CHAPTER III

RESULTS

3.1 DNA Extraction

Genomic DNA was extracted from the muscle of *P.monodon*. The quality and quantity of extracted DNA were determined by electrophoresis on 0.8% agarose gel (w/v) comparing to an undigested lamda DNA. High molecular weight genomic DNA was located at approximately 23.1 kb, indicating good quality of extracted genomic DNA (Fig.3.1). The concentration of genomic DNA was spectrometically determined at 260 nm. The OD_{260}/OD_{280} ratio of extracted DNA ranged from 1.8-2.0 indicating that the purity of DNA samples was acceptible.

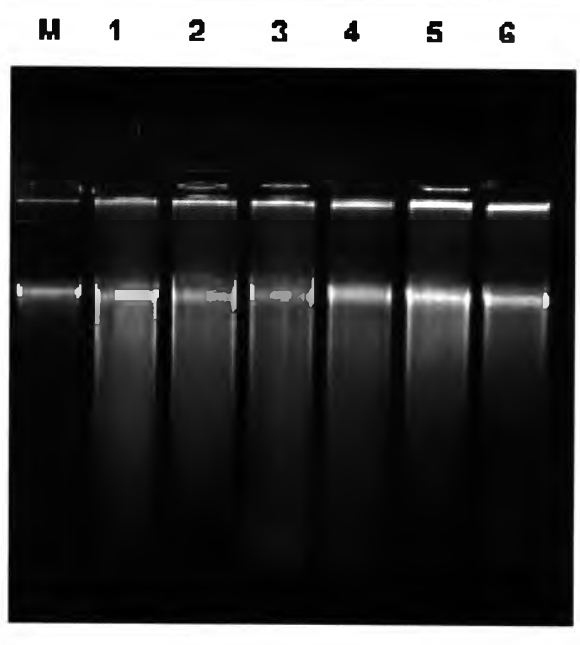

Figure 3.1 A 0.8% ethidium bromide strained agarose gel showing the quality of genomic DNA extracted from the muscle of *P.monodon*. Genomic DNA was individually extracted from muscle of *P.monodon* (lane 1-6). Lane M is 100 ng of undigested lamda DNA marker.

3.2 RNA Extraction

RNA was extracted from haemocyte and gill of *P.monodon*. The quality and quantity of extracted RNA were determined by electrophoresis on 1.2% agarose gel (w/v). The extracted RNA showed 18s, 5.8s and 5s rRNA which located at approximately 1.9, 0.16 and 0.12 Kbp, respectively, indicating good quality of RNA (Fig. 3.2). The concentration of RNA was spectrometically determined at 260 nm. The OD_{260}/OD_{280} ratio of extracted RNA was 2.0 indicating that the purity of RNA samples was acceptible. RNA was used as the template for 1st strand cDNA systhesis, which was then used as the template for semiquantitative RT-PCR.

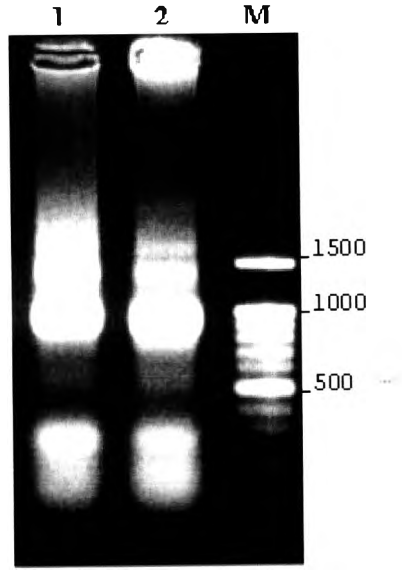

Figure 3.2 A 1.2% ethidium bromide strained agarose gel showing the quality of RNA extracted from haemocyte and gill of *P.monodon* (lane 2 and 3, respectively). Lane M is λ*Hin*dIII marker.

3.3 PCR amplification of MnSOD, Cu/ZnSOD and AK

Partial sequences of Mn-SOD, AK and Cu/Zn-SOD genes were amplified from 1st strand cDNA templates of hepatopancreas, muscle, haemocytes, heart, digestive tract and gills. PCR products were determined by electrophoresis on 1.2% agarose gel (w/v) (Fig.3.3).

DNA fragments at the size of 500 bp were obtained from PCR amplification of AK in all target tissues. High intensities of the DNA bands were observed in gill, heart and haemocytes indicating high expression levels in these tissues whereas moderate intensities were found from hepatopancreas and muscle, respectively. Faint band of DNA product was detected in sample from digestive tract revealing low expression of AK in this tissue.

For Cu/Zn-SOD amplification, DNA products at the size of 600 bp were amplified from degenerated primers in all tissues. PCR amplification of samples from muscle and haemocyte showed high intensities of DNA bands while that of hepatopancreas and heart were clearly lower. Only faint band was detected in digestive tract. The size of DNA products was about 2 folds larger than the expected DNA fragment (the expected size was 272 bp), indicating a high possibility that the product was not Cu/Zn-SOD. After sequencing and BLAST analysis, this PCR product was identified as cytochrome c oxidase subunit 3 of *P.monodon* with 89 % similarity. (Fig.A4, Appendix B).

For Mn-SOD gene amplification, PCR products were amplified at the size of 700 bp showing the same level of band intensity in all 6 tissues indicating high expression level of this gene in all tissues of the shrimps.

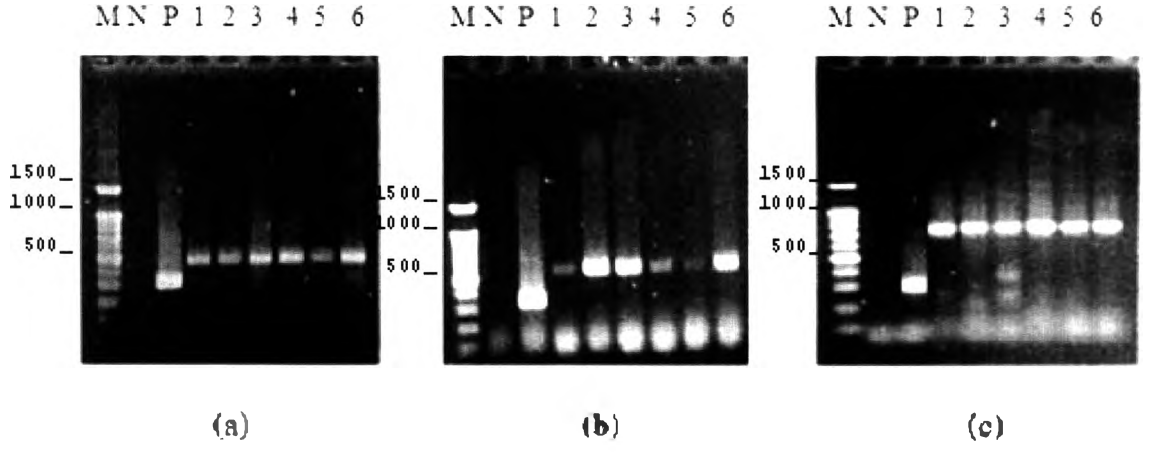

Figure 3.3 PCR products of AK (a), Cu/Zn-SOD (b) and Mn-SOD genes (c) separated in 1.2% agarose gel. PCR products amplified from hepatopancreas, muscle, haemocytes, heart, digestive tract and gill (lane 1-6, respectively. Lane M is 100 bp marker, lane N is negative control and lane P is positive control.

3.4 Polymorphisms of Mn-SOD and AK genes

Variation of partial DNA sequences of Mn-SOD and AK were determined in a number of tissues including hepatopancreas, muscle, heart, haemocyte, digestive tract and gill. Purified PCR products of Mn-SOD and AK genes were subjected to SSCP analysis.

3.4.1 Polymorphisms of Mn-SOD gene

PCR products of Mn-SOD at the size of 731 bp from all 6 tissues were separated in 10% acrylamine gel at 300 V. for 10 hours. Four bands of single strand DNA were detected (Fig 3.4).

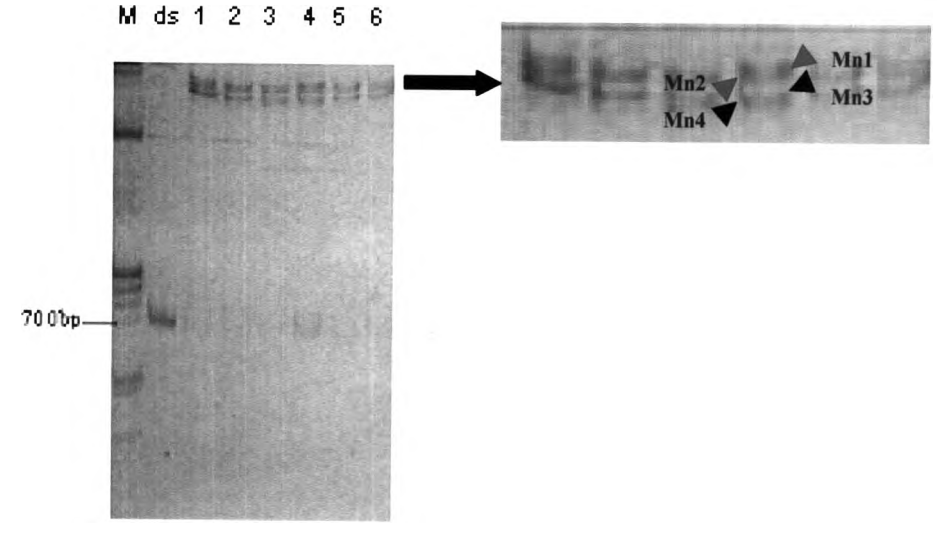

Figure 3.4 DNA sequence of Mn-SOD determined on 10% acrylamide gel by SSCP technique and stained with silver solution.

Lane M	=	100 bp ladder
Lane ds	=	Double stand DNA of Mn-SOD
Lane 1-6	=	Single stand DNA of Mn-SOD from hepatopancreas, muscle, heart, haemocyte, digestive tract and gill of *P.monodon*, respectively.

3.4.2 Polymorphisms of AK gene

PCR products of AK at the size of 538 bp were separated in 10% acrylamine gel at 300 V. for 10 hours. Two 2 bands were detected from all 6 tissues (Fig 3.2). These 2 bands were light and heavy strands of DNA fragment, indicating that there was no polymorphism of AK gene.

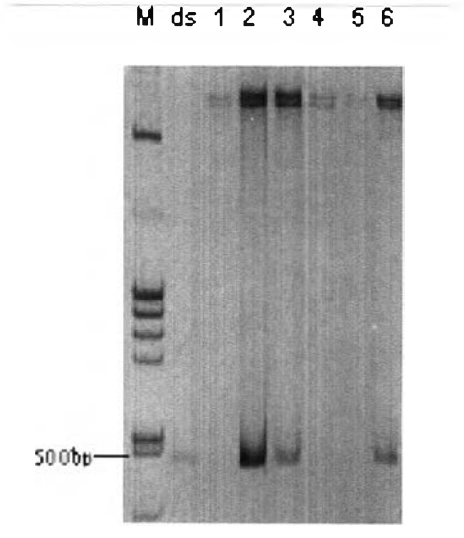

Figure 3.5 Nucleotide sequence of AK determine on 10% acrylamide gel by SSCP technique and stained with silver solution.

Lane M	=	100 bp ladder
Lane ds	=	Double stand DNA of AK
Lane 1-6	=	Single stand DNA of AK from hepatopancreas, muscle, heart, haemocyte, digestive tract and gill of *P.monodon*, respectively.

3.5 Variation of Mn-SOD DNA sequences

Four bands of Mn-SOD separated by SSCP were isolated, reamplified, cloned, and subjected to sequence analysis. The comparisons of DNA and amino acid sequences from all 4 fragments were shown in Fig 3.6 and 3.7. There were 12 positions of amino acid residues that were different among Mn-SOD DNA sequences. Protein sequence alignment of deduced *P.monodon* Mn-SOD obtained from this study and Mn-SOD retrieved from GenBank databases were shown in Fig 3.9. As the results, All 4 Mn-SOD fragments were different from each other and they were also different from the sequences reported elsewhere. This indicates the high polymorphism of Mn-SOD in *P.monodon*.

```
Mn3_reverse_complement      CAGAGGCTGCCAATGA-TGTGAATGCAA-TGAATGCC-CTTCTACCAGCT
Mn4_reverse_complement      CAGAGGCTGCCAATGA-TGTGAATGCAA-TGAATGCC-CTTCTACCAGCT
Mn1_reverse_complement      CAAAGGCTGCCAATGAGTGTGAATGCAAGTGAATGCCGCTTCTACCAGCT
Mn2_reverse_complement      CAGAGGCTGCCAATGA-TGTGAATGCAA-TGAAGGCC-CTTCTACCAGCT
                            ** ************ *********** **** *** ************

Mn3_reverse_complement      ATCAAGTTCAATGGAGGTGGCCACTTGAACCACACCATCTTCTGGACCAA
Mn4_reverse_complement      ATCAAGTTCAATGGAGGTGGCCACTTGAACCACACCATCTTCTGGACCAA
Mn1_reverse_complement      ATCAAGTTCAATGGAGGTGCCCACTTGAACCACACCATCTTCTGGACCAA
Mn2_reverse_complement      ATCAAGTTCAATGGAGGTGCCCACTTGAACCACACCATCTTCTGGACCAA
                            ****************** ******************************

Mn3_reverse_complement      CATGGCTCCTGATGCTGGTGGTGAGCCAGAAGGAGCAATTGCACAAGCCA
Mn4_reverse_complement      CATGGCTCCTGATGCTGGTGGTGAGCCAGAAGGAGCAATTGCACAAGCCA
Mn1_reverse_complement      CATGGCTCCTGATGCTGGTGGTGAGCCAGAAGGAGCAATTGCACAAGCCA
Mn2_reverse_complement      CATGGCTCCTGATGCTGGTGGTGAGCCAGAAGGAGCAATTGCACAAGCCA
                            **************************************************

Mn3_reverse_complement      TTGACGATAGCTTTGGATCATTCCAGTCCTTTAAGGACAAATTTTCTGCT
Mn4_reverse_complement      TTGTCGATAGCTTTGGATCATTCCAGTCCTTTAAGGACAAATTTTCTGCT
Mn1_reverse_complement      TTGATGATAGCTTTGGATCATTCCAGTCCTTTAAGGACAAATTTTCTGCT
Mn2_reverse_complement      TTGATGATAGCTTTGGATCATTCCAGTCCTTTAAGGACGAATTTTCTGCT
                            ***  ********************************** ***********

Mn3_reverse_complement      GCCAGCGTTGGAGTGAAAGGCTCTGGCTGGGGATGGCTCGGGTATTGCCC
Mn4_reverse_complement      GCCAGCGTTGGAGTGAAAGGCTCTGGCTGGGGATGGCTCGGGTATTGCCC
Mn1_reverse_complement      GCCAGCGTTGGAGTGAAAGGCTCTGGCTGGGGATGGCTCGGGTATTGCCC
Mn2_reverse_complement      GCCAGCGTTGGAGTGAAAGGCTATGGCTGGGGATGGCTCGGGTATTGCCC
                            ********************** *************************

Mn3_reverse_complement      CAATAACAACAAGCTTGAGATCGCCACTTGCCAGAACCAGGATCCCTTGC
Mn4_reverse_complement      CAATAACAACAAGCTTGAGATCGCCACTTGCCAGAACCAGGATCCCTTGC
Mn1_reverse_complement      CAATAACAACAAGCTTGAGATCGCCACTTGCCAGAACCAGGATCCCTTGC
Mn2_reverse_complement      CAATAACAACAAGCTTGAGATCGCCACTTGCCAGAACCAGGATCCCTTGC
                            **************************************************

Mn3_reverse_complement      AGATCACTCATGGCCTGGTTCCATTGCTCGGTCTTGA
Mn4_reverse_complement      AGATCACTCATGGCCTGGTTCCATTGCTCGGTCTTGA
Mn1_reverse_complement      AGATCACTCATGGCCTGGTTCCATTGCTCGGTCTTGA
Mn2_reverse_complement      AGATCACTCATGGCCTGGTTCCATTGCTCGGTCTTGA
                            ************************************
```

Figure 3.6 Multiple alignments of Mn-SOD DNA fragments of *P. monodon*

```
Mn3             SEAANDVNAMNALLPAIKFNGGGHLNHTIFWTNMAPDAGGEPEGAIAQAIDDSFGSFQSF
Mn4             SEAANDVNAMNALLPAIKFNGGGHLNHTIFWTNMAPDAGGEPEGAIAQAIVDSFGSFQSF
Mn2             SEAANDVNAMKALLPAIKFNGGAHLNHTIFWTNMAPDAGGEPEGAIAQAIDDSFGSFQSF
Mn1             SKAANECECKMPLLPAIKFNGGAHLNHTIFWTNMAPDAGGEPEGAIAQAIDDSFGSFQSF
                *:***: :.  .**********.*************************** *********

Mn3             KDKFSAASVGVKGSGWGWLGYCPNNNKLEIATCQNQDPLQITHGLVPLLGL
Mn4             KDKFSAASVGVKGSGWGWLGYCPNNNKLEIATCQNQDPLQITHGLVPLLGL
Mn2             KDEFSAASVGVKGYGWGWLGYCPNNNKLEIATCQNQDPLQITHGLVPLLGL
Mn1             KDKFSAASVGVKGSGWGWLGYCPNNNKLEIATCQNQDPLQITHGLVPLLGL
                **:********** ************************************
```

Figure 3.7 Multiple alignment of deduced amino acid sequence of Mn-SOD of *P. monodon*

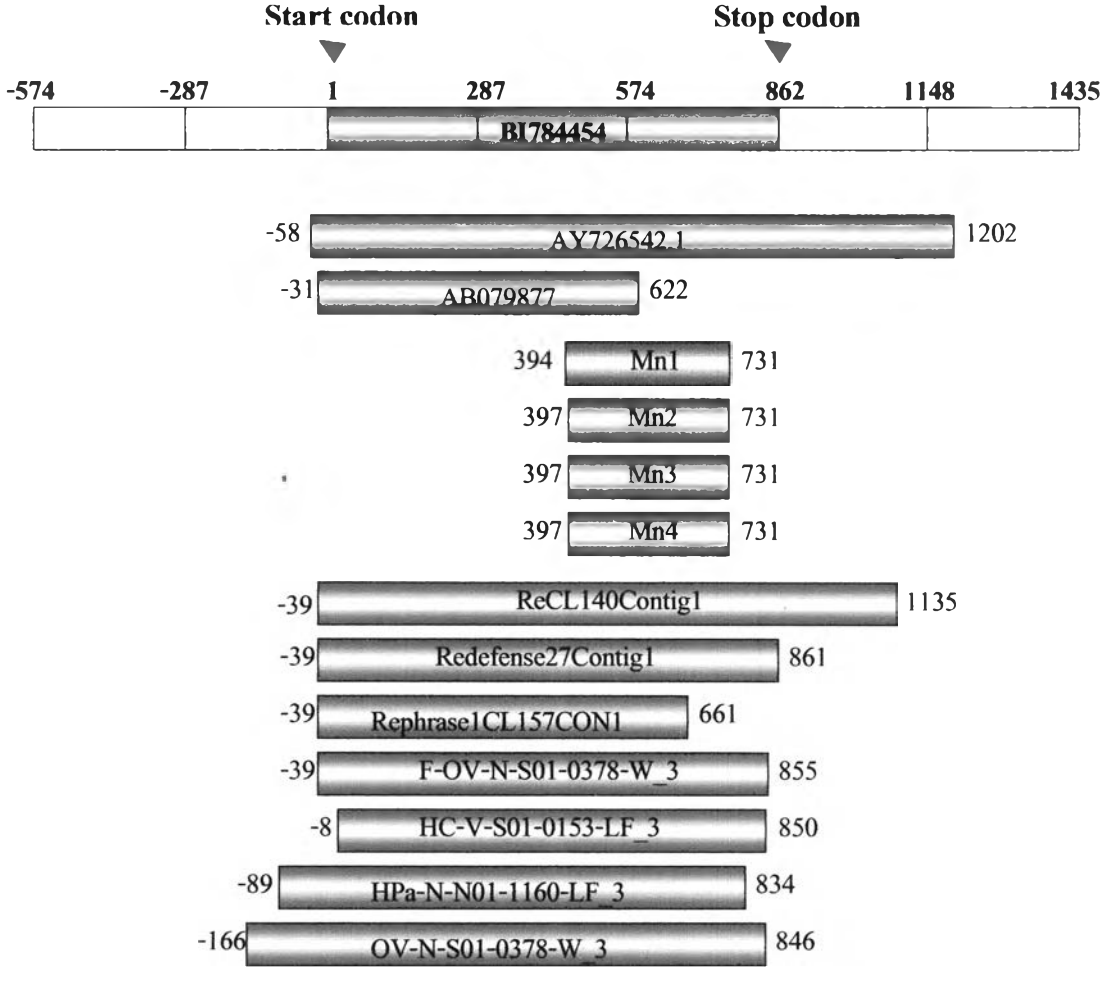

Figure 3.8 Schematic representation of the structure of full length of Mn-SOD gene. Complete coding sequence (nucleotide 1-862) construct from the sequence of Genbank (Red boxes), PCR products of Mn-SOD specific primer (Green boxes) and partail

sequence from EST cDNA library of *P.monodon* (Pink boxes). Colorlaess boxes represent 5' and 3' untranslated regions

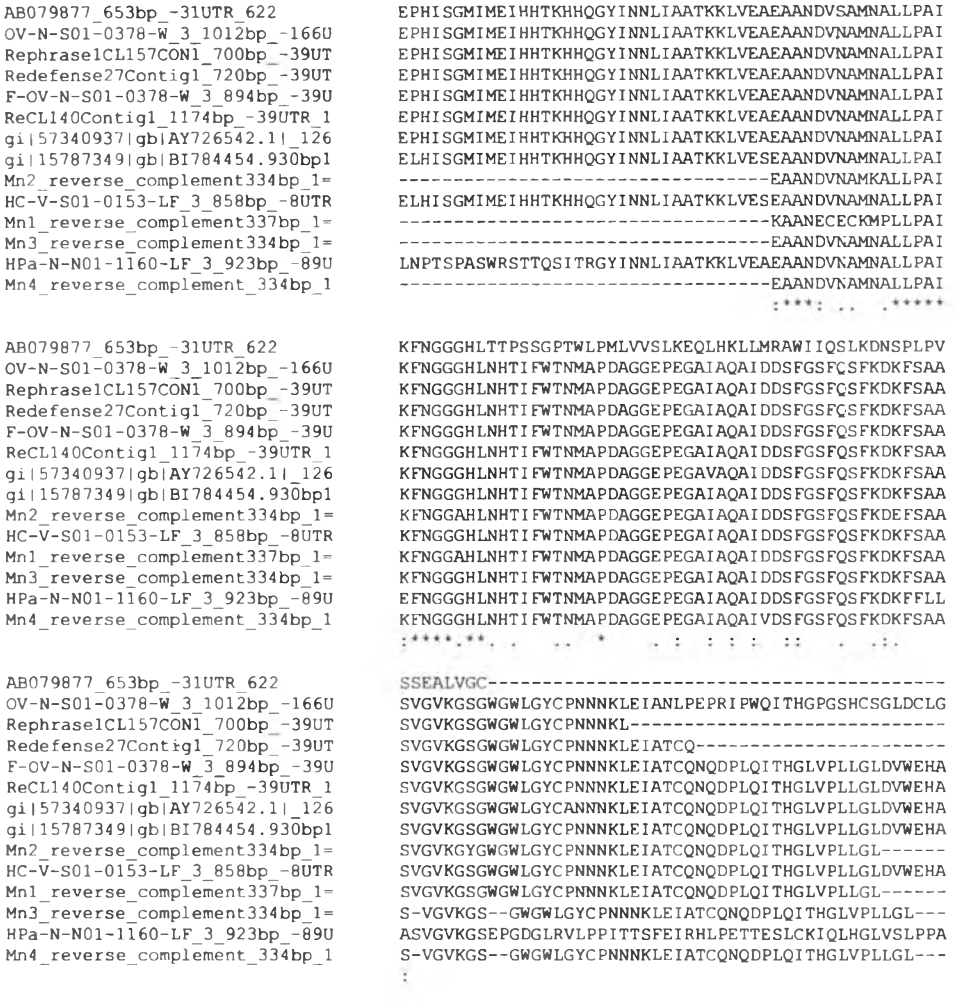

Figure 3.9 Multiple alignment deduce amino acid of Mn-SOD of *P. monodon* retrieved from Genbank, PCR product amplified with Mn-SOD specific primer and EST library of *P.monodon*.

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

Expression levels of stress responsive genes including Mn-SOD, AK, HSP70, HSP90, DADI and thioredoxin peroxidase genes in the shrimps under oxidative, osmotic, and handling stress conditions were quantitatively analyzed using semiquantitative RT-PCR. Two important steps were carried out: optimization of PCR conditions for each gene and quantitative measurement of gene expression.

3.6.1 Optimization of Semi quantitative PCR condition

Prior to quantitative measurement, appropriate PCR condition for each gene including actin gene, which is a reference gene, was optimized. The condition for semi-quantitative detection was chosen on the criteria that the PCR product was on the log phase of amplification. These PCR condition needed to be optimized included annealing temperature, number of cycles, DNA template and $MgCl_2$ concentrations.

Primers for β-actin amplification used in this study were adopted as described earlier by Traisresilp (2002). The sequence of this genes and primer location was shown in Fig. 3.10. Primers for HSP70 and HSP90 genes were adopted from protocol described by Buathong (2003), and primers for DADI and Thioredoxin peroxidase were adopted from protocol described by from Buaklin (2005). After optimization, the appropriate conditions for semi-quantitative RT-PCR for Mn-SOD, AK, HSP70, HSP90, DADI, Thioredoxin peroxidase and actin genes were obtained and the results were shown in table 3.1

Appropriate conditions for semi-quantitative RT-PCR of Mn-SOD, AK, HSP70, HSP90, DADI, Thioredoxin peroxidase and Actin genes were as follow: annealing temperature were 60, 60, 65, 55, 55, 55, and 55°C, respectively; template concentrations were 100, 100, 25, 100, 100, 100, and 5 ng, respectively; the number of PCR cycles were 25, 25, 25, 25, 30, 30, and 25 cycles, respectively. (Fig.3.11-3.24)

Table 3.1 Summary of the optimal conditions of semi-quantitative RT-PCR for determining the expression of stress responsive genes

Gene	Template concentration (ng)	Annealing temperature (°C)	Number of PCR cycle	$MgCl_2$ concentrations (mM)
Mn-SOD	100	60	25	2
Arginine kinase	100	60	25	2
HSP70	25	65	25	2
HSP90	100	55	25	2
DADI	100	55	30	2
Thioredoxin peroxidase	100	55	30	2
Actin	5	55	25	2

```
1    GGGACTGGGGTACTCCTACACTCATAAACCAACGACATCATGTGTGACGACGAGGATCTTACTGC   65
66   CCTTGTGGTTGACAATGGCTCCGGCCTTTGCAAGGCCGGCTTCGCCGGAGACGACGCCCCTCGTG  130
131  CCGTCTTCCCCTCCATCGTCGGCCGTGCCCGTCATCAGGGTGTGATGGTCGGTATGGGTCAGAAG  195
                                                Actin 1
197  GACGCCTACGTCGGTGATGAGGCCCAGAGCAAACGTGGTATCCTCACCCTCAAGTACCCCATTGA  260
261  ACACGGTATCATCACCAACTGGGATGACATGGAGAAGATCTGGTACCATACTTTCTACAATGAGC  325
326  TCCGTGTTGCCCCCGAGGAGTCCCCCACACTTCTCACTGAGGCTCCCCTCAACCCCAAGGCCAAC  390
391  CGTGAGAAGATGACTCAGATCATGTTCGAGTCCTTCAATGTACCTGCCACTTACATTACCATCCA  455
456  GGCTGTGCTCTCCCTCTACGCCTCTGGTCGTACTACCGGTGAGGTTTGCGACTCTGGTGATGGTG  520
                    Actin 2
521  TGACTCACTTTGTCCCCGTCTATGAAGGTTTCGCTCTTCCTCATGCTATCCTTCGTCTCGATCTT  585
586  GCTGGTCGTGACCTGACCCACTATCTGATGAAGATCATGACTGAGCGTGGCTACTCCTTCACCAC  650
651  CACCGCTGAACGTGAAATCGTTCGTGACATCAAGGAGAAGCTTTGCTACATTGCCCTTGACTTCG  715
716  AGAGTGAGATGAACGTTGCTGCTGCTTCCTCCTCCTTGGACAAGTCATACGAGCTTCCCGACGGC  780
781  CAGGTCATCACCATTGGTAACGAGCGTTTCCGCTGCGCTGAAGCTCTGTTCCAGCCTTCCTTCCT  845
846  TGGTATGGAATCTGCTGGTATTCAGGAAACCGTCCACAGCTCCATCATGAGGTGTGACATTGACA  910
911  TCAGGAAGGACCTGTTCGCCAATATCGTCATGTCTGGTGGTACCACCATGTACCCTGGTATTGCT  975
976  GACCGCATGCAGAAGGAAATCACTGCTCTTGCTCCTTCCACCATCAAGATCAAGATCATTGCTCC 1040
1041 TCCTGAGCGTAAGTACTCCGTCTGGATCGGTGGTTCCATCCTGTCTTCTCTGTCCACCTTCCAGT 1105
1106 CCATGTGGATCACCAAGGATGAGTACGAAGAGTCTGGTCCCGGCATTGTCCACCGCAAGTGCTTC 1170
1171 TAAATGGAGATTGACAACTTTTACTACAGTTGATAATAAAATTCCGAAACATC              1223
```

Figure 3.10 DNA sequence of β-actin gene. The highlight shows the position of Actin1 and Actin2 primer.

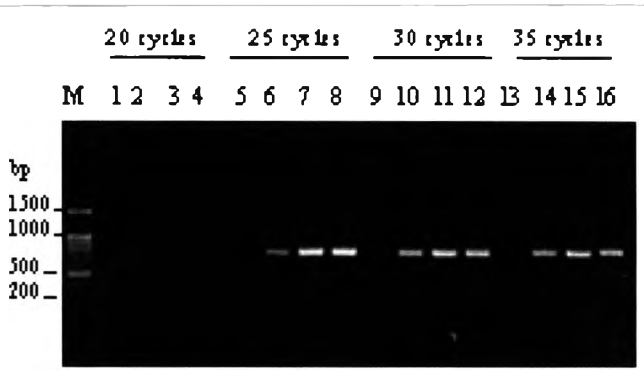

Figure 3.11 PCR product of Mn-SOD amplification determined on 1.2% agarose gel and stained with Ethidium bromide. PCR reaction was conducted on 20 cycles (lane 1-4), 25 cycles (lane 5-8), 30 cycles (lane 9-12) and 35 cycles (lane 13-16). The template concentration in each reaction was 10, 100, 500, and 1000 ng, respectively (1-4). A 100 bp DNA standard was shown in lane M.

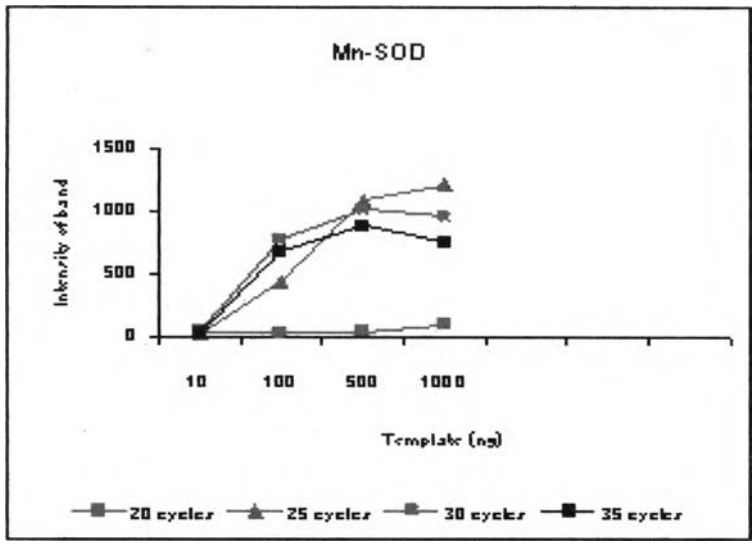

Figure 3.12 Relationship between PCR products of Mn-SOD gene amplified from haemocyte cell of *P.monodon* and various amount of template used in PCR reaction.

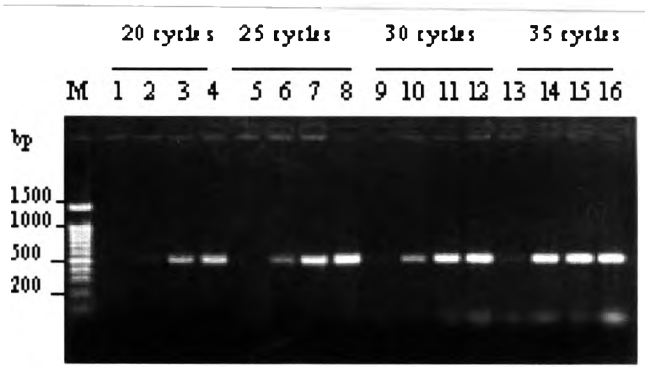

Figure 3.13 PCR product of AK amplification determined on 1.2% agarose gel and stained with Ethidium bromide, PCR reaction was conducted on 20 cycles (lane 1-4), 25 cycles (lane 5-8), 30 cycles (lane 9-12) and 35 cycles (lane 13-16). The template concentration in each reaction was 10, 100, 500, and 1000 ng, respectively (1-4). A 100 bp DNA standard was shown in lane M.

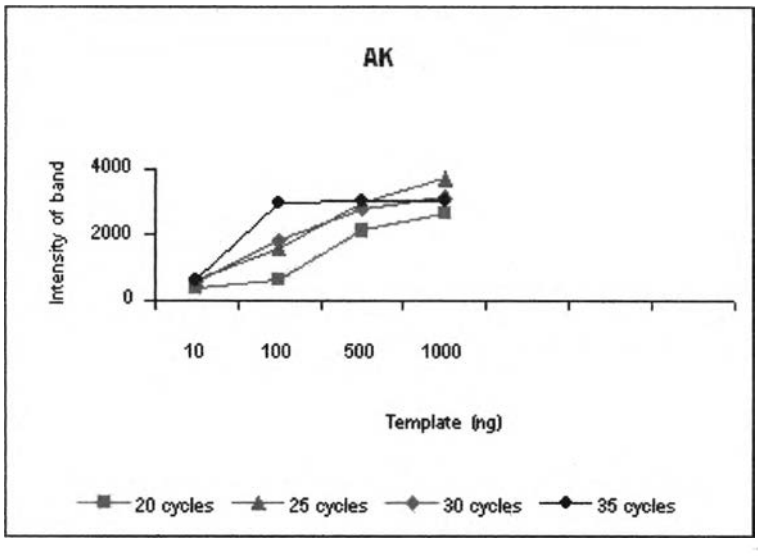

Figure 3.14 Relationship between PCR products of AK gene amplified from haemocyte cell of *P.monodon* and various amount of template used in PCR reaction.

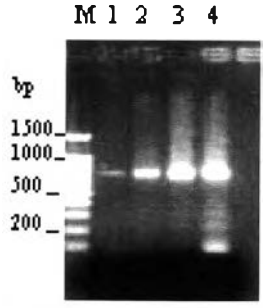

Figure 3.15 PCR product of HSP70 amplification determined on 1.2% agarose gel and stained with Ethidium bromide, PCR reaction was conducted on 20 cycles (lane 1), 25 cycles (lane 2), 30 cycles (lane 3) and 35 cycles (lane 4). The template concentration in each reaction was 25 ng. A 100 bp DNA strandard was shown in lane M.

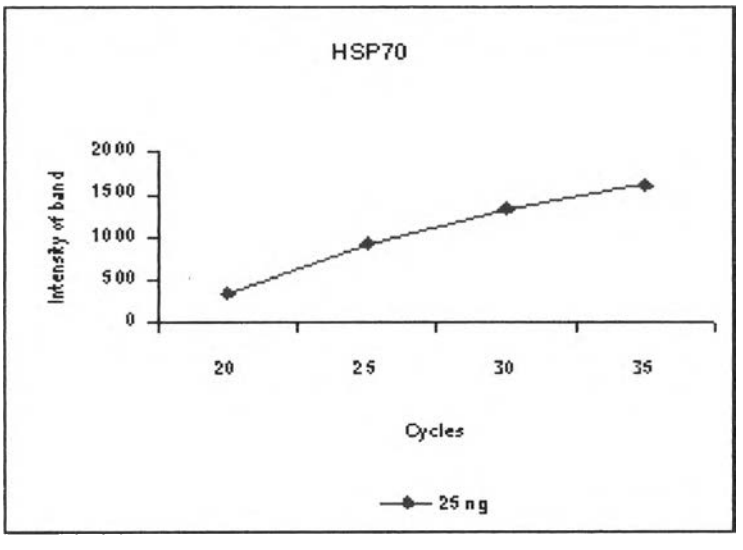

Figure 3.16 Relationship between PCR products of HSP70 gene amplified from haemocyte cell of *P.monodon* and amount of template used in PCR reaction

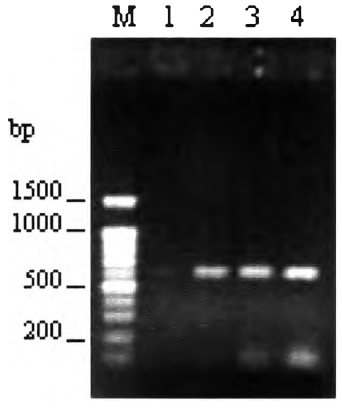

Figure 3.17 PCR product of HSP90 amplification determined on 1.2% agarose gel and stained with Ethidium bromide, PCR reaction was conducted on 20 cycles (lane 1), 25 cycles (lane 2), 30 cycles (lane 3) and 35 cycles (lane 4). The template concentration in each reaction was 100 ng. A 100 bp DNA standard was shown in lane M.

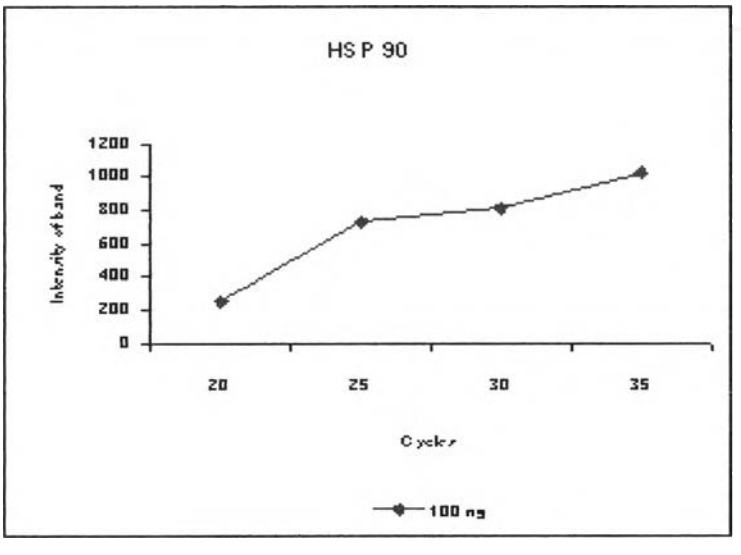

Figure 3.18 Relationship between PCR products of HSP90 gene amplified from haemocyte cell of *P.monodon* and amount of template used in PCR reaction.

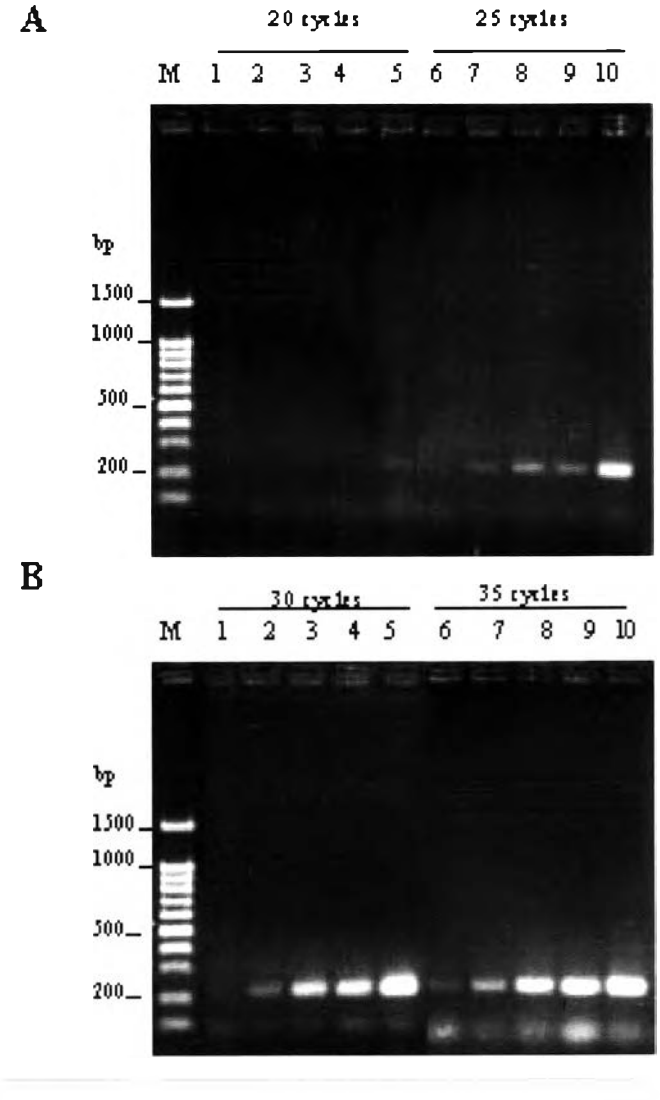

Figure 3.19 PCR product of DADI amplification determined on 1.2% agarose gel and stained with Ethidium bromide, PCR reaction was conducted on 20 cycles (lane 1-4), 25 cycles (lane 5-8) (A) , 30 cycles (lane 1-4) and 35 cycles (lane 5-8) (B). The template concentration in each reaction was 10, 50, 100, 500, and 1000 ng. A 100 bp DNA standard was shown in lane M.

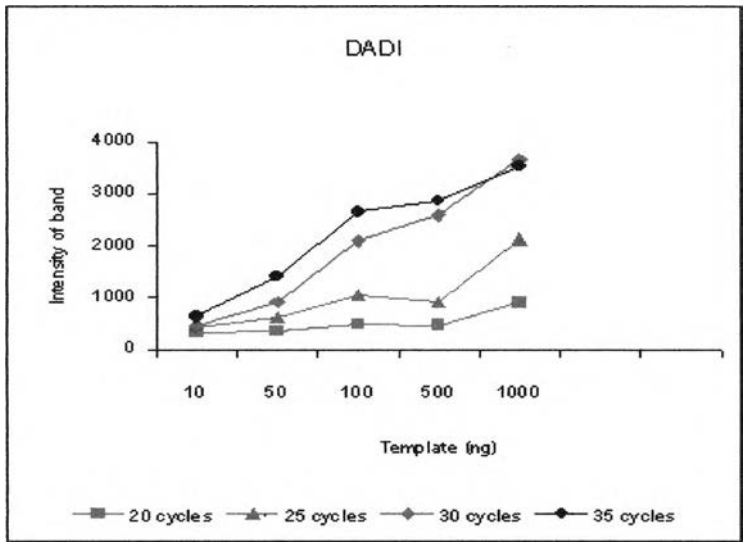

Figure 3.20 Relationship between PCR products of DADI gene amplified from haemocyte cell of *P.monodon* and various amount of template used in PCR reaction.

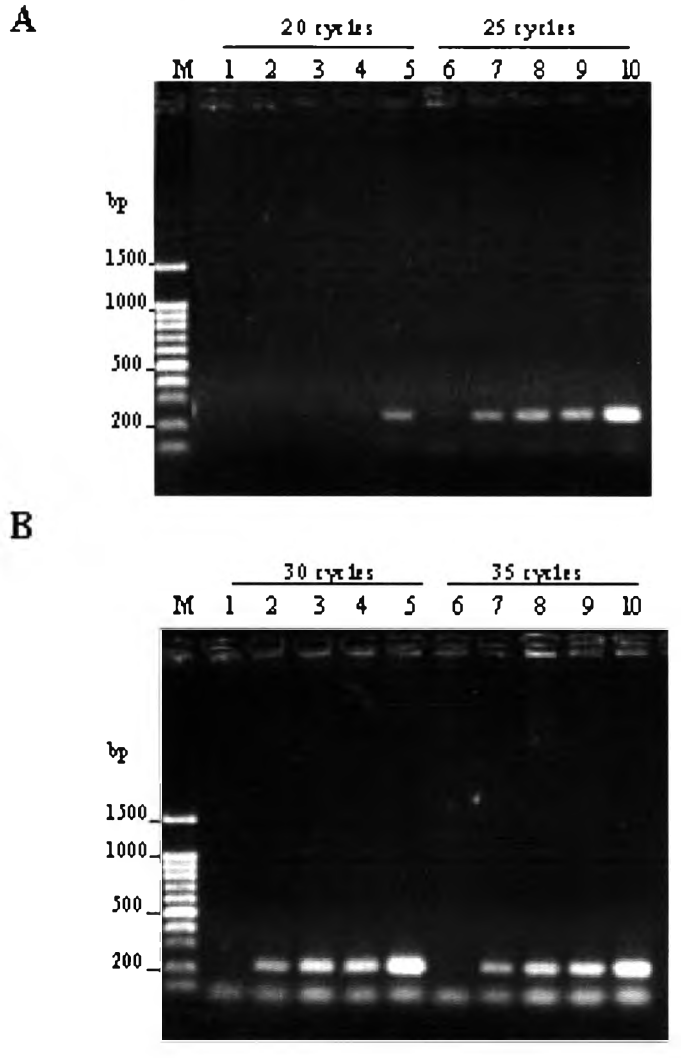

Figure 3.21 PCR product of Thioredoxin peroxidase amplification determined on 1.2% agarose gel and stained with Ethidium bromide, PCR reaction was conducted on 20 cycles (lane 1-4), 25 cycles (lane 5-8) (A), 30 cycles (lane 1-4) and 35 cycles (lane 5-8) (B). The template concentration in each reaction was 10, 50, 100, 500, and 1000 ng. A 100 bp DNA standard was shown in lane M.

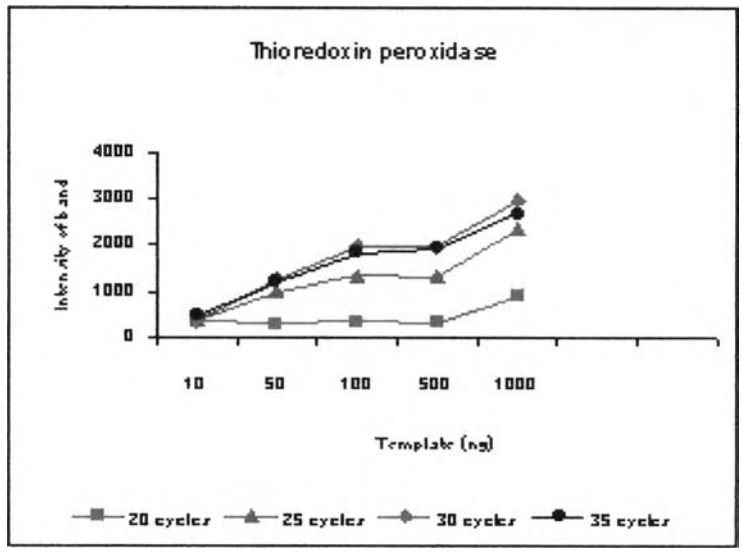

Figure 3.22 Relationship between PCR products of Thioredoxin peroxidase gene amplified from haemocyte cell of *P.monodon* and various amount of template used in PCR reaction.

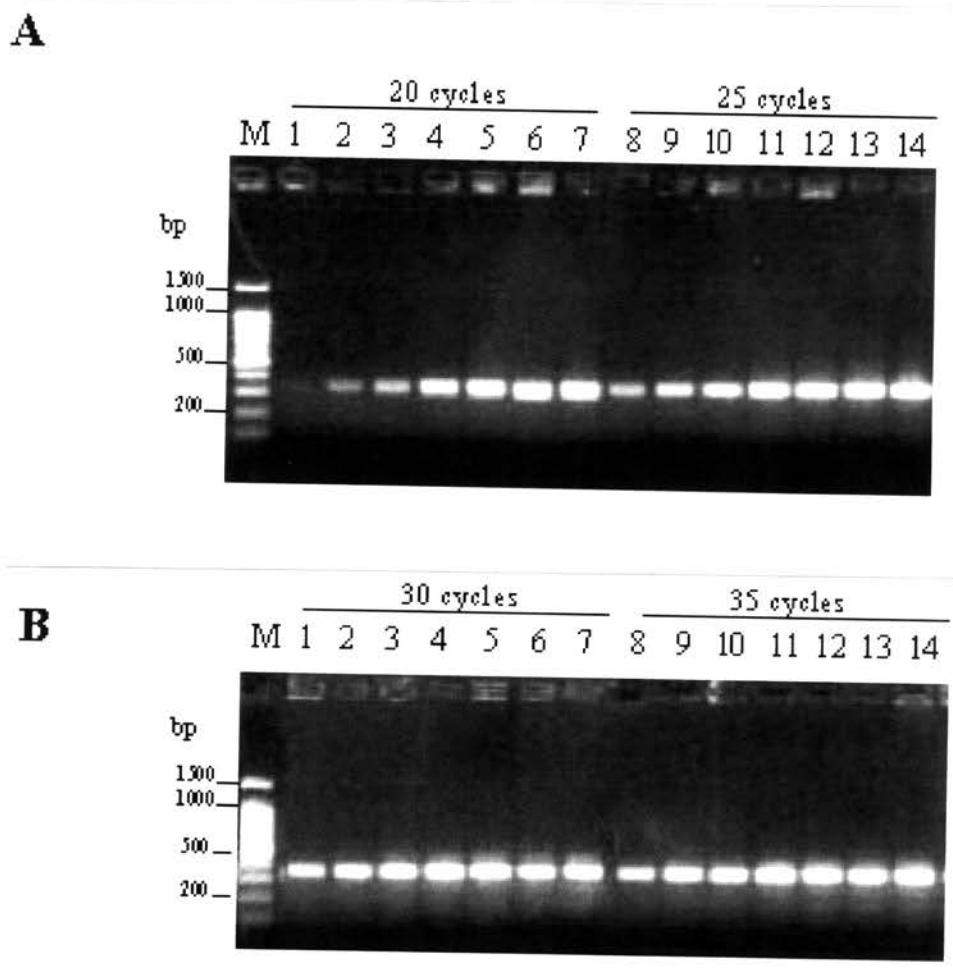

Figure 3.23 PCR product of β-actin amplification determined on 1.2% agarose gel and stained with Ethidium bromide, PCR reaction was conducted on 20 cycles (lane 1-7), 25 cycles (lane 8-14) (A), 30 cycles (lane 1-7) and 35 cycles (lane 8-14) (B). The template concentration in each reaction was 1, 5, 10, 50, 100, 200, and 500 ng, respectively (1-7). A 100 bp DNA standard was shown in lane M.

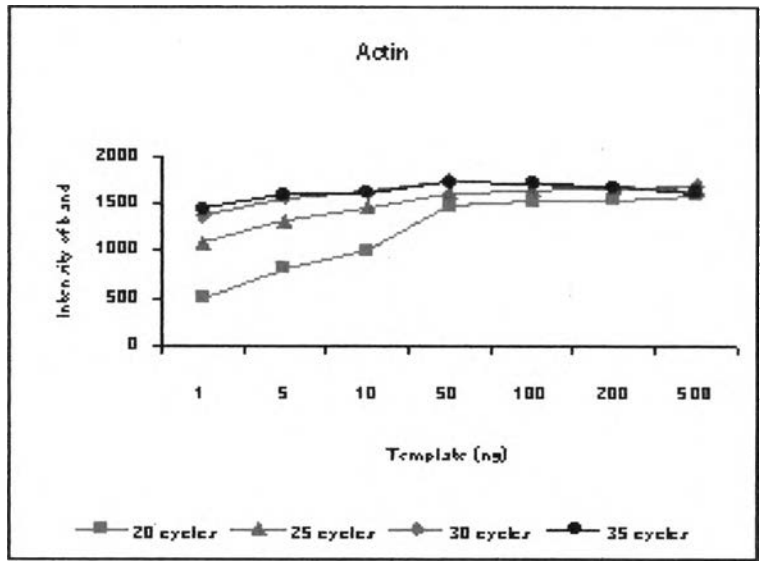

Figure 3.24 Relationship between PCR products of β-actin gene amplified from haemocyte cell of *P.monodon* and various amount of template used in PCR reaction.

3.7 Survival rate of shrimps during osmotic stress

Shrimps acclimated to seawater at the salinity of 15 ppt were stressed by immediately placing in seawater at the salinity of 0, 15 (control), 30, 45, and 60 ppt (Fig.3.25). The survival rates of the shrimps from each salinity condition were recorded during 72 h of experiment. The result revealed that complete mortalities of the shrimps were observed in the treatments with the salinity of 0 and 60 ppt after 12 h of experiment while the percentage of survival rates of the shrimps in the water at salinity of 15, 30, and 45 ppt were still significantly high after 72 h of experiment. It is quite interesting to note that the survival rate of the shrimps at 30 ppt was lower than that of 15 and 45 ppt. This is probably because the number of samples was not high and also because of unfortunate mortality of the shrimp during moulting, which usually happens when, water salinity was altered.

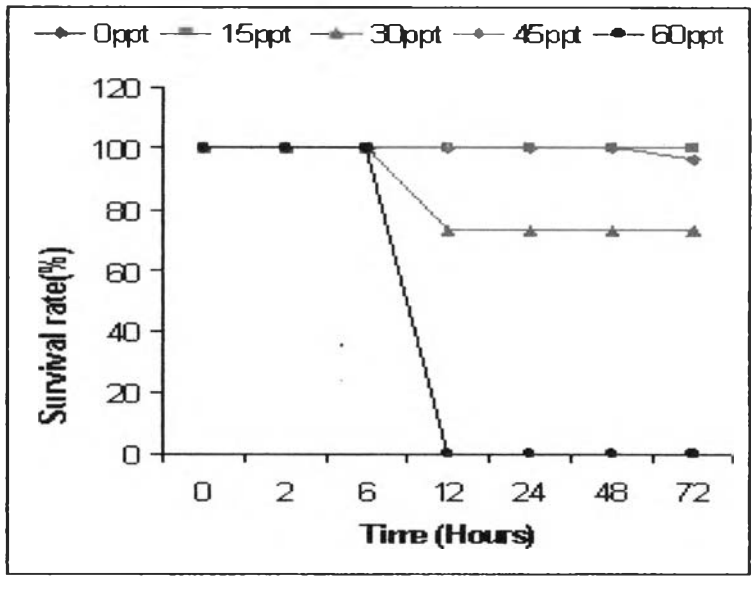

Figure 3.25 Suvival rate of shrimps stressed with salinity changes. Shrimps acclimated in seawater at 15 ppt were shocked with water at salinity of 0, 15, 30, 45, and 60 ppt.

3.8 Expression level of stress responsive genes during osmotic stress

According to the result of survival rate of the shrimps stressed by salinity changes, shrimps stressed with 15, 30, and 45 ppt, which provided high survival rate, were subjected to quantitative analysis of the expression levels of candidate stress responsive genes.

3.8.1 Expression Level of AK gene in gill

As shown in table 3.2, the expression levels of AK gene in the gills of the shrimps stressed with 30 and 45 ppt were significantly lower than that of 15 ppt (control shrimps) ($p<0.05$). The expression level decreased according to the increasing levels of salinities. At 72 h, similar result was obtained with decreasing of the expression level of AK, however, it showed no statistically difference among treatments. This indicates that the expression of AK gene was down regulated by the increasing level of water salinity.

Table 3.2 The ratio of AK gene and β-actin gene in gill of *P.monodon* after stressed with salinity change at 15, 30, and 45 ppt for 2, 6, 12, 24, 48 and 72 h (N=3).

POST EXPOSURE TIME (HOUR)	SALINITY (ppt)		
	15	30	45
No stress	1.01±0.15	1.01±0.15	1.01±0.15
2	1.91±1.02	0.89±0.64	0.94±0.55
6	1.50±0.54	0.91±0.48	0.94±0.33
12	1.50±0.62	1.02±0.37	0.69±0.12
24	1.63±0.30[2]	0.92±0.34[1]	0.60±0.20[1]
48	1.22±0.10[2]	0.74±0.22[1]	0.83±0.17[1]
72	1.90±1.06	0.98±0.30	0.69± 0.14

3.8.2 Expression Level of Mn-SOD gene in gill

The expression levels of Mn-SOD gene in the gills of the shrimps stressed with 45 ppt were significantly higher than that of 15 and 30 ppt ($p<0.05$) (table 3.3). The expression level increased according to the increasing levels of salinities at 48 h and after 72 h of experiment. This indicates that the expression of Mn-SOD gene was induced by the increasing level of water salinity.

Table 3.3 The ratio of Mn-SOD gene and β-actin gene in gill of *P.monodon* after stressed with salinity change at 15, 30, and 45 ppt for 2, 6, 12, 24, 48 and 72 h (N=3).

POST EXPOSURE TIME (HOUR)	SALINITY (ppt)		
	15	30	45
No stress	1.25±0.33	1.25±0.33	1.25±0.33
2	1.24±0.71	0.61±0.33	1.01±0.55
6	0.63±0.12	0.65±0.31	0.97±0.16
12	0.74±0.48	0.63±0.14	0.89±0.06
24	0.71±0.35	0.60±0.14	0.91±0.31
48	0.66±0.16[1]	0.48±0.10[1]	0.95±0.11[2]
72	0.63±0.13[1]	0.56±0.11[1]	1.00±0.17[2]

3.8.3 Expression Level of HSP70 gene in gill

No significant difference of the expression levels of HSP70 gene was detected in the gills of the shrimps in all treatments (table 3.4).

Table 3.4 The ratio of HSP70 gene and β-actin gene in gill of *P.monodon* after stressed with salinity change at 15, 30, and 45 ppt for 2, 6, 12, 24, 48 and 72 h (*N*=3).

POST EXPOSURE TIME (HOUR)	SALINITY (ppt)		
	15	30	45
No stress	2.85± 1.57	2.85±1.57	2.85±1.57
2	1.73±0.63	1.28± 0.65	1.10±0.94
6	1.19± 0.06	1.23±0.44	0.96±0.31
12	1.45± 0.82	1.08±0.49	0.85±0.27
24	1.23±0.22	1.15± 0.52	0.88±0.47
48	1.21±0.50	0.97±0.37	0.92±0.33
72	1.19± 0.50	1.04±0.40	0.96±0.38

3.8.4 Expression Level of HSP90 gene in gill

No significant difference of the expression levels of HSP90 gene was detected in the gills of the shrimps in all treatments (table 3.5).

Table 3.5 The ratio of HSP90 gene and β-actin gene in gill of *P.monodon* after stressed with salinity change at 15, 30, and 45 ppt for 2, 6, 12, 24, 48 and 72 h (*N*=3).

POST EXPOSURE TIME (HOUR)	SALINITY (ppt)		
	15	30	45
No stress	0.94±0.46	0.94±0.46	0.94±0.46
2	1.27±0.76	0.87±0.46	0.70±0.14
6	1.14±0.14	0.90±0.40	0.80±0.07
12	1.23±0.43	1.01±0.35	0.74±0.10
24	1.23±0.32	1.08±0.35	0.67±0.12
48	1.26±0.59	0.92±0.27	0.82±0.04
72	1.20±0.56	1.07±0.33	0.89±0.22

3.8.5 Expression Level of DADI gene in gill

No significant difference of the expression levels of DADI gene was detected in the gills of the shrimps in all treatments (table 3.6).

Table 3.6 The ratio of DADI gene and β-actin gene in gill of *P.monodon* after stressed with salinity change at 15, 30, and 45 ppt for 2, 6, 12, 24, 48 and 72 h (*N*=3).

POST EXPOSURE TIME (HOUR)	SALINITY (ppt)		
	15	30	45
No stress	1.23±0.38	1.23±0.38	1.23±0.38
2	1.04±0.34	1.17±0.63	0.70±0.13
6	0.85±0.11	1.07±0.48	0.74±0.14
12	0.96±0.44	1.13±0.32	0.73±0.18
24	1.09±0.25	1.24±0.23	0.70±0.19
48	0.98±0.39	1.03±0.21	0.75±0.12
72	1.11±0.64	1.17±0.29	0.79±0.15

3.8.6 Expression Level of Thioredoxin peroxidase gene in gill

No significant difference of the expression levels of Thioredoxin peroxidase gene was detected in the gills of the shrimps in all treatments (table 3.7).

Table 3.7 The ratio of Thioredoxin peroxidase gene and β-actin gene in gill of *P.monodon* after stressed with salinity change at 15, 30, and 45 ppt for 2, 6, 12, 24, 48 and 72 h (*N*=3).

POST EXPOSURE TIME (HOUR)	SALINITY (ppt)		
	15	30	45
No stress	2.20±1.06	2.20±1.06	2.20±1.06
2	0.97±0.21	1.03±0.42	1.28±0.43
6	0.84±0.19	1.12±0.45	1.33±0.43
12	0.80±0.32	1.11±0.20	1.25±0.31
24	0.90±0.39	1.18±0.26	1.23±0.50
48	0.80±0.22	1.01±0.26	1.34±0.33
72	0.89±0.41	1.14±0.33	0.98±0.25

3.9 Survival rate of shrimps during oxidative stress

Survival rate of the shrimps challenged by *V.harveyi* to induce oxidative stress was observed in comparison with normal shrimps. As result shown in Fig 3.26, mortality of the shrimps was initially detected after 24 h of vibrio challenge. Almost 50% mortality and complete mortality were detected in the shrimps challenged by *V.harveyi* after 48 and 72 h of post challenge, respectively, while the complete survival of normal shrimps was still observed after 24 h of post challenge and remained high (>80%) after 72 h of post challenge.

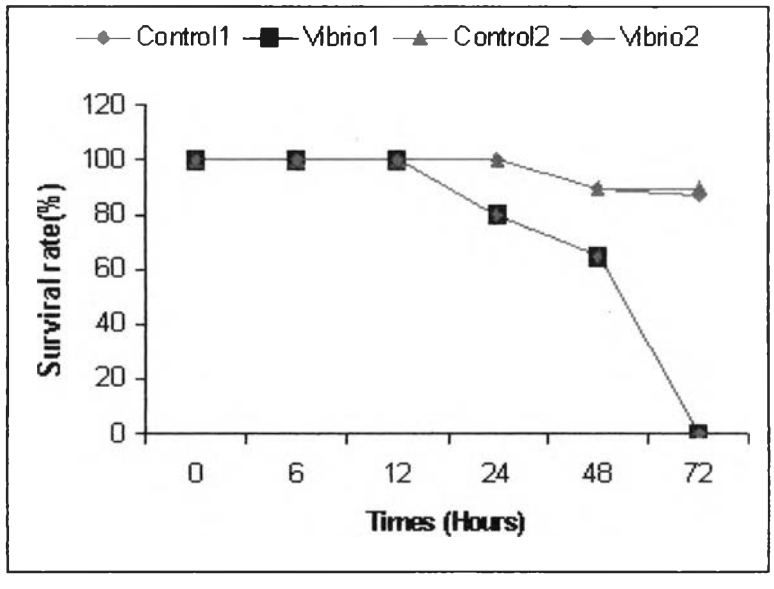

Figure 3.26 Suvival rate of shrimp stressed with *V.harveyi* administration at 10^6 CFU/ml

3.10 Expression level of stress responsive genes during oxidative stress

Oxidative stress in shrimps was induced by infection of *V.harveyi*. As the result of vibrio challenge, high mortality of the shrimps was detected after 48 h of post challenge. Therefore, shrimps challenged by *V.harveyi* within 24 h were subjected to quantitative analysis of the expression levels of candidate stress responsive genes. Both gills and haemocytes were target tissues in this analysis.

3.10.1 Expression Level of Mn-SOD gene in haemocyte and gill

The expression levels of Mn-SOD gene in both haemocyte and gill of the shrimps stressed with *V.harveyi* were significantly higher than normal shrimps within the first hour of post challege ($p<0.05$). Similar results were still observerd at 6, 12, and 24 h of post challenge (table 3.8). Noticably, at 24 h, the expression level of challenged shrimps appeared to be higher than that of control shrimps, however, it was not statistically different between control and challenged shrimps. The result indicates that the expression of MnSOD gene in both haemocyte and gill of *P.monodon* can be induced by *V.harveyi* challenge.

Table 3.8 The ratio of Mn-SOD gene and β-actin gene in haemocyte (A) and gill (B) of *P.monodon* after stressed with *Vibrio* exposure for 0, 6, 12, 24, 48 and 72 h (*N*=3).

A

Post Exposure Time (hour)	HAEMOCYTE	
	Control	*Vibrio*
0	0.46±0.07[1]	0.71±0.15[2]
6	0.41±0.08[1]	0.56±0.03[2]
12	0.49±0.04[1]	0.63±0.14[2]
24	0.65±0.29	0.82±0.25

B

Post Exposure Time (hour)	GILL	
	Control	*Vibrio*
0	0.83±0.06[1]	1.05±0.12[2]
6	0.79±0.11[1]	1.03±0.17[2]
12	0.83±0.19[1]	1.02±0.08[2]
24	0.79±0.06[1]	0.96±0.12[2]

3.10.2 Expression Level of AK gene in haemocyte and gill

Significant differences of the expression levels of AK gene in both haemocyte and gill of the shrimps stressed with *V.harveyi* were detected between normal and challenged shrimps ($p<0.05$). However, the changes of the expression levels showed no correlation to vibrio or time of challenge. (table 3.9). Therefore, repeat experiment on the expression of AK gene relating to bacterial infection might be needed.

Table 3.9 The ratio of AK gene and β-actin gene in haemocyte (A) and gill (B) of *P.monodon* after stressed with *Vibrio* exposure for 0, 6, 12, 24, 48 and 72 h (N=3).

A

Post Exposure Time (hour)	HAEMOCYTE	
	Control	*Vibrio*
0h	0.82±0.10[2]	0.67±0.04[1]
6h	0.38±0.03[1]	0.65±0.04[2]
12h	0.58±0.07[1]	0.73±0.11[2]
24h	1.18±0.33[2]	0.68±0.04[1]

B

Post Exposure Time (hour)	GILL	
	Control	*Vibrio*
0	0.91±0.07	0.88±0.11
6	0.88±0.10[1]	1.08±0.12[2]
12	1.00±0.11	0.96±0.10
24	1.16±0.07[2]	0.96±0.12[1]

3.10.3 Expression Level of HSP70 gene in haemocyte and gill

The decrease of the expression levels of HSP70 gene in haemocyte of the shrimps stressed with *V.harveyi* were detected clearly within the first hour and throughout the post challege while that of the gill were detected at 24 h of post challenge ($p<0.05$) (table 3.10). The result of gene expression from both tissues indicates the down regulation of HSP70 gene during vibrio infection.

Table 3.10 The ratio of HSP70 gene and β-actin gene in haemocyte (A) and gill (B) of *P.monodon* after stressed with *Vibrio* exposure for 0, 6, 12, 24, 48 and 72 h (N=3).

A

Post Exposure Time (hour)	HAEMOCYTE	
	Control	*Vibrio*
0	1.30±0.10[2]	0.69±0.18[1]
6	1.00±0.09[2]	0.70±0.25[1]
12	1.11±0.26[2]	0.62±0.33[1]
24	1.33±0.31[2]	0.67±0.23[1]

B

Post Exposure Time (hour)	GILL	
	Control	*Vibrio*
0	1.03±0.14	1.02±0.21
6	0.89±0.14	0.97±0.24
12	0.91±0.18	0.95±0.19
24	1.23±0.15[2]	1.02±0.16[1]

3.10.4 Expression Level of HSP90 gene in haemocyte and gill

Similar result as observed earlier in the expression of HSP70 gene was obtained. The decrease of the expression levels of HSP90 gene in haemocyte of the shrimps stressed with *V.harveyi* were detected throughout the post challege except at 6 h which the expression levels of HSP90 gene of challenged shrimps were significantly higher than normal shrimps ($p<0.05$) (table 3.11A). Similar result was also obtained from gill sample where down regulation of HSP90 gene was detected after 12 h of post challenge ($p<0.05$)(table 3.11B). The majority of the expression result from both tissues indicates the down regulation of HSP90 gene during vibrio infection.

Table 3.11 The ratio of HSP90 gene and β-actin gene in haemocyte (A) and gill (B) of *P.monodon* after stressed with *Vibrio* exposure for 0, 6, 12, 24, 48 and 72 h (N=3).

A

Post Exposure Time (hour)	HAEMOCYTE	
	Control	*Vibrio*
0	1.48±0.09[2]	0.29±0.02[1]
6	0.61±0.29[1]	0.98±0.19[2]
12	0.99±0.21[2]	0.39±0.08[1]
24	1.09±0.26[2]	0.67±0.23[1]

B

Post Exposure Time (hour)	GILL	
	Control	*Vibrio*
0	1.09± 0.24	0.84±0.08
6	0.95±0.09	0.93±0.11
12	1.01±0.12[2]	0.68±0.06[1]
24	1.09±0.12[2]	0.82±0.16[1]

3.10.5 Expression Level of DADI gene in haemocyte and gill

No significant difference of the expression level of DADI gene was detected between the haemocyte of challenged and normal shrimps. (p<0.05) (table 3.12A). Similar result was obtained in the gill except at 12 h of post challenge where the expression level of DADI gene in normal shrimps was significantly higher than that of challenged shrimps. (p<0.05) (table 3.12B). It can be indicated from the result that the expression of DADI gene in haemocyte and gill of the shrimps slightly responds to vibrio infection.

Table 3.12 The ratio of DADI gene and β-actin gene in haemocyte (A) and gill (B) of *P.monodon* after stressed with *Vibrio* exposure for 0, 6, 12, 24, 48 and 72 h (N=3).

A

Post Exposure Time (hour)	HAEMOCYTE	
	Control	*Vibrio*
0	1.37±0.14	1.19±0.24
6	1.27±0.05	1.31±0.25
12	1.29±0.13	1.20±0.22
24	1.40±0.42	1.28±0.10

B

Post Exposure Time (hour)	GILL	
	Control	*Vibrio*
0	0.80±0.22	0.84±0.06
6	1.27±0.20	1.08±0.14
12	1.28±0.09[2]	1.04±0.19[1]
24	1.29±0.16	1.41±0.24

3.10.6 Expression Level of Thioredoxin peroxidase gene in haemocyte and gill

Repression of thioredoxin peroxidase gene was detected from the haemocyte while induction was detected from the gill of challenged shrimps ($p<0.05$) (table 3.13). Significant difference was detected within 6 h of post challenge from haemocyte but the difference in gill was detected within 12 h of post challenge. These contradictory results were probably due to the difference on gene regulation in different tissues. Further investigation is, therefore, needed.

Table 3.13 The ratio of Thioredoxin peroxidase gene and β-actin gene in haemocyte (A) and gill (B) of *P.monodon* after stressed with *Vibrio* exposure for 0, 6, 12, 24, 48 and 72 h (N=3).

A

Post Exposure Time (hour)	HAEMOCYTE	
	Control	*Vibrio*
0	0.56±0.04[2]	0.44±0.04[1]
6	0.60±0.10[2]	0.49±0.03[1]
12	0.56±0.09	0.52±0.05
24	0.63±0.17	0.51±0.04

B

Post Exposure Time (hour)	GILL	
	Control	*Vibrio*
0	0.83±0.07[1]	0.98±0.05[2]
6	0.82±0.10[1]	1.03±0.17[2]
12	0.81±0.10[1]	1.02±0.08[2]
24	0.79±0.06	0.86±0.13

3.11 Survival rate of shrimps during handling stress

Survival rate of the shrimps physically stressed by disturbing water and moving the shrimps out of water was recorded in comparison with uninterfered shrimps. As result shown in Fig 3.27, mortality of the shrimps was initially detected at 48 h of handling stress. Less than 15% mortality was detected in the stressed shrimps after 72 h of experiment.

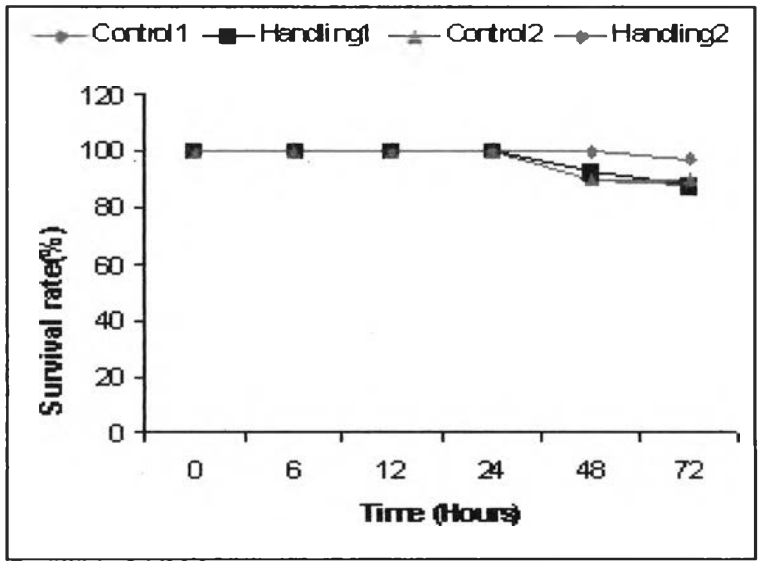

Figure 3.27 Suvival rate of shrimp stressed by disturbing water for 5 minutes (4 times in every 30 minutes) and moving the shrimps out of water for 1 minute.

3.12 Expression level of stress responsive genes during handling stress

3.12.1 Expression Level of Mn-SOD gene in haemocyte and gill

The expression levels of Mn-SOD gene from both haemocytes and gills of stressed shrimps appeared to be induced. However, only at 6, 48, and 72 h of post stress in haemocyte and 48 h of post stress in gill were significantly different ($p<0.05$) (table 3.14). This indicates that Mn-SOD gene can slightly respond handling stress.

Table 3.14 The ratio of Mn-SOD gene and β-actin gene in haemocyte (A) and gill (B) of *P.monodon* after stressed with handling stress for 0, 6, 12, 24, 48 and 72 h (*N*=3).

A

Post Exposure Time (hour)	HAEMOCYTE	
	Control	Handling
0	0.46±0.07	0.43±0.15
6	0.41±0.08[1]	0.86±0.13[2]
12	0.49±0.04	0.49±0.14
24	0.65±0.29	0.94±0.11
48	0.40±0.07[1]	0.86±0.24[2]
72	0.56±0.14[1]	1.02±0.19[2]

B

Post Exposure Time (hour)	GILL	
	Control	Handling
0	0.83±0.06	0.80±0.13
6	0.79±0.11	0.82±0.09
12	0.83±0.19	0.87±0.09
24	0.79±0.06	0.90±0.12
48	0.79± 0.08[1]	0.95±0.12[2]
72	0.89±0.07	0.81±0.16

3.12.2 Expression Level of AK gene in haemocyte and gill

In haemocyte, the expression level of AK gene in stressed shrimps increased within 6-12 h but decreased at 72 h of experiment ($p<0.05$) (table 3.15A). In gill, no significant difference was detected between treatment except at 24 h where the expression level of AK gene in stressed shrimps was significantly decreased ($p<0.05$) (table 3.15B).

Table 3.15 The ratio of AK gene and β-actin gene in haemocyte (A) and gill (B) of *P.monodon* after stressed with handling stress for 0, 6, 12, 24, 48 and 72 h (N=3).

A

Post Exposure Time (hour)	HAEMOCYTE	
	Control	Handling
0	0.82±0.10	1.15±0.55
6	0.38±0.03[1]	0.89±0.17[2]
12	0.58±0.07[1]	1.04±0.35[2]
24	1.18±0.33	1.14±0.16
48	0.91±0.14	0.90±0.16
72	1.31±0.18[2]	0.84±0.27[1]

B

Post Exposure Time (hour)	GILL	
	Control	Handling
0	0.91±0.07	0.97±0.05
6	0.88±0.10	1.00±0.21
12	1.00± 0.11	0.94±0.05
24	1.16±0.07[2]	0.98±0.12[1]
48	1.11±0.11	1.07±0.08
72	1.14±0.11	1.13±0.16

3.12.3 Expression Level of HSP70 gene in haemocyte and gill

Similar results were obtained from both haemocyte and gill. The expression level of HSP70 gene in stressed shrimps significantly decreased from the first hour to 6 h in haemocyte and only 24 h in gill.but decreased at 72 h of experiment ($p<0.05$) (table 3.16). This indicates the slightly down regulation of HSP70 gene in handlind stressed shrimps.

Table 3.16 The ratio of HSP70 gene and β-actin gene in haemocyte and gill of *P.monodon* after stressed with handling stress for 0, 6, 12, 24, 48 and 72 h ($N=3$).

A

Post Exposure Time (hour)	HAEMOCYTE	
	Control	Handling
0	1.30±0.10[2]	0.85±0.31[1]
6	1.00±0.09[2]	0.87±0.11[1]
12	1.11±0.26	1.08±0.59
24	1.33±0.31	1.18±0.12
48	1.08±0.15	0.96±0.15
72	1.17±0.20	1.20±0.36

B

Post Exposure Time (hour)	GILL	
	Control	Handling
0	1.03±0.14	0.89±0.13
6	0.89±0.14	1.01±0.12
12	0.91±0.18	0.99±0.26
24	1.23±0.15[2]	1.07±0.09[1]
48	0.91±0.05	0.89±0.17
72	0.82±0.08	0.92±0.10

3.12.4 Expression Level of HSP90 gene in haemocyte and gill

There were significant differences between normal and stressed shrimps ($p<0.05$) (table 3.17), however, the results from both haemocyte and gill were not conclusive probably due to high variation between replications.

Table 3.17 The ratio of HSP90 gene and β-actin gene in haemocyte (A) and gill (B) of *P.monodon* after stressed with handling stress for 0, 6, 12, 24, 48 and 72 h (N=3).

A

Post Exposure Time (hour)	HAEMOCYTE	
	Control	Handling
0	1.48±0.09[2]	0.91±0.15[1]
6	0.61±0.29[1]	0.98±0.12[2]
12	0.99±0.21	0.69±0.38
24	1.09±0.26	1.23±0.08
48	0.79±0.05[2]	0.51±0.07[1]
72	1.23±0.27	1.03±0.70

B

Post Exposure Time (hour)	GILL	
	Control	Handling
0	1.09±0.24	1.13±0.14
6	0.95±0.09	0.93±0.18
12	1.01±0.12	1.17±0.19
24	1.09±0.12	0.97±0.07
48	1.02±0.20[2]	0.72±0.05[1]
72	1.16±0.11[1]	1.39±0.24[2]

3.12.5 Expression Level of DADI gene in haemocyte and gill

Significantly higher expression levels of DADI gene from most (but not all) stressed shrimps were obtained from both haemocyte and gill ($p<0.05$) (table 3.18). Although the results were not absolute, it can imply that DADI gene is inducible by handling stress.

This indicates the slightly down regulation of HSP70 gene in handlind stressed shrimps.

Table 3.18 The ratio of DADI gene and β-actin gene in haemocyte (A) and gill (B) of *P.monodon* after stress with handling stress for 0, 6, 12, 24, 48 and 72 h (N=3).

A

Post Exposure Time (hour)	HAEMOCYTE	
	Control	Handling
0	1.37±0.14	1.32±0.25
6	1.27±0.05[2]	0.81±0.12[1]
12	1.29±0.13	1.49±0.29
24	1.40±0.42	1.22±0.21
48	1.47±0.13[2]	1.20±0.17[1]
72	1.34±0.10	1.30±0.23

B

Post Exposure Time (hour)	GILL	
	Control	Handling
0	0.80±0.22[1]	1.08±0.14[2]
6	1.27±0.20	1.01±0.21
12	1.28±0.09[2]	0.90±0.09[1]
24	1.29±0.16	1.23±0.17
48	1.22±0.12[2]	0.93±0.11[1]
72	1.39±0.09[2]	1.09±0.09[1]

3.12.6 Expression Level of Thioredoxin peroxidase gene in haemocyte and gill

In haemocyte, the expression levels of Thioredoxin peroxidase gene from stressed shrimps decreased significantly in almost all the time of experiment ($p<0.05$) (table 3.19A). However, in gill, the expression seemed to be no difference between treatment except at 24 h where the expression level was significantly higher in stressed shrimps ($p<0.05$) (table 3.19B).

Table 3.19 The ratio of Thioredoxin peroxidase gene and β-actin gene in haemocyte and gill of *P.monodon* after stressed with handling stress for 0, 6, 12, 24, 48 and 72 h ($N=3$).

A

Post Exposure Time (hour)	HAEMOCYTE	
	Control	Handling
0	0.56±0.04[2]	0.43±0.08[1]
6	0.60±0.10[2]	0.42±0.04[1]
12	0.56±0.09	0.51±0.10
24	0.63±0.17	0.50±0.05
48	0.58±0.08[2]	0.48±0.04[1]
72	0.63±0.13	0.60±0.15

B

Post Exposure Time (hour)	GILL	
	Control	Handling
0	0.83±0.07	0.84±0.10
6	0.82±0.10	0.82±0.09
12	0.81±0.10	0.87±0.09
24	0.79±0.06[1]	0.97±0.17[2]
48	0.84±0.05	0.92±0.10
72	0.89±0.07	0.86±0.16