CHAPTER V

RESULTS

4.1 Determination of the appropriate thermal treatment in shrimp

To determine the non-lethal level of thermal change, shrimps were initially exposed to various temperatures (15, 27 (ambient), 30, 33, and 35°C) for 6 h. The non-lethal temperatures were indicated by the survival rate of the shrimps. The result of survival rate analysis as illustrated in Table 4.1 and Figure 4.1 revealed that complete mortality of the shrimps exposed to the temperature at 10, 40, and 45°C were observed, while the survival rates of the shrimps were ranged from 94.85 to 100 % after exposing to the temperature at 15, 27 (ambient), 30, 33, and 35°C for 14 h. No mortality was detected from the shrimps exposed to ambient temperature (27°C) and 30°C throughout the experiment. The no significant difference of the survival rates from the control and thermal-treated shrimps were detected at the temperature after thermal shock ($P<0.05$).

Table 4.1 Percentage of survival rate from shrimps after the exposure of thermal stress for 6 h. The values are means±SD, ($N=2$).

Exposure time (h.)	Survival rate (%)							
	10°C	15°C	27°C (ambient)	30°C	33°C	35°C	40°C	45°C
0	0	100	100	100	100	100	0	0
2	0	98.70±1.84	100	100	100	97.5±1.84	0	0
4	0	97.85±4.04	100	100	97.5±4.04	97.5±4.04	0	0
6	0	97.85±4.04	100	100	97.5±4.04	97.5±4.04	0	0
8	0	96.15±5.44	100	100	95.0±5.44	95.0±5.44	0	0
10	0	96.15±5.44	100	100	95.0±5.44	95.0±5.44	0	0
12	0	94.85±7.28	100	100	95.0±7.28	95.0±7.28	0	0
14	0	94.85±7.28	100	100	95.0±7.28	95.0±7.28	0	0

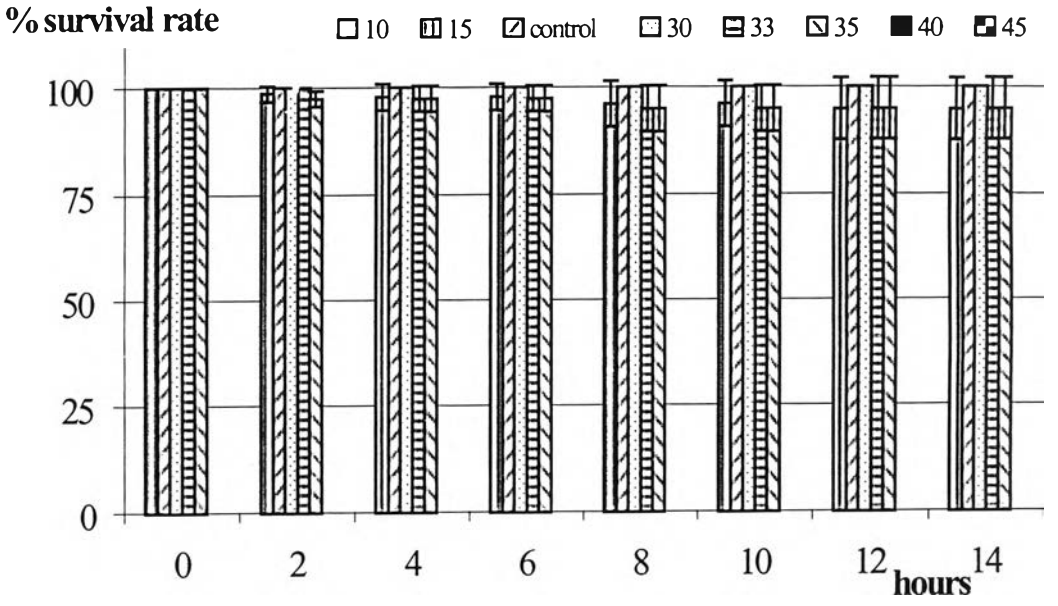


Figure 4.1 Survival rates of the shrimps after exposure to various levels of temperature for 14 h. All data were derived from Table 4.1. The lines above the bar represent standard derivation (*N*=2).

As the result, no significant difference between the survival rate of the shrimps from control and the ones with the thermal shock at 15, 30, 33, and 35°C. For the time of exposure, the survival rate of the shrimps from these treatments remained no significant difference from the control until 14 h after thermal shock. Therefore, it can be indicated that the thermal shock at 15, 30, 33, and 35°C for 6 h were the appropriate conditions for using in further experiments.

4.2 Physiological responses of the shrimps exposed to thermal shock

The levels of total protein concentration from haemolymph and plasma glucose concentration from the shrimps induced by different temperatures were measured. The accumulated protein synthesis in the haemocyte lysate and tissue homogenates were analyzed using SDS-PAGE, followed by Western blot analysis.

4.2.1 Plasma Protein concentration

Protein concentrations of the haemolymph from the survived shrimps exposed to thermal shock were measured. Total protein concentration of the haemolymph from the thermal-induced shrimps varied vastly. The levels ranged from low concentration in shrimps exposed to 15°C and increased corresponding to the temperature and the time of post exposure (Table 4.2 and Figure 4.2). Minimum level of plasma protein (15.7 mg/ml) was found in cold shock shrimps within an hour after the exposure and maximum level (75.0 mg/ml) was found in shrimps exposed to 35°C. The protein levels responded to the thermal shock within the first hour of post exposure and maintained the same levels for more than 72 h. The shrimps maintained at 27°C, which was an ambient temperature were regarded as control shrimps. Protein concentrations of the shrimps exposed to 15°C were significantly lower than that of other treatments ($P<0.05$). Protein concentrations of the shrimps exposed to 30, 33, and 35°C were significantly higher than that of control and cold shock treatment ($P<0.05$). Protein concentrations of the shrimps exposed to 35°C were slightly higher according to the raising temperature but no statistically significant difference was detected. It is quite noticeable that at cold shock condition, plasma protein concentration tended to increase according to time after thermal shock while it tended to decrease in the shrimps exposed to heat shock.

Table 4.2 Protein concentration in the haemolymph of shrimps after thermal treatments for 6 h. The values are means±SD, ($N=5$).

Exposure time (h.)	Plasma protein concentration (mg/ml)				
	15°C	27°C (ambient)	30°C	33°C	35°C
0	15.7±4.35	45.4±4.77	66.4±10.04	74.0±15.13	75.0±16.72
3	16.2±6.49	44.6±4.53	64.7±17.36	70.6±19.44	72.9±9.66
6	18.7±8.28	42.9±4.53	60.5±20.57	64.8±7.270	66.1±18.36
12	20.8±8.35	42.1±5.05	68.1±7.760	68.6±10.40	69.0±14.73
24	24.8±9.22	40.5±4.49	66.3±9.610	65.9±14.90	66.6±19.37
72	26.0±9.77	39.4±4.47	60.2±14.09	61.1±9.340	60.3±15.66

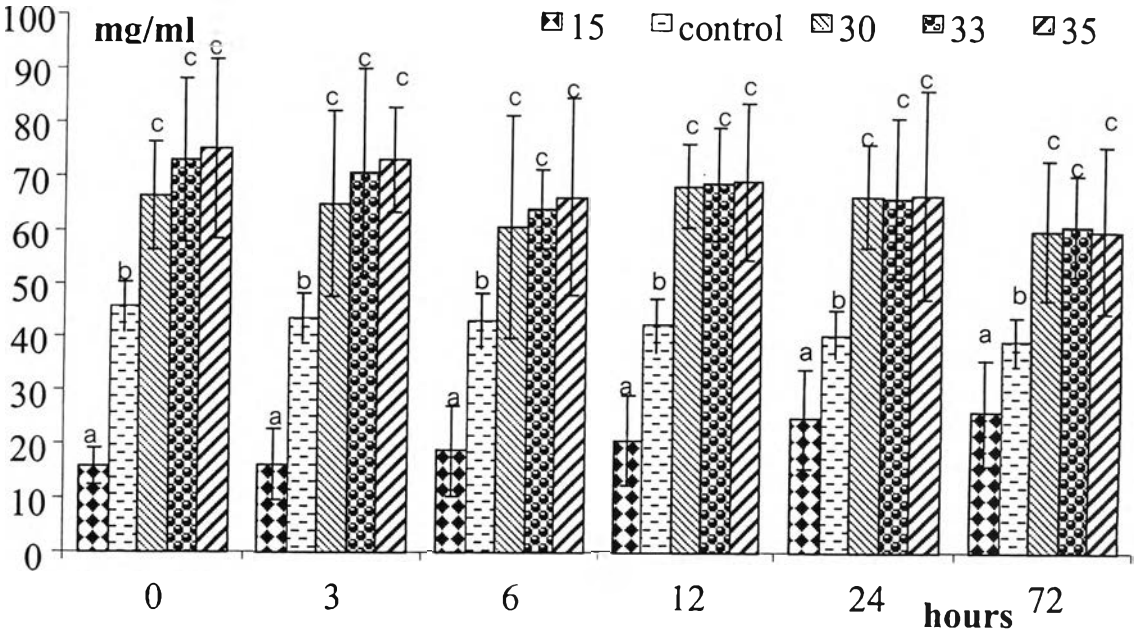


Figure 4.2 Protein concentration in the haemolymph of shrinps after thermal treatments for 6 h (N=5). All datas were derived from Table 4.2. The lines above the bar represent standard derivation. Values with significantly difference are indicated by different letters (P<0.05 by Duncan's new multiple range test).

4.2.2 Plasma glucose concentration

Glucose concentrations were measured in haemolymph of *P. monodon* exposed to the temperature at 15, 27, 30, 33, and 35°C for 6 h. Significantly higher levels of plasma glucose were found in shrimps exposed to the temperatures of 30, 33, and 35°C when compared to control shrimps and the shrimps exposed to temperature of 15°C (Table 4.3 and Figure 4.3). The highest level (101.77 mg%) of plasma glucose was detected from the shrimps exposed to 35°C within 12 hours of post exposure. The lowest level (22.52 mg%) of plasma glucose was detected from the shrimps exposed to 15°C within 12 h of post exposure. The average level of plasma glucose from control shrimps through out the experiment was 34.33 mg%.

Plasma glucose detected in control shrimps (maintained at 27°C) was in the level between that of the cold and heat shocks. Glucose levels from control shrimps showed no significant change throughout the experiment. Glucose levels from cold shock shrimps tended to decrease within 12 h and increased afterward, but, no significant difference was detected. On the other hand, glucose levels from heat shock shrimps (exposed to 30, 33, and 35°C) significantly increased within 12 h of post thermal exposure and significantly decreased afterward (P<0.05).

Table 4.3 Glucose concentration in haemolymph of *P. monodon* after thermal shock at 15, 27, 30, 33, and 35°C for 6 h (*N*=5). The values are means±SD.

Exposure time (h.)	Glucose concentration (mg%)				
	15°C	27°C (ambient)	30°C	33°C	35°C
0	28.78±5.59	34.78±4.98	35.81±11.51	37.78±4.81	37.27±14.65
3	26.63±6.47	32.212±1.89	64.03±16.52	67.89±14.23	78.77±16.37
6	37.90±6.52	34.33±4.19	70.81±7.07	89.70±20.56	94.89±2.50
12	22.52±17.18	37.37±7.45	78.79±8.67	92.65±14.93	101.77±11.90
24	41.48±10.55	34.82±6.04	70.96±9.41	84.89±10.80	94.97±11.09
72	31.82±11.4	34.44±5.75	56.17±15.68	52.89±16.52	60.82±10.80
Average	**31.52±4.20**	**34.33±0.85**	**56.09±14.93**	**70.79±21.21**	**77.92±24.74**

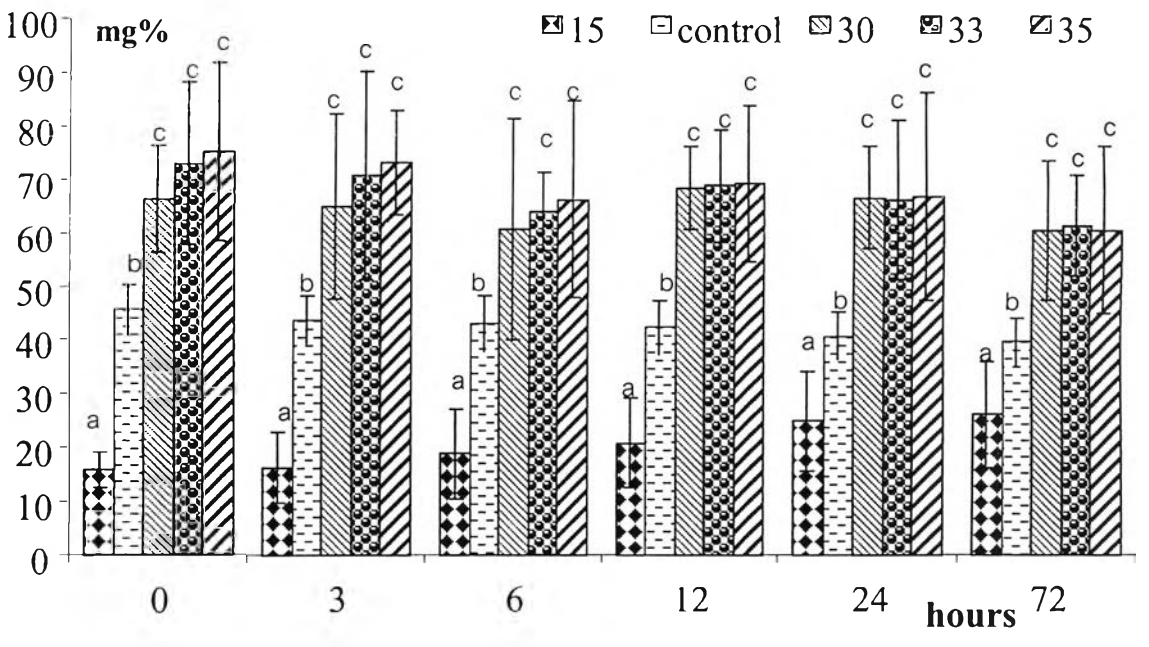


Figure 4.3 Glucose concentrations in the haemolymph of shrimps after thermal treatments at 15, 27, 30, 33, and 35°C for 6 h. (*N*=5). All datas were derived from Table 4.4. The lines above the bar represent standard derivation. Values with significantly difference are indicated by different letters ($P<0.05$ by Duncan's new multiple range test).

4.3 Analysis of proteins from haemocyte lysate and tissue extracts from the shrimps exposed to thermal shock using SDS-PAGE

4.3.1 Cold shock

The results of protein analysis from the haemocyte lysate of shrimps induced with cold shock at 15°C for 6 h were shown in Figure 4.4. Two major bands with the size of approximately 70 and 81 kDa were clearly seen in every sample. When compared with control, less number and intensity of peptide bands were observed in the samples at 0 to 6 h of post induction. However, the intensity and the number of peptide bands from the samples at 12 to 72 h of post induction appeared to increase up to the same level of control. This result was in correspondent with the protein concentration of the haemolyph from cold shock shrimps.

The results of protein analysis in gill, muscle, hepatopancreas, digestive tract and heart extracts were shown in Figure 4.5-4.9, respectively. Two major bands (70 and 81 kDa) were still seen in every sample, except in muscle extract. Three major bands at the same molecular weight level were detected in muscle extract instead of 2 bands. The protein profiles of the samples were various depending on the type of tissue extracts. A number of major peptide bands appeared in tissue extracts especially from muscle, hepatopancreas, and digestive tract was higher than that of haemocyte samples. However, no obvious or consistent difference between peptide bands from control and treatment samples was clearly detected from the tissue extracts.

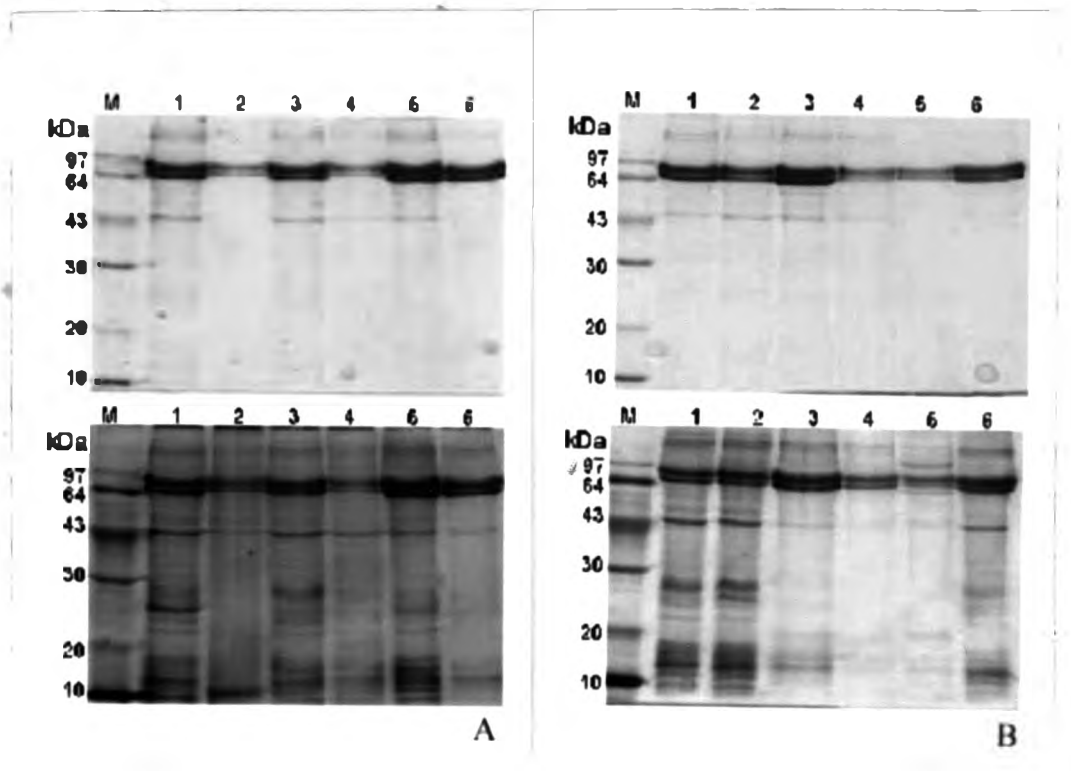


Figure 4.4 Protein analysis of haemocyte lysate from shrimps treated at 15°C for 6 h. Samples were collected at 0, 3, 6, 12, 24, and 72 h of post exposure and separated in 12% SDS-PAGE followed by staining with coomassie stain (upper gel) and silver stain (lower gel).

Lane M: Low molecular weight markers

Lane 1, 3 and 5: control group

Lane 2, 4 and 6: shrimp stress response

Gel A: Samples were collected at 0, 3, and 6 h

Gel B: Samples were collected at 12, 24, and 72 h

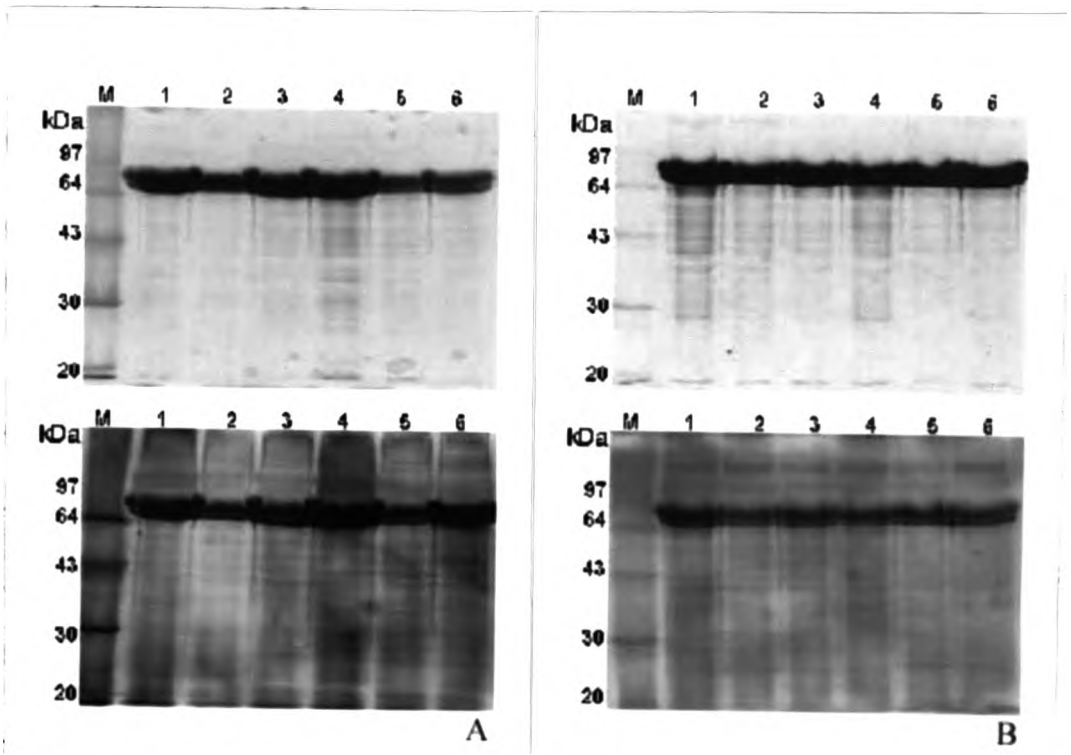


Figure 4.5 Protein analysis of gill extracts from shrimps treated at 15°C for 6 hours. Samples were collected at 0, 3, 6, 12, 24, and 72 h of post exposure and separated in 12% SDS-PAGE followed by staining with coomassie stain (upper gel) and silver stain (lower gel).

Lane M: Low molecular weight markers

Lane 1, 3 and 5: control group

Lane 2, 4 and 6: shrimp stress response

Gel A: Samples were collected at 0, 3, and 6 h

Gel B: Samples were collected at 12, 24, and 72 h

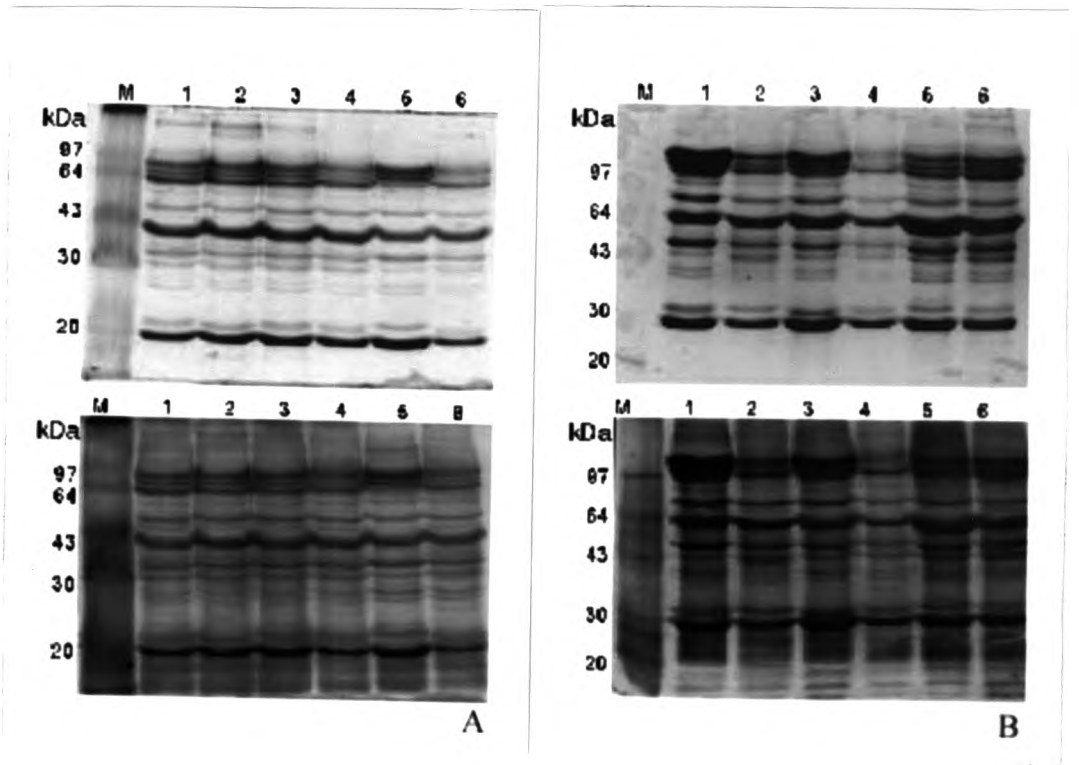


Figure 4.6 Protein analysis of muscle extracts from shrimps treated at 15°C for 6 hours. Samples were collected at 0, 3, 6, 12, 24, and 72 h of post exposure and separated in 12% SDS-PAGE followed by staining with coomassie stain (upper gel) and silver stain (lower gel).

Lane M: Low molecular weight markers

Lane 1, 3 and 5: control group

Lane 2, 4 and 6: shrimp stress response

Gel A: Samples were collected at 0, 3, and 6 h

Gel B: Samples were collected at 12, 24, and 72 h

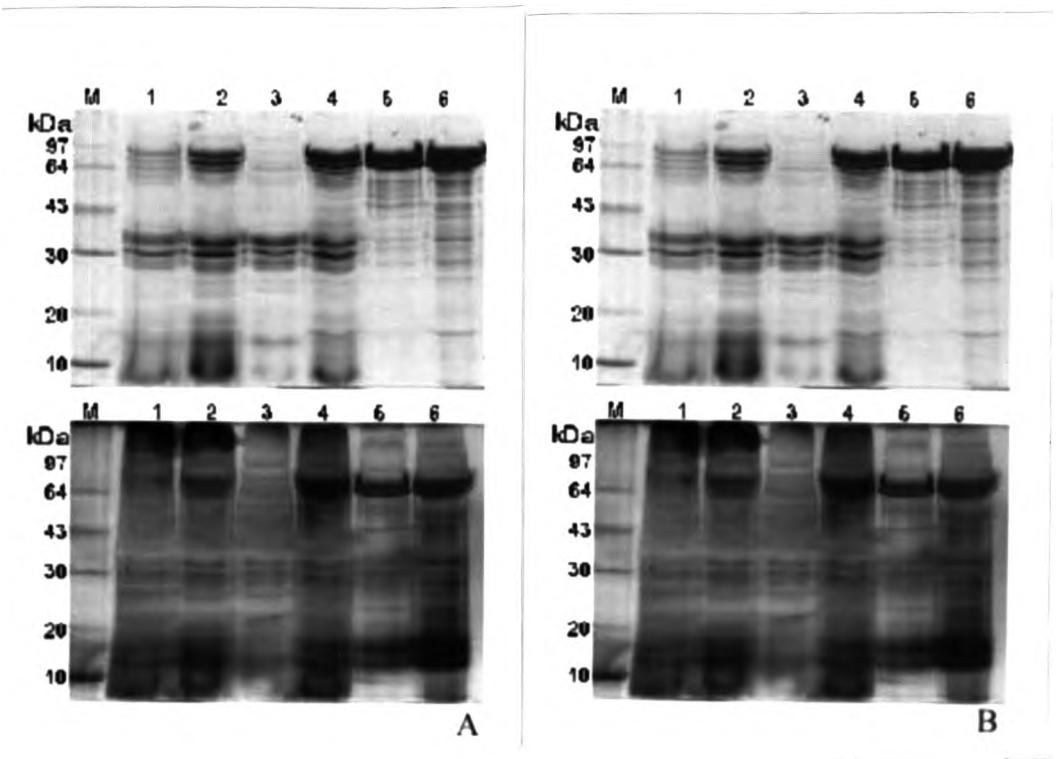


Figure 4.7 Protein analysis of hepatopancreas extracts from shrimps treated at 15°C for 6 hours. Samples were collected at 0, 3, 6, 12, 24, and 72 h of post exposure and separated in 12% SDS-PAGE followed by staining with coomassie stain (upper gel) and silver stain (lower gel).

Lane M: Low molecular weight markers

Lane 1, 3 and 5: control group

Lane 2, 4 and 6: shrimp stress response

Gel A: Samples were collected at 0, 3, and 6 h

Gel B: Samples were collected at 12, 24, and 72 h

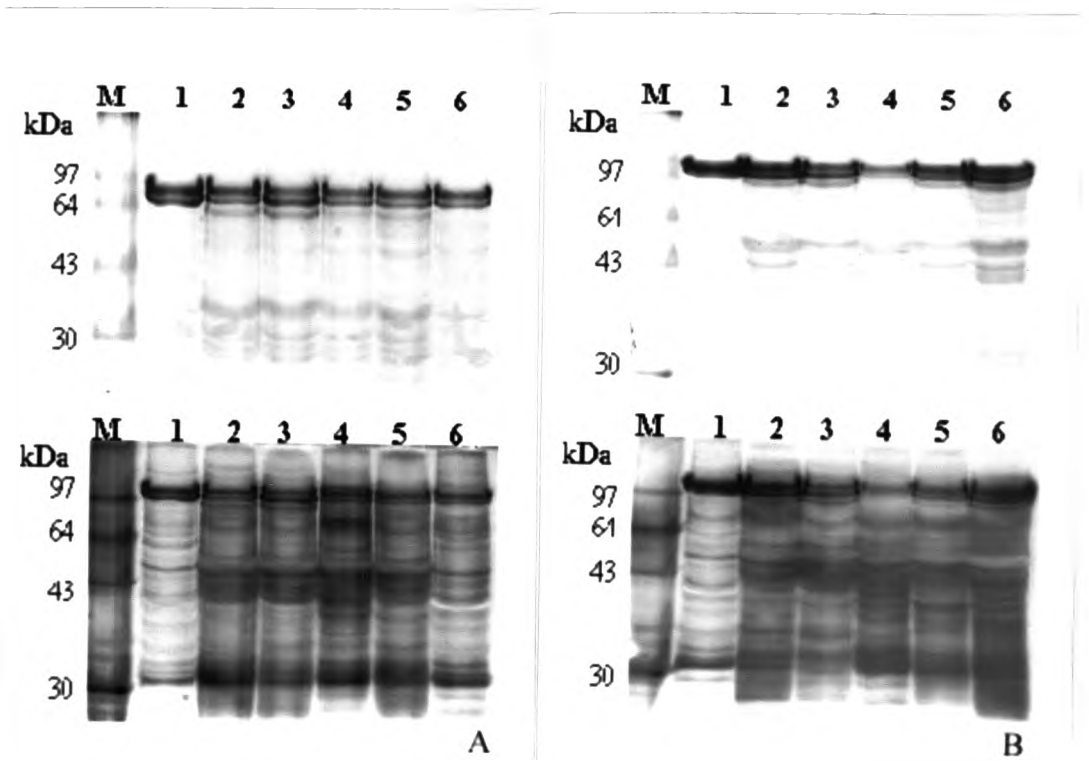


Figure 4.8 Protein analysis of digestive tract extracts from shrimps treated at 15°C for 6 hours. Samples were collected at 0, 3, 6, 12, 24, and 72 hour of post exposure and separated in 12% SDS-PAGE followed by staining with coomassie stain (upper gel) and silver stain (lower gel).

Lane M: Low molecular weight markers

Lane 1, 3 and 5: control group

Lane 2, 4 and 6: shrimp stress response

Gel A: Samples were collected at 0, 3, and 6 h

Gel B: Samples were collected at 12, 24, and 72 h

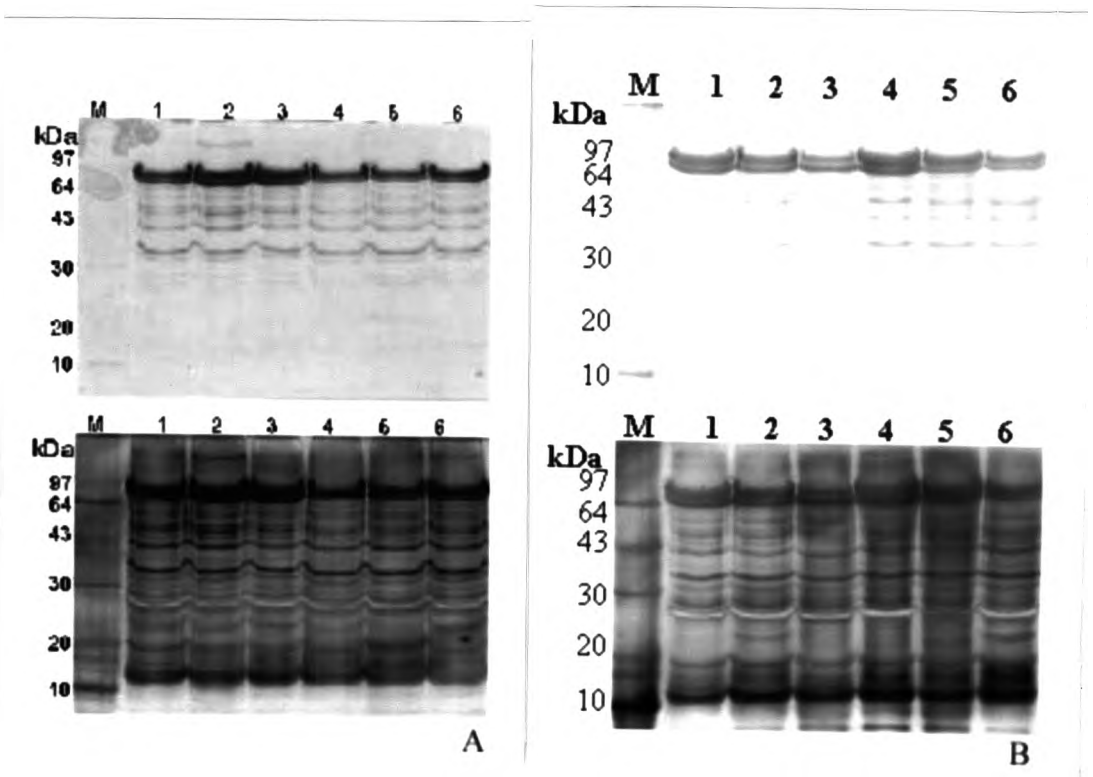


Figure 4.9 Protein analysis of heart extracts from shrimps treated at 15°C for 6 hours. Samples were collected at 0, 3, 6, 12, 24, and 72 h of post exposure and separated in 12% SDS-PAGE followed by staining with coomassie stain (upper gel) and silver stain (lower gel).

Lane M: Low molecular weight markers

Lane 1, 3 and 5: control group

Lane 2, 4 and 6: shrimp stress response

Gel A: Samples were collected at 0, 3, and 6 h

Gel B: Samples were collected at 12, 24, and 72 h

4.3.2 Heat shock

The results of protein analysis from the haemocyte lysate of shrimps induced with heat shock at 30, 33, and 35°C for 6 h were shown in Figure 4.10. Two major bands with the size of approximately 70 and 81 kDa were also observed in most samples. The accumulated proteins of tissue extracts from all heat shock condition were shown in Figure 4.11-4.15, respectively. Similar to the protein profiles of samples detected in cold shock shrimps, no obvious or consistent differences between peptide bands from control and treatment samples were clearly detected from the tissue extracts of heat shock shrimps. Estimating by molecular weight and the amount that found in many tissue extracts, those two major bands were expected to be the trace of haemocyanin which was the main protein found in the haemolymph of most invertebrates.

The results indicated that the change of protein profiles from *P. monodon* treated with both cold and heat shock were not detectable by SDS-PAGE technique.

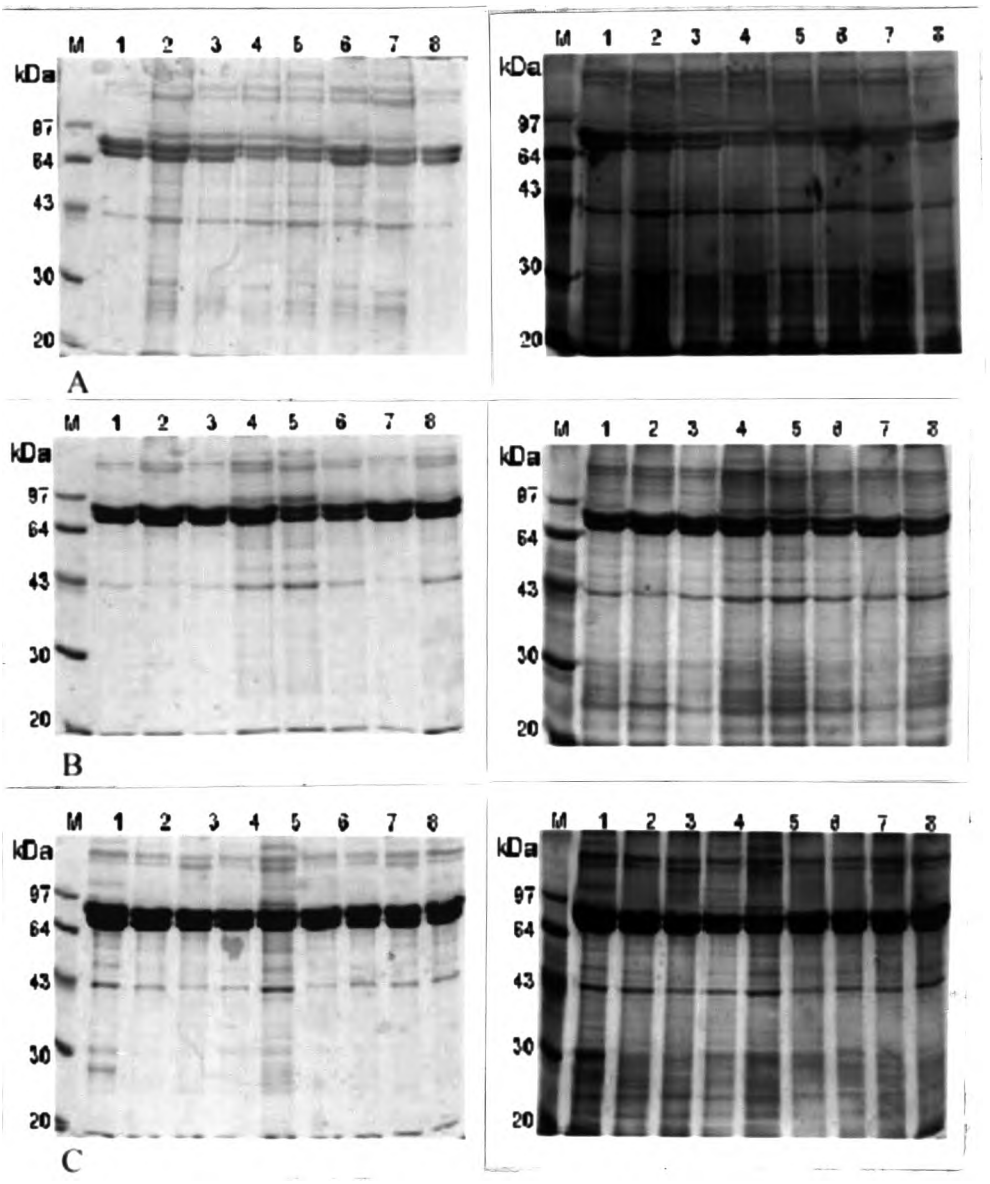


Figure 4.10 Protein analysis of haemocyte lysate from shrimps treated at 30, 33, and 35°C for 6 h. Samples were collected at 0, 3, 6, 12, 24, and 72 h of post exposure and separated in 12% SDS-PAGE, followed by staining with coomassie stain (left) and silver stain (right).

Lane M: Low molecular weight markers

Lane 1 and 5: control group

Lane 2 and 6: thermal shock shrimp at 30°C

Lane 3 and 7: thermal shock shrimp at 33°C

Lane 4 and 8: thermal shock shrimp at 35°C

Gel A: Samples were collected at 0 and 3 hours, Gel B: Samples were collected at 6 and 12 h, and Gel C: Samples were collected at 24 and 72 h.

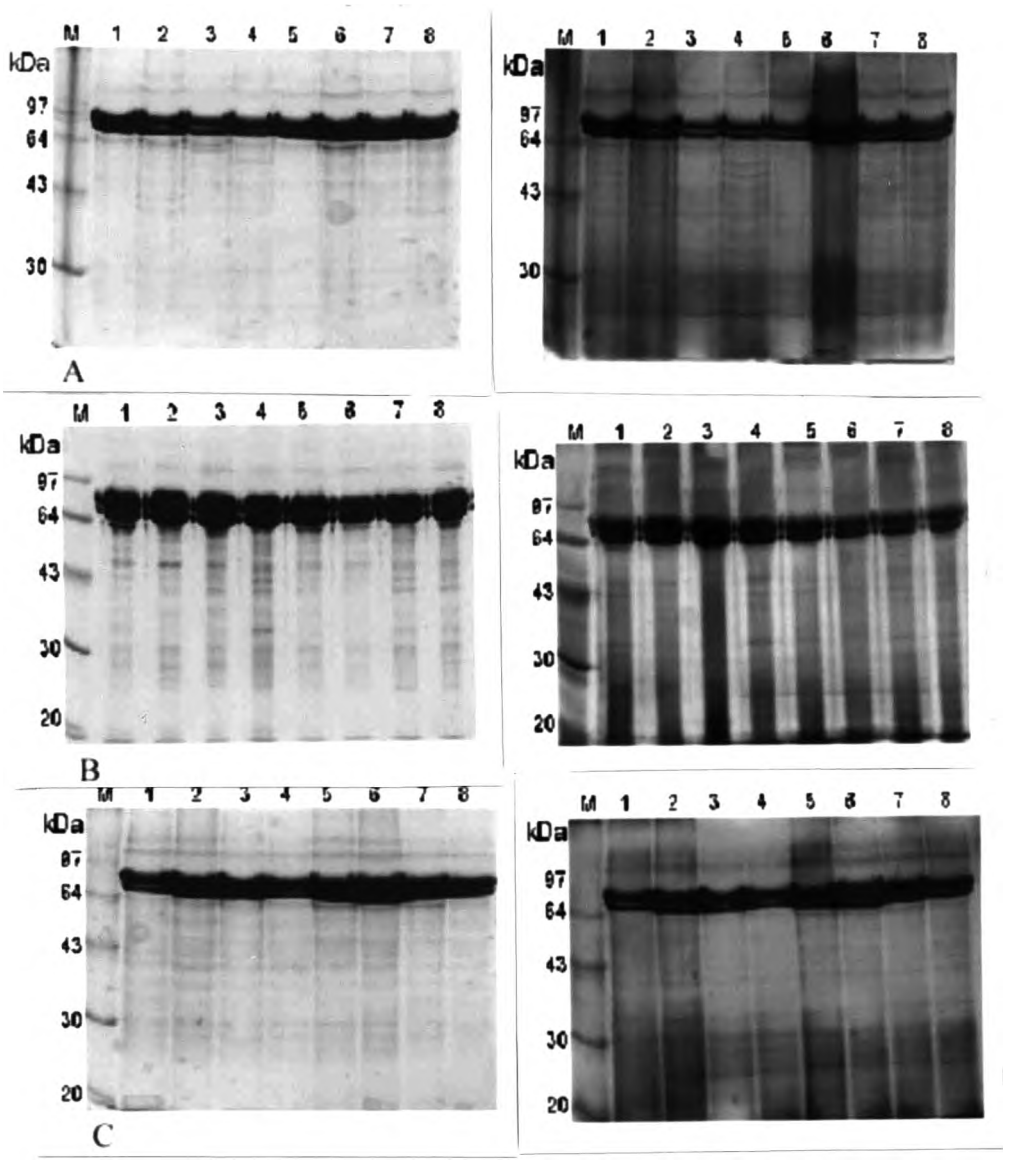


Figure 4.11 Protein analysis of gill extracts from shrimps treated at 30, 33, and 35°C for 6 hours. Samples were collected at 0, 3, 6, 12, 24, and 72 h of post exposure and separated in 12% SDS-PAGE, followed by staining with coomassie stain (left) and silver stain (right).

Lane M: Low molecular weight markers

Lane 1 and 5: control group

Lane 2 and 6: thermal shock shrimp at 30°C

Lane 3 and 7: thermal shock shrimp at 33°C

Lane 4 and 8: thermal shock shrimp at 35°C

Gel A: Samples were collected at 0 and 3 h, Gel B: Samples were collected at 6 and 12 h, and Gel C: Samples were collected at 24 and 72 h

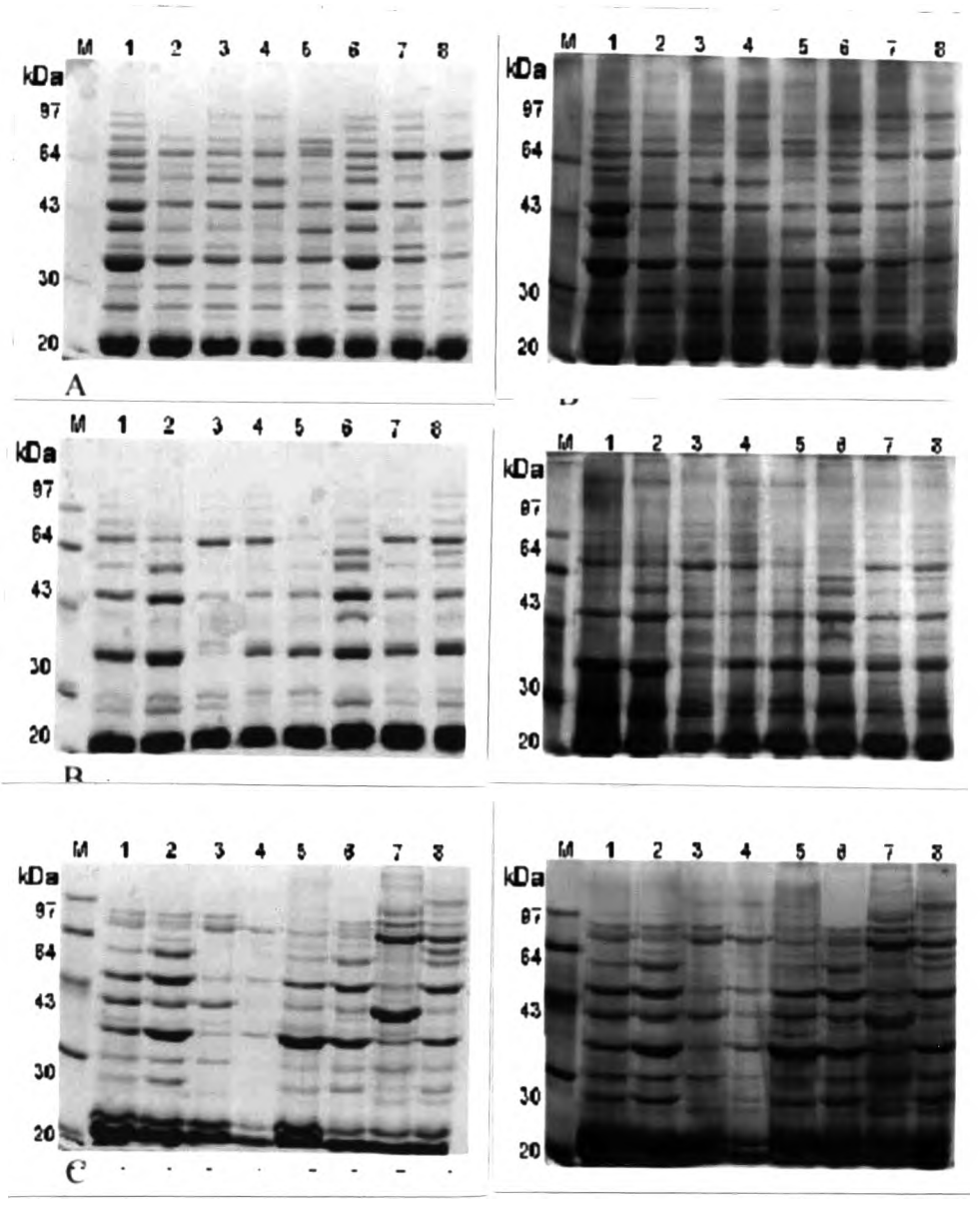


Figure 4.12 Protein analysis of muscle extracts from shrimps treated at 30, 33, and 35°C for 6 h. Samples were collected at 0, 3, 6, 12, 24, and 72 h of post exposure and separated in 12% SDS-PAGE, followed by staining with coomassie stain (left) and silver stain (right).

Lane M: Low molecular weight markers

Lane 1 and 5: control group

Lane 2 and 6: thermal shock shrimp at 30°C

Lane 3 and 7: thermal shock shrimp at 33°C

Lane 4 and 8: thermal shock shrimp at 35°C

Gel A: Samples were collected at 0 and 3 h, Gel B: Samples were collected at 6 and 12 h, and Gel C: Samples were collected at 24 and 72 h.

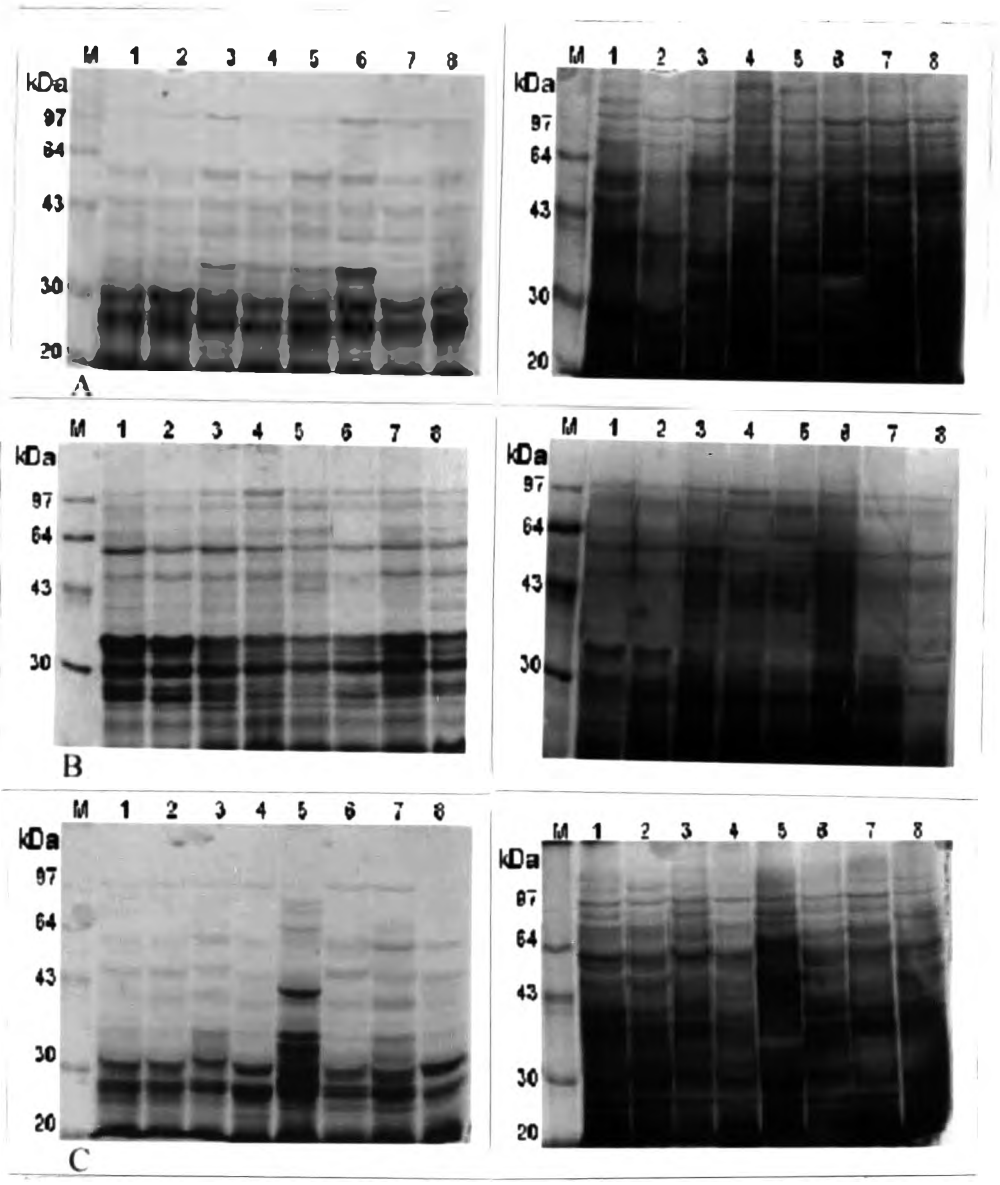


Figure 4.13 Protein analysis of hepatopancreas extracts from shrimps treated at 30, 33, and 35°C for 6 h. Samples were collected at 0, 3, 6, 12, 24, and 72 h of post exposure and separated in 12% SDS-PAGE, followed by staining with coomassie stain (left) and silver stain (right).

Lane M: Low molecular weight markers

Lane 1 and 5: control group

Lane 2 and 6: thermal shock shrimp at 30°C

Lane 3 and 7: thermal shock shrimp at 33°C

Lane 4 and 8: thermal shock shrimp at 35°C

Gel A: Samples were collected at 0 and 3 h, Gel B: Samples were collected at 6 and 12 h, and Gel C: Samples were collected at 24 and 72 h.

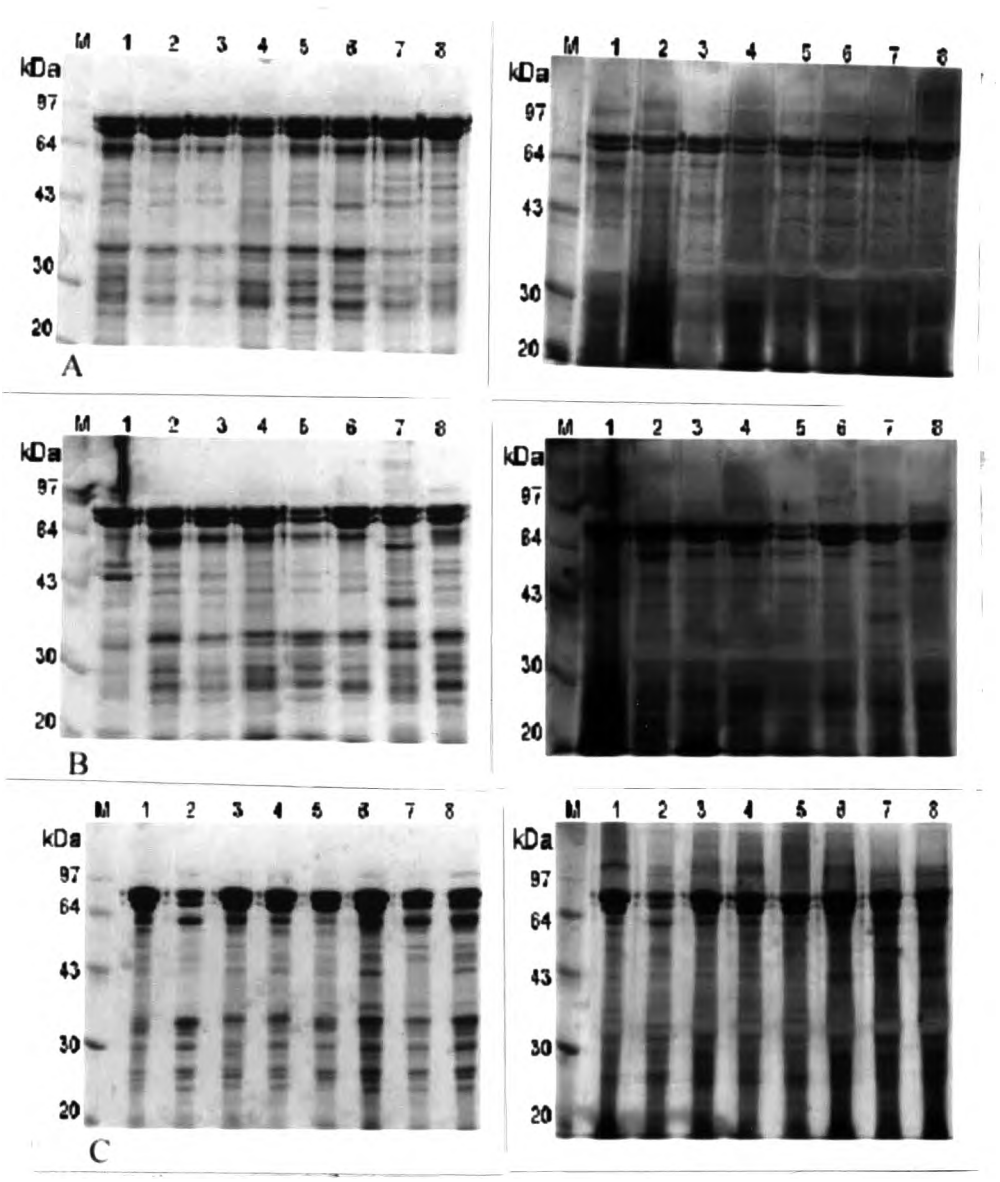


Figure 4.14 Protein analysis of digestive tract extracts from shrimps treated at 30, 33, and 35°C for 6 h. Samples were collected at 0, 3, 6, 12, 24, and 72 h of post exposure and separated in 12% SDS-PAGE, followed by staining with coomassie stain (left) and silver stain (right).

Lane M: Low molecular weight markers

Lane 1 and 5: control group

Lane 2 and 6: thermal shock shrimp at 30°C

Lane 3 and 7: thermal shock shrimp at 33°C

Lane 4 and 8: thermal shock shrimp at 35°C

Gel A: Samples were collected at 0 and 3 h, Gel B: Samples were collected at 6 and 12 h, and Gel C: Samples were collected at 24 and 72 h.

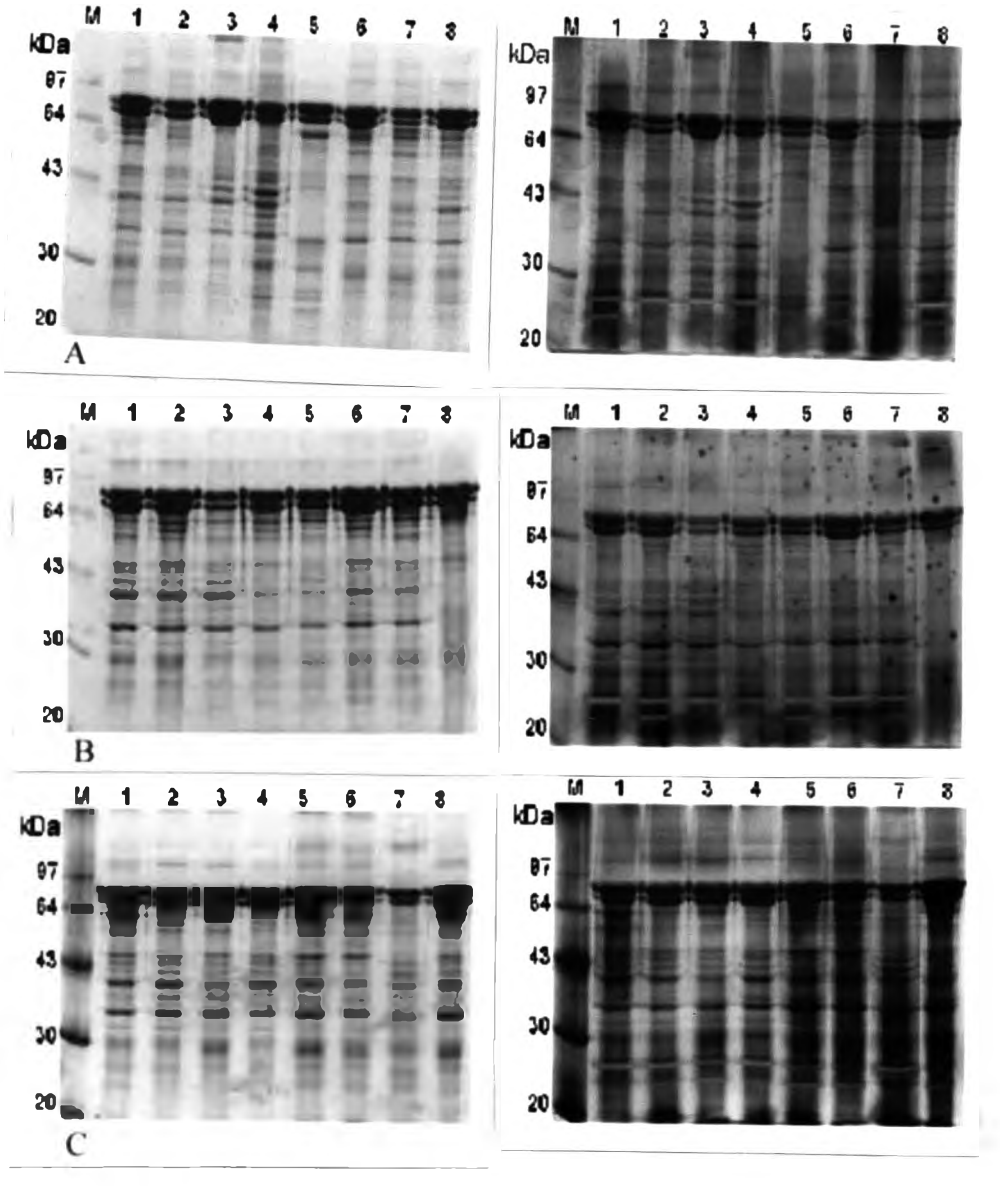


Figure 4.15 Protein analysis of heart extracts from shrimps treated at 30, 33, and 35°C for 6 h. Samples were collected at 0, 3, 6, 12, 24, and 72 h of post exposure and separated in 12% SDS-PAGE, followed by staining with coomassie stain (left) and silver stain (right).

Lane M: Low molecular weight markers

Lane 1 and 5: control group

Lane 2 and 6: thermal shock shrimp at 30°C

Lane 3 and 7: thermal shock shrimp at 33°C

Lane 4 and 8: thermal shock shrimp at 35°C

Gel A: Samples were collected at 0 and 3 hours, Gel B: Samples were collected at 6 and 12 h, and Gel C: Samples were collected at 24 and 72 h.

4.4 Western blot analysis

Haemocyte lysates from shrimps treated with cold and heat shock were subjected to Western blot analysis to detect the presence of HSP60, HSP70 and HSP90 using cross activity of monoclonal antibodies against Human, Bovine and Water mold, respectively.

The results from Western blotting analysis of the haemocyte lysates from the shrimps from control, cold and heat shock experiments showed a considerably clear signal of cross reaction of monoclonal anti-HSP70 antibody and the proteins at 76 kDa. The intensity of the bands increased in coordination with the temperature and time of induction and the intensity of the band from induced shrimp samples appeared to be higher than control (Figure 4.16). The intensities of the bands from the samples at 12 h of post exposure appeared to higher than the ones at 6 h of post exposure and control, respectively, indicating the increase of HSP70 accumulation after thermal shock due to time and temperature level. This indicated that HSP70 was present in normal shrimps and could be induced by both cold and heat shock. For the detection of HSP60 and HSP90 in the haemocyte lysate of thermal treated shrimps, no positive recognition between proteins samples and antibodies was detected (no Figure show). This was probably because of low cross reactivity and low sensitivity of the antibodies used in this study, causing no detectable bands of HSP60 and HSP90. It should be noted that molecular weight of HSP70 detected by Western blot was very close to the major bands of haemocyanin as mentioned earlier. Therefore, the change of HSP70 could not probably be observed by SDS-PAGE with only non-specific staining such as coomassie and silver staining.

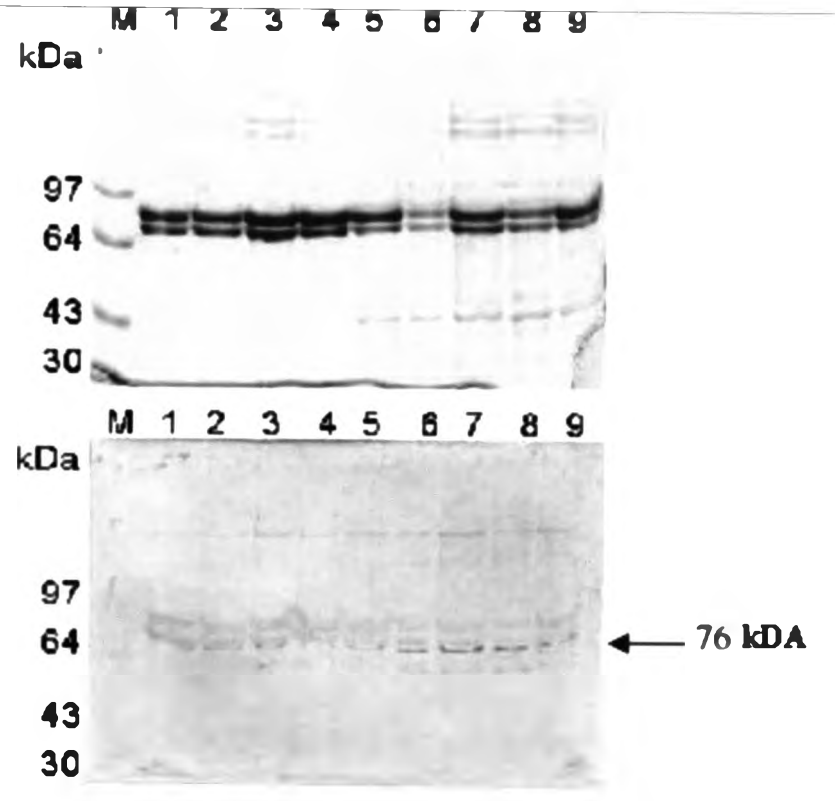


Figure 4.16 Western blot analysis in the haemocyte lysate of thermal shock shrimps at 15, 30, 33, and 35°C temperature using mouse anti-HSP70. Samples were collected at 6 and 12 h of post thermal exposure. Upper figure was Coomassie stained gel and lower figure was membrane probed with mouse anti-HSP70.

M: Low molecular weight marker

Lane 1: control shrimp

Lane 2 and 6: shrimp thermally shocked at 15°C for 6 and 12 h

Lane 3 and 7: shrimp thermally shocked at 30°C for 6 and 12 h

Lane 4 and 8: shrimp thermally shocked at 33°C for 6 and 12 h

Lane 5 and 9: shrimp thermally shocked at 35°C for 6 and 12 h

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

Bacterial tolerance in the shrimps pre-treated with thermal shock was determined by the administration of *V. harveyi* to the thermal pre-treated and normal shrimps. The virulence test of *V. harveyi* for determining the LC_{50} in *P. monodon* was initially conducted. The appropriate concentration of *V. harveyi* for challenge test was then used. The survival rate of the thermal pre-treated shrimps was recorded and compared to that of the normal shrimps.

4.5.1 Acute virulence test of *Vibrio harveyi* in *P. monodon*

Prior to the bacterial challenge test in thermal shock experiments, the virulence of the *V. harveyi* strain 1526 was tested in *P. monodon*.

Shrimps were exposed to *V. harveyi* by suspension administration ranging from 10^6 to 10^9 CFU/ml. The results were illustrated in Table 4.4. No mortality from the shrimps of all treatments was detected within the first 12 h of exposure. Complete survival was still observed in control shrimps within 96 h of exposure. Fifty percent mortality (LC_{50}) of the shrimps exposed to *V. harveyi* at the concentrations of 10^6, 10^7, 10^8, and 10^9 CFU/ml were detected at 6, 5, 3, and 2 days, respectively.

Table 4.4 Survival rate of shrimps exposed to various concentrations of the *V. harveyi* strain 1526.

Hours	Survival rate (%)				
	Control	10^6CFU/ml	10^7CFU/ml	10^8CFU/ml	10^9CFU/ml
0	100	100	100	100	100
3	100	100	100	100	100
6	100	100	100	100	100
12	100	100	100	100	100
24	100	100	90	90	90
48	100	90	70	60	50
72	100	90	60	50	30
96	100	80	60	30	20
120	90	90	50	20	10
144	80	30	20	0	0
168	80	10	0	0	0

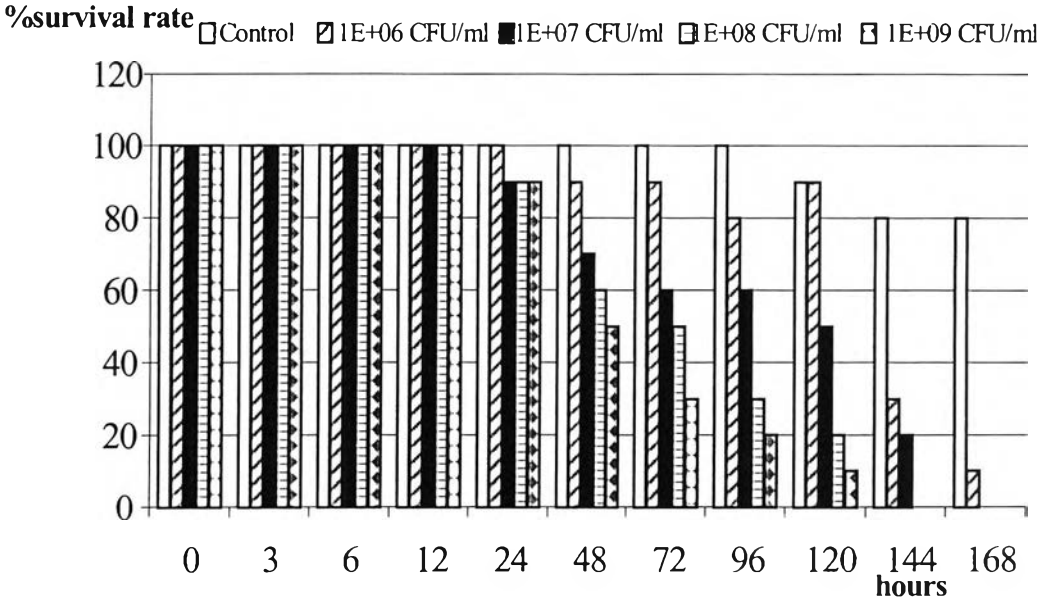


Figure 4.17 Survival rates of shrimps exposed to various concentrations of the *V. harveyi* strain 1526.

4.5.2 *V. harveyi* challenge test in the shrimps pre-treated with thermal shock

Following the result of the acute virulence test in 4.5.1, *V. harveyi* concentration at 10^8 CFU/ml which was LC_{50} at 72 h was applied by suspension administration to 2 groups of shrimps; normal shrimps and the shrimps pre-treated at 35°C for 6 h. The shrimps neither treated by heat nor challenged by *V. harveyi* were regarded as control. The survival rates from the shrimps in each group were shown in Table 4.5 and Figure 4.18. The significant difference of survival rates from the shrimps in each group was detected within 2 days of *V. harveyi* exposure ($P<0.05$). During 15 days of exposure, the survival rates of the shrimps from every group decreased in correlation with time of exposure. The survival rate of the shrimps without pre-heat treatment decreased dramatically. Only 2% of survival rate was detected after 15 days of exposure. Pre-heated shrimps obtained significant higher survival rate than that of the shrimps without pre-heat treatment ($P<0.05$). Control shrimps gave the highest survival rate during 15 days of *V. harveyi* exposure.

It was indicated by the result that the *V. harveyi* tolerance in *P. monodon* could be induced by heat shock at 35°C for 6 h.

Table 4.5 Survival rates of pre-heat induced shrimps exposed to 10^8CFU/ml of *V. harveyi* compared to un-induced and control shrimps.

Days	Survival rate (%)		
	Control shrimps	**Heat induced shrimps**	**Un-induced shrimps**
1	100	100	100
2	94	86	80
3	94	82	56
4	94	78	54
5	88	70	46
6	86	66	34
7	84	52	30
8	70	44	22
9	68	36	12
10	68	36	12
11	60	26	6
12	60	22	6
13	54	16	4
14	52	12	4
15	50	12	2

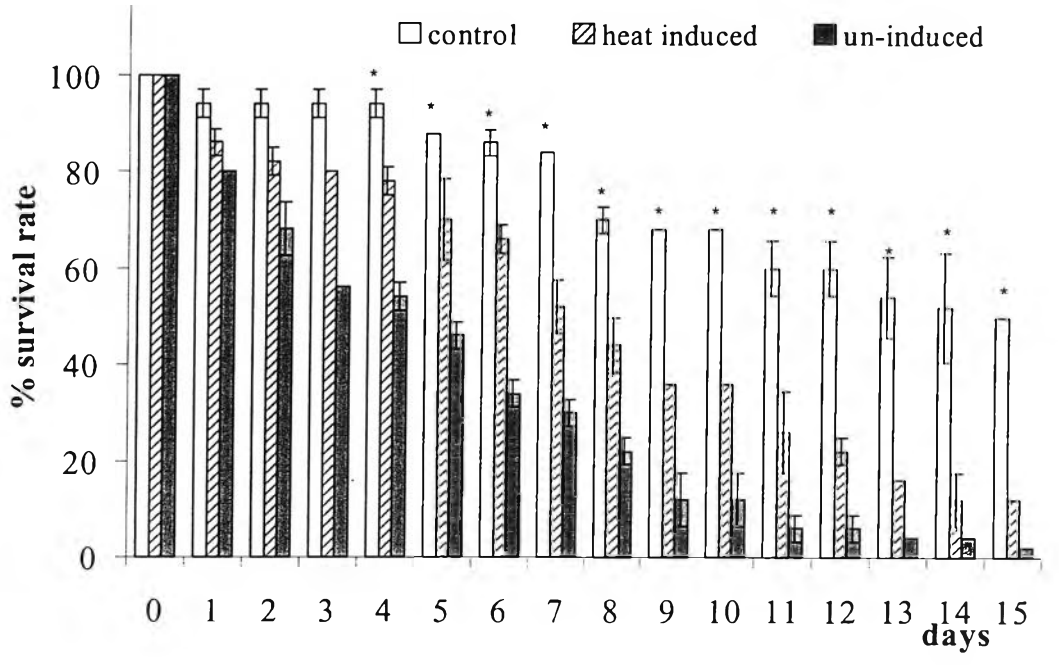


Figure 4.18 Survival rates of pre-heat induced, un-induced and normal shrimps exposed to 10^8 CFU/ml of *V. harveyi* (N=2). All data were derived from Table 4.6. The lines above the bar represent standard derivation. Significantly different at days 4-15 (P<0.05 by ANOVA) was indicated by the symbol (*).

4.6 Physiological responses in pre-heat induced shrimps exposed to *V. harveyi*

Protein and glucose concentrations in the haemolymph of pre-heat induced shrimps after exposing to *V. harveyi* were monitored in comparison with that of un-induced and control shrimps. The result was shown in Table 4.6-4.7 and Figure 4.18-4.19.

Higher protein concentration (65.7 mg/ml) was clearly detected in the haemolymph of pre-heat induced shrimps within the first hour of post exposure when compared to that of un-induced (51.2 mg/ml) and control shrimps (51.5 mg/ml) (P<0.05). The highest protein level (72.6 mg/ml) was detected within 12 h of post exposure and began to decrease to the same level as in un-induced and normal shrimps (51.3 mg/ml) at 168 h of post exposure while there was no significant difference between the protein levels of un-induced and control shrimps throughout the post exposure time. This indicated that the protein concentration from the haemolymph of the shrimps was induced by heat treatment and there was no induction on plasma protein level by *vibrio* exposure.

Table 4.6 Protein concentration of the haemolymph from heat-induced shrimps after *vibrio* exposure (*N*=6).

Hours	Protein concentration (mg/ml)		
	Control shrimps	Heat induced shrimps	Un-induced shrimps
0	51.5±14.2	65.7±7.30	51.2±12.0
3	54.2±14.0	68.4±4.70	54.8±8.80
6	52.9±11.1	72.6±10.1	56.9±8.30
12	50.7±14.8	69.7±10.6	62.9±9.90
24	54.1±11.6	67.5±11.0	54.3±10.4
72	50.5±11.8	62.9±8.50	52.0±11.3
120	54.2±12.3	58.2±8.00	52.7±8.80
168	51.5±14.7	51.3±16.3	54.1±11.6

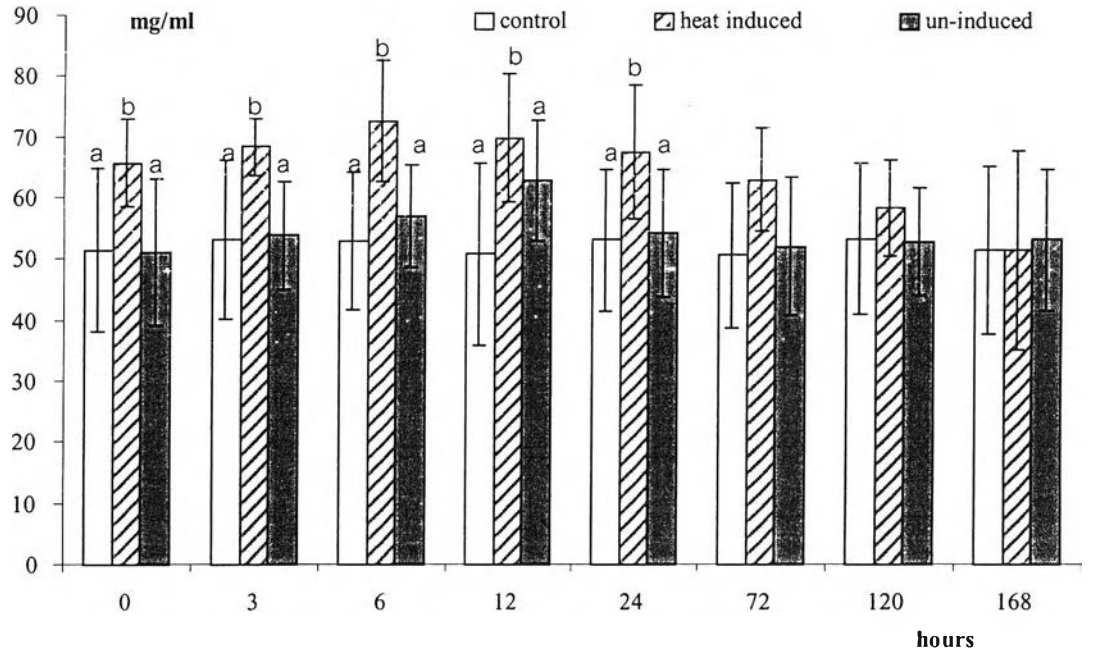


Figure 4.19 Protein concentration of the haemolympg from heat-induced shrimp after *vibrio* exposure (*N*=6). All data were derived from Table 4.6. The lines above the bar represent standard derivation. Values that are significantly different have different superscripts ($P<0.05$ by Duncan's new mutiple range test).

Table 4.7 Glucose concentration of the haemolymph from heat-induced shrimps after *vibrio* exposure (*N*=6).

Hours	Glucose concentration (mg%)		
	Control shrimps	Heat induced shrimps	Un-induced shrimps
0	46.12±5.22	54.72±6.83	51.93±6.27
3	45.52±8.83	60.58±8.13	50.59±5.91
6	47.01±8.00	67.44±6.28	47.76±6.87
12	46.12±7.35	71.31±8.19	48.79±7.98
24	45.37±6.44	62.52±7.65	47.31±6.08
72	45.22±8.11	46.56±5.19	46.12±6.37
120	44.63±8.85	47.31±9.74	48.05±7.59
168	45.23±8.87	46.56±5.02	46.12±4.83

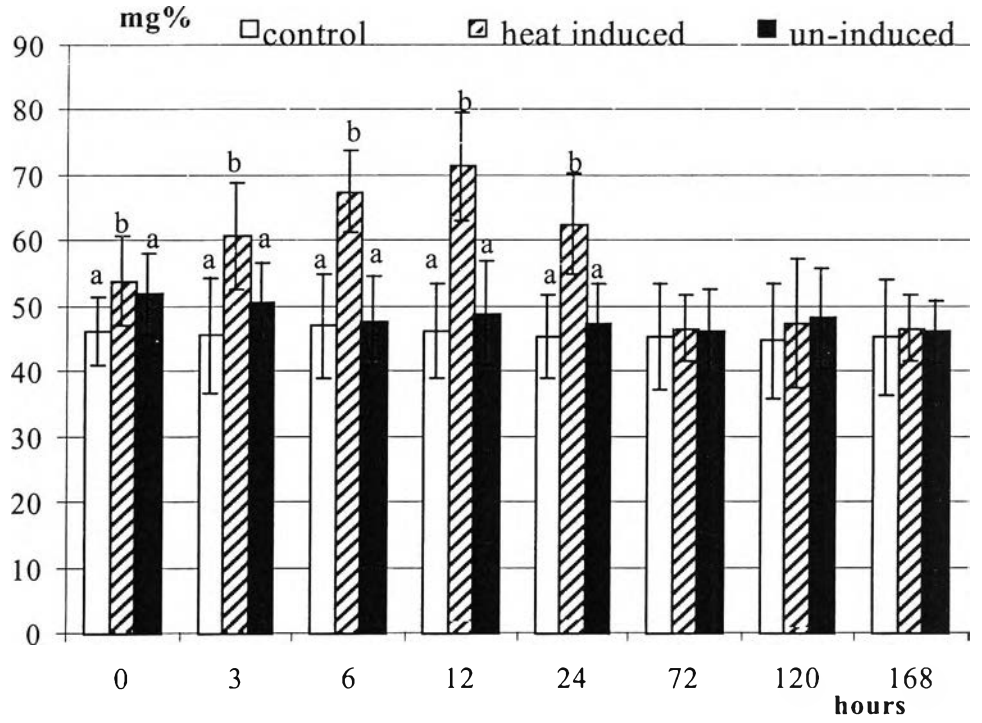


Figure 4.20 Plasma glucose concentration of the haemolympg from heat-induced shrimp after *vibrio* exposure (*N*=6). All data were derived from Table 4.7. The lines above the bar represent standard derivation. Values that are significantly different have different superscripts ($P<0.05$ by Duncan's new mutiple range test).

Similar results were obtained from the detection of glucose level in the haemolymph. Higher glucose concentration (60.58 mg%) was clearly detected in the haemolymph of pre-heat induced shrimps within 3 h when compared to un-induced and control shrimps. Glucose level increased to the highest level (71.31 mg%) at 12 h of post exposure and began to decrease the levels (46.56 mg%) close to the levels obtained from un-induced (45.22 mg%) and control shrimps (46.12 mg%) after 72 h of post exposure and throughout the experiment ($P<0.05$). No significant difference between the glucose levels of un-induced and control shrimps throughout the post exposure time. This indicated that the glucose concentration from the haemolymph of the shrimps was also induced by heat treatment but not by *vibrio* exposure. It could be noted that the increase of glucose level was slightly slower than that of protein level but was able to return to normal level quicker.

4.7 Protein analysis in heat induced shrimps by SDS-PAGE

Protein of haemocyte lysate, gill, muscle, hepatopancreas, digestive tract and heart extracts from heat induced shrimps after *vibrio* exposures were analyzed on 12% denatured polyacrylamide gel. The results were shown in Figure 4.21-4.26.

Similar results as obtained in Figure 4.4-4.15 were detected. Major bands which were suspected as haemocyanin were still observed in most tissues. No distinctive bands among tissue samples from the shrimps in each group were detected. No consistence difference was obtained between the normal shrimps and the shrimps exposed to *vibrio*.

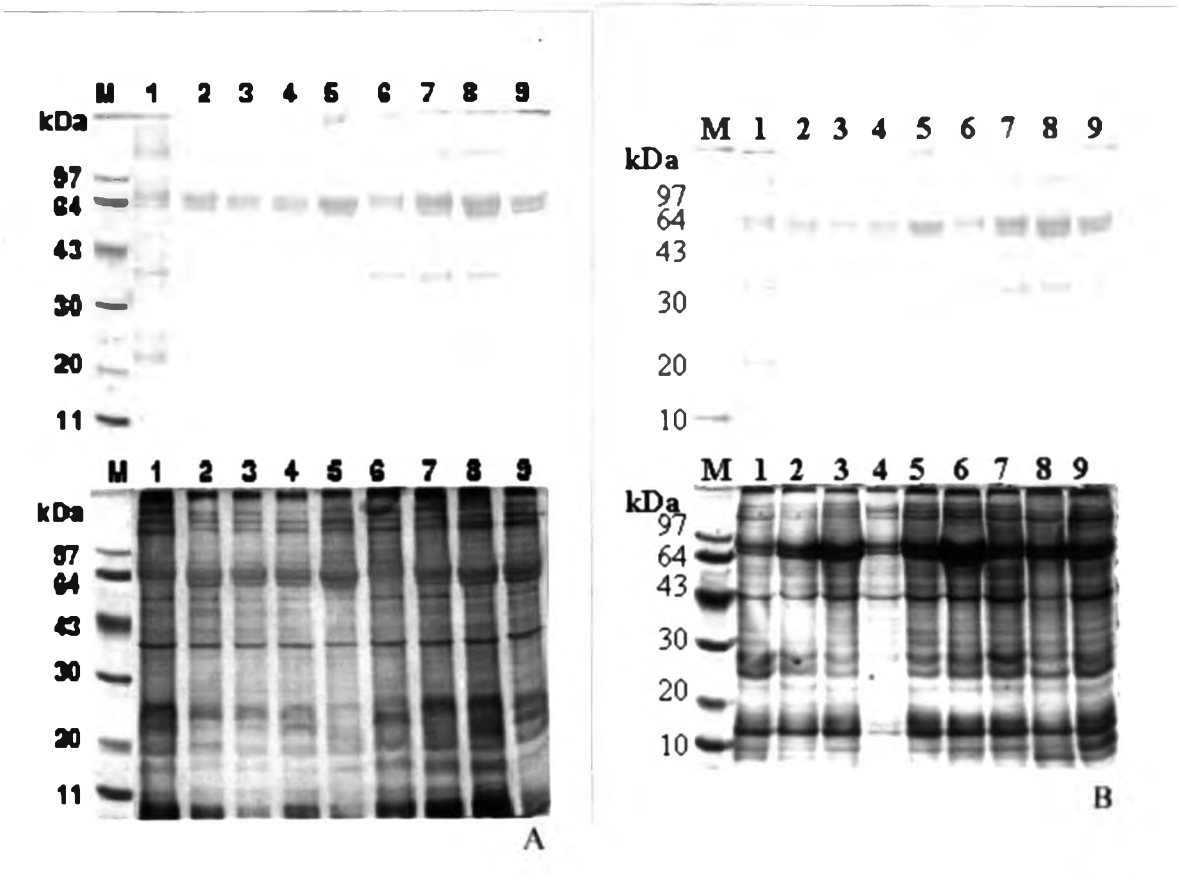


Figure 4.21 Haemocyte lysates of shrimp after challenged by *V. harveyi*. Samples were analyzed on 12% SDS-PAGE and stained with Coomassie (upper gels) and silver (lower gels). A indicates the gels loaded with samples from heat induced shrimps and B are gels loaded with samples from un-induced shrimps. Lane M is Low molecular weight marker, Lane 1 is control, and Lane 2-9 are samples at 0, 3, 6, 24, 72, 120, and 168 h, respective)

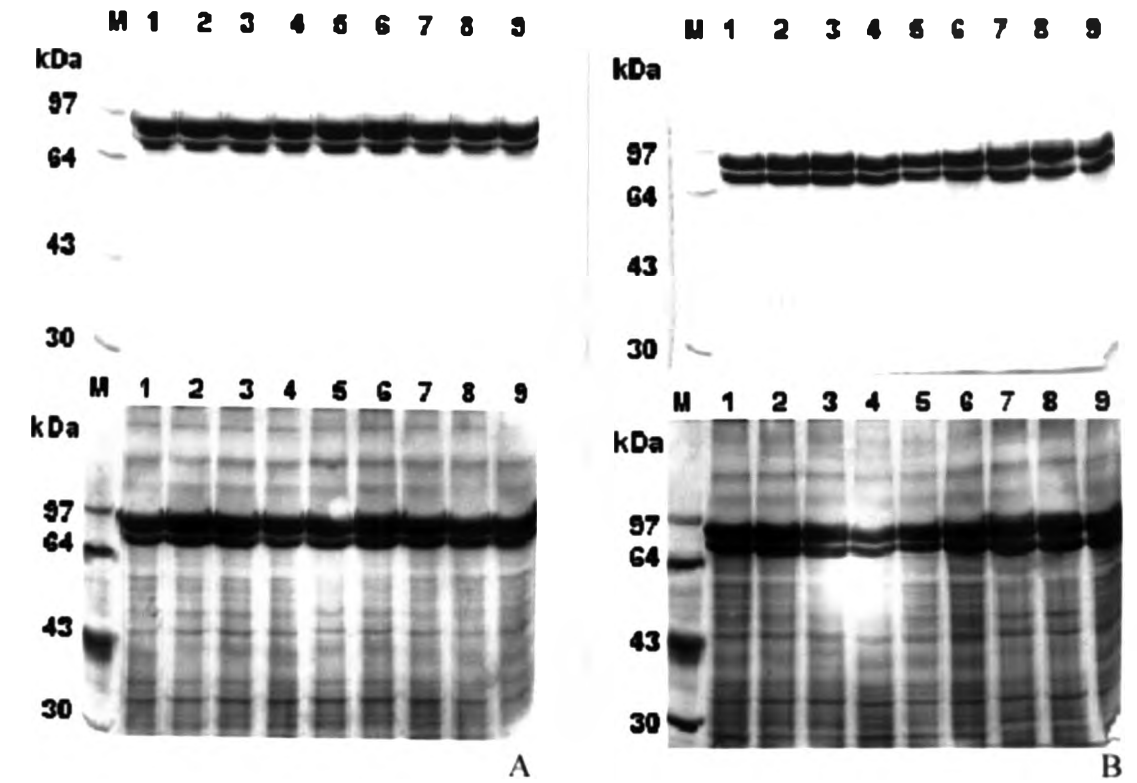


Figure 4.22 Gill extracts of shrimp after challenged by *V. harveyi*. Samples were analyzed on 12% SDS-PAGE and stained with Coomassie (upper gels) and silver (lower gels). A indicates the gels loaded with samples from heat induced shrimps and B are gels loaded with samples from un-induced shrimps. Lane M is Low molecular weight marker, Lane 1 is control, and Lane 2-9 are samples at 0, 3, 6, 24, 72, 120, and 168 h, respective)

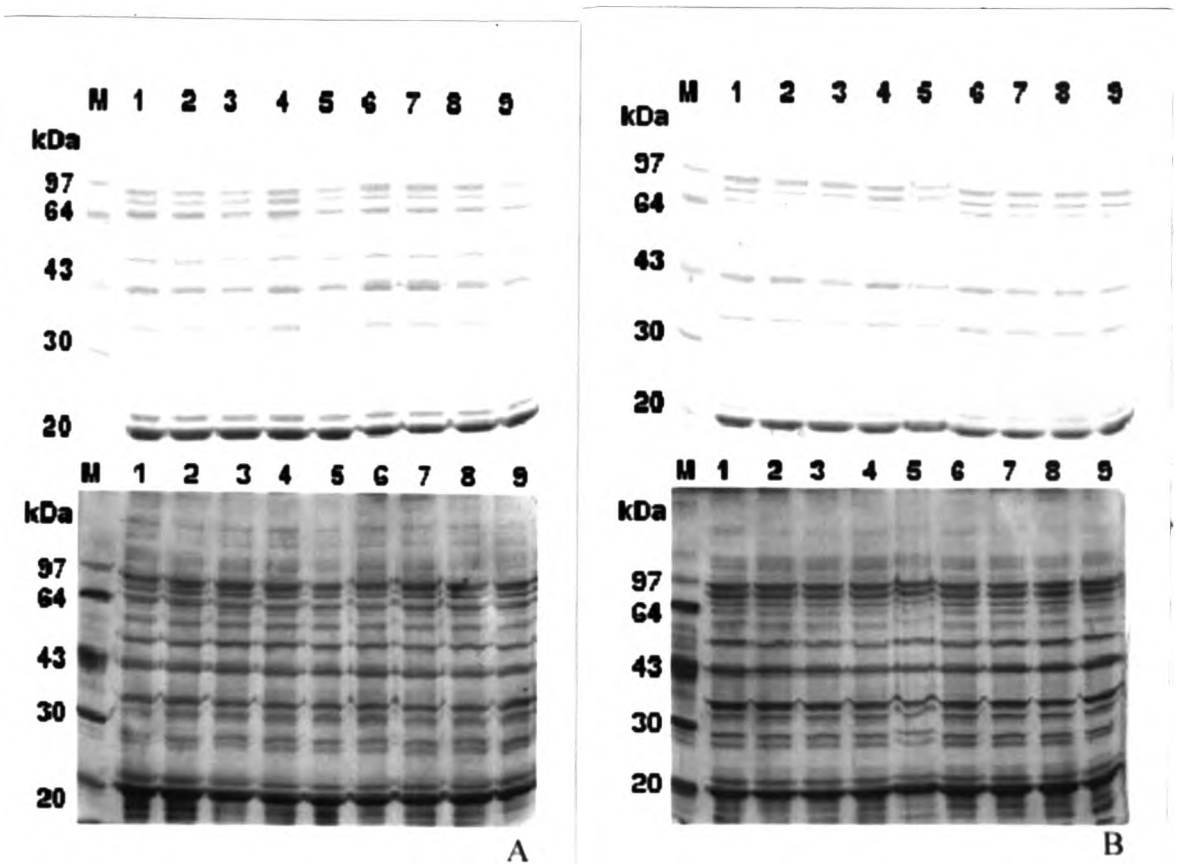


Figure 4.23 Muscle extracts of shrimp after challenged by *V. harveyi*. Samples were analyzed on 12% SDS-PAGE and stained with Coomassie (upper gels) and silver (lower gels). A indicates the gels loaded with samples from heat induced shrimps and B are gels loaded with samples from un-induced shrimps. Lane M is Low molecular weight marker, Lane 1 is control, and Lane 2-9 are samples at 0, 3, 6, 24, 72, 120, and 168 h, respective)

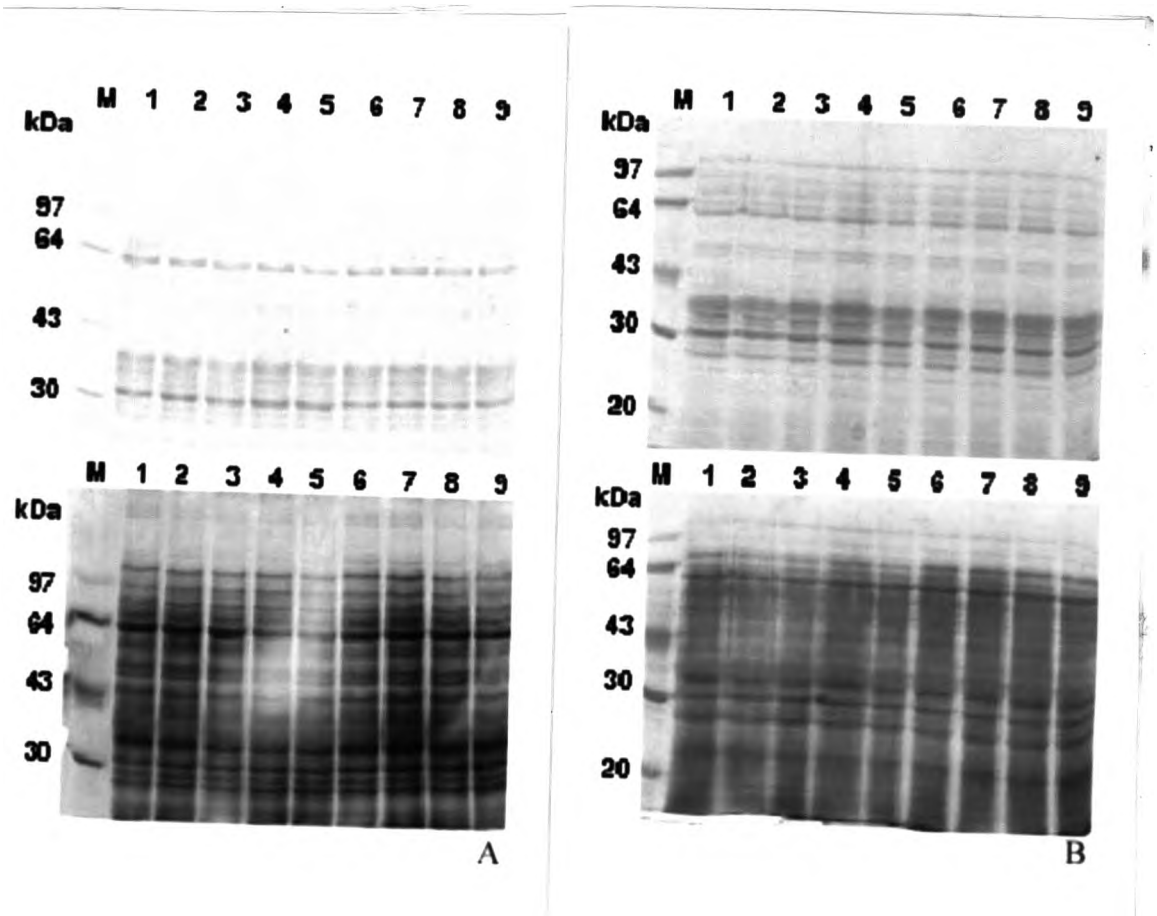


Figure 4.24 Hepatopancreas extracts of shrimp after challenged by *V. harveyi*. Samples were analyzed on 12% SDS-PAGE and stained with Coomassie (upper gels) and silver (lower gels). A indicates the gels loaded with samples from heat induced shrimps and B are gels loaded with samples from un-induced shrimps. Lane M is Low molecular weight marker, Lane 1 is control, and Lane 2-9 are samples at 0, 3, 6, 24, 72, 120, and 168 h, respective)

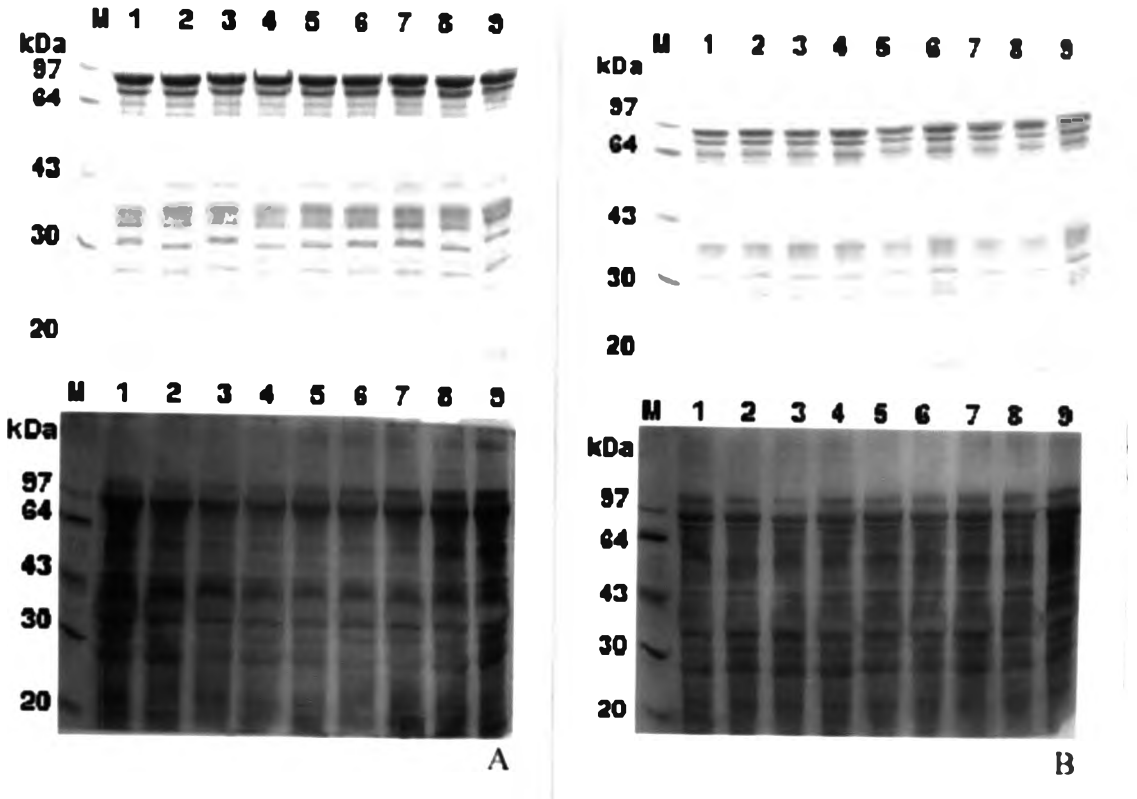


Figure 4.25 Digestive tract extracts of shrimp after challenged by *V. harveyi*. Samples were analyzed on 12% SDS-PAGE and stained with Coomassie (upper gels) and silver (lower gels). A indicates the gels loaded with samples from heat induced shrimps and B are gels loaded with samples from un-induced shrimps. Lane M is Low molecular weight marker, Lane 1 is control, and Lane 2-9 are samples at 0, 3, 6, 24, 72, 120, and 168 h, respective)

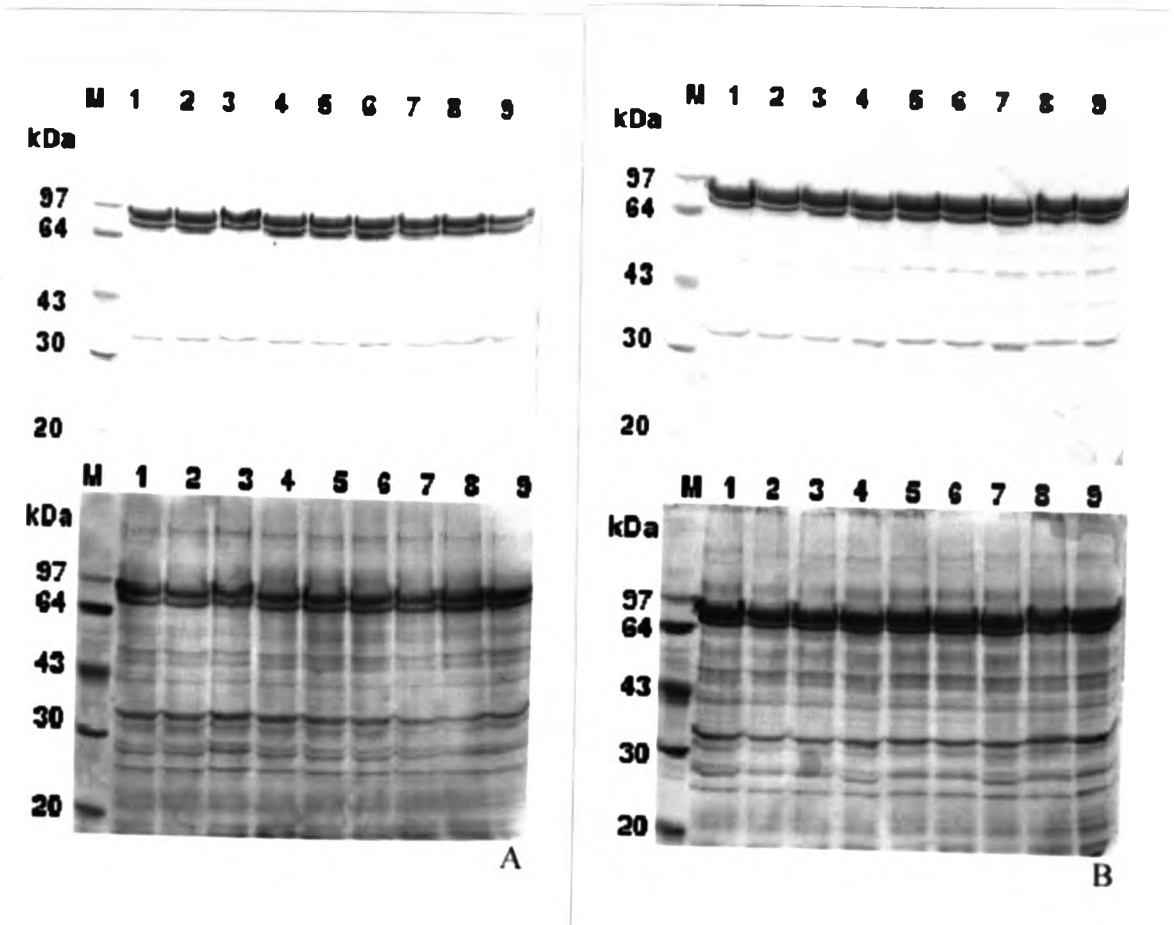


Figure 4.26 Heart extracts of shrimp after challenged by *V. harveyi*. Samples were analyzed on 12% SDS-PAGE and stained with Coomassie (upper gels) and silver (lower gels). A indicates the gels loaded with samples from heat induced shrimps and B are gels loaded with samples from un-induced shrimps. Lane M is Low molecular weight marker, Lane 1 is control, and Lane 2-9 are samples at 0, 3, 6, 24, 72, 120, and 168 h, respective)

4.8 Determination of the genes expressed in response to heat shock in *P. monodon*

Heat-induced genes expressed in the haemocytes of the shrimps in response to heat shock were detected. The expression levels of the genes were differentially displayed using RAP-PCR technique.

Shrimps heated at 15, 27 (ambient), 30, 33, and 35°C for 6 h were bled at 0, 6, 12, 24, and 72 h of post treatment. The average total RNA obtained from the haemocytes of the shrimps was approximately 1.5-2.0 μg per individual. RNAs were analyzed in a 1.5% agarose gel. Two predominant bands of (18S and 28S rRNA) ribosomal RNAs, discrete bands of low melecular weight RNAs, and high molecular weight RNAs were observed (Figure 4.27). Total RNAs were then subjected to first stranded cDNA reverse transcription.

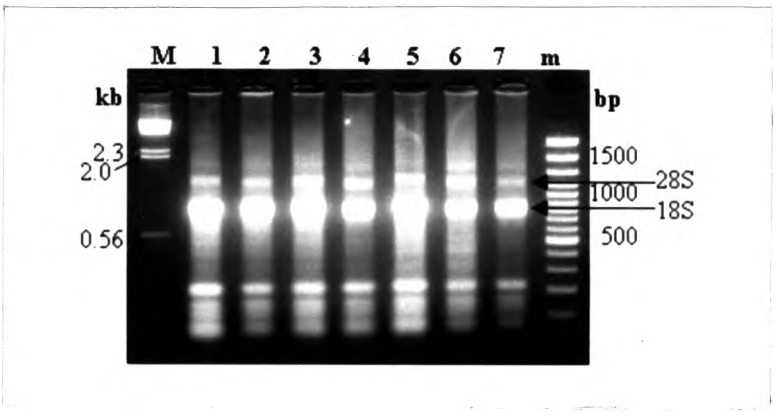


Figure 4.27 Total RNAs extracted from the haemocytes of *P. monodon* and analyzed on 1.5% agarose gel.

Lane M: λ/*Hin*dIII markers

Lane m: 100 bp ladder markers

Lane 1-7: Total RNA (2 μg each) extracted from haemocytes

The arbitrarily decanucleotide primers (UBC122, UBC128, UBC135, UBC158, UBC174, UBC228, UBC268, UBC299, UBC457, and UBC459) were coupled with UBC119 and used for PCR amplification in control and heat shock shrimps. RAP-PCR products were analyzed from the scorable bands ranging from 100 to more than 1000 bp. The results of DNA fragments which revealed up- or down-regulation correlated with heat shock temperature or differential displayed between control and heat shock shrimps were identified and subjected to sequence analysis (Figure 4.28-4.37).

The amplification using UBC119 and UBC122 obtained 1 DNA fragments (320 bp) of up-regulating genes and 1 DNA fragments (360 bp) of down-regulating genes. The amplification of UBC119 and UBC128 revealed 2 DNA fragments (120 and 380 bp) which displayed differentially between control and treatment shrimps and 1 DNA fragments (500 bp) of up-regulating genes. With the combination of UBC119 and UBC135, 2 bands (520 and 900 bp) showed differential display and 1 bands showed down-regulation characteristic. The combination of UBC119 and UBC158 was resulted in 1 DNA fragment of differential display (420 bp), 1 DNA fragment of up-regulation (320 bp) and 1 DNA fragment of down-regulation (360 bp). The amplification using UBC119 and UBC174 showed 1 differentially displayed band (520 bp) and 1 up-regulated band (320 bp). The amplification using UBC119 and UBC228 obtained 1 differential displayed band (420 bp) and 1 up-regulated band (320 bp). For UBC119 and UBC 299 combination, 2 up-regulated bands (260 and 320 bp) were detected. Only 1 up-regulated band (380 bp) was observed in the UBC119 and UBC457 combination. For UBC119 and UBC459 combination, 2 up-regulated bands (260 and 320 bp) were detected.

The results of RAP-PCR from 10 primer combinations revealed that 7 DNA fragments were displayed differentially between control and heat shock shrimps. Ten DNA fragments were up-regulated and 3 DNA fragments were down-regulated in corresponding to the heat shock temperature. Details were summarized in Table 4.8.

Table 4.8 Summary of heat-related genes expressed differentially in the shrimps induced at 30, 33, and 35°C for 6 h. The expressed genes were detected by RAP-PCR analysis.

Pattern	Size (bp)	Primer combination
1) Differential display:	120 and 380	UBC119+UBC128
The expression undetectable in control	520 and 900	UBC119+UBC135
shrimps but present in heat shock shrimps	420	UBC119+UBC158
	520	UBC119+UBC174
	420	UBC119+UBC228
2) Up-regulation:	320	UBC119+UBC122
The expression increased in correlation with	500	UBC119+UBC128
the increase of heat shock temperature	320	UBC119+UBC158
	320	UBC119+UBC174
	320	UBC119+UBC228
	260 and 320	UBC119+UBC299
	380	UBC119+UBC457
	260 and 320	UBC119+UBC459
3) Down-regulation:	360	UBC119+UBC122
The expression decreased in correlation	400	UBC119+UBC135
with the increase of heat shock temperature	360	UBC119+UBC158

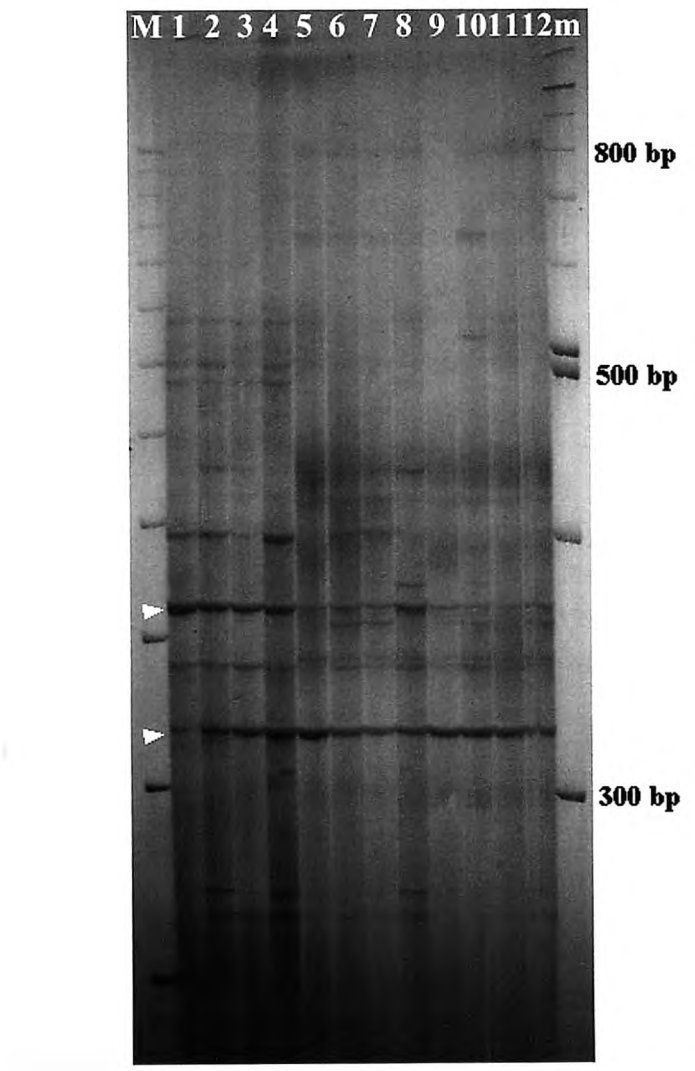


Figure 4.28 RAP-PCR patterns of *P. monodon* using primers UBC119 and UBC122 eletrophoretically analyzed by a 4.5% denaturing acrylamide gel. White arrows indicate DNA bands expressed differentially

Lane M: 50 bp ladder marker

Lane m: 100 bp ladder marker

Lane 1,5 and 9: control shrimps at 0, 6, and 12 h

Lane 2, 6 and 10: 30°C induced shrimps at 0, 6, and 12 h

Lane 3, 7 and 11: 33°C induced shrimps at 0, 6, and 12 h

Lane 4, 8 and 12: 35°C induced shrimps at 0, 6, and 12 h

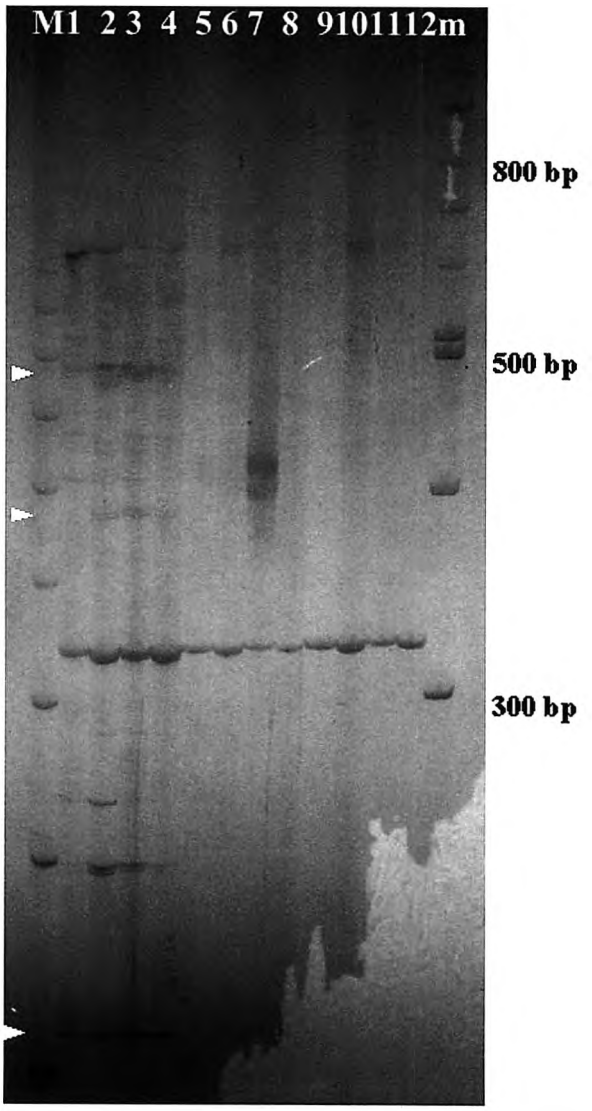


Figure 4.29 RAP-PCR patterns of *P. monodon* using primers UBC119 and UBC128 eletrophoretically analyzed by a 4.5 % denaturing acrylamide gel. White arrows indicate DNA bands expressed differentially

Lane M: 50 bp ladder marker

Lane m: 100 bp ladder marker

Lane 1,5 and 9: control shrimps at 0, 6, and 12 h

Lane 2, 6 and 10: 30°C induced shrimps at 0, 6, and 12 h

Lane 3, 7 and 11: 33°C induced shrimps at 0, 6, and 12 h

Lane 4, 8 and 12: 35°C induced shrimps at 0, 6, and 12 h

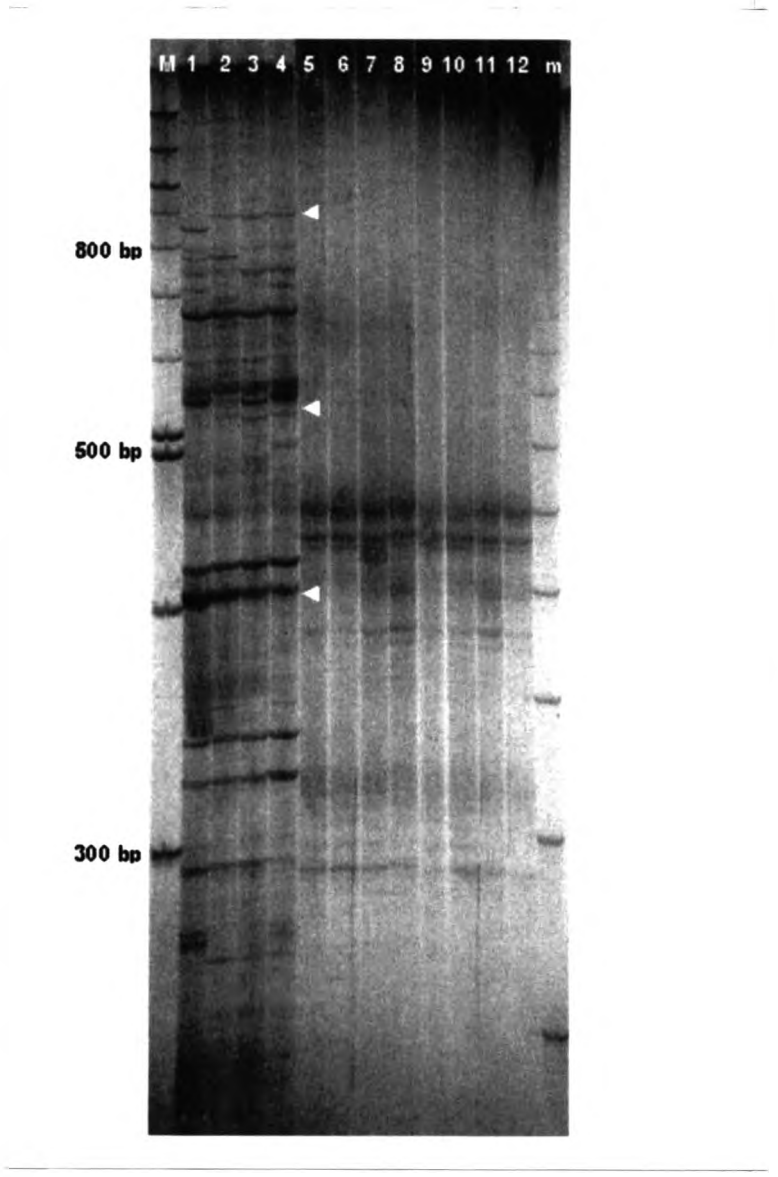


Figure 4.30 RAP-PCR patterns of *P. monodon* using primers UBC119 and UBC135 eletrophoretically analyzed by a 4.5 % denaturing acrylamide gel. White arrows indicate DNA bands expressed differentially

Lane M: 50 bp ladder marker

Lane m: 100 bp ladder marker

Lane 1,5 and 9: control shrimps at 0, 6, and 12 h

Lane 2, 6 and 10: 30°C induced shrimps at 0, 6, and 12 h

Lane 3, 7 and 11: 33°C induced shrimps at 0, 6, and 12 h

Lane 4, 8 and 12: 35°C induced shrimps at 0, 6, and 12 h

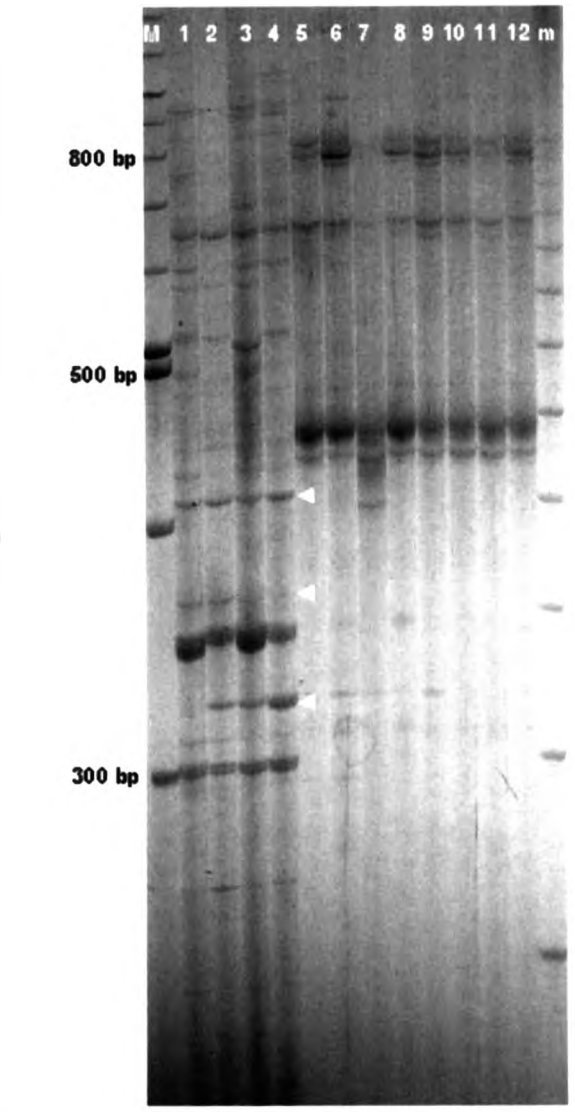


Figure 4.31 RAP-PCR patterns of *P. monodon* using primers UBC119 and UBC158 eletrophoretically analyzed by a 4.5 % denaturing acrylamide gel. White arrows indicate DNA bands expressed differentially

Lane M: 50 bp ladder marker

Lane m: 100 bp ladder marker

Lane 1,5 and 9: control shrimps at 0, 6, and 12 h

Lane 2, 6 and 10: 30°C induced shrimps at 0, 6, and 12 h

Lane 3, 7 and 11: 33°C induced shrimps at 0, 6, and 12 h

Lane 4, 8 and 12: 35°C induced shrimps at 0, 6, and 12 h

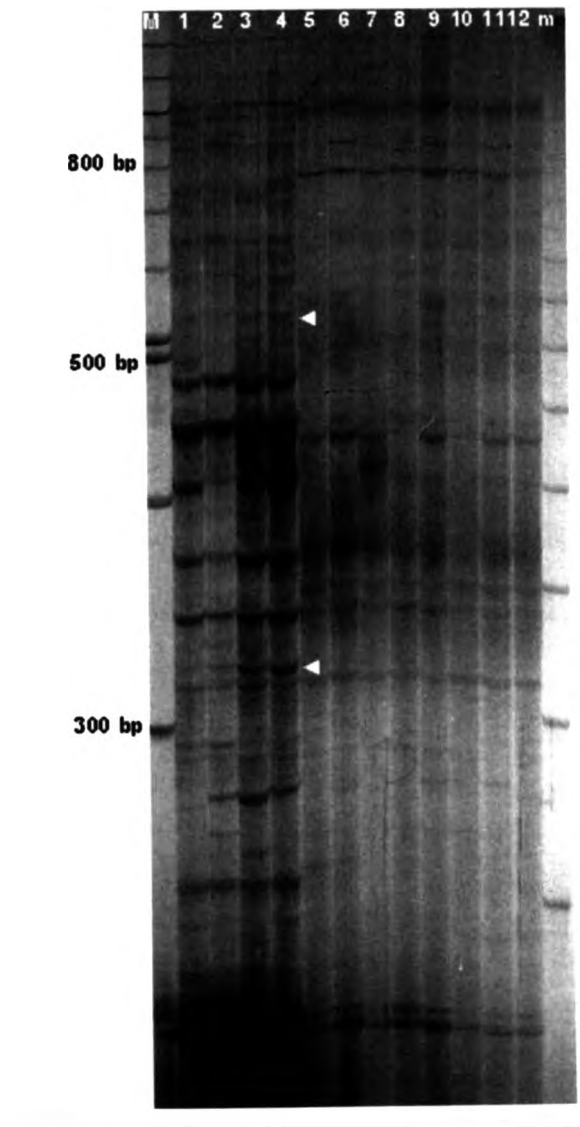


Figure 4.32 RAP-PCR patterns of *P. monodon* using primers UBC119 and UBC174 eletrophoretically analyzed by a 4.5 % denaturing acrylamide gel. White arrows indicate DNA bands expressed differentially

Lane M: 50 bp ladder marker

Lane m: 100 bp ladder marker

Lane 1,5 and 9: control shrimps at 0, 6, and 12 h

Lane 2, 6 and 10: 30°C induced shrimps at 0, 6, and 12 h

Lane 3, 7 and 11: 33°C induced shrimps at 0, 6, and 12 h

Lane 4, 8 and 12: 35°C induced shrimps at 0, 6, and 12 h

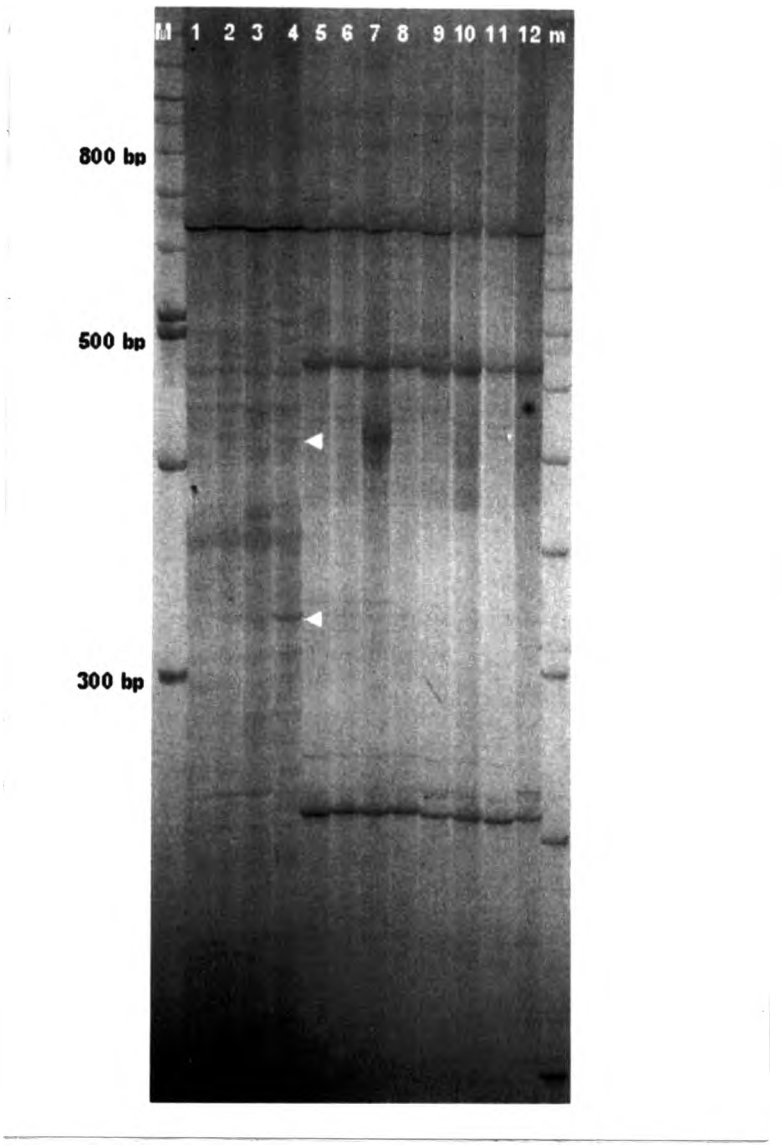


Figure 4.33 RAP-PCR patterns of *P. monodon* using primers UBC119 and UBC228 eletrophoretically analyzed by a 4.5 % denaturing acrylamide gel. White arrows indicate DNA bands expressed differentially

Lane M: 50 bp ladder marker

Lane m: 100 bp ladder marker

Lane 1,5 and 9: control shrimps at 0, 6, and 12 h

Lane 2, 6 and 10: 30°C induced shrimps at 0, 6, and 12 h

Lane 3, 7 and 11: 33°C induced shrimps at 0, 6, and 12 h

Lane 4, 8 and 12: 35°C induced shrimps at 0, 6, and 12 h

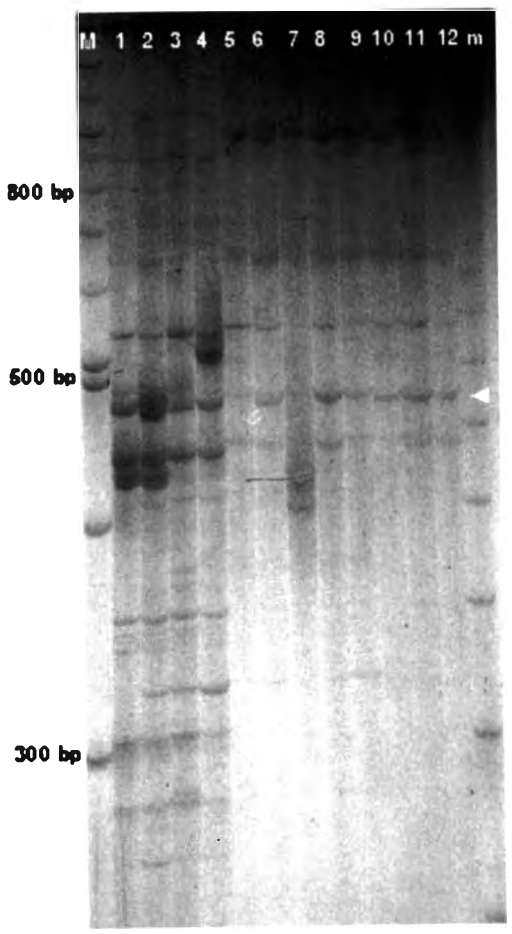


Figure 4.34 RAP-PCR patterns of *P. monodon* using primers UBC119 and UBC268 eletrophoretically analyzed by a 4.5 % denaturing acrylamide gel. White arrows indicate DNA bands expressed differentially

Lane M: 50 bp ladder marker

Lane m: 100 bp ladder marker

Lane 1,5 and 9: control shrimps at 0, 6, and 12 h

Lane 2, 6 and 10: 30°C induced shrimps at 0, 6, and 12 h

Lane 3, 7 and 11: 33°C induced shrimps at 0, 6, and 12 h

Lane 4, 8 and 12: 35°C induced shrimps at 0, 6, and 12 h

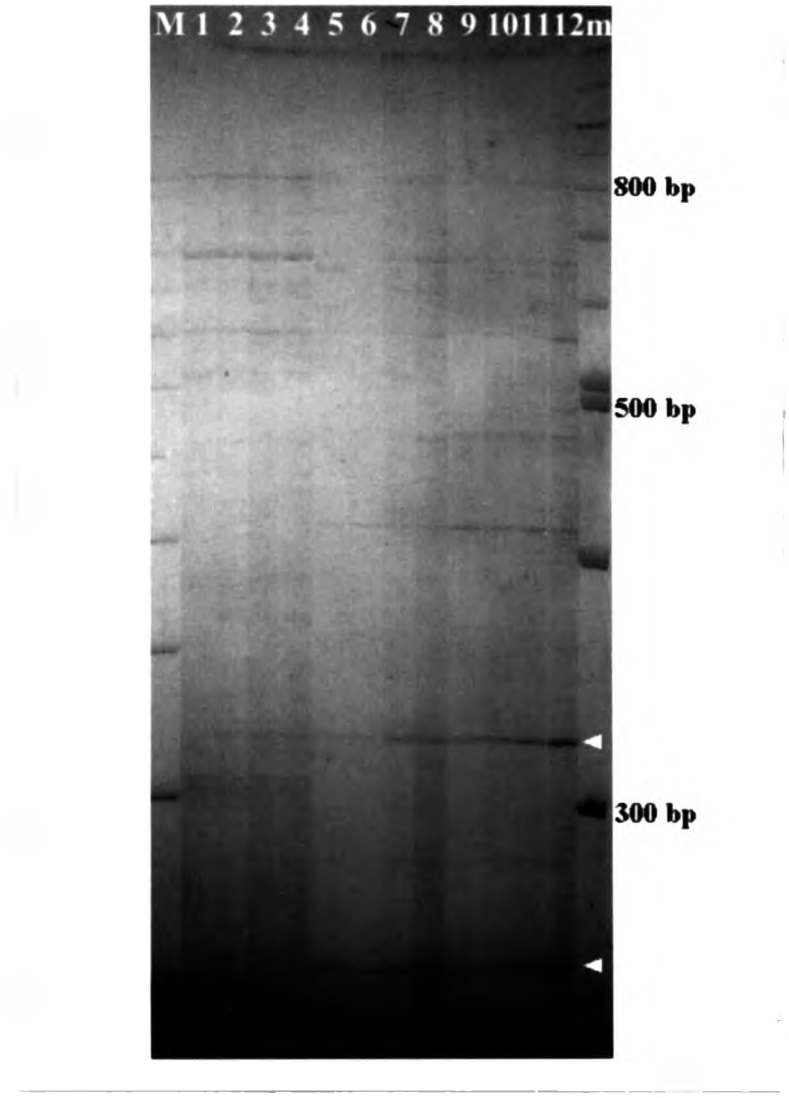


Figure 4.35 RAP-PCR patterns of *P. monodon* using primers UBC119 and UBC299 eletrophoretically analyzed by a 4.5 % denaturing acrylamide gel. White arrows indicate DNA bands expressed differentially

Lane M: 50 bp ladder marker

Lane m: 100 bp ladder marker

Lane 1,5 and 9: control shrimps at 0, 6, and 12 h

Lane 2, 6 and 10: 30°C induced shrimps at 0, 6, and 12 h

Lane 3, 7 and 11: 33°C induced shrimps at 0, 6, and 12 h

Lane 4, 8 and 12: 35°C induced shrimps at 0, 6, and 12 h

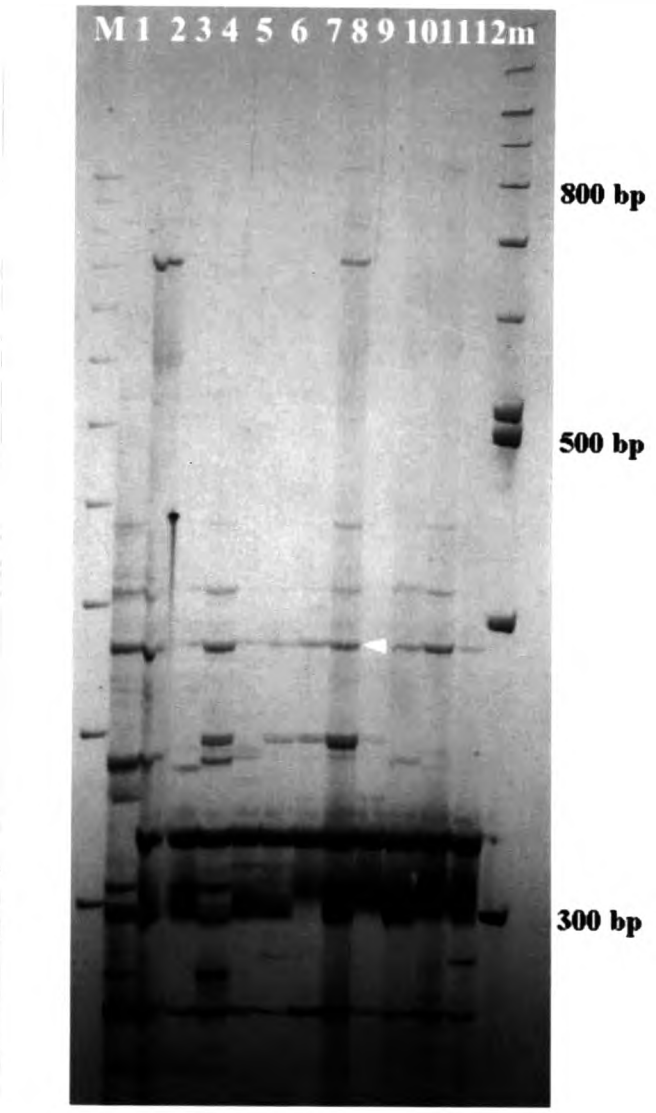


Figure 4.36 RAP-PCR patterns of *P. monodon* using primers UBC119 and UBC457 eletrophoretically analyzed by a 4.5 % denaturing acrylamide gel. White arrows indicate DNA bands expressed differentially

Lane M: 50 bp ladder marker

Lane m: 100 bp ladder marker

Lane 1,5 and 9: control induced shrimps at 0, 6, and 12 h

Lane 2, 6 and 10: 30°C induced shrimps at 0, 6, and 12 h

Lane 3, 7 and 11: 33°C induced shrimps at 0, 6, and 12 h

Lane 4, 8 and 12: 35°C induced shrimps at 0, 6, and 12 h

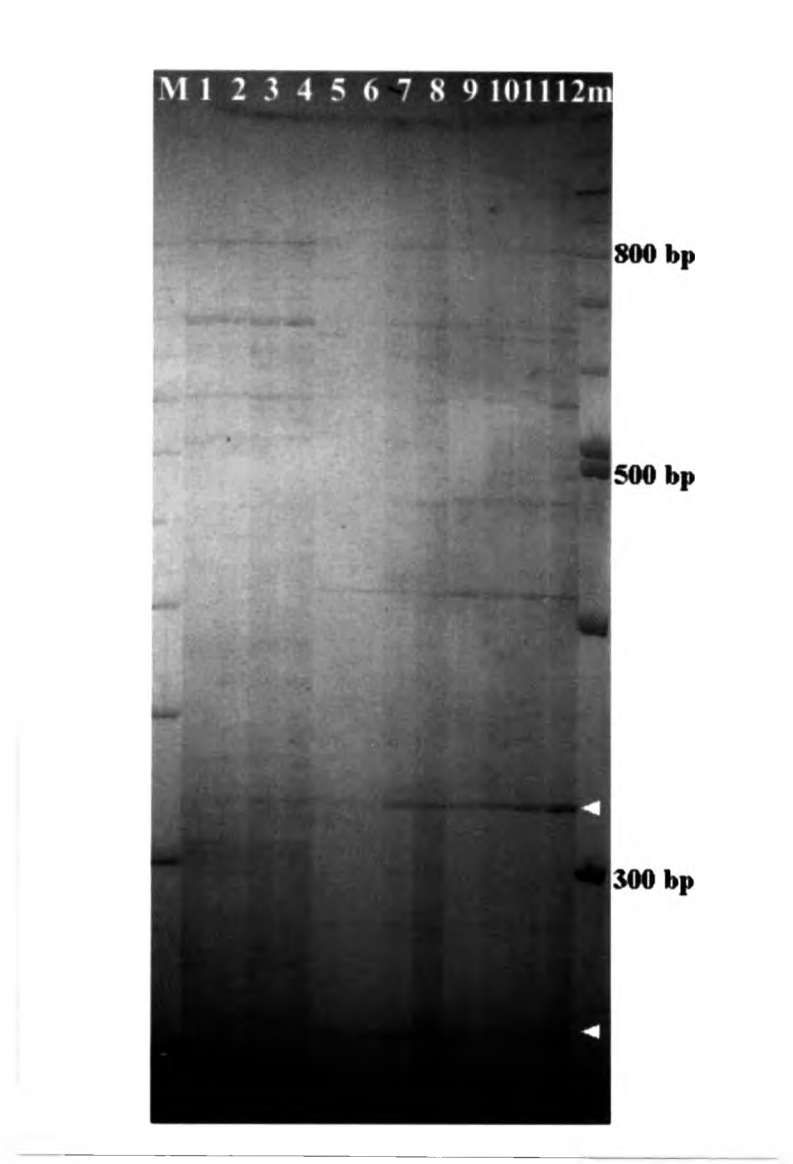


Figure 4.37 RAP-PCR patterns of *P. monodon* using primers UBC119 and UBC459 eletrophoretically analyzed by a 4.5 % denaturing acrylamide gel. White arrows indicate DNA bands expressed differentially

Lane M: 50 bp ladder marker

Lane m: 100 bp ladder marker

Lane 1,5 and 9: control shrimps at 0, 6, and 12 h

Lane 2, 6 and 10: 30°C induced shrimps at 0, 6, and 12 h

Lane 3, 7 and 11: 33°C induced shrimps at 0, 6, and 12 h

Lane 4, 8 and 12: 35°C induced shrimps at 0, 6, and 12 h

4.9 Sequence analysis of RAP-PCR markers

A number of cDNA markers (7 differential displayed markers, 10 up-regulated markers, and 3 down-regulated markers) generated from RAP-PCR were isolated and cloned. DNA inserts from some of recombinant clones (clones no.36, 37, 38, and 4) were shown in Figure 4.38. All clones were subjected to sequence analysis.

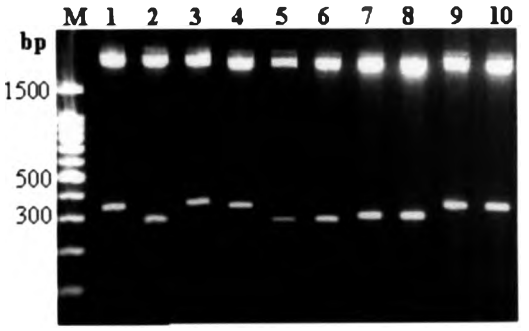


Figure 4.38 DNA inserts from some of recombinant clones on 1% agarose gel. Plasmids from each clone were purified and digested with *Eco*RI

Lane M: 100 bp ladder marker

Lane 1-4: recombinant clone no. 36

Lane 5-6: recombinant clone no. 37

Lane 7-8: recombinant clone no. 38

Lane 9-10: recombinant clone no. 4

The DNA sequences obtained from each marker (appendix D) were compared to the DNA and protein sequences reported in GenBank for gene identification using BLAST*N* and BLAST*X*. The results from sequence comparison showed no significant similarity (E<-4) to any known genes in the GenBank excepted clones RAP4, RAP9, and RAP21 (Figure D.29-D.31) which were indicated as *P. monodon* clone TUZX4-6:86 microsatellite sequence, *P. (Litopenaeus) vannamei* microsatellite TUMXLv10.221 sequence, and ENSANGP00000010415 (*Anopheles gambiae* str. PEST) and Niemann-Pick type C1 disease protein (*Oryctolagus cuniculus*), respectively.

Some of the markers (RAP12, RAP16, RAP22, and RAP58) were further investigated in quantitative analysis using semi-quantitative RT-PCR.

Table 4.9 Sequence identification of the markers obtained from RAP-PCR. The sequences were blasted with GENBANK database.

No.	Name of primer	Pattern	Product of	Heat shock temp. (°C)	Post exposure time (h.)	Size (bp)	BLAST*N*	BLAST*X*
1	-	Up-regulation	UBC119+UBC135	33	0	400	unknown	unknown
2	-	Up-regulation	UBC119+UBC135	33	0	408	unknown	unknown
3	-	Up-regulation	UBC119+UBC268	30	12	400	unknown	unknown
4	-	Up-regulation	UBC119+UBC268	33	0	318	*P. monodon* clone TUZX4-6:86 microsatellite	unknown
5	-	Up-regulation	UBC119+UBC268	27	12	471	unknown	unknown
6	-	Up-regulation	UBC119+UBC135	35	6	413	unknown	unknown
7	-	Up-regulation	UBC119+UBC128	35	6	435	unknown	unknown
8	-	Up-regulation	UBC119+UBC128	33	6	396	unknown	unknown

Table 4.9 (cont.)

No.	Name of primer	Pattern	Product of	Heat shock temp. (°C)	Post exposure time (h.)	Size (bp)	BLAST*N*	BLAST*X*
9	-	Up-regulation	UBC119+UBC128	33	6	434	*P. vannamei* microsatellite TUMXLv10.221	unknown
10	-	Down-regulation	UBC119+UBC228	35	0	356	unknown	unknown
11	-	Up-regulation	UBC119+UBC457	35	0	340	unknown	unknown
12	RAP12	Up-regulation	UBC119+UBC457	35	0	262	unknown	unknown
13	-	Up-regulation	UBC119+UBC457	35	0	317	unknown	unknown
14	-	Up-regulation	UBC119+UBC459	35	0	258	unknown	unknown
15	-	Up-regulation	UBC119+UBC459	35	0	298	unknown	unknown
16	RAP16	Up-regulation	UBC119+UBC228	35	0	319	unknown	unknown
17	-	Up-regulation	UBC119+UBC299	30	0	318	unknown	unknown

Table 4.9 (cont.)

No.	Name of primer	Pattern	Product of	Heat shock temp. (°C)	Post exposure time (h.)	Size (bp)	BLAST*N*	BLAST*X*
19	-	Up-regulation	UBC119+UBC457	30	0	318	unknown	unknown
20	-	Up-regulation	UBC119+UBC457	33	0	270	unknown	unknown
21	RAP21	Up-regulation	UBC119+UBC459	27	12	408	unknown	Niemann-Pick type C1 disease protein [*Oryctolagus cuniculus*]
22	-	Down-regulation	UBC119+UBC459	30	0	258	unknown	unknown
24	-	Up-regulation	UBC119+UBC122	33	6	595	unknown	unknown
26	-	Up-regulation	UBC119+UBC228	35	0	317	unknown	unknown
28	-	Up-regulation	UBC119+UBC299	35	0	335	unknown	unknown
29	-	Differential display	UBC119+UBC128	30	0	318	unknown	unknown

Table 4.9 (cont.)

No.	Name of primer	Pattern	Product of	Heat shock temp. (°C)	Post exposure time (h.)	Size (bp)	BLAST*N*	BLAST*X*
30	-	Down-regulation	UBC119+UBC128	27	3	318	unknown	unknown
32	-	Up-regulation	UBC119+UBC459	30	12	320	unknown	unknown
58	RAP58	Up-regulation	UBC119+UBC158	35	0	335	unknown	unknown
62	-	Down-regulation	UBC119+UBC158	35	0	258	unknown	unknown

4.10 Quantitative analysis of genes expressed in the haemocytes from heat and *vibrio* treated *P. monodon*

The expression levels of thermal induced genes obtained from RAP-PCR and stress related genes (PO and HSP genes) were determined using semi-quantitative RT-PCR method on the shrimps either treated with heat shock (35°C, 6 h), *Vibrio* exposure (10^8 CFU/ml), or heat shock and *Vibrio* exposure. The expression levels of target genes were detected in comparison with control shrimps at 0, 6, 12, 24, and 72 h after *Vibrio* exposure. The expression of β-actin gene was used as transcriptional control. The expression levels of target genes were determined on the ratio of the intensity bands of PCR products and that of β-actin gene.

4.10.1 Optimization of PCR condition for semi-quantitative analysis

Prior to quantitative measurement, appropriate conditions for PCR amplification of control (β-actin) and target markers (PO, HSP, and RAP-PCR genes) were optimized. The appropriate PCR condition for semi-quantitative detection was chosen on the criteria that the PCR product should be on the log phase of amplification. These PCR conditions required optimization which included annealing temperature, number of cycles, DNA template and $MgCl_2$ concentrations.

For the PCR condition of β-actin amplification, the results (Figure 4.39) indicated that the condition of using cDNA template at 500 ng and $MgCl_2$ concentration at 1.5 mM with 25 PCR cycles were suitable for the amplification of β-actin gene.

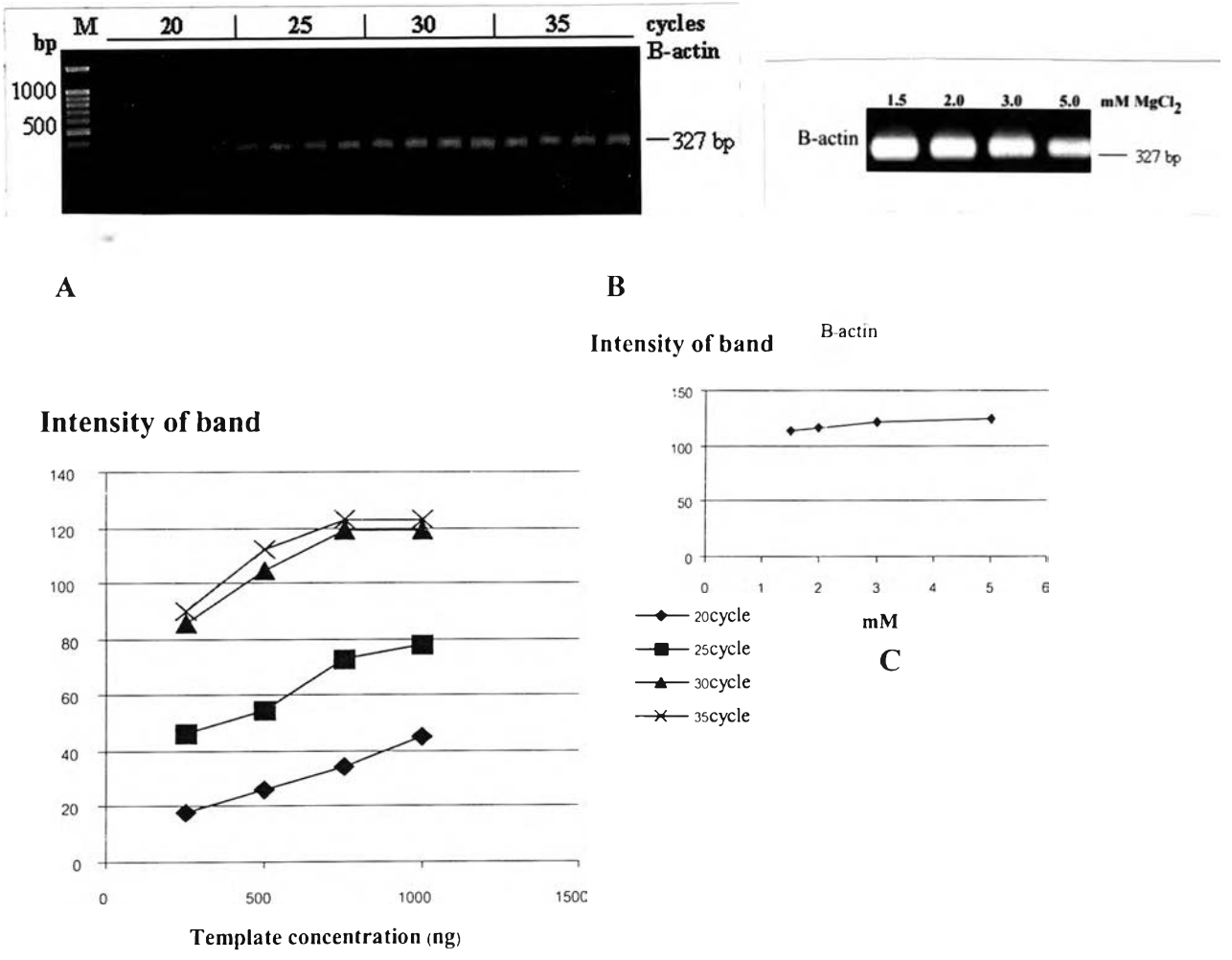
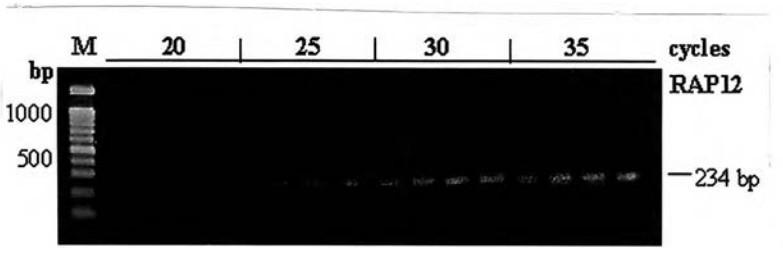
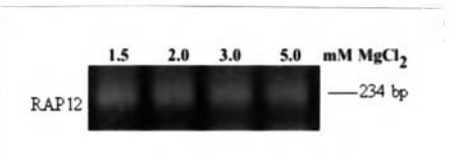


Figure 4.39 The optimization of PCR condition for β-actin amplification. Differences in cycle numbers (A), $MgCl_2$ concentrations (B and C), and template concentrations (D) were determined.

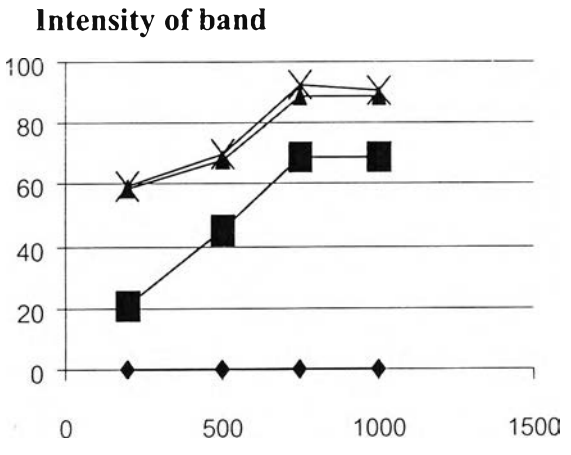
The optimization of PCR condition for quantifying differential displayed genes were shown in Figures 4.40-4.43, respectively. It was indicated that the optimal template and $MgCl_2$ concentrations for PCR condition of all 4 RAP-PCR transcripts (RAP12, RAP16, RAP21, and RAP58) were 200, 200, 500, and 500 ng of DNA templates, respectively. $MgCl_2$ concentration at 1.5 μM and the numbers of PCR cycles at 25 cycles were applied to PCR conditions of all 4 genes.



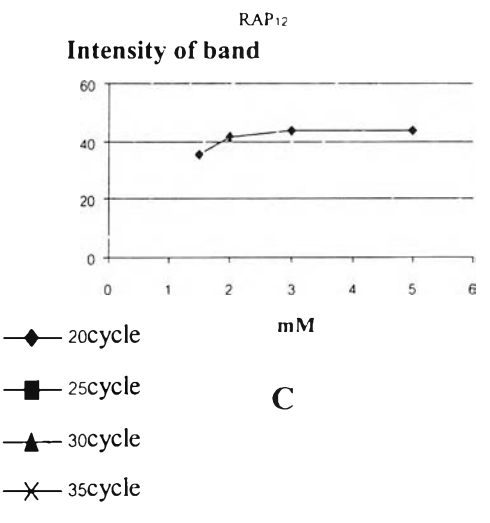
A



B

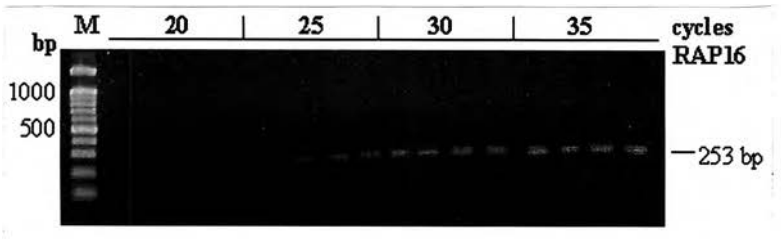


D

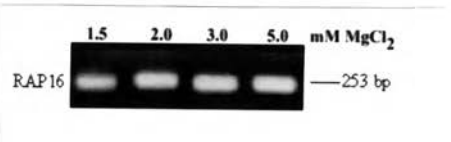


C

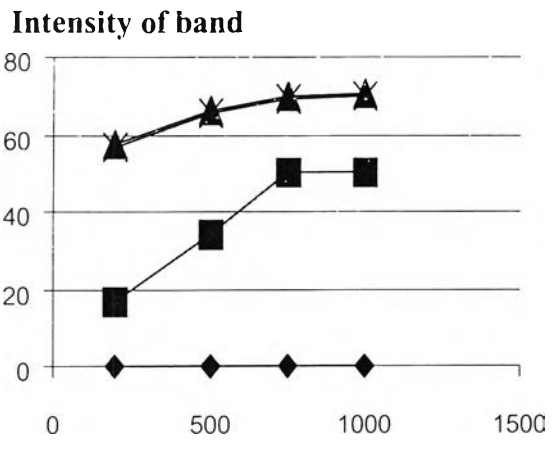
Figure 4.40 The optimization of PCR condition for RAP12 amplification. Differences in cycle numbers (A), $MgCl_2$ concentrations (B and C), and template concentrations (D) were determined.



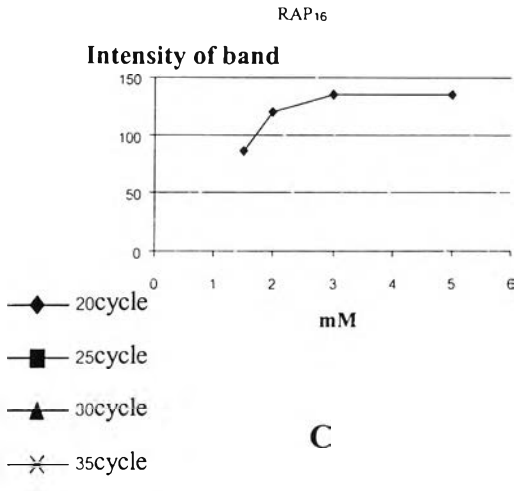
A



B

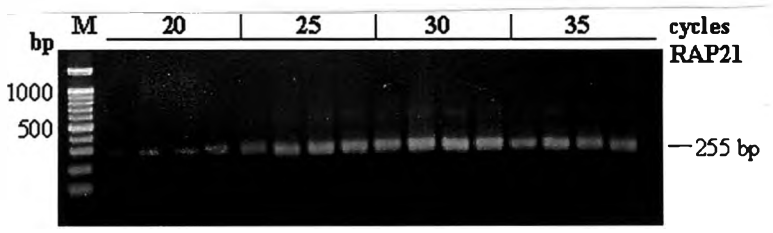


D

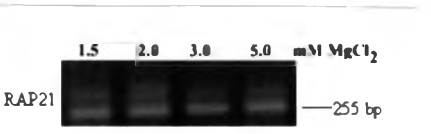


C

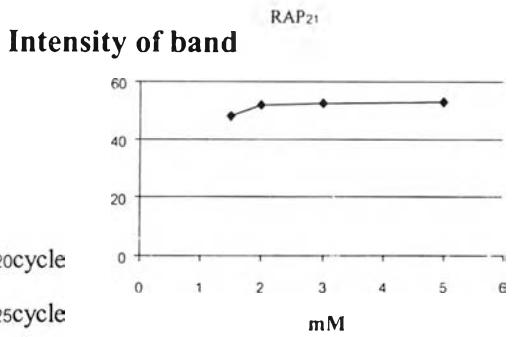
Figure 4.41 The optimization of PCR condition for RAP16 amplification. Differences in cycle numbers (A), $MgCl_2$ concentrations (B and C), and template concentrations (D) were determined.



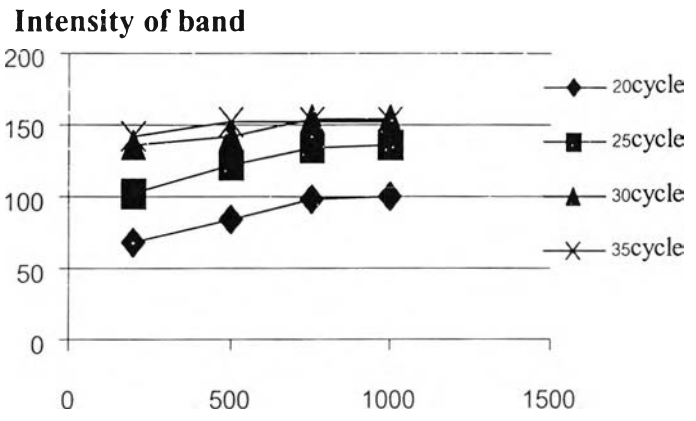
A



B

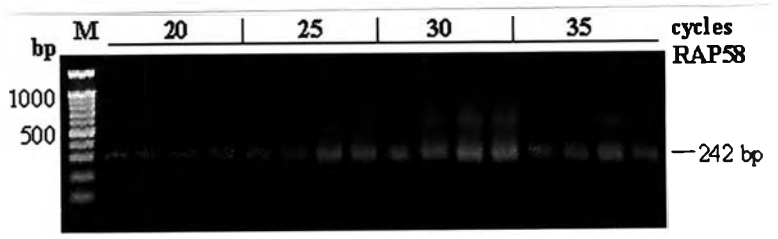


C

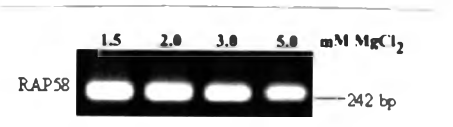


D

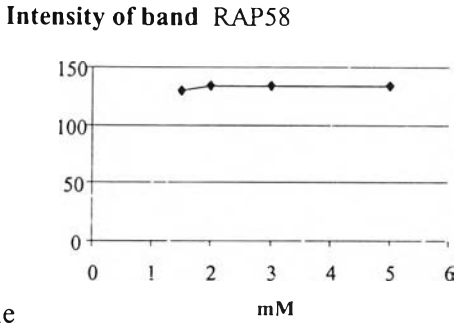
Figure 4.42 The optimization of PCR condition for RAP21 amplification. Differences in cycle numbers (A), $MgCl_2$ concentrations (B and C), and template concentrations (D) were determined.



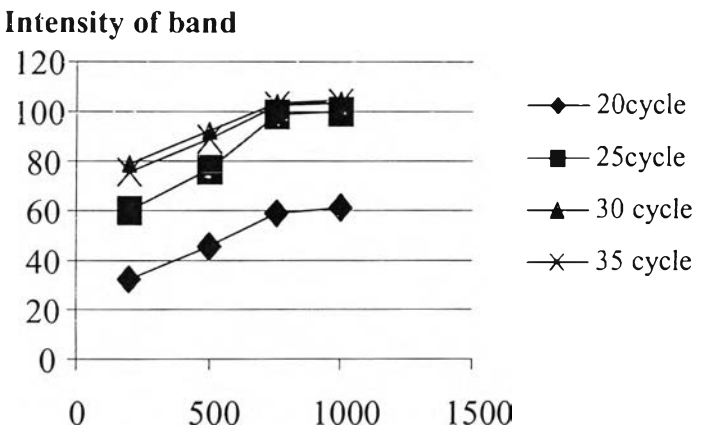
A



B



C



D

Figure 4.43 The optimization of PCR condition for RAP58 amplification. Differences in cycle numbers (A), $MgCl_2$ concentrations (B and C), and template concentrations (D) were determined.

As the result shown in Figure 4.44, the optimal PCR condition of prophenoloxidase were to use DNA template concentration at 100 ng, $MgCl_2$ concentration at 1.5 µM and the PCR cycles of 25. This condition was adopted from the experiment described by Chaicharn (2003).

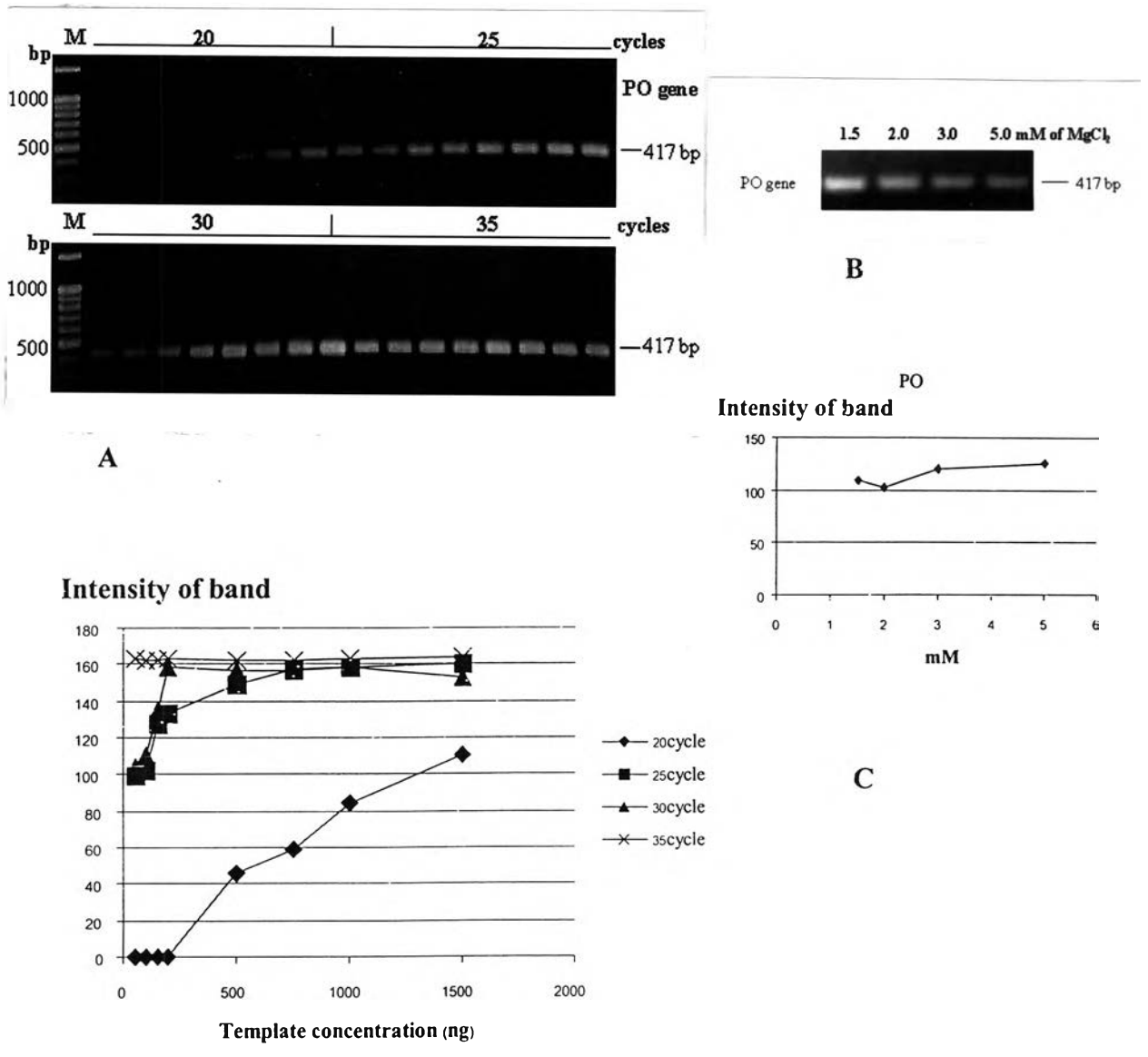


Figure 4.44 The optimization of PCR condition for phenoloxidase amplification. Differences in cycle numbers (A), $MgCl_2$ concentrations (B and C), and template concentrations (D) were determined.

The PCR condition optimized for quantitative analysis of heat shock protein genes (HSP60, HSP70, and HSP90) (Figures 4.45-4.47, respectively) were to use DNA template concentration at 500, 50, and 50 ng, respectively. $MgCl_2$ concentration at 1.5 μM and the PCR cycles of 25.

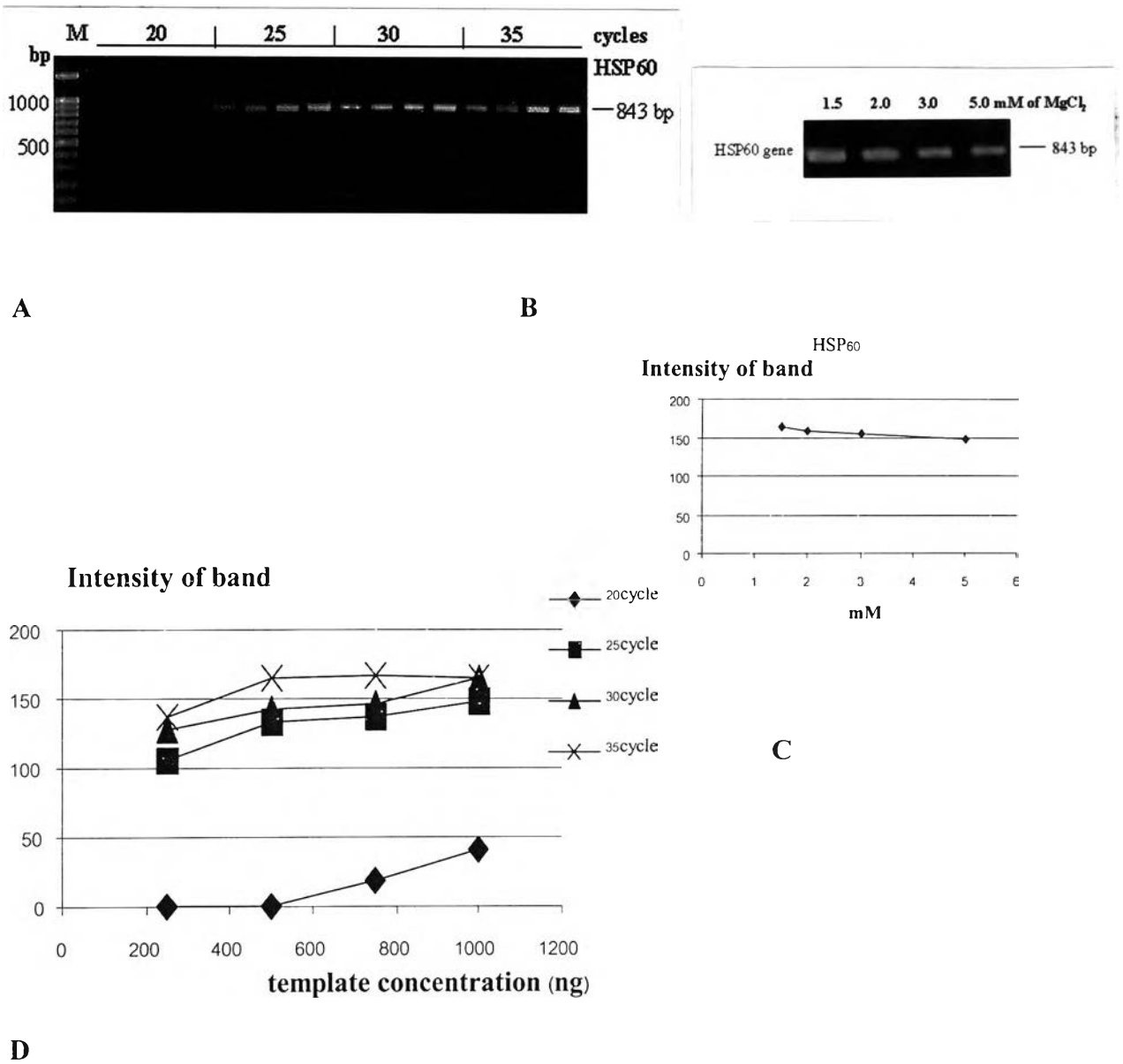
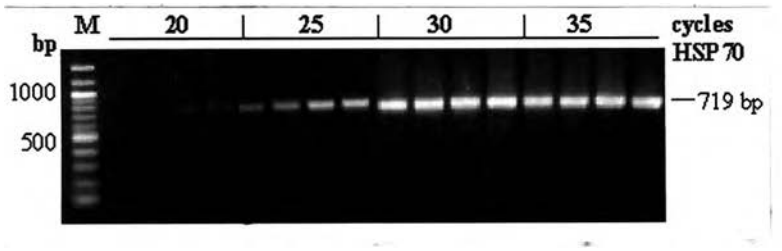
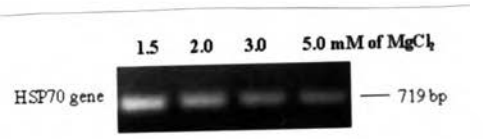


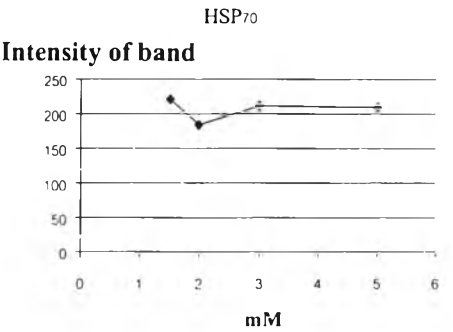
Figure 4.45 The optimization of PCR condition for HSP60 amplification. Differences in cycle numbers (A), $MgCl_2$ concentrations (B and C), and template concentrations (D) were determined.



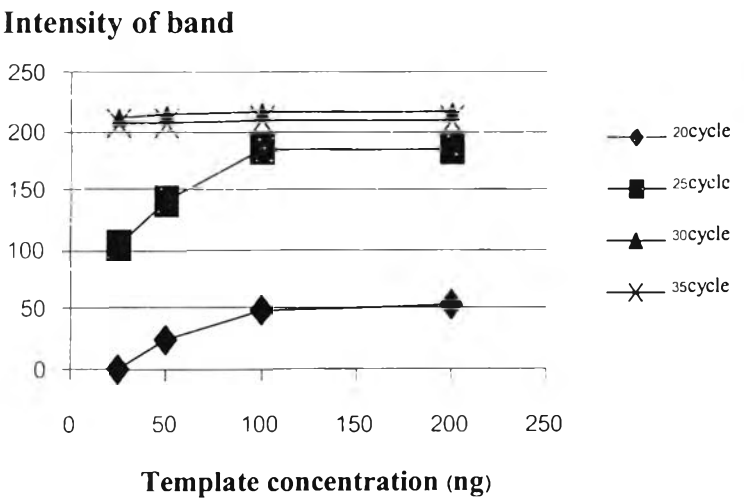
A



B

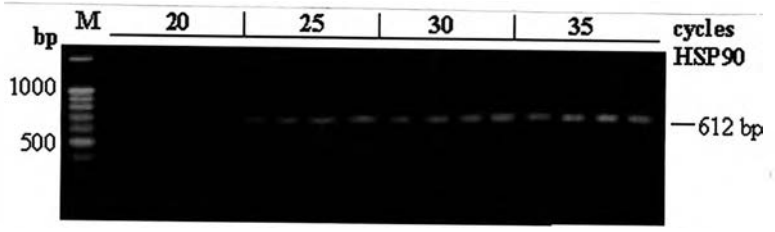


C

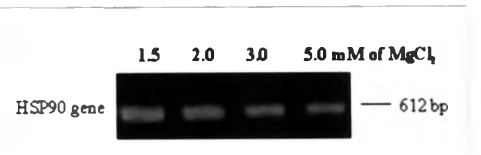


D

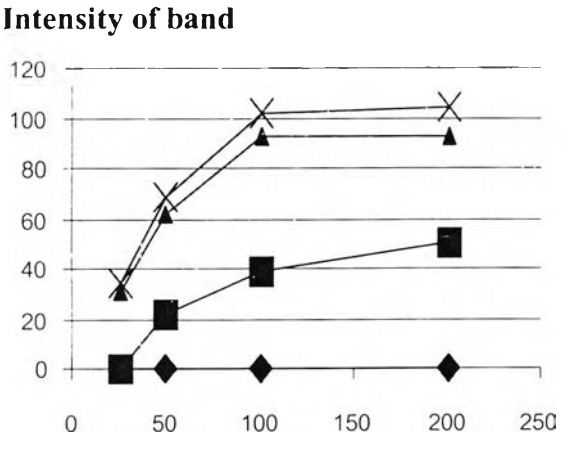
Figure 4.46 The optimization of PCR condition for HSP70 amplification. Differences in cycle numbers (A), $MgCl_2$ concentrations (B and C), and template concentrations (D) were determined.



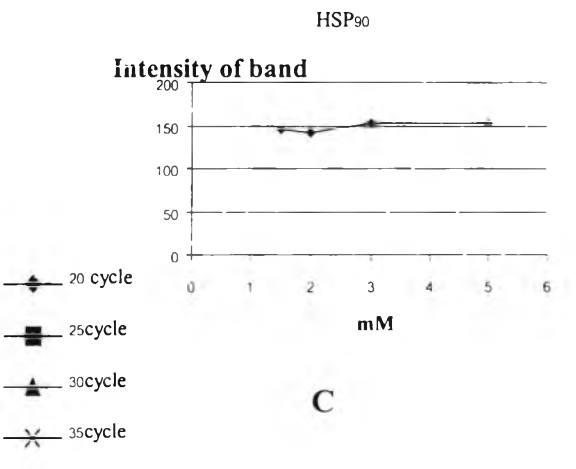
A



B



D



C

Figure 4.47 The optimization of PCR condition for HSP90 amplification. Differences in cycle numbers (A), $MgCl_2$ concentrations (B and C), and template concentrations (D) were determined.

Table 4.10 Summary of the optimal PCR conditions used in semi-quantitative analyses for determining the expression levels of thermal-induced genes.

Gene	Template concentration (ng)	Cycle	$MgCl_2$ concentration (mM)
HSP genes			
-HSP60	500	25	1.5
-HSP70	50	25	1.5
-HSP90	50	25	1.5
RAP-PCR genes			
-RAP12	500	25	1.5
-RAP16	500	25	1.5
-RAP21	200	25	1.5
-RAP58	200	25	1.5
Immune related gene			
-Phenoloxidase	100	25	1.5
Constitutive gene			
-β-actin	500	25	1.5

4.10.2 Expression level of stress related genes

Following the adjustment of PCR conditions, the expression levels of HSP60, HSP70, HSP90, and PO genes were determined on the pre-heat and *Vibrio* treated *P. monodon*.

4.10.2.1 Expression level of HSP60 gene

The results of the experiment on the shrimps showed in Figure 4.48-4.49 and Table 4.11. No significant difference was detected on the expression level of HSP60 gene from the shrimps in all treatments ($P<0.05$). It was indicated from the result that the expression of HSP60 gene in *P. monodon* was not induced by the heat shock at 35°C for 6 h and/or *vibrio* exposure.

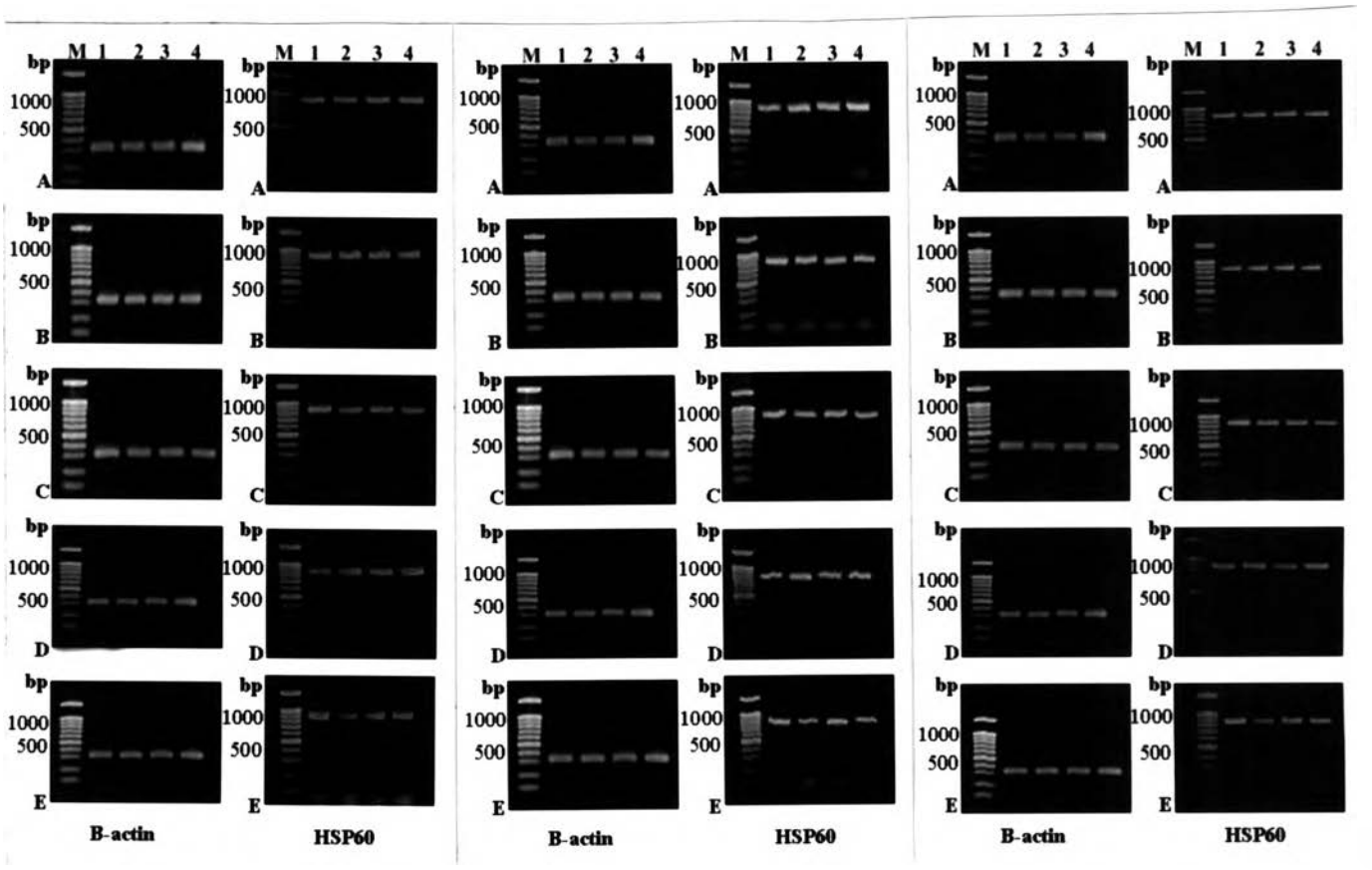


Figure 4.48 The expression levels of HSP60 gene transcripts between control and heat shock, heat induced, and un-induced treatments in comparison with β–actin. Samples were obtained from 3 shrimps and analysed by 1.5% agarose gel electrophoresis. A, B, C, D, and E are the results at 0, 6, 12, 24, and 72 h of *Vibrio* exposure. Lane M is 100 bp markers, Lane 1 to 4 represent control, heat shock, heat induced, and un-induced shrimps, respectively.

Table 4.11 The ratios of HSP60 gene and β-actin genes from *P. monodon* (*N*=3).

Post exposure time (hour)	No *vibrio* exposure		*Vibrio* exposure	
	control shrimp	heat induced shrimp	heat induced shrimp	un-induced shrimp
0	0.93±0.12	0.96±0.1	1.02±0.05	0.91±0.07
6	0.89±0.05	0.93±0.1	0.98±0.03	0.94±0.07
12	1.00±0.01	1.05±0.08	1.04±0.08	1.03±0.1
24	0.96±0.09	1.00±0.1	1.01±0.06	0.96±0.05
72	0.89±0.1	0.89±0.08	0.91±0.02	0.89±0.07

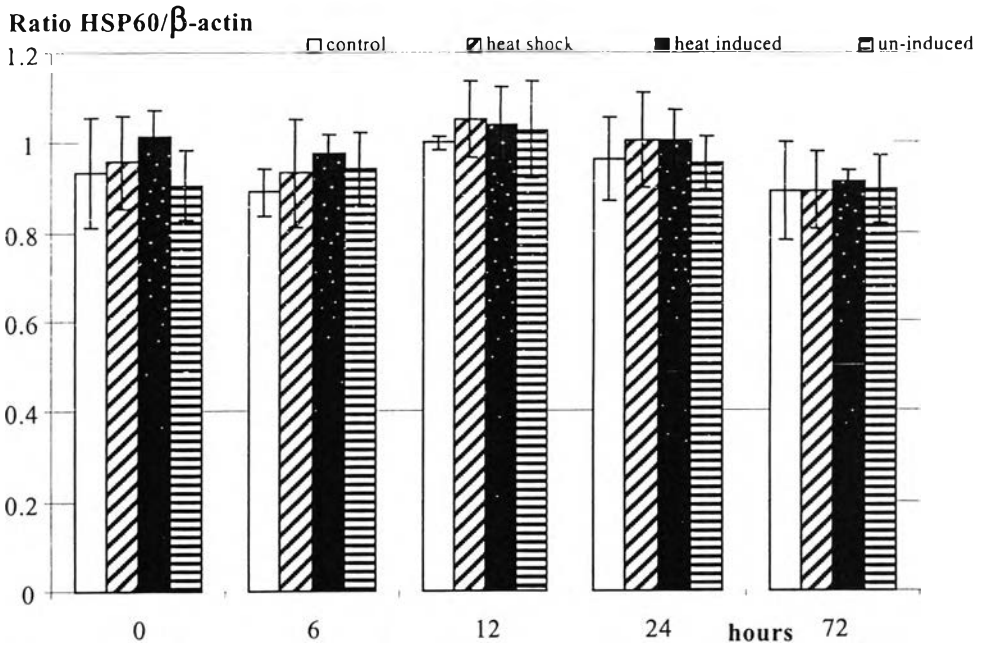


Figure 4.49 The ratios of HSP60 gene and β-actin genes from *P. monodon* (*N*=3). All data were derived from Table 4.11. The lines above the bar represent standard derivation.

4.10.2.2 Expression level of HSP70 gene

The results were shown in Figure 4.50-4.51 and Table 4.12. It was indicated that significant differences on the expression level of HSP70 gene between the experiment shrimps were detected since the first hour of post *vibrio* exposure ($P<0.05$). The expression levels of HSP70 gene in heat shock shrimps were significantly higher than that of un-induced shrimps. The expression level decreased according to time and no significant difference was detected at 24 h of post exposure while the expression levels in the heat induced shrimps exposed to *vibrio* were still higher than that in un-induced shrimps at 72 h of post exposure. The expression levels in un-induced shrimps showed no significant difference when compared with that in control shrimps. From this result, it was indicated that the expression of HSP70 genes was induced by heat shock at 35°C for 6 h, but it was uninducible by *vibrio* exposure. However, the expression of HSP70 gene in heat shock shrimps remained in high level for longer period of time when the shrimps were exposed to *vibrio*.

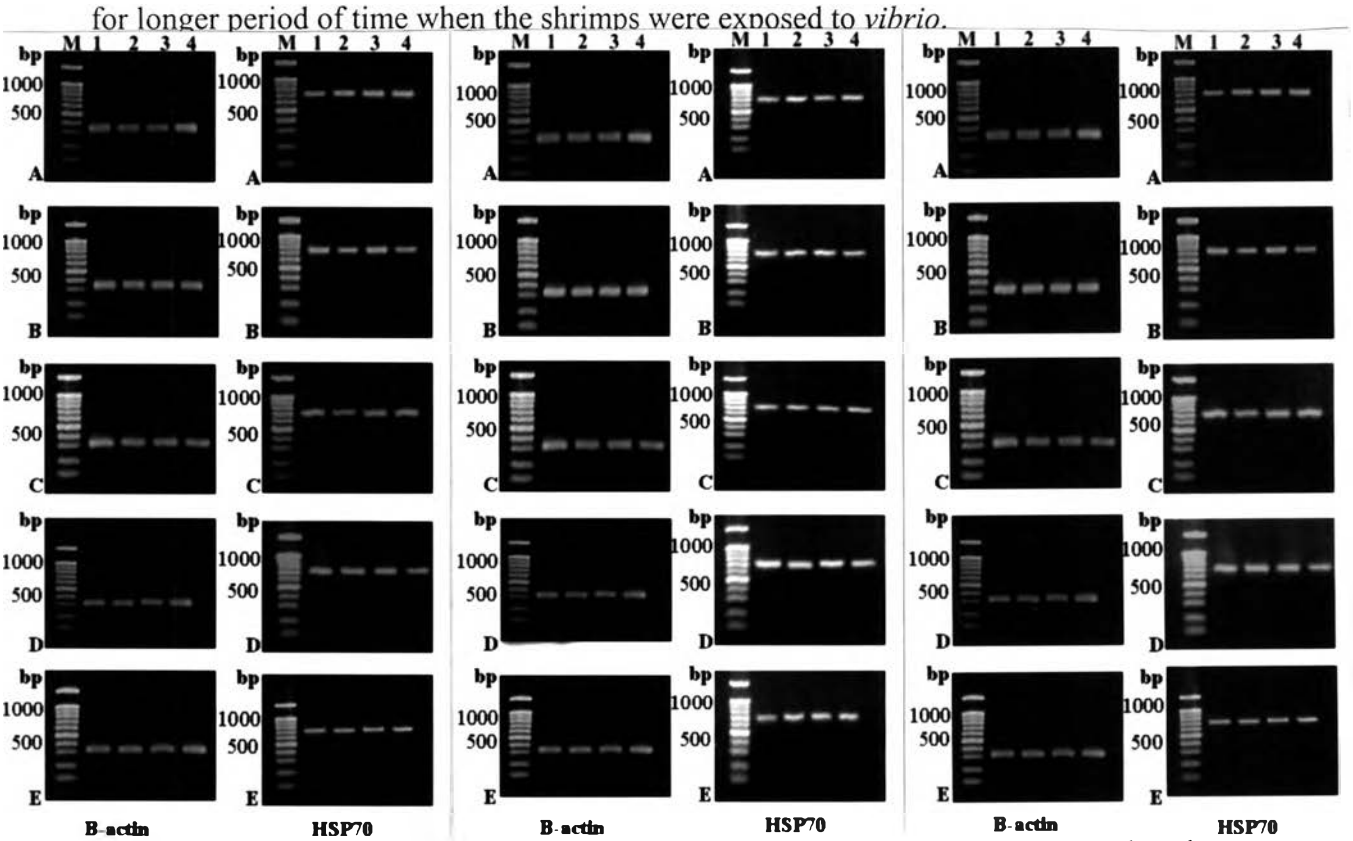


Figure 4.50 The expression levels of HSP70 gene transcripts between control and heat shock, heat induced, and un-induced treatments in comparison with β–actin. Samples were obtained from 3 shrimps and analysed by 1.5% agarose gel electrophoresis. A, B, C, D, and E are the results at 0, 6, 12, 24, and 72 h of *Vibrio* exposure. Lane M is 100 bp markers, Lane 1 to 4 represent control, heat shock, heat induced, and un-induced shrimps, respectively.

Table 4.12 The ratios of HSP70 gene and β-actin genes from *P. monodon* were treated with heat shock, heat induced, and un-induced (*N*=3).

Post exposure time (hour)	No *vibrio* exposure		*Vibrio* exposure	
	control shrimp	heat induced shrimp	heat induced shrimp	un-induced shrimp
0	0.98±0.12	1.27±0.1	1.31±0.05	1.06±0.07
6	0.93±0.05	1.24±0.1	1.26±0.03	0.97±0.07
12	1.01±0.01	1.14±0.08	1.23±0.08	0.97±0.1
24	1.03±0.09	1.09±0.1	1.17±0.06	0.92±0.05
72	1.01±0.1	1.09±0.08	1.15±0.02	0.97±0.07

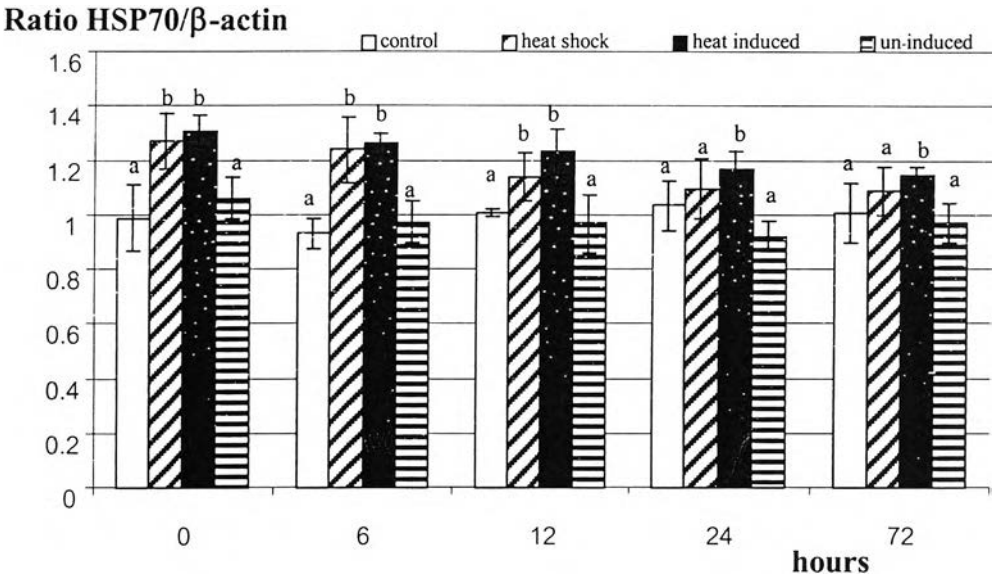
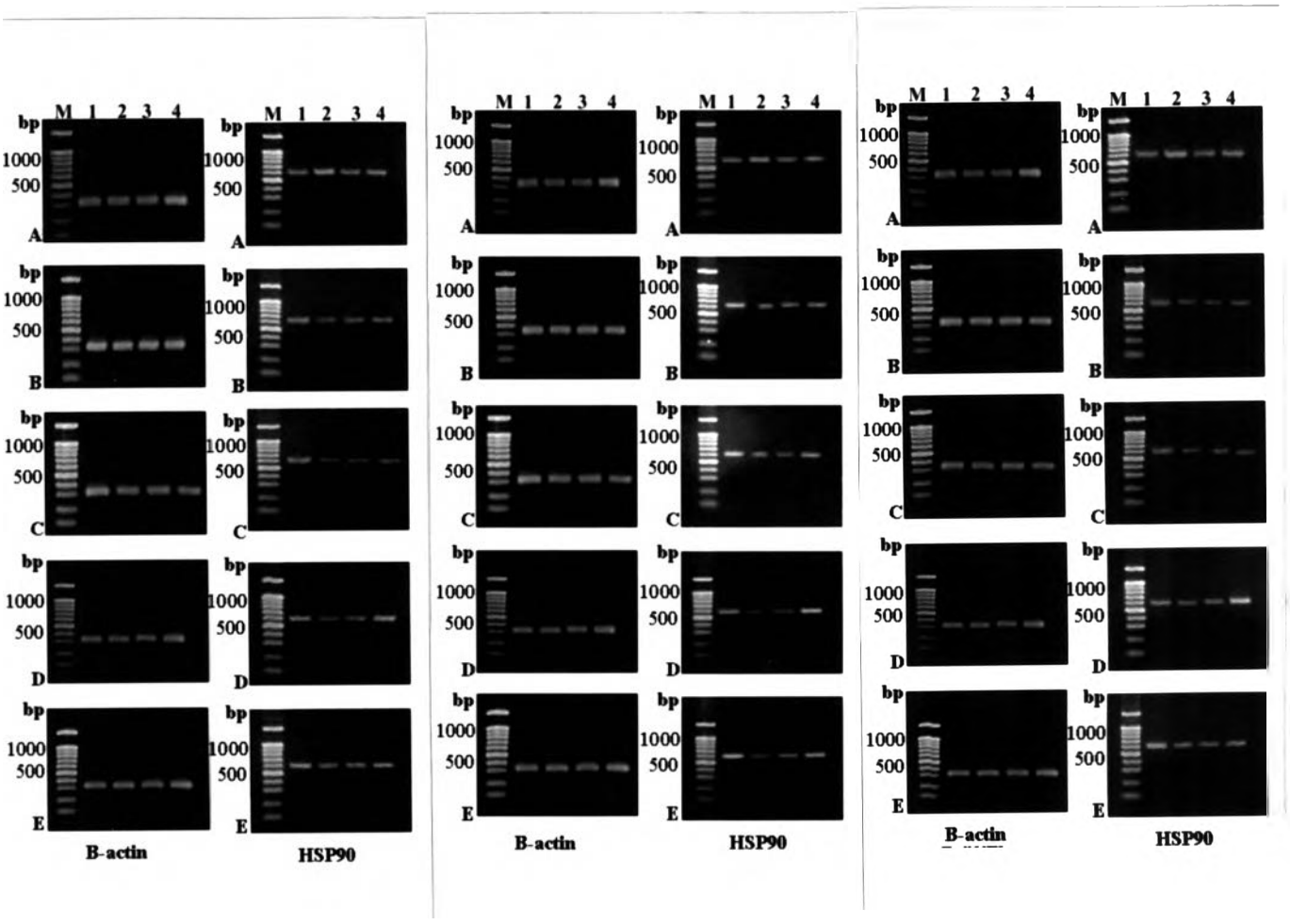


Figure 4.51 The ratios of HSP70 gene and β-actin genes from *P. monodon* were treated with heat shock, heat induced, and un-induced (*N*=3). All data were derived from Table 4.12. The lines above the bar represent standard derivation. Values that are significantly different have different superscripts ($P<0.05$ by Duncan's new multiple range test).

4.10.2.3 Expression level of HSP90 gene

The results of the experiment on the shrimp were shown in Figure 4.52-4.53 and Table 4.14. Similar results as obtained in the expression of HSP70 gene were observed. The expression levels of HSP90 in heat induced shrimps were much higher than that of un-induced shrimps ($P<0.05$).



Set 1 **Set 2** **Set 3**

Figure 4.52 The expression levels of HSP90 gene transcripts between control and heat shock, heat induced, and un-induced treatments in comparison with β–actin. Samples were obtained from 3 shrimps and analysed by 1.5% agarose gel electrophoresis. A, B, C, D, and E are the results at 0, 6, 12, 24, and 72 h of *Vibrio* exposure. Lane M is 100 bp markers, Lane 1 to 4 represent control, heat shock, heat induced, and un-induced shrimps, respectively.

Table 4.13 The ratios of HSP90 gene and β-actin genes from *P. monodon* were treated with heat shock, heat induced, and un-induced (*N*=3).

Post exposure time (hour)	No *vibrio* exposure		*Vibrio* exposure	
	control shrimp	heat induced shrimp	heat induced shrimp	un-induced shrimp
0	0.90±0.1	1.20±0.1	1.05±0.05	0.89±0.07
6	0.77±0.05	0.96±0.1	0.99±0.03	0.79±0.07
12	0.76±0.01	0.94±0.08	0.94±0.08	0.79±0.1
24	0.82±0.09	0.91±0.1	0.91±0.06	0.83±0.05
72	0.81±0.1	0.88±0.08	0.90±0.02	0.82±0.07

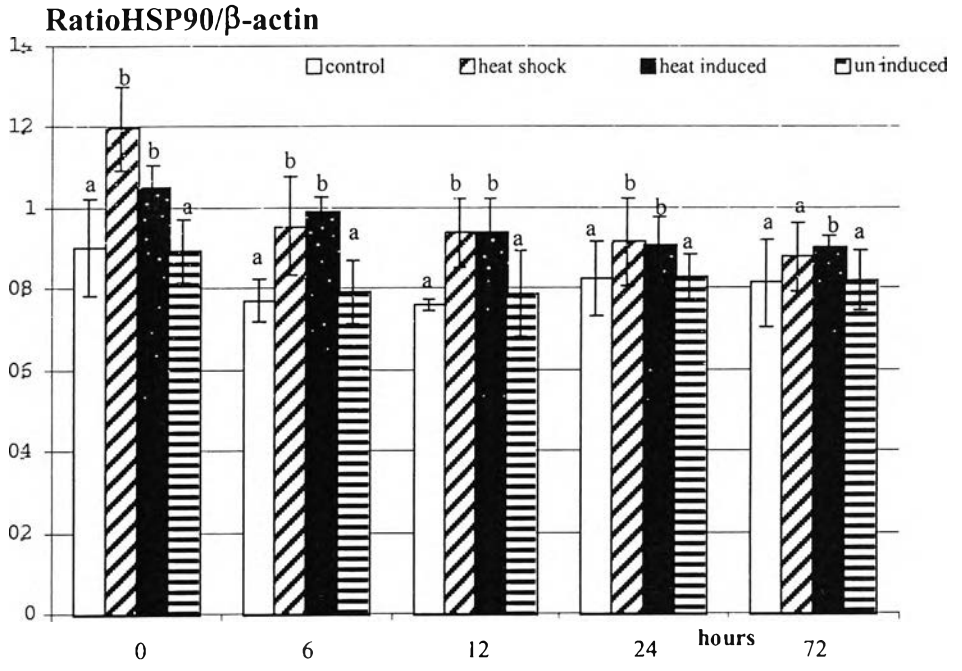


Figure 4.53 The ratios of HSP90 gene and β-actin genes from *P. monodon* were treated with heat shock, heat induced, and un-induced (*N*=3). All data were derived from Table 4.14. The lines above the bar represent standard derivation. Values that are significantly different have different superscripts ($P<0.05$ by Duncan's new multiple range test).

4.10.2.4 Expression level of PO gene

The results of the expression level of PO gene in the shrimps were shown in Figure 4.54-4.55 and Table 4.14. The results revealed that the significant differences of the PO expression levels between the shrimps were detected after 6 hour of post exposure ($P<0.05$). Significantly higher expression levels of PO gene in *vibrio*-treated shrimps were detected in comparison with that of control and no *vibrio* exposed shrimps at 6 hours of post exposure. The highest expression level was observed at 12 hours of post exposure then started to decrease until it was close to the normal level at 72 hours of post exposure. No significant difference of the PO gene expression level was detected between heat treated and no treated shrimps. This was indicated that the expression of PO gene in *P. monodon* was obviously induced by *vibrio* exposure but it was not inducible by the heat treatment.

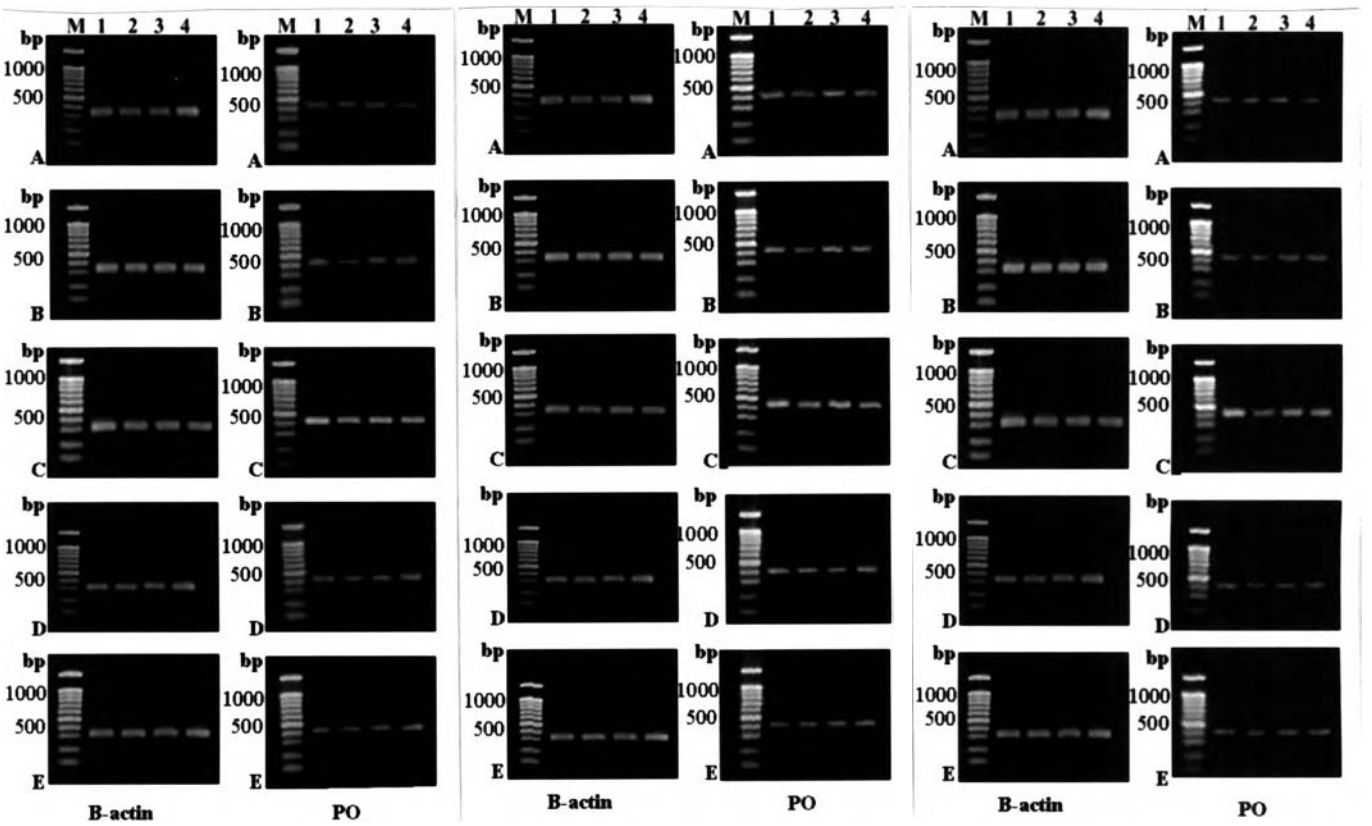


Figure 4.54 The expression levels of PO gene transcripts between control and heat shock, heat induced, and un-induced treatments in comparison with β–actin. Samples were obtained from 3 shrimps and analysed by 1.5% agarose gel electrophoresis. A, B, C, D, and E are the results at 0, 6, 12, 24, and 72 h of *Vibrio* exposure. Lane M is 100 bp markers, Lane 1 to 4 represent control, heat shock, heat induced, and un-induced shrimps, respectively.

Table 4.14 The ratios of PO gene and β-actin genes from *P. monodon* were treated with heat shock, heat induced, and un-induced (*N*=3).

Post exposure time (hour)	No *vibrio* exposure		*Vibrio* exposure	
	control shrimp	heat induced shrimp	heat induced shrimp	un-induced shrimp
0	0.65±0.1	0.67±0.1	0.70±0.05	0.73±0.08
6	0.65±0.05	0.62±0.1	0.81±0.03	0.86±0.07
12	0.72±0.01	0.71±0.08	0.92±0.08	0.91±0.1
24	0.72±0.09	0.74±0.1	0.87±0.07	0.87±0.06
72	0.67±0.01	0.74±0.08	0.79±0.02	0.80±0.08

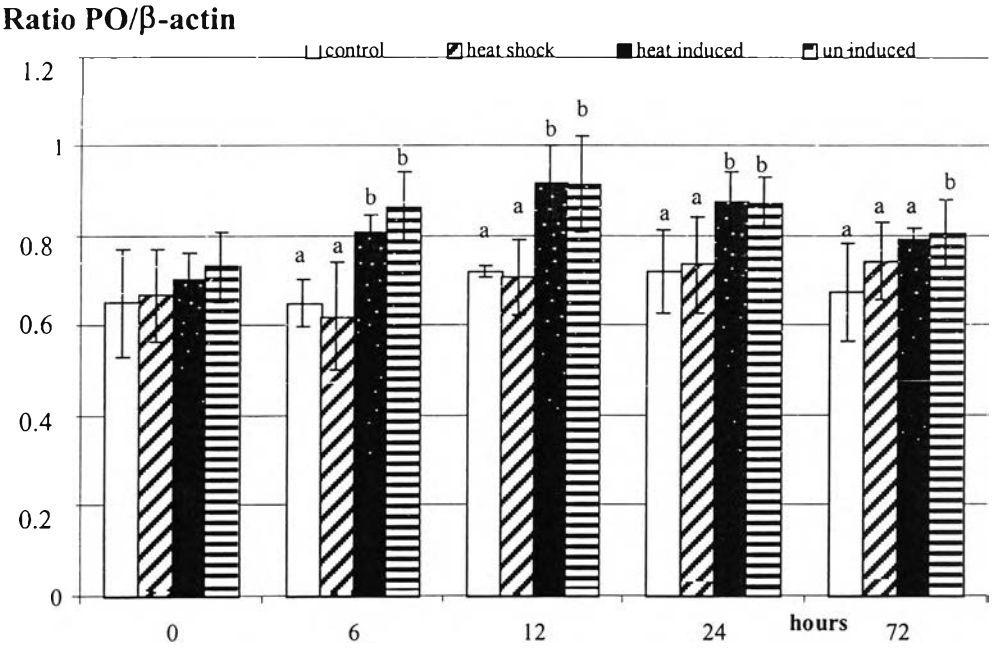


Figure 4.55 The ratios of PO gene and β-actin genes from *P. monodon* were treated with heat shock, heat induced, and un-induced (*N*=3). All data were derived from Table 4.14. The lines above the bar represent standard derivation. Values that are significantly different have different superscripts ($P<0.05$ by Duncan's new multiple range test).

4.10.3 Expression levels of genes obtained from RAP-PCR

The expression levels of genes obtained from RAP-PCR were also determined in the heat and *Vibrio* treated *P. monodon*. The genes appeared to respond to the heat induction were selected. These included RAP12, RAP16, RAP21 and RAP58 transcripts.

4.10.4.1 Expression level of RAP12 gene

As shown in Figure 4.56-4.57 and Table 4.15, significant differences on the expression level of RAP12 gene from the heat induced shrimps were detected at the first hour of post exposure. The highest level was detected at 24 hours and decreased after 72 hours of post exposure. With the heat induce shrimps but received no *vibrio* exposure, similar result was obtained. The higher expression level was significantly detected from 12 to 72 hours of post exposure. It appeared that RAP12 gene was not induced by exposing the shrimps with *vibrio*. However, *vibrio* treatment seemed to enhance the expression level of RAP12 in heat induced shrimps.

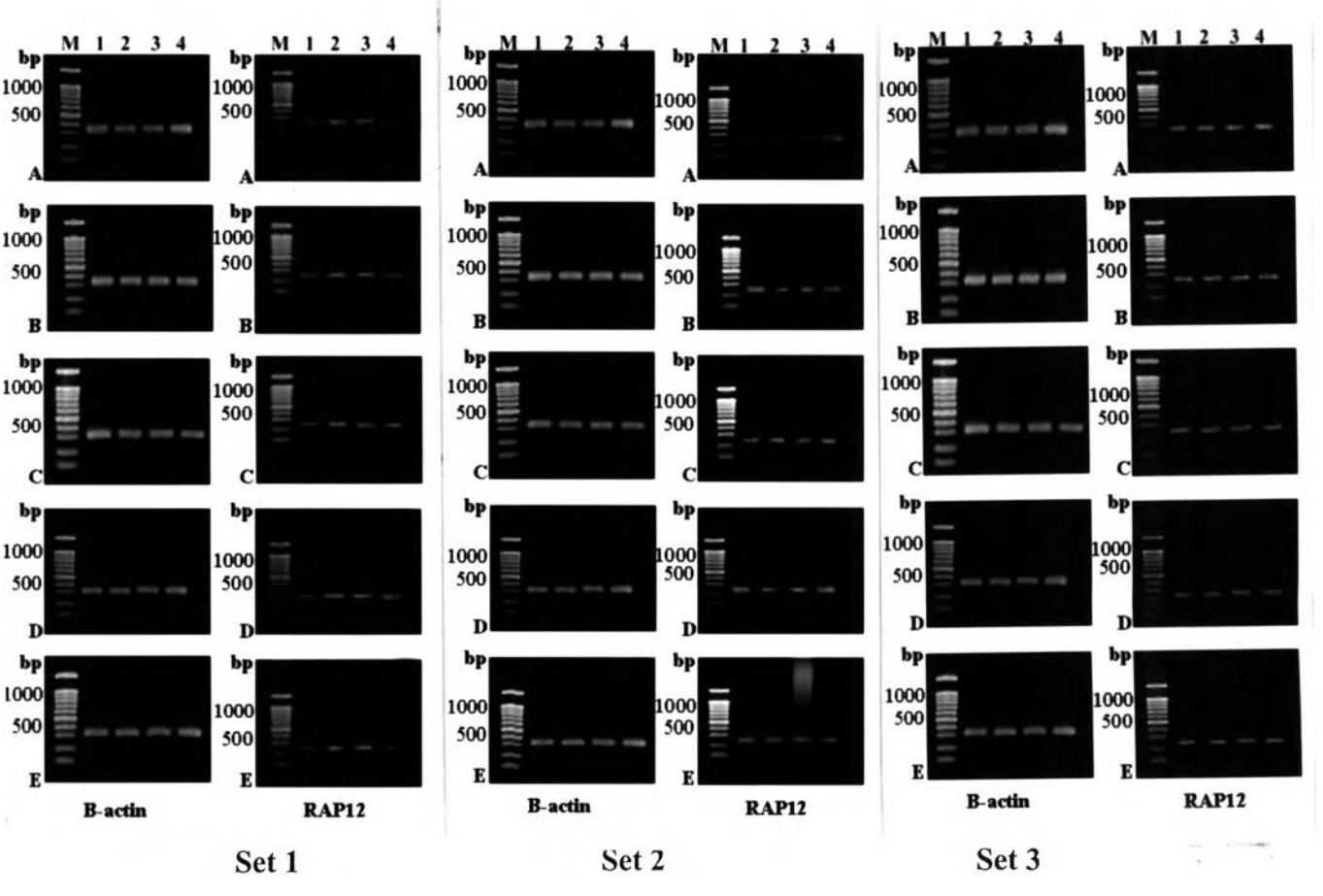


Figure 4.56 The expression levels of RAP12 gene transcripts between control and heat shock, heat induced, and un-induced treatments in comparison with β–actin. Samples were obtained from 3 shrimps and analysed by 1.5% agarose gel electrophoresis. A, B, C, D, and E are the results at 0, 6, 12, 24, and 72 h of *Vibrio* exposure. Lane M is 100 bp markers, Lane 1 to 4 represent control, heat shock, heat induced, and un-induced shrimps, respectively.

Table 4.15 The ratios of RAP12 gene and β-actin genes from *P. monodon* were treated with heat shock, heat induced, and un-induced (*N*=3).

Post exposure time (hour)	No *vibrio* exposure		*Vibrio* exposure	
	control shrimp	heat induced shrimp	heat induced shrimp	un-induced shrimp
0	0.61±0.1	0.63±0.1	0.68±0.06	0.62±0.08
6	0.63±0.05	0.65±0.1	0.69±0.04	0.60±0.07
12	0.60±0.01	0.66±0.08	0.73±0.08	0.64±0.1
24	0.63±0.09	0.76±0.1	0.75±0.07	0.63±0.06
72	0.62±0.1	0.71±0.09	0.66±0.03	0.61±0.08

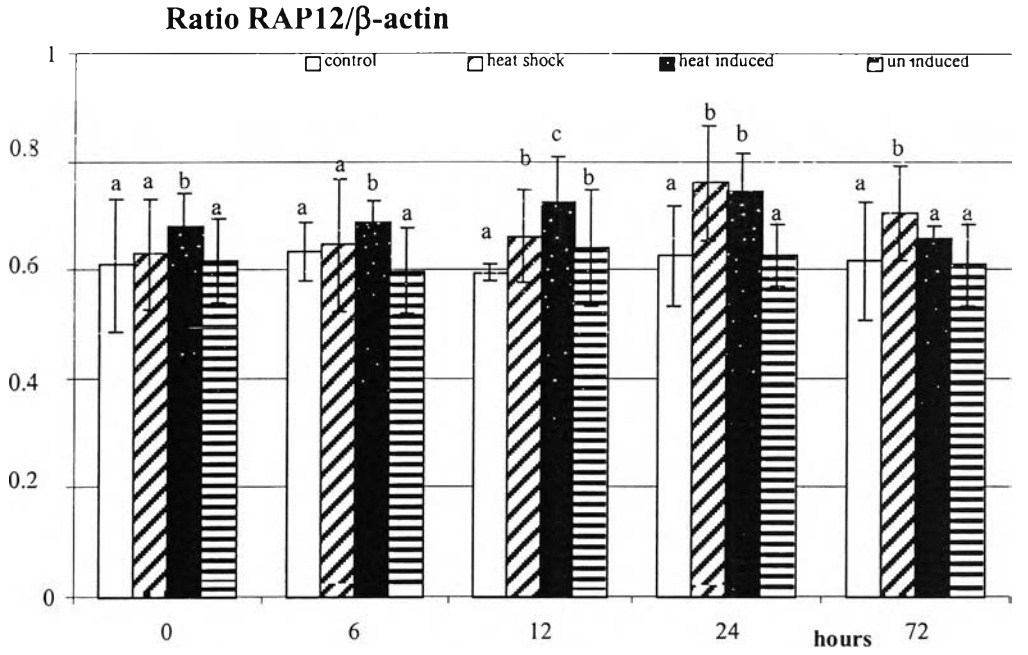


Figure 4.57 The ratios of RAP12 gene and β-actin genes from *P. monodon* were treated with heat shock, heat induced, and un-induced (*N*=3). All data were derived from Table 4.15. The lines above the bar represent standard derivation. Values that are significantly different have different superscripts ($P<0.05$ by Duncan's new multiple range test).

4.10.4.2 Expression level of RAP16 gene

The results of RAP16 expression were shown in Figure 4.58-4.59 and Table 4.16. The significant differences of the expression levels were detected from 6 to 72 hours of post exposure. Significantly higher expression level of RAP16 gene were seen in heat induced shrimps whereas no significant difference was detected in control shrimps and the shrimps exposed to *vibrio* ($P<0.05$). This indicated that RAP16 was induced by heat shock and its expression was not influenced by *vibrio*.

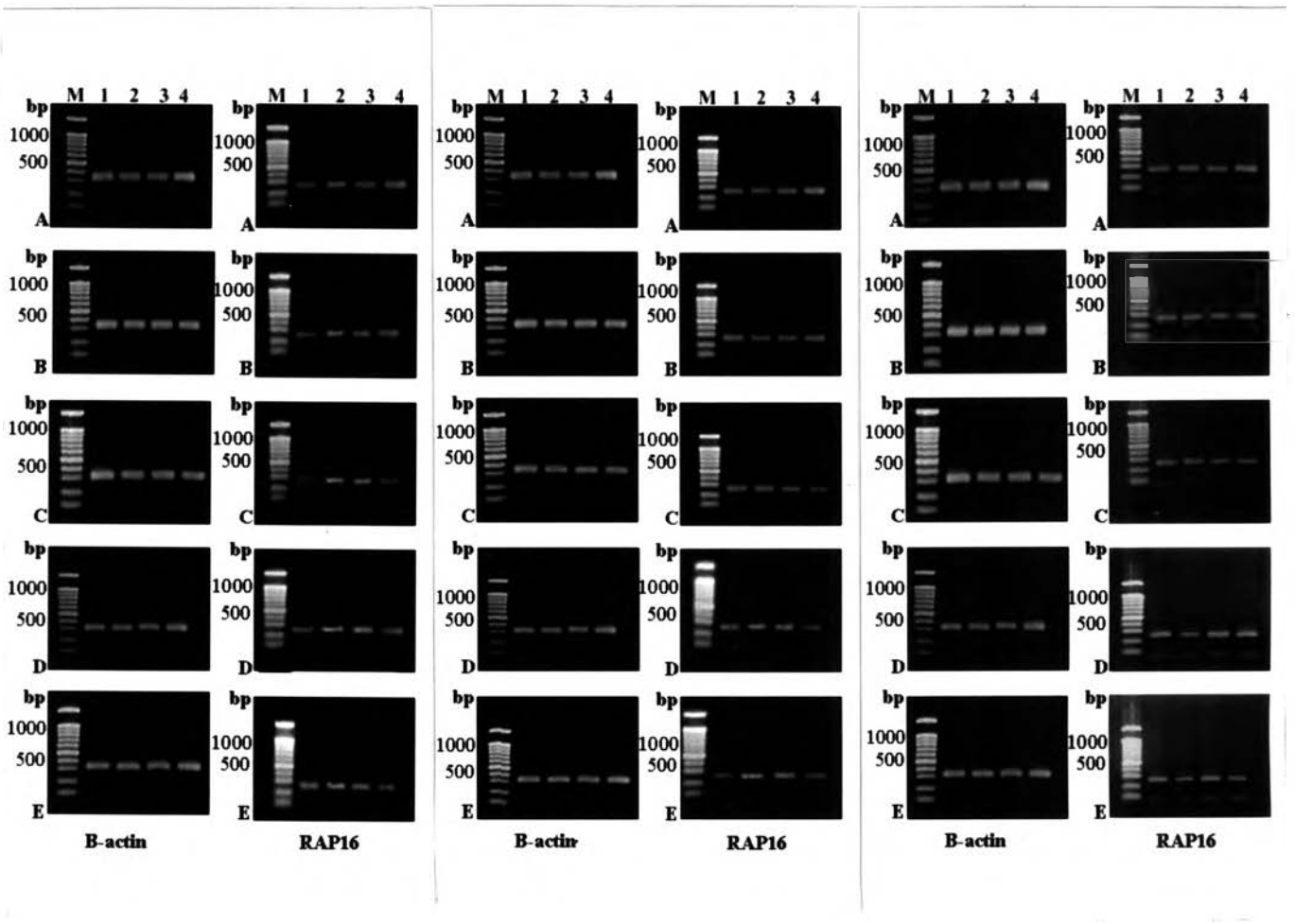


Figure 4.58 The expression levels of RAP16 transcripts between control and heat shock, heat induced, and un-induced treatments in comparison with β–actin. Samples were obtained from 3 shrimps and analysed by 1.5% agarose gel electrophoresis. A, B, C, D, and E are the results at 0, 6, 12, 24, and 72 h of *Vibrio* exposure. Lane M is 100 bp markers, Lane 1 to 4 represent control, heat shock, heat induced, and un-induced shrimps, respectively.

Table 4.16 The ratios of RAP16 gene and β-actin genes from *P. monodon* were treated with heat shock, heat induced, and un-induced (*N*=3).

Post exposure time (hour)	No *vibrio* exposure		*Vibrio* exposure	
	control shrimp	heat induced shrimp	heat induced shrimp	un-induced shrimp
0	0.65±0.06	0.66±0.08	0.68±0.07	0.65±0.09
6	0.63±0.08	0.72±0.07	0.77±0.07	0.67±0.09
12	0.66±0.07	0.77±0.07	0.78±0.08	0.66±0.06
24	0.64±0.08	0.85±0.07	0.86±0.09	0.65±0.07
72	0.61±0.09	0.76±0.08	0.81±0.07	0.62±0.1

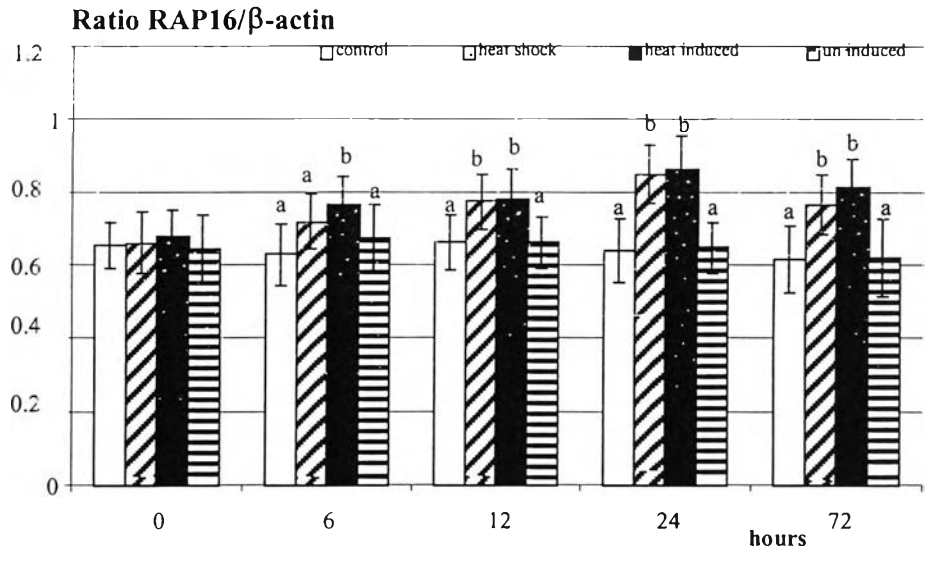
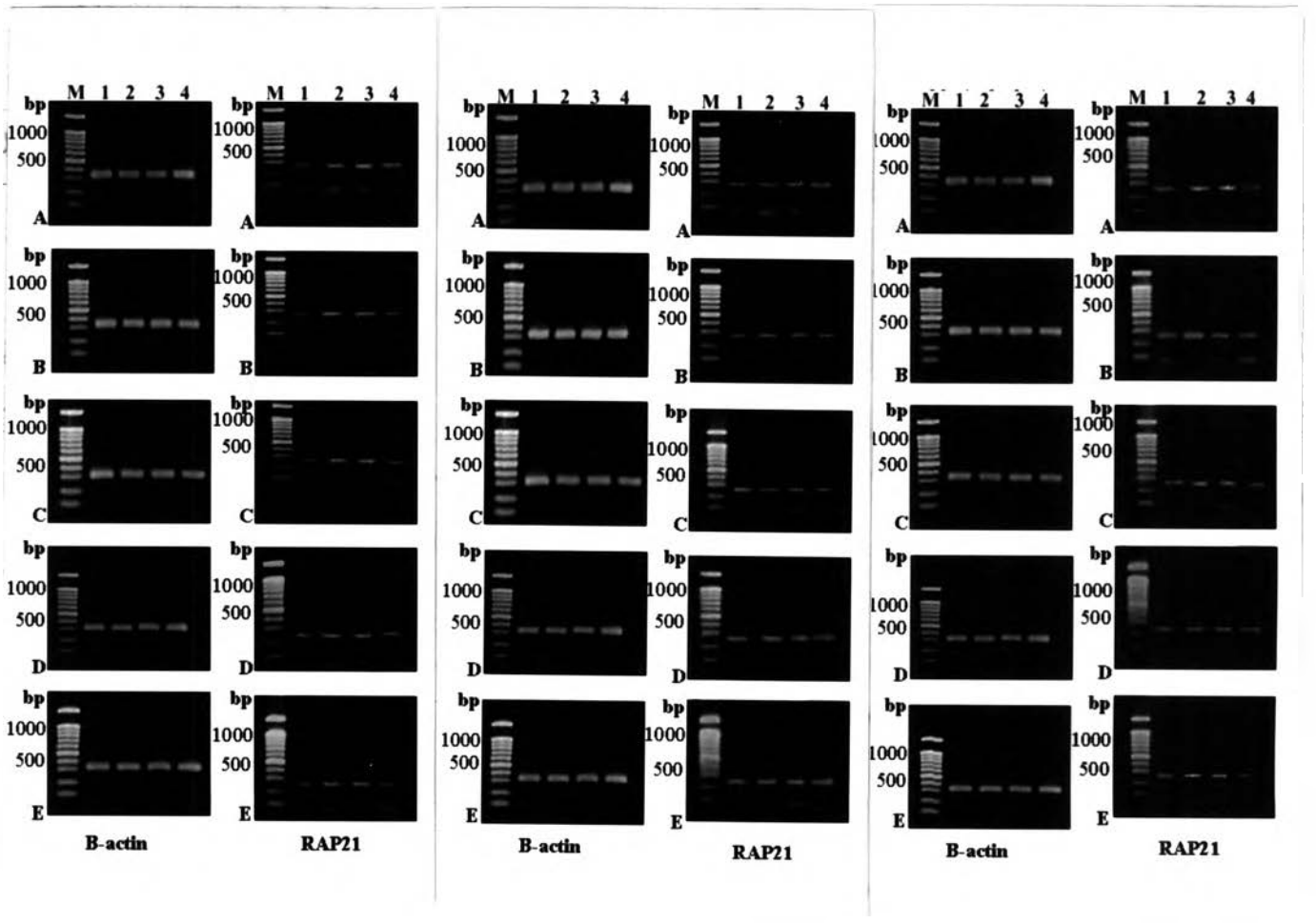


Figure 4.59 The ratios of RAP16 gene and β-actin genes from *P. monodon* were treated with heat shock, heat induced, and un-induced (*N*=3). All data were derived from Table 4.16. The lines above the bar represent standard derivation. Values that are significantly different have different superscripts ($P<0.05$ by Duncan's new multiple range test).

4.10.4.3 Expression level of RAP21 gene

Similar results as obtained from RAP16 gene were detected. The highest level of expression was observed at 24 hours of post exposure ($P<0.05$). The expression level of RAP21 in shrimps exposed to *vibrio* appeared to be slightly enhanced, however, after 6 hours of post exposure, the levels were not significantly different from that of control shrimps. The results were shown in Figure 4.60-4.61 and Table 4.17.



Set 1 **Set 2** **Set 3**

Figure 4.60 The expression levels of RAP21 gene transcripts between control and heat shock, heat induced, and un-induced treatments in comparison with β–actin. Samples were obtained from 3 shrimps and analysed by 1.5% agarose gel electrophoresis. A, B, C, D, and E are the results at 0, 6, 12, 24, and 72 h of *Vibrio* exposure. Lane M is 100 bp markers, Lane 1 to 4 represent control, heat shock, heat induced, and un-induced shrimps, respectively.

Table 4.17 The ratios of RAP21 gene and β-actin genes from *P. monodon* were treated with heat shock, heat induced, and un-induced (*N*=3).

Post exposure time (hour)	No *vibrio* exposure		*Vibrio* exposure	
	control shrimp	heat induced shrimp	heat induced shrimp	un-induced shrimp
0	0.61±0.07	0.63±0.07	0.64±0.06	0.62±0.05
6	0.59±0.06	0.66±0.07	0.66±0.1	0.62±0.09
12	0.60±0.06	0.69±0.02	0.68±0.01	0.63±0.09
24	0.62±0.07	0.82±0.01	0.81±0.01	0.67±0.04
72	0.64±0.01	0.76±0.05	0.72±0.09	0.66±0.04

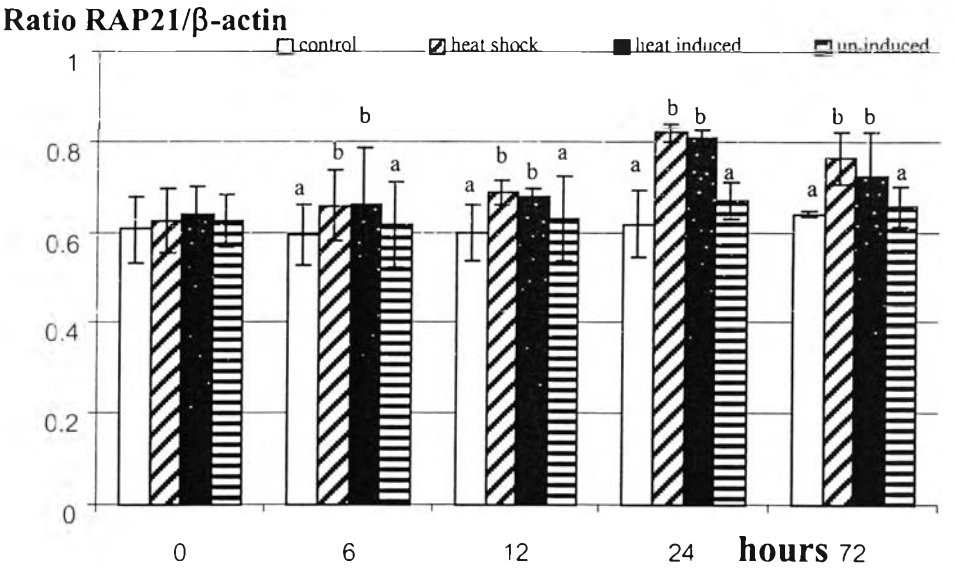


Figure 4.61 The ratios of RAP21 gene and β-actin genes from *P. monodon* were treated with heat shock, heat induced, and un-induced (*N*=3). All data were derived from Table 4.17. The lines above the bar represent standard derivation. Values that are significantly different have different superscripts ($P<0.05$ by Duncan's new multiple range test).

4.10.4.4 Expression level of RAP58 gene

The results of the expression level of RAP58 gene were shown in Figure 4.62-4.63 and Table 4.18. Higher expression level of RAP58 was detected in heat induced shrimps at 12 hours of post exposure while the significantly higher level was detected in pre-heated shrimps exposed to *vibrio* when compared to the expression level from un-induced shrimps($P<0.05$). It was indicated from the results that the expression of RAP58 gene was induced by heat shock and also enhanced by *vibrio* in pre-heated shrimps while *vibrio* exposure alone was not induced the expression of RAP58 gene.

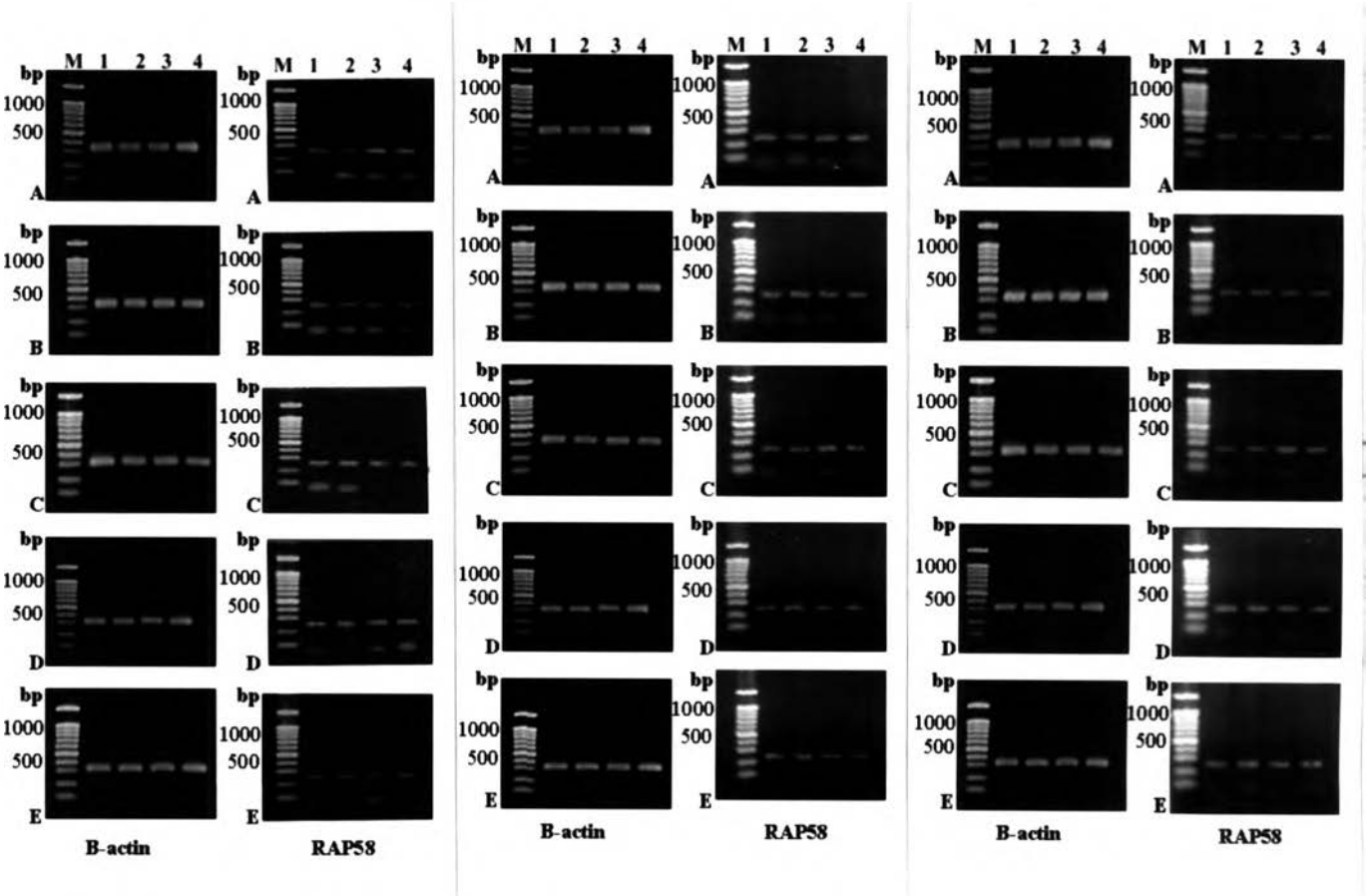


Figure 4.62 The expression levels of RAP58 gene transcripts between control and heat shock, heat induced, and un-induced treatments in comparison with β–actin. Samples were obtained from 3 shrimps and analysed by 1.5% agarose gel electrophoresis. A, B, C, D, and E are the results at 0, 6, 12, 24, and 72 h of *Vibrio* exposure. Lane M is 100 bp markers, Lane 1 to 4 represent control, heat shock, heat induced, and un-induced shrimps, respectively.

Table 4.18 The ratios of RAP58 gene and β-actin genes from *P. monodon* were treated with heat shock, heat induced, and un-induced (*N*=3).

Post exposure time (hour)	No *vibrio* exposure		*Vibrio* exposure	
	control shrimp	heat induced shrimp	heat induced shrimp	un-induced shrimp
0	0.54±0.1	0.51±0.1	0.56±0.06	0.49±0.08
6	0.54±0.05	0.52±0.1	0.59±0.04	0.49±0.08
12	0.54±0.01	0.60±0.08	0.67±0.08	0.57±0.1
24	0.56±0.09	0.67±0.1	0.70±0.07	0.59±0.06
72	0.56±0.1	0.60±0.09	0.64±0.03	0.54±0.08

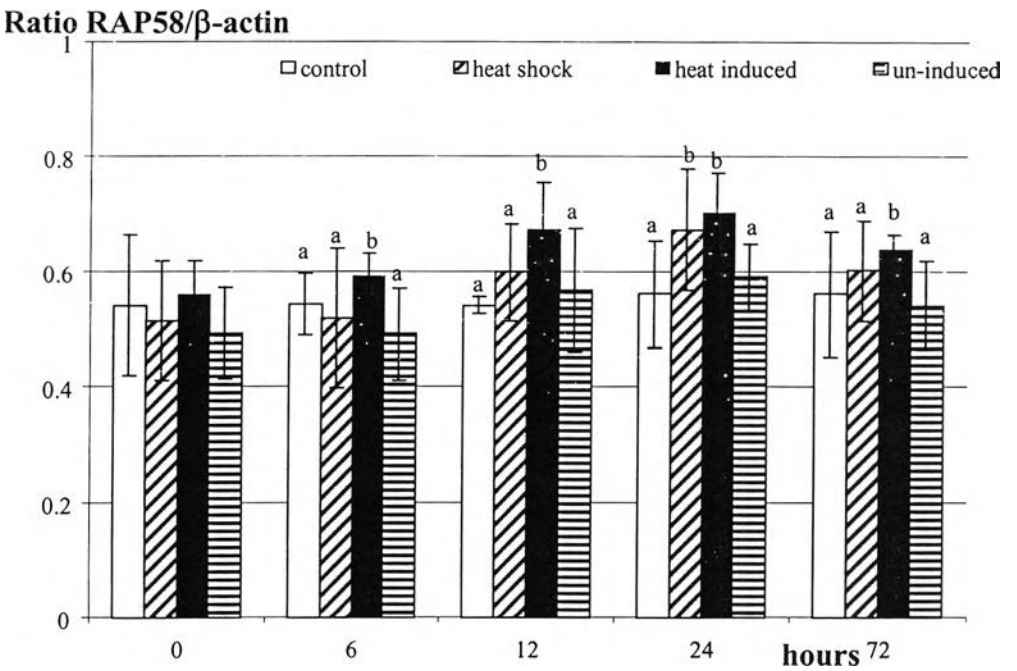


Figure 4.63 The ratios of RAP58 gene and β-actin genes from *P. monodon* were treated with heat shock, heat induced, and un-induced (*N*=3). All data were derived from Table 4.18. The lines above the bar represent standard derivation. Values that are significantly different have different superscripts ($P<0.05$ by Duncan's new multiple range test).