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Acclimation-dependent expression of heat shock protein 70 in Pacific abalone (*Haliotis discus hannai* Ino) and its acute response to thermal exposure*

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Abstract Heat shock protein 70 (Hsp70) is one important member of heat shock protein (Hsp) family that is responsible for various stresses, especially thermal stress. Here we examined the response of Hsp70 gene to both chronic and acute thermal exposure in Pacific abalone (*Haliotis discus hannai* Ino). For the chronic exposure, abalones were maintained at 8, 12, 20, and 30°C for four months and their mRNA levels were measured. The highest mRNA level of Hsp70 gene relative to actin gene was detected in the 30°C-acclimated group, followed by the 8°C-acclimated group and then the 12°C- and 20°C-acclimated groups. After the long-term acclimation, gills from each of the above acclimation groups were dissected and exposed to different temperatures between 8°C and 38°C for 30 min. Hsp70 expression in gills acclimated to different temperatures responded differentially to the same temperature exposure. The incubation temperature that induced maximum Hsp70 mRNA expression was higher in the higher temperature acclimation groups than lower temperature groups. Pacific abalones could alter the expression pattern of Hsp70 gene according to environmental thermal conditions, through which they deal with the stress of thermal variations.

Keyword: *Haliotis discus hannai* Ino; heat shock; cold stress; Hsp70; biomarker

1 INTRODUCTION

Heat shock protein 70 (Hsp70) is one of the most important proteins involved in chronic temperature acclimation and acute response of animals to temperature challenge in invertebrates (Feder and Krebs, 1998, Feder and Hofmann, 1999). Under these conditions, Hsp70 may play key roles in *de novo* protein folding, refolding of misfolded and aggregated proteins, and other regulatory processes (Gething and Sambrook, 1992; Hartl, 1996; Hartl and Hayer-Hartl, 2002). Hsp70 gene and its expression have been reported in molluscs such as oysters (Rathinam et al., 2000; Boutet et al., 2003; Zhang et al., 2003), scallops (Song et al., 2006; Gao et al., 2007), mussels (Franzellitti and Fabbri, 2005), and abalones (Farcy et al., 2007). Hsp70 may play similar roles in temperature stress as it has been found that heat shock significantly increases Hsp70 expression in the Pacific abalone *H. discus hannai* (Cheng et al., 2007).

The Pacific abalone is cold-water gastropod naturally distributed in temperate coasts of Northwest Pacific Ocean. Its natural habitats range from Shandong and Liaodong Peninsulas of China, Korea Peninsula, Japan, and Far East waters of Russia, where the waters are relatively cold (Lv, 1978; Hara and Sekino, 2005). Farming of the Pacific abalone in China was initiated in 1980s, and the farming activities remain only restricted to the coastal areas within or close to their natural distribution ranges in the first 20 years (Nie and Wang, 2004; Zhang et al., 2004). Since the beginning of this century, Pacific abalone farming has been gradually extending from northern Yellow Sea to East China Sea, where the temperature is at most time significantly higher than that in the natural habitats. In the past decade, a new

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farming model is becoming prevalent. In this model, abalone seeds and/or adults are transferred directly from northern waters to warm waters in Fujian Province in late October for overwintering. In late April of the next year, they are moved back to northern waters. Although the abalones suffer dramatic temperature changes from about 12°C to 24°C when moved to the south and from 24°C to 10°C when moved to the north, significant improvement in growth and survival rate suggests that the abalones could well adapt to such challenges. To investigate the underlying mechanisms of temperature acclimation, we examined the Hsp70 expression in chronic temperature acclimation and in response to acute temperature shocks.

2 MATERIAL AND METHOD

2.1 Experimental animals

The abalones used in the present study were derived from a selectively bred population named P-97 that had been linearly selected for four generations since 1997. Experimental abalones were produced from 18 sirs and 34 dams in April 2008 in a hatchery in Shandong Province and cultured there till being moved to Fujian Province for overwintering. They were transferred back to Rongcheng City, Shandong Province in the early spring of 2009 and cultured there prior to the experiments.

2.2 Influences of long-term temperature acclimation on Hsp70 expression

Thirty abalones were randomly selected for each of the four groups cultured at 8, 12, 20, and 30°C, respectively, in recirculation systems for four months. Three repeats were set for each group. The average shell length of the abalones used was about 5.8±0.3 cm. During the chronic temperature exposure, 40% of the water in the system was replaced everyday and the water was completely changed once every two weeks. The animals were fed with kelps (*Laminaria japonica*) once each day.

2.3 Effects of acute thermal stress on Hsp70 expression

To measure the response of Hsp70 expression to acute thermal exposure, we examined the Hsp70 mRNA levels in gill pieces after chronic thermal exposure. A piece of gills was removed from each animal and its Hsp70 expression was determined using the protocol given below. The gills were used because it has been reported that Hsp70 mRNA in gills exhibited similar trends with that of the muscles but at much higher levels when exposed to heat shocks (Cheng et al., 2007).

In the first set of experiments, to determine the optimal incubation time for detection of maximum Hsp70 expression in response to acute thermal exposure, we used small gill pieces of about 3 mg dissected from four abalones of the 20°C-acclimated group. They were transferred to Eppendorf tubes containing 1.0 mL 30°C seawater and were incubated for 10, 15, 20, 30, 45 min, respectively. Immediately after the treatment, the gill pieces were moved to Eppendorf tubes containing 0.5 mL RNAwait (Solarbio, Beijing, China) for subsequent Hsp70 expression measurement. The gill pieces were transferred to tubes containing RNAwait immediately after dissection (i.e. without acute thermal exposure) as controls.

In the second set of experiments, gill pieces dissected from four animals of each of the four temperature acclimated groups were incubated in Eppendorf tubes containing 1 mL seawater at a series of temperatures for 30 min (see Table 1 for temperature series). The gill pieces were then transferred to Eppendorf tubes containing 0.5 mL RNAwait for subsequent RNA extraction and measurement of Hsp70 mRNA level. Hsp70 mRNA levels of the gill pieces from the same animals incubated at the same temperature as their long-term acclimations were used as controls.

2.4 Measurement of Hsp70 mRNA levels

Total RNA was extracted from gill pieces using Fastagene RNAfast 200 Kit (Fastagen, Shanghai, China) according to the manufacturer's protocol. The quality of RNA was checked on 1.2% denaturing agarose gel. For reverse transcription, the cDNA was synthesized in a 25-μL reaction volume containing 2 μg of DNase I-treated total RNA, 1× Moloney murine leukemia virus (MMLV) buffer, 0.5 mmol/L deoxyribonucleotide triphosphate (dNTP), 0.4 mmol/L oligo-dT, 20 U of RNase inhibitor

Table 1 Acute exposure temperature series for each temperature acclimation group

Acclimated temperature (°C)	Acute exposure temperature (°C)									
8	8	12	/	20	22	24	26	30	34	/
12	/	12	16	20	22	24	26	30	34	/
20	8	12	/	20	22	24	26	30	34	/
30	8	12	/	20	/	24	26	30	34	38

(Promega, Madison, WI, USA), 200 U of MMLV reverse transcriptase (Promega) and 10 mmol/L Oligo dT-18.

Primers for Hsp70 and *β*-actin genes were designed using Primer Primer 5.0 (http://www.premierbiosoft.com/primerdesign) based on the the full length cDNA sequences deposited in GenBank: Hsp70F: GAGAGCAAAGAGAACCCTG; Hsp70R: TCTCT ACGGGCTCCAGTGT; *β*-actinF: GGTATCCTCAC CCTCAAGT; *β*-actinR: GGGTCATCTTTTCACGG TTG. The fluorescent real-time RT-PCR assays were carried out on an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems) with triplicate in 20 μL reaction volume containing 10 μL SYBER Green Realtime PCR Master mix (TOYOBO), 1 μL forward and reverse primer mix, 1 μL template cDNA, and 8 μL H_2O. After incubation for 10 min at 95°C, the reaction continued for 40 cycles at 95°C for 15 s and 60°C for 1 min.

The standard curves were established using a series of diluted samples (10-fold serial dilutions: 1×, 10×, 100×, 1 000×, 10 000×) for both Hsp70 and *β*-actin. The slopes of the two standard curves were calculated according to the formula $E=10^{(-1/slope)}$ where E is amplification efficiency. Melt curve analysis was performed to confirm that only a single product was amplified.

The relative mRNA levels of Hsp70 were calculated based on the Ct values of Hsp70 and *β*-actin normalized to that of the cDNA standard. One-way ANOVA procedure of GLM of SAS software (SAS Institute, 1990) was used to determine whether there is a significant variation in the real-time RT-PCR data.

3 RESULT

3.1 Hsp70 expression in different chronic temperature-acclimated groups

After exposure to different temperatures for 4 months, the Hsp70 mRNA levels relative to *β*-actin were dramatically different in different temperature-acclimated groups (Fig.1). Levels of Hsp70 mRNA in animals cultured at optimal temperatures (12°C and 20°C) were much lower than those cultured at extreme temperatures (8°C and 30°C). Abalones acclimated to 30°C exhibited the highest Hsp70 mRNA level relative to *β*-actin, followed by the 8°C-acclimated group, both of which were significantly higher than those in the 12°C- and 20°C-acclimated groups ($P<0.05$). The Hsp70 levels

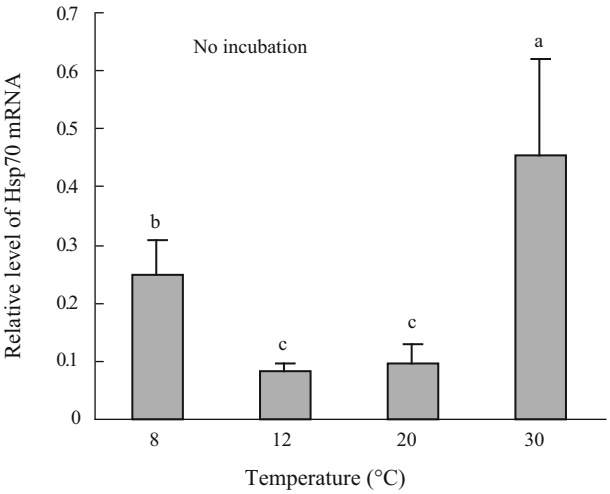

Fig.1 The mRNA level of Hsp70 gene of abalones acclimated to different temperatures for 4 months

All values are expressed as mean±SD ($n=4$), and different letters above the bars indicate significant difference ($P<0.05$).

in the 30°C-acclimated group were 4.7-fold and 2.5-fold higher than that in the 20°C-acclimated group respectively. No significant difference was found between the 20°C- and 12°C-acclimated groups ($P>0.05$).

3.2 Determination of optimal incubation time for acute thermal stress

To determine the optimal incubation time for response of Hsp70 expression, we compared the Hsp70 mRNA level at different exposure time. The results showed that Hsp70 mRNA level increased with temperature exposure time and reached the peak at 30 min but decreased thereafter (Fig.2). The Hsp70 mRNA level at 30 min was about 3 times higher than that of the control. Therefore, 30 min was chosen as the incubation time for the acute thermal exposure experiments.

3.3 Effects of long-term acclimation on acute thermal exposure-induced Hsp70 expression

Hsp70 mRNA level responded differentially to acute thermal exposure in different temperature-acclimated groups. In the 8°C-acclimated group, no significant change in Hsp70 mRNA level was observed when exposed to any temperature for 30 min compared with the control (Fig.3a). Acute exposure to 26°C and 30°C in the 12°C-acclimated group and to 30°C and 34°C in the 20°C-acclimated group significantly increased the Hsp70 expression (Fig.3b, 3c), both with peaks at 30°C ($P<0.05$). A

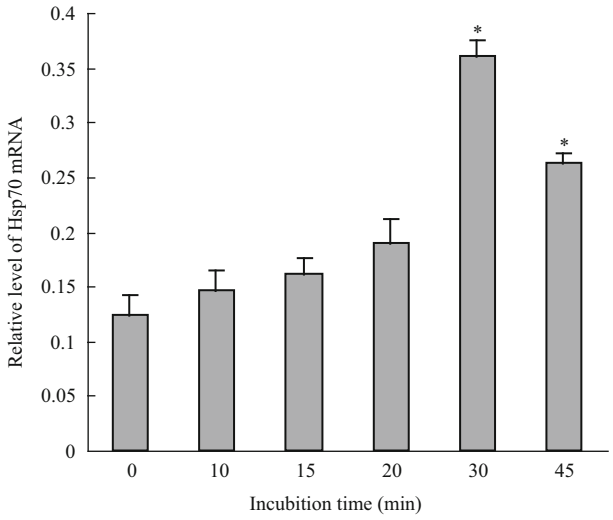

Fig.2 Changes of relative level of Hsp70 mRNA in gill pieces isolated from the 20°C-acclimated group in response to acute exposure to 30°C for different time

Values are expressed as mean±SD (n=4). Asterisks above bars indicate statistical significance (P<0.05).

very robust response, as high as 5.2-fold of the control, was observed in the 30°C-acclimated group when exposed to 34°C (P<0.05; Fig.3d). In contrary to heat shock, at the temperatures tested, gill pieces showed no response to cold shock (Fig.3b, 3c, and 3d).

4 DISCUSSION

In this study, we examined the Hsp70 gene expression in the Pacific abalones and found that animals chronically exposed to extreme temperatures exhibited higher Hsp70 levels than those exposed to favorable temperatures. We further showed that thermal history also had significant effects on subsequent in-vitro acute exposure to different temperatures.

Heat shock protein 70 is one of the most important factors involved in acute response to environmental changes, especially temperature (Snyder et al., 2001; Mayer and Bukau, 2005). For example, in *H. discus hannai*, transient expression of Hsp70 can be induced by heat shock at 32°C for 1h, reached the peak at 12 h, and decreased to normal levels thereafter gradually (Cheng et al., 2007). However, in this study, we observed higher Hsp70 levels even when animals were acclimated to extreme temperatures (8°C and 30°C) for 4 months. Similar phenomenon has also been observed in cold-acclimated South African abalones, *Haliotis midae*. The Hsp70 content in the muscles of the abalones cultured at 22°C for one month was dramatically increased than that of the control group maintained at 16°C (Vosloo and Vosloo, 2010). Substantial growth when cultured at 30°C suggested that the abalones well adapted to this extreme temperature. This seemed to contradict with the high Hsp70 levels at 30°C. Thus, the exact functions of Hsp70 gene at extreme conditions remain to be exploited.

We also found that high Hsp70 expression was induced by culturing the abalones at 8°C, which is close to the biological zero point (Kikuchi and Uki, 1974) and obviously is not favorable for the growth and survival of the animals. Similar phenomenon has also been reported in other animals such as the Atlantic salmon (Takle et al., 2005). Thus, it seems to be common that the so-called heat shock proteins are also ‘cold shock proteins’ that can be induced by cold shock.

To examine the influence of thermal history on subsequent response of abalones to acute temperature exposure, we employed an in-vitro model using dissected gill pieces. Use of marine animal tissues to study their response to temperature stress has been reported in the purple sea urchins, *Strongylocentrotus purpuratus*, which showed that sea urchin tube feet responded intensively to acute temperature shock. (Osovitz and Hofmann, 2005). We also found that the gill pieces were very sensitive to heat shock and Hsp70 mRNA level can be increased to three times of the control in as short as 30 min. In fact, the use of gill pieces in-vitro has several advantages over whole animals such as more homogenous response and less need for whole animals. Gill pieces can be used as a good biomarker for acute response studies in abalones.

It is interesting that the response of the gill pieces to temperature shock was significantly affected by the thermal history of the donor. If the donor was cultured at 12°C, the gill pieces only respond to a temperature shock of (14–18)°C while gill pieces from the donor cultured at 20°C responded intensively to a temperature shock of (10–14)°C. On the contrary, if the donor was cultured at 30°C, a temperature shock of only 4°C can induce Hsp70 mRNA level in 30 min and up to 5.2 times of that of the control. Gill pieces from donors cultured at high temperature are more sensitive to temperature shock and resulted in more intensive responses. It is possible that the response of Hsp70 gene expression to temperature shock is affected by the basic metabolism level;

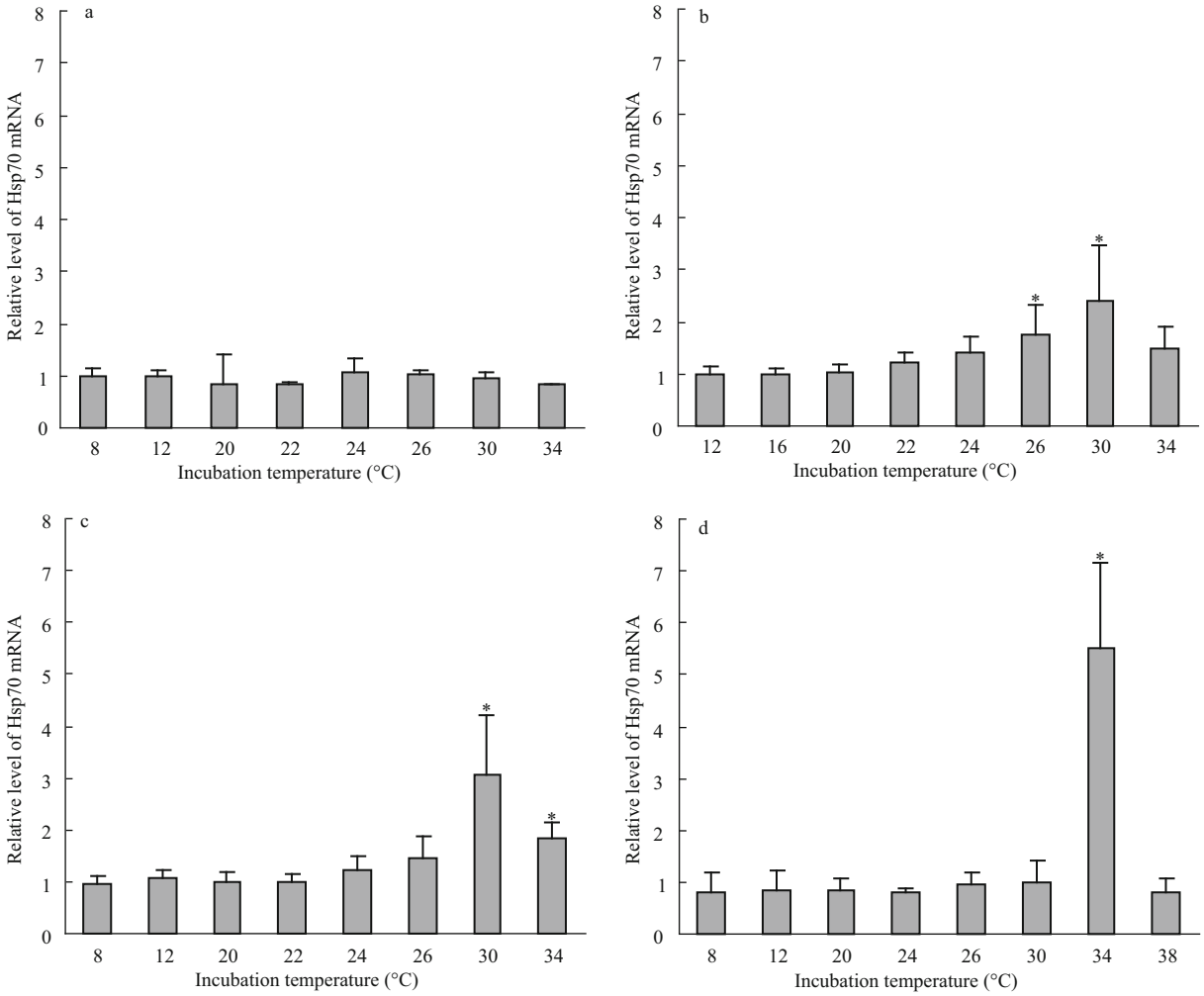

Fig.3 Expression of Hsp70 in response to acute thermal exposure in 8°C(a), 12°C(b), 20°C(c) and 30°C(d) -acclimated groups

Values are Hsp70 mRNA levels relative to that of the control for each specific temperature-acclimated group. Asterisks above the bars indicate the value is significantly different from the control ($P<0.05$). Values are expressed as mean±SD.

higher basic metabolism level tends to induce faster and more intensive response. However, When interpreting the above results, we must keep in mind that although β-actin, the so-called housekeeping gene, is often used as internal reference gene, its expression is also affected by the physiological status of the donor (Ruan and Lai, 2007). In our case, the expression of β-actin may vary with the physiological conditions of donors cultured at different temperatures. Obviously, new and more solid evidence with more reference genes is absolutely needed to support this hypothesis.

5 CONCLUSION

1. In-vivo experiments showed that when cultured under long-term hot and cold stresses, the animals maintained high Hsp70 level in their gills. The results may suggest the potential functions of Hsp70 in adaptation of animals to unfavorable temperatures.
2. Gill pieces in-vitro responded to heat shock quickly and robustly and can be used as a biomarker for acute response studies in abalones.
3. The sensitivity of gill pieces in-vitro in response to heat shock is affected by the thermal history of the donor. If the donor was cultured at high temperature, its gills tend to respond to high temperature shock and result in fast and robust responses.

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