comet cells were then analyzed using Comet Score software (TriTek Corp 2006). DNA tail lengths were measured from the exposed haemocytes, DNA tail moment was calculated (tail length x % DNA in tail), and used as parameters for determining the degree of DNA damage.

Viability of haemocyte and the values of tail length and tail moment were analyzed using the statistical package in SPSS Version 11.5 for Window. The difference of parameter among groups of treatment for each experiment was tested for normality and variance homogeneity using Shapiro-Wilk and Levene's test. For the genotoxicity test, the parameter was averaged tail length and tail moment. Significant different among group of treatments was examined using Duncan test at $P<0.05$.

3.7 Cloning and characterization of xenobiotic inducible genes in *P. monodon*

Genes reported in various animals to respond significantly to pesticide exposure or recognized as sensitive biomarkers for organophosphate pesticides were selected and subjected to cloning and characterization. These genes include carboxylesterase, cytochrome P450, beta glucuronidase, glutathione-s-transferase, heat shock protein 90, heat shock protein 70, and vitellogenin. DNA fragments from each gene were amplified by PCR using specific primers and/or degenerate primers designed from conserved regions reported in closest species. Templates used in PCR reaction were genomic DNA and first strand cDNA. After DNA fragments were obtained and subjected to BLAST analysis to confirm sequence identity, more specific primers were designed for amplifying full-length sequence of the gene using RACE-PCR technique. Protocols for each process were conducted as follow.

3.7.1 Primer design

To amplify fragments of these genes in *P. monodon*, degenerate and specific primers for PCR amplification were carried out. Primer designed specifically for each target gene is shown in Table 3.1.

For cytochrome P450, degenerate primers were designed from conserved regions of *CYP4C* from *Penaeus setiferus*, *Carcinus maenas*, and *Homarus americanus* reported in GenBank (Accession number AF072854, AY32846, and AAC32832, respectively). Primers for carboxylesterase, beta glucuronidase,

glutathione-s-transferase, and vitellogenin genes were designed from EST cDNA sequences from haemocyte and hepatopancreas libraries (HC-H-S01-1056-LF, HPa-N-S01-0351-LF, HPa-N-N01-0261-LF, and DQ288843, respectively). Primers for Heat Shock Protein 70 and Heat Shock Protein 90 genes were adopted from the study of Buethong (2004).

Table 3.1 Sequence, length and the melting temperature of primers designed for characterized genes

Gene	Sequence	Length (bp)	Tm
1. Carboxylesterase	F: 5' TAGACTCAAGGACCAAAGGA 3'	20	52.0
	R: 5' GCAGTAGCCCACGGACACAG 3'	20	61.2
2. Cytochrome P450	F1: 5' TT(T,C)ATGTT(T,C)GA(A,G) GG(A,T,C,G)CA(T,C)GA(T,C)AC 3'	23	46.0
	F2: 5' GA(A,G)AA(T,C)TG(T,C)AT (A,T,C)AA(A,G)GA(A,G)GC 3'	20	56.0
	R1: 5' (T,C)TC(A,T,C,G)GG(A,G)TT (A,T,C,G) GG(A,G)AA(T,C)TG (T,C)TC 3'	21	46.0
	R2: 5' GG(A,T,C,G)AC(A,G)TA(A,T,C,G) GC(A,G)TA(A,T,C,G)GG(A,G)TG 3'	20	62.0
3. Beta glucuronidase	F: 5' GGTAAATCGCTACTATTCCTGG 3'	22	55.9
	R: 5' CAACTCTGCTTGGTATTCTTCA 3'	22	55.1
4. Glutathione-s-transferase	F: 5' AGGTCGGTAATGCTAACGGC 3'	20	59.5
	R: 5' CGGGTCGGACGGGTAGAGTG 3'	20	64.8
5. Heat Shock Protein70	F: 5' CCTCTATCACTCGTGCTCGC 3'	20	61.9
	R: 5' GTCCCTCTGCTTCTCATCGT 3'	20	59.8
6. Heat Shock Protein90	F: 5' TCCACGAGGATTCCACCAACC 3'	21	51.2
	R: 5'TCGGCATCCGCCTTTGTCTCA 3'	21	51.2
7. Vitellogenin	F: 5' TCACCCAGAGGACGAACC 3'	18	59.6
	R: 5' GGAGATGAAGCGAAGTGTT 3'	19	55.4
8. Elongation factor - 1 alpha	F: 5' ATGGTTGTCAACTTTGCCCC 3'	20	60
	R: 5' TTGACCTCCTTGATCACACC 3'	20	60

3.7.2 PCR amplification

PCR was conducted to amplify fragments of target gene using genomic DNA extracted from pleopod and first strand cDNA created from total RNA extracted from haemocyte, gill, and hepatopancreas of *P. monodon*. PCR reaction was performed in a final volume of 25 µl, which composed of 1X buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.2 µM Forward Primer, 0.2 µM Reverse Primer, 1 U of DyNAzyme^{TM} II DNA Polymerase (Finnzymes), 200 to 1000 ng of first strand cDNA or 50 ng of genomic DNA as template. The typical PCR profiles was predenaturing at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 sec, 50-65 °C for 45 sec (depending on the melting temperature of the primer), and 72 °C for 45 sec, and a final extension at 72 °C for 5 min. Amount of first strand cDNA template and annealing temperature for each gene are shown in Table 3.2.

Table 3.2 Template and annealing temperature for gene amplification

Gene	First Strand cDNA (ng)	Annealing Temperature (°C)	PCR product (bp)
1. Carboxylesterase	500	55	204
2. Cytochrome P450	1,000	45 and 50	336, 401, 168, and 234
3. Beta glucuronidase	500	55	196
4. Glutathione-s-transferase	1,000	55	225
5. Heat Shock Protein70	500	55	719
6. Heat Shock Protein90	500	55	612
7. Vitellogenin	500	55	416
8. Elongation factor -1 alpha	200	55	500

3.8 Identification of the genes differentially expressed during chlorpyrifos exposure

Known and novel genes expressed in response to chlorpyrifos were identified using mRNA differential display reverse transcription polymerase chain reaction (mRNA DDRT-PCR). The method described in this study was based on the method of Welsh et al. (1992).

3.8.1 Exposure of shrimp to chlorpyrifos

Shrimp were exposed to chlorpyrifos in 3 concentration series: 0, 0.681, and 6.81 μg/l. Shrimp were then collected from each treatment after 72 hr of exposure (N=2). Hepatopancreas were dissected and subjected to RNA isolation and first strand cDNA synthesis.

3.8.2 First strand cDNA synthesis

Total RNA was extracted from hepatopancreas of exposed shrimp as described in 3.10.4, followed by mRNA purification using oligo(dT)-cellulose (Amersham Biosciences ®, UK). Three kinds of the first strand cDNA templates for mRNA DDRT-PCR were synthesized from 1.0 μg of purified mRNA using one of 3 one-base anchored oligo-dT primers (i.e., 5'-TTTTTTTTTTT-N-3': N is either A, G, or C). The reaction was performed in the final volume of 20 μl, at 42 °C, for 90 min using Improm II ™ reverse transcription kit condition (1 U of Improm II ™ reverse transcription, 2 μl of 1x Improm II ™ reaction buffer, 2.5 mM $MgCl_2$, 0.5 mM dNTP mix, 0.5 μg Oligo dT, and 2 U of recombinant RNasin ® Ribonuclease inhibitor).

3.8.3 DDRT-PCR

Arbitrary primers used in this study are shown in Table 3.3. The mRNA DDRT-PCR reaction was performed in the final volume of 25μl which composed of 1X buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.5 μM arbitrary primer, 0.5 μM one-base anchored oligo-dT primer (identical to the one used for 1st strand cDNA synthesis), 1 U of DyNAzyme™ II DNA Polymerase (Finnzymes), 200 ng of first strand as template. The PCR profiles was predenaturing at 94 °C for 1 min, followed by 40 cycles of 94 °C for 1 min, 42 °C for 1 min, and 72 °C for 1.5 min, and a final extension at 72 °C for 7 min.

Table 3.3 Arbitrary primers used for mRNA DDRT-PCR

Gene	Sequence (5'→3')	Length (bp)
1. OPA01	CAGGCCCTTC	10
2. OPA02	TGCCGAGCTG	10
3. OPA07	GAAACGGGTG	10
4. OPA09	GTAACGCCAT	10
5. OPA11	CAATCGCCGT	10
6. OPA14	TCTGTGCTGG	10
7. OPA15	TTCCGAACCC	10
8. OPA16	AGCCAGCGAA	10
9. OPA17	GACCGCTTGT	10
10. OPA18	AGGTGACCGT	10
11. OPB01	GTTTCGCTCC	10
12. OPB02	TGATCCCTGG	10
13. OPB03	CATCCCCCTG	10
14. OPB04	GGACTGGAGT	10
15. OPB07	GGTGACGCAG	10
16. OPB08	GTCCACACGG	10
17. OPB09	TGGGGGACTC	10
18. OPB10	CTGCTGGGAC	10
19. OPB12	CCTTGACGCA	10
20. OPB16	TTTGCCCGGA	10
21. UBC101	GCGCCTGGAG	10
22. UBC119	ATTGGGCGAT	10
23. UBC122	GTAGACGAGC	10
24. UBC128	GCATATTCCG	10
25. UBC 135	AAGCTCCGAG	10
26. UBC 158	TAGCCGTGGC	10
27. UBC 159	GAGCCCGTAG	10
28. UBC 169	ACGACGTAGG	10
29. UBC 174	AACGGGCAGC	10
30. UBC191	CGATGGCTTT	10

3.8.4 Polyacrylamide gel electrophoresis.

The products of DDRT-PCR were analyzed using polyacrylamide gel electrophoresis. Denaturing polyacrylamide gel (4.5% w/v) was used for the separation and purification of single strand fragments of DNA product. The gel was polymerized in the presence of urea that suppresses base pairing in nucleic acids. Denatured DNA migrated through the gel at the rate that was almost completely independent of its base composition and sequence.

Six microliters of PCR products were electrophoresis on Model SA Adjustable Sequencing Gel Electrophoresis system (GibcoBRL). All gels were assembled using sets of 17 x 32 cm, 0.4-mm spacers and 24 well sharktooth combs. Polyacrylamide solution, 19:1 acrylamide: bis-acrylamide, containing 1x TBE electrophoresis buffer and 7 M urea, was used to fractionate of single strand molecules. PCR products were mixed with 2 volumes of denaturing solution (98% w/v formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, and 10 mM EDTA in water), heating at 95 °C for 5 min and placing immediately on ice. Electrophoresis proceeded at constant watts (35 watts) at room temperature for 2 h. The gel was further subjected to silver staining to detect the mobility of the DNA fragment.

3.8.5 Silver staining

After electrophoresis, the gel plate was carefully removed and placed into plastic tray containing fix stop solution (10% gracial acetic acid) for 30 min. The gel was washed 3 times with deionized water for 3 min by lifting out from the tray between each wash. The gel was then placed into the covered tray containing 1.5 l of 0.1% silver nitrate and shaken for 30 min at room temperature. After silver staining, the gel was transferred into the tray containing deionized water, shaken well for 10 s, and immediately placed in the tray containing chilled developing solution (30% NaCO3, Sodiumthiosulphate, 0.55% formaldehyde). Once the band of DNA started to appear, the gel was immediately transferred into freshly prepared developing solution and shaken until all DNA bands were visualized. The gel was directly added with one liter of fix stop solution and shaken for 3 min. The stained gel was soaked with deionized water twice for 3 min and dried at 56 °C for 2-3 h.

3.8.6 Analysis of DDRT-PCR

By comparing the electrophoretic migration of the PCR products, PCR products shown differential display of gene among groups of treatment were selected for reamplification. The selected DNA fragments were purified from the gel and re-amplified using the identical primer set. The re-amplified DNA fragments are applied to agarose gel electrophoresis and then purified for cloning and sequencing analysis. The obtained nucleotide sequences are subjected to BLAST search (NCBI) to identify homologous nucleotide sequence. Finally, the obtained results are analyzed and further compare to identify the sensitive indicator for exposure to chlorpyrifos at the broad-range concentration.

3.9 Expression analysis of the genes in chlorpyrifos-exposed shrimp

Expression levels of target genes, including cytochrome P450, glucuronidase, glutathione-s-transferase, heat shock protein 70, heat shock protein 90, vitellogenin, OPA07G350-27-1 (LDL receptor member LR3), UBC101C-1,000-D-3 (esterase), UBC119A-650-F-5 (*CYP330A1*), glutathione-s-transferase, OPA18G-600-4-1 (ubiquitin-like-7), OPA01G-415-1 (leucine zipper protein 5), and OPA02G-450-2 (sequence of unknown gene) in chlorpyrifos-exposed shrimp were analyzed using semi-quantitative RT-PCR analysis. Elongation factor 1 alpha was used as internal control gene.

3.9.1 Exposure of shrimp to chlorpyrifos

Acclimated shrimp were exposed to chlorpyrifos in 5 exposure series: 0, 0.0681, 6.81, 13.62, and 27.24 µg/l. Exposed shrimp (N=3) were collected from each treatment after 12, 24, 48, 72, and 96 hr of exposure and their hepatopancreas were subjected to the total RNA extraction (3.10.1.2), DNase treatment (3.10.2), and first strand cDNA synthesis (3.10.4).

3.9.2 Semi-quantitative analysis

3.9.2.1 Primer design

Primers designed specifically for each target gene for semi-quantitative RT-PCR are shown in Table 3.4.

Table 3.4 Sequence, length and the melting temperature of primers designed for characterized genes

Gene	Sequence	Length (bp)	Tm
1. Cytochrome P450	F: 5' CACGACACCACCACGGCGGC 3' R: 5' TGTTCTCGGGCAGGAAGCGG3'	20 20	78.5 74.4
2. Beta glucuronidase	F: 5' GGTAAATCGCTACTATTCCTGG 3' R: 5' CAACTCTGCTTGGTATTCTTCA 3'	22 22	55.9 55.1
3. Heat Shock Protein70	F: 5' CCTCTATCACTCGTGCTCGC 3' R: 5' GTCCCTCTGCTTCTCATCGT 3'	20 20	61.9 59.8
4. Heat Shock Protein90	F: 5' TCCACGAGGATTCCACCAACC 3' R: 5'TCGGCATCCGCCTTTGTCTCA 3'	21 21	51.2 51.2
5. Vitellogenin	F: 5' TCACCCAGAGGACGAACC 3' R: 5' GGAGATGAAGCGAAGTGTT 3'	18 19	59.6 55.4
6. OPA07G350-27-1 (LDL receptor member LR3)	F: 5' CTCACGCACCCAAGAGG3' R: 5' CGGTCCCATTATAGTTCACAT 3'	17 21	54.3 54.4
7. UBC101C-1,000-D-3 (Esterase)	F: 5' CAACCATCCTGCCACGCTAC 3' R: 5' GCCCGAAGAACATTTCCAAG 3'	20 20	61.2 59.7
8. UBC119A-650-F-5 (*CYP330A1*)	F: 5' GTGACGAGACGGCGAAGAAA 3' R: 5' ACTGAGTGCGGGAGGCTGAA 3'	20 20	61.0 62.8
9. Glutathione-s-transferase	F: 5' AGGTCGGTAATGCTAACGGC 3' R: 5' CGGGTCGGACGGGTAGAGTG 3'	20 20	59.5 64.8
10. OPA18G-600-4-1 (Ubiquitin-like-7)	F: 5' AGAAATGTTGCCGCTCACCC 3' R: 5' ACTCTGCCACCCTTCCCACT 3'	20 20	62.3 60.6
11. OPA01G-415-1 (Leucine zipper protein 5)	F: 5' CAAGACCAGGACAAGGAAGAAT3' R: 5' AACAGAGGTTGCGGTGTAAGTG 3'	22 22	57.7 59.5
12. OPA02G-450-2 (sequence of unknown gene)	F: 5' GGGGAGTTTAGGAAGATGAGT 3' R: 5' TTATTGGTATCGCTCTGTGGT 3'	21 21	54.4 55.1
13. Elongation factor	F: 5' ATGGTTGTCAACTTTGCCCC 3' R: 5' TTGACCTCCTTGATCACACC 3'	20	60

Gene	Sequence	Length (bp)	Tm
-1 alpha		20	60

3.9.2.2 Optimization of PCR condition

Prior to the quantitative analysis, the appropriate PCR conditions including temperature, template concentration, number of cycles, and $MgCl_2$ concentration for each of target genes and reference gene were verified based on the criteria that the PCR product must be on the log phase of amplification.

PCR was performed in a PCR thermal cycler (Hybraid Limited, England). The PCR reaction was based on the standard condition consisted of 1X PCR buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl, and 0.1% Triton X-100), 0.2 mM each of dNTPs, and 1 unit of DyNAzyme^{TM} II DNA Polymerase (Finnzymes) in a final volume of 25 µl reaction. The standard PCR profiles consisted of predenaturing step at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 sec, 50-65 °C for 45 sec (depending on the melting temperature of the primers), and 72 °C for 45 sec, and a final extension at 72 °C for 5 min. The condition was optimized as follow.

First, the annealing temperature for each target gene was adjusted within several degrees to obtain the best intensity and specificity of the target band. Then, PCR reactions with various concentrations of DNA templates (between 50 to 1000 ng) and amplified in 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 applications of $MgCl_2$ and primer concentration, ranged from 1.5, 2, 3, and 5 mM for $MgCl_2$ and 0.05, 0.1, 0.15 and 0.2 µM for primers, were determined. The concentrations that gave the highest yield and specificity were chosen.

3.9.2.3 Semi-quantitative RT-PCR

Semi-quantitative RT-PCR for each target gene was conducted using the optimized condition as shown in Table 3.5. The PCR product was analyzed using agrose gel electrophoresis. The intensity of target band was examined using the

Quantity I Program (BioRad). The expression ratio of target gene and elongation factor 1 alpha gene was analyzed using statistical package in SPSS Version 11.5 for Window. The difference in expression ratio among groups of treatment was tested for normality and variance homogeneity using Shapiro-Wilk and Levene's test. Significant different among group of treatments was examined using Duncan test at $P<0.05$.

Table 3.5 Optimal condition for Semi-quantitative RT-PCR of genes in hepatopancreas of chlorpyrifos-exposed shrimp

Gene	Template (ng)	$MgCl_2$ (mM)	Primer (µM)	Annealing Temperature (°C)	PCR Cycle Number	PCR Product (bp)
1. Cytochrome P450 (CYP4C39)	1,000	1.5	0.1	65	23	355
2. Beta glucuronidase	750	1.0	0.15	60	28	196
3. Heat Shock Protein70	100	1.0	0.2	60	28	719
4. Heat Shock Protein90	750	1.5	0.1	50	30	612
5. Vitellogenin	1,000	1.0	0.15	50	30	416
6. OPA07G350-27-1 (LDL receptor member LR3)	1,000	1.0	0.1	50	30	232
7. UBC101C-1,000-D-3 (esterase)	750	1.0	0.1	55	28	410
8. UBC119A-650-F-5 (*CYP330A1*)	750	1.5	0.1	55	28	349
9. Glutathione-s-transferase	750	1.5	0.1	65	28	255
10. OPA18G-600-4-1 (ubiquitin-like-7)	1,000	1.5	0.1	65	30	217
11. OPA01G-415-1 (leucine zipper protein 5)	1,000	1.5	0.2	55	30	200
12. OPA02G-450-2 (sequence of unknown gene)	1,000	1.5	0.1	55	30	266
13. Elongation factor -1 alpha	100	1.5	0.1	55	23	500

3.10 Basic techniques for molecular study

3.10.1 Nucleic acid extraction

3.10.1.1 Genomic DNA extraction

Genomic DNA was extract from a piece of target tissue using a phenol-chloroform-proteinase K method (Klinbunga et al., 1996). A piece of frozen or fresh tissue was placed in an ice-chilled microcentrifuge tube containing 500 µl of the extraction buffer (100 mM Tris-HCl, 100 mM EDTA, 200 mM NaCl, pH 8). The tissue was briefly homogenized with a micropestel and added with SDS (10%) and RNase A (10 mg/ml) solutions to a final concentration of 1 % (w/v) and 100 µg/ml, respectively. The mixture was incubated at 37 °C for 1 h. At the end of incubation period, proteinase K solution was added to the final concentration of 300 µg/ml and further incubated at 55 °C for 3-4 h. An equal volume of buffer-equlibrated phenol: chloroform: isoamylalcohol (25:24:1) was added and gently mixed for 10 min. The solution was centrifuged at 10,000, rpm for 10 min at room temperature. The aqueous phase was transferred into a new microcentrifuge tube. The solvent exchange process was repeated once with phenol: chloroform: isoamylalcohol (25:24:1) and once with chloroform: isoamylalcohol (24:1). The aqueous phase was transferred into a new microcentrifuge tube. One-tenth volume of 3 M sodium acetate, pH 5.2 was added to the aqueous solution. Two volume of chilled absolute ethanol was added and gently mixed to precipitate genomic DNA. The mixture was kept at -80 °C for 30 min. The precipitated DNA was recovered by centrifugation at 12,000 rpm for 10 min at room temperature and washed twice with 1 ml of 70% ethanol. After centrifugation, the supernatant was removed. The DNA pellet was air-dried and resuspended in 50-80 µl of TE buffer (10 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA). The DNA solution was incubated at 37 °C for 1-2 h and kept at 4 °C until use.

3.10.1.2 RNA extraction

Total RNA was isolated from gill, haemocyte, and hepatopancreas using TRI REAGENT® (Molecular Research Center,INC). For haemocyte RNA extraction, haemocyte pellet (5-10 x 10^6 cells) was added to 0.2 ml of TRI REAGENT® and homogenized with a glass homogenizer to obtain the find homogenate. Eight hundred µl of TRI REAGENT® was further added and mixed vigorously. For gill and hepatopancreas, the tissue was ground in the mortar containing liquid nitrogen to

obtain the fine power and transferred to microcentrifuge tube containing 500 µl of TRI REAGENT®. The solution was homogenized using a glass homogenizer and added with 500 µl of TRI REAGENT®. The homogenate was vigorously mixed. Following the homogenization, the insoluble materials were removed from the solution by centrifugation at 12,000 g for 5 min at 4 °C. The clear supernatant was transferred to the new microcentrifuge tube and stored at room temperature for 5 min to permit the complete dissociation of nucleoprotein complexes. The solution was added with 0.2 ml chloroform per 1 ml of TRI REAGENT® and mixed vigorously for 15 min. The mixture was stored at room temperature for 15 min and centrifuged at 12,000 g for 15 min at 4 °C. After centrifugation, the upper clear supernatant containing RNA was transferred to the new centrifuge tube. The aqueous phase was added with 0.5 ml isopropanal and stored at room temperature to precipitate RNA. The RNA pellet was obtained by centrifugation at 12,000 g for 10 min at 4 °C. The obtained RNA pellet was washed by removal of the supernatant and adding 1 ml of 75% ethanol. The mixture was subsequently centrifuged at 12,000 g for 5 min. After centrifugation, 75% ethanol was removed and 1 ml absolute ethanol was added. The RNA pellet in absolute ethanol was kept at -80 °C until use.

3.10.2 DNAse treatment

Prior to reverse transcription of RNA to first strand cDNA, contaminated genomic DNA in RNA sample was eliminated using RQI Rnase free Dnase. The treatment was performed in a volume of 10 µl containing 10 µg of total RNA, 1X RQI Rnase free Dnase buffer (40 mM Tris-HCl pH 8.0, 10 mM $MgSO_4$, and 10 mM $CaCl_2$), and 5 units of RQI Rnase free Dnase. The mixture was incubated at 37 °C for 30 min. A volume of 500 µl of TRI REAGENT® (Molecular Research Center, INC) was added and mix vigorously at the end of incubation period to stop the Dnase activity. The mixture was further subjected to the protocol of RNA extraction as described in 3.8.2 to remove the Dnase from RNA sample.

3.10.3 Determination of Nucleic acid

3.10.3.1 Spectrophotometry

DNA and RNA can be quantified by measuring the absorbance at the wavelength of 260 nm (A_{260}). One A_{260} unit for double strand DNA, single strand

RNA, and oligonucleotide equals to 50, 40, and 33 μg/ml, respectively (Sambrook et al., 2001). The concentration of nucleic acid is calculated using the following equation:

Nucleic acid concentration (μg/ml) = A_{260} x absorbability coefficient x Dilution factor

The quality of nucleic acid was estimated by the ratio of A_{260}/ A_{280}. The isolated DNA that was free from RNA and protein, the A_{260}/ A_{280} ratio should be > 1.7.

3.10.3.2 Agarose gel electrophoresis

DNA and RNA can be determined by agarose gel electrophoresis using 1.2 to 1.75 % agarose gel. Generally, agarose gel was prepared by adding agarose powder into 1x TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid, and 2.0 mM EDTA), melt in microwave oven until completely dissolved, and then poured into the gel mould with an appropriate comb. The gel was left to solidify for at least 30 min at room temperature. The comb was gently removed and the gel was transferred into the electrophoretic chamber. TBE (1x) was added to cover the gel. Five μl of PCR products was thoroughly mixed with one-tenth volume of 10x loading dye (0.25% bromophenol blue and 25% ficoll) and carefully applied into the gel slot. Two hundred μg of 100 bp DNA ladder and/or *λ-Hind* III was used as standard DNA marker. Electrophoresis was carried out at constant voltage of 100 volts until tracking dye reach about 1 cm from the lower edge of the gel. After electrophoresis, the gel was stained with ethidium bromide (0.5 μg/ml) for 3 min and destained to remove unbound ethidium bromide by submerging in water for 20 min. The DNA fragments were visualized under the UV light using UV transiluminator. The visible bands of DNA on the stained gel were photographed using camera Pentax K1000 (Asahi Opt. Co, Ltd).

3.10.4 First strand cDNA synthesis using Reverse Transcription Polymerase Chain Reaction

Total RNA extracted from target tissues was used as template for synthesizing first strand cDNA in reverse transcription. The reaction is performed in the final

volume of 20 µl, at 42 °C, for 90 min using Improm II ™ reverse transcription kit condition (1 U of Improm II ™ reverse transcription, 2 µl of 1x Improm II ™ reaction buffer, 2.5 mM $MgCl_2$, 0.5 mM dNTP mix, 0.5 µg Oligo dT, and 2 U of recombinant RNasin® Ribonuclease inhibitor). The obtained first strand cDNA template was kept at -20 °C until use.

3.10.5 Isolation and characterization of the full length cDNA of target genes using rapid amplification of cNDA ends-polymerase chain reaction (RACE-PCR)

3.10.5.1 First strand cDNA synthesis

Total RNA extracted from hepatopancreas shrimp using TRI REAGENT® (Molecular Research Center, INC) as described in 3.8.1.2 was subjected to mRNA purification using oligo(dT)-cellulose (Amersham Biosciences ®, UK). The purified mRNA was further reversed transcribed to RACE-Ready cDNA using a BD SMART™ RACE cDNA Amplification Kit (BD Clontech). The reverse transcription was performed by mixing of 1.5 µg of hepatopancreas mRNA, 1 µl of 5' CDS primer and 1 µl of 10 µM SMART II A oligonucleotide for 5' RACE-PCR and 1.5 µg of hepatopancreas mRNA, 1 µl of 3' CDS primer A oligonucleotide for 3' RACE-PCR. The solution were gently mixed and briefly centrifuged. The reaction was incubated at 70 °C for 2 min and immediately placed on ice for 2 min. The reaction tube was briefly centrifuged and added with 2 µl of 5X first strand buffer, 1 µl of 20 mM DTT, 1 µl of dNTP Mix (10 mM each) and 1 µl of PowerScript Reverse Transcriptase. The reaction was gently pipetting and briefly centrifuged. The reaction was then incubated at 42 °C for 1.5 h. The first strand reaction products were diluted with 125 µl of Tricine-EDTA buffer and heated at 72 °C for 7 min.

Table 3.6 Primer sequence for first strand cDNA synthesis and RACE PCR

Primer	Sequence
SMART II Oligonucleotide	5' AAGCAGTGGTATCAACGCAGAGTACGCGGG 3'
3' RACE CDS Primer A	5' AAGCAGTGGTATCAACGCAGAGTAC(T)30N-1N 3' (N=A,C,G, or T; N-1=A, C, OR G)
5' RACE CDS Primer	5' (T)25N-1N 3'
10X Universal Primer A Mix (UPM)	Long: 5' CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAAC GCAGAGT 3' (0.4 μM) Short: 5' CTAATACGACTCACTATAGGGC 3' (2 μM)
Nested Universal Primer A (NUP)	5' AAGCAGTGGTATCAACGCAGAGT 3' (10 μM)

3.10.5.2 RACE-PCR

3.10.5.2.1 Primer design

Gene specific primers (GSPs), including carboxylesterase, cytochrome P450, and glutathione-s-transferase were design from the obtained nucleotide sequence resulting from cloning and sequencing analysis. Primer designed specifically for each target gene is shown in Table 3.7.

Table 3.7 Sequence, length and the melting temperature of gene specific primers for RACE PCR

Gene	Sequence	Length (bp)	Tm
1. Carboxylesterase	3' RACE F: 5'ATTTTCTACCCGACTACCCCGCCGTTCT 3'	28	73.5
	5'RACE R: 5' CGCCGAGGTCACGGATGTTGTCTTG 3'	25	73.4
2. Cytochrome P450	3' RACE F: 5' CACGACACCACCACGGCGGC 3'	20	78.5
	5'RACE R: 5'CTCCTGGTGGACCCGAGCCTGTATCT 3'	26	80.7

Gene	Sequence	Length (bp)	Tm
3. Glutathione-s-transferase	3' RACE F: 5'TGGGCACGCTCTACCACAGGTTCGGGGA 3'	28	80.5
	5'RACE R: 5'AGGGCACGAGTCTTCGGGTCGGACGGGT 3'	28	81.3

3.10.5.2.2 PCR amplification

Master mix of 5' and 3' RACE PCR reaction was prepared in a volume of 42.75 µl for each reaction. The mixture contained 5 µl of 10X Advantage 2 PCR buffer, 1 µl of dNTP mix (10 µM each) and 1 µl of 50X Advantage 2 polymerase mix. The PCR reaction condition for 5' and 3' cDNA ends are shown in Table 3.8.

PCR reaction was performed for 20 cycles of 94 °C for 30 sec, 68 °C for 30 sec, and 72 °C for 3 min. The 5' and 3' RACE-PCR products were analyzed using agarose gel electrophoresis. The positive product was eluted and subjected to cloning and sequencing analysis as described in 3.10.6.

Table 3.8 Component of RACE-PCR

Component	5' RACE-Sample (µl)	3' RACE-Sample (µl)
5' RACE-Ready cDNA	1.25	--
5' GSP (10 µM)	1	--
3' RACE-Ready cDNA	--	1.25
3' GSP (10 µM)	--	1
UPM	0.5	0.5
H2O	1	1
Master mix	42.75	42.75
Final volume	50	50

3.10.6 Cloning and Sequencing

3.10.6.1 DNA preparation

The target bands of DNA product were excised from agarose gel using a sterile razor, placed in a microcentrifuge tube, and weighed. DNA was eluted from agarose gel using QIAquick gel extraction kit (QIAGEN) following manufacturer protocol. Three gel volume of QG buffer added and incubated at 50 °C for 10 min. During the incubation period, the mixture was vortexed briefly every 2-3 min. One gel volume of isopropanol was added into the mixture after the gel was completely dissolved. The mixture was gently mixed and transferred to the QIAquick spin column placed on the collection tube and centrifuged at 13,000 rpm for 1 min at room temperature. The flow-through in the collection tube was discarded. Another 0.5 ml of QG buffer was added to the QIAquick column and centrifuged at 13,000 rpm for 1 min. The flow-through solution was discarded. A volume of 0.75 ml PE buffer was added to the QIAquick column and centrifuged at 13,000 rpm for 1 min. The flow-through solution was discarded. The column was recentrifuged to remove the trace amount of washing solution. The QIAquick column was placed onto a new microcentrifuge tube. DNA was eluted out by addition of 10-15 µl of elution buffer (10 mM Tris-HCl, pH 8.5) to the center of the QIA quick membrane and left for 1-2 min at room temperature before centrifugation at 13,000 rpm for 2 min. The eluted sample was kept at -20 °C until use.

3.10.6.2 Preparation of competent cells

Competent *E. coli* strain JM109 cells were prepared following Ausubel et al. (1989) with modification. A single colony of *E. coli* was inoculated into 5 ml of sterile LB broth (1% Bacto try ptone, 0.5% Bacto yeast extract, and 0.% NaCl) and incubated at 37 °C overnight with shaking. The culture was sub-inoculated by adding 1 ml of the culture into 50 ml of LB broth and incubated with vigorous shaking at 37 °C until the OD_{600} was approximately 0.4-0.6. The culture was then placed on ice for 30 min and centrifuged at 3,000 rpm for 10 min. The supernatant was discarded before the cell pellet was resuspended with 30 ml of chilled $MgCl_2$-$CaCl_2$ solution (80 Mm $MgCl_2$ and 20 Mm $CaCl_2$) and kept on ice for 45 min. The solution was then centrifuged at 3,000 rpm for 10 min. The supernatant was discarded and cell pellet was resuspended with 2 ml of chilled 0.1 M $CaCl_2$ solution containing (15%

glycerol). The cell suspension was divided into 200 µl aliquots in microcentrifuge tubes and stored at -80 °C for subsequently used.

3.10.6.3 Ligation of PCR product to pGEM-T easy vector

The ligation of DNA product to pGEM-T easy vector was performed in a final volume of 10 µl containing 3 µl of gel-eluted PCR product, 25 ng of pGEM-T easy vector, 5 µl of 2x rapid ligation buffer (60 mM Tris-HCl pH 7.8, 20 mM DTT, 2 mM ATP and 10% PEG 8000) and 3 Weiss unit of T4 DNA ligase. The ligation solution was gently mixed by pipetting and incubated at 4 °C overnight.

3.10.6.4 Transformation of ligation product to competent cells

A volume of 200 µl of competent cells was thawed on ice for 5 min and added with 5 µl of ligated DNA product. The solution was gently mixed by pipetting and placed on ice for 30 min. The mixture was placed in a 42 °C water bath for 45 sec and immediately removed and placed on ice for 5 min. The solution was added in a test tube containing 1 ml of SOC medium (2% Baco tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) The solution was incubated at 37 °C with vigorous shaking for 1.5 h. The solution was transferred to a microcentrifuge tube and centrifuged at 8,000 rpm for 1 min at room temperature. The supernatant was discarded and the cell pellet was resuspended with 100 µl of SOC medium. The cell solution was spread on LB agar 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 were observed as white colony whereas the clones without inserted DNA were blue colony.

3.10.6.5 Detection of recombinant clone using Colony PCR

The recombinant colony was screened for the size of inserted DNA using colony PCR. The PCR was performed in a volume of 25 µl containing 1x buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100), 1.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP, dTTP, 0.2 µM of pUC1 (5'-CCGGCTCGTATGTTGTGTGGA-3') and pUC2 (5'-GTGCTGCAAGGCGATTAAGTTGG-3') primers and 0.5 U of DyNAzyme™ II DNA Polymerase (Finnzymes). Individual of recombinant colony was picked using

micropipette tip and mixed in the amplification reaction. The PCR profiles was predenatured at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 sec, 55 °C for 60 sec, and 72 °C for 90 sec, and a final extension at 72 °C for 7 min. The resulting PCR product was analyzed using agarose gel electrophoresis.

3.10.6.6 Digestion of the amplified DNA insert from colony PCR

The recombinant clones showing expectected size of target DNA insert were subjected to *Hind*III and *Rsa*I digestion to examine sequence pattern of DNA insert as a criterion for clone selection for plasmid DNA preparation. The digestion was performed in a volume of 15 µl reaction containing restriction enzyme buffer (buffer E; 6 mM Tris-HCl, pH 7.5, 6 mM MgCl2, 100 mM NaCl, and 1 mM DTT for *Hind*III and buffer C; 10 mM Tris-HCl, pH 7.9, 10 mM MgCl2, 50 mM NACl, and 1 mM DTT for *Rsa*I), 5 µl of colony PCR product, and 2 unit of *Hind*III or *Rsa*I. The mixture was incubated at 37 °C for 3-4 h. The digestion pattern was analyzed using agaroe gel electrophoresis.

3.10.6.7 Plasmid DNA preparation and restriction enzyme digestion

A recombinant clone was inoculated into 3 ml of LB broth containing 50 µg/ml ampicillin and incubated at 37 °C overnight with vigorous shaking. The cultured cells were transferred to a microcentrifuge tube and harvested by centrifugation at 12,000 rpm for 1 min at room temperature. Plasmid DNA was isolated using QIAprep® Miniprep Kit (QIAGEN GmbH, D-40724 Hilden). The supernatant was discarded and the cell pellet was resuspended with 250 µl of P1 resuspension buffer (containing Rnase A) by vortexing. The mixure was added with 250 µl of P2 lysis buffer (containing sodium hydroxide), gently mixed and placed on ice for 10 min. A volume of 350 µl of N3 Neutralization buffer (containing guadinine hydrochloride) was added and gently mixed. The mixture was centrifuged at 15,000 rpm for 15 min at 10 °C to separate cell debris. The supernatant was transferred to a new microcentrifuge tube and one additional centrifugation at 15,000 rpm for 10 min at 10 °C was conduct to remove the residue cell debris. The supernatant was transfer to the QIAprep column placed on the collection tube and centrifuged at 13,000 rpm for 1 min at room temperature. The flow-through solution was discarded and a volume of 750 µl of PE wash buffer (containing ethanol) was added to wash plasmid

DNA. The column was centrifuged at 13,000 rpm for 1 min at room temperature. The flow-through was discarded and one additional centrifugation at 14,000 rpm for 2 min at room temperature was conduct to remove the residue of PE buffer. The column was placed on a new microcentrifuge tube and a volume of 50 µl of elution buffer (10 mM Tris-HCl, pH 8.5) was added. The column was stored for 2 min at room temperature and centrifuged at 14,000 rpm for 2 min to elute plasmid DNA.

The insert size of recombinant plasmid was examined by digestion with *ECo*RI. The digestion was performed in a volume of 15 µl reaction containing 1x restriction buffer (90 mM Tris-HCl; pH 7.5, 10 mM NaCl, and 50 mM MgC12), 1 µg of recombinant plasmid and 2-3 units of *ECo*RI and incubated at 37 °C for 3-4 h. The resulting digestion was analyzed using agarose gel electrophoresis.

3.10.6.8 DNA Sequencing and analysis

The cloned DNA fragments were sequences by automated DNA sequencer using M13 forward and/or M13 reverse primers as the sequencing primer by MACROGEN (Korea). The obtained nucleotide sequences were subjected to BLAST search (NCBI) to identify homologous nucleotide sequence.

3.11 Statistical Analysis

Variables of each experiment were typically analyzed using the statistical package in SPSS Version 11.5 for Window. The difference of each variable among groups of treatment was tested for normality and variance homogeneity using Shapiro-Wilk and Levene's test. Significant different among group of treatments was examined using Duncan test at $P<0.05$.