GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Effects of various salinities on *Na⁺-K⁺-ATPase*, *Hsp70* and *Hsp90* expression profiles in juvenile mitten crabs, *Eriocheir sinensis*

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Genet. Mol. Res. 11 (2): 978-986 (2012)
Received April 13, 2011
Accepted November 10, 2011
Published April 19, 2012
DOI http://dx.doi.org/10.4238/2012.April.19.3

ABSTRACT. *Eriocheir sinensis* is a euryhaline crab migrating from sea to freshwater habitats during the juvenile stage. We used quantitative real-time polymerase chain reaction (qRT-PCR) to investigate the gene expression profile of *Na⁺-K⁺-ATPase*, *Hsp70* (heat shock protein 70) and *Hsp90* in megalopa exposed to salinities of 0, 2, 5, 10, and 15 parts per thousand. Both low and high salinities markedly stimulated expression of *Na⁺-K⁺-ATPase*, *Hsp70* and *Hsp90* genes of Chinese mitten crab megalopa; salinity had different effects on *Na⁺-K⁺-ATPase*, *Hsp70* and *Hsp90* levels depending on the duration of salinity stress, implying that *Na⁺-K⁺-ATPase*, *Hsp70* and *Hsp90* may play an important role in salinity tolerance in this crab species.

Key words: *Eriocheir sinensis*; *Na⁺-K⁺-ATPase*; Salinity stress; qRT-PCR

INTRODUCTION

The Chinese mitten crab (CMC) *Eriocheir sinensis* is a native species in Eastern Asia and can tolerate a wide range of ambient salinities from 0.5 to 40 ppt. During their complex life cycle, adult CMCs migrate downstream towards estuarine waters to release planktonic larvae (Herborg et al., 2003). The larval development undergoes 5 zoeal stages Z1, Z2, Z3, Z4, and Z5, and a megalopa stage (Kim and Hwang, 1995; Montú et al., 1996) takes place in estuarine and marine coastal waters. After metamorphosis, juvenile CMCs start the upstream migration towards their limnic parental habitats (Herborg et al., 2003). Therefore, the CMCs are prone to be affected by salinities at various stages during its growth, leading to high mortality. As a result, in recent years, applied researchers have increasingly focused on the characterization of proteins playing key roles in salinity regulation in CMCs. For example, the effects of long-term exposure to different salinities on the activity of *Na^+-K^+-ATPase* in the gills (Torres et al., 2007) have been studied in CMC juveniles.

Special ion transport-related enzymes have been inferred to participate in the crab's osmoregulatory system, of which *Na^+-K^+-ATPase* has been studied the most extensively. *Na^+-K^+-ATPase* is capable of transporting Na^+ out of cells and K^+ into the cell against their respective concentration gradients, while the sodium pump sustains the osmotic balance of the cell, transports nutrients into the cell and helps sustain electrical activity in excitable cells (Skou and Esmann, 1992). Many studies have indicated that *Na^+-K^+-ATPase* plays a major role in active Na^+ uptake as well as in systemic ion regulation and cellular water balance in euryhaline crabs (Towle, 1981). It is the primary mediator of ion transport across cellular membranes (Neufeld et al., 1980).

Cells generally respond to stress by changing gene expression and upregulating the production of a group of highly conserved proteins known as the heat shock proteins (HSPs) in the blood and tissues. It is now recognized that upregulation in response to stress is universal to all cells and not restricted to heat stress (Chiang et al., 1989; Welch, 1993). Thus, other stressors such as anoxia ischemia, toxins, protein degradation, hypoxia, acidosis, and microbial damage will also lead to upregulation of HSPs (Feder and Hofmann, 1999). One physiological stress where HSP induction has been shown to play a significant role is in relation to salinity shock. A broad spectrum of aquatic animals have been shown to react to salinity stress through HSP production, particularly HSP70 (Deane et al., 2002). For example, there was a significant rise in HSP70 levels in the Atlantic salmon while transferring them to high-salinity sea water (Pan, et al., 2000). Additionally, it was reported that both low and high salinities significantly changed the levels of the mRNAs of HSP70 and HSP90 in abdominal muscle (Spees et al., 2002). The effects of salinity fluctuation on the Hsp70 activity of juvenile *Fenneropenaeus chinensis* cultured in sea water with a salinity of 20 have been studied (Ding et al., 2009).

Although considerable research has been devoted to various marine species, less attention has been given to CMC megalopa. This paper therefore describes a study of the involvement of *Na^+-K^+-ATPase*, *Hsp70* and *Hsp90* in the osmoregulation of CMCs megalopa during short-term exposure to different salinities.

MATERIAL AND METHODS

Animal materials

Megalopas of CMC (*E. sinensis*) were artificially cultivated, challenged with 0, 2, 5, 10, and 15 ppt salinity stress, and sampled at 24, 48, 72, and 96 h. Samples cultivated at a salinity of 5 ppt served as the control. The megalopas were cultivated in beakers at 20°C and a density

of 1 individual/100 mL with natural sunlight. Sampling was carried out at 10:00 am every day.

RNA purification and first-strand synthesis of cDNA

Total RNA was extracted from the samples using the TRIzol reagent (TaKaRa, Shiga, Japan) and stored at -80°C until use. First-strand cDNA synthesis was performed using M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA) to transcribe poly(A) mRNA with oligo-dT and random 6-mer primers. The reaction conditions were according to manufacturer instructions. The cDNA was maintained at -20°C until used as a template for quantitative real-time PCR (qRT-PCR).

Quantification of mRNA expression

qRT-PCR was carried out with an ABI StepOnePlus detection system (Applied Biosystems, Foster City, CA, USA). Amplifications were performed on a 96-well plate with a 20-μL reaction volume containing 10 μL 2X SYBR Premix Taq™, 0.8 μL PCR forward primer (10 μM), 0.8 μL PCR reverse primer (10 μM), 2.0 μL cDNA temple, and 6.4 μL diethylpyrocarbonate water (DEPC-water). The thermal profile for SYBR Green qRT-PCR consisted of an initial step at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. As the preliminary trial of the experiment had shown that β-actin gene had a steady expression in this experimental species, it was used as the housekeeping gene in all qRT-PCR assays, where primers for the β-actin gene were designed on the basis of the β-actin gene sequence (GenBank accession No. FL589653). Primers used to amplify the β-actin gene were β-actin-RT-F (5'-GCC GTG ACC TCA CAG ACT ACC T-3') and β-actin-RT-R (5'-TTG ATG TCT CGC ACG ATT TCT C-3'). Primers used to amplify *Na^+-K^+-ATPase* were Na^+-K^+-ATPase-RT-F (5'-ACT GGC TGT TGG AGA TGT CA-3') and Na^+-K^+-ATPase-RT-R (5'-ACG AGG AGG TAG CGA GGA T-3'). Primers used to amplify *Hsp70* were Hsp70-RT-F (5'-TCGTCACCGTTCCAGCCTACT-3') and Hsp70-RT-R (5'-CGC AGC ACA TTG AGA CCA GAG A-3'). Primers used to amplify Hsp90 were Hsp90-RT-F (5'-GAT GCT GAC AAG AAC GAC AAG T-3') and Hsp90-RT-R (5'-GGT GAA GCC AGA TGA GAG GAG-3'). The standard curve and the gene expression levels were analyzed automatically by the system, as was the setting of the baseline. A melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one product was amplified and detected, and qRT-PCR observations at a salinity of 5 ppt were calculated to derive the means and standard deviations.

RESULTS

Na^+-K^+-ATPase

Na^+-K^+-ATPase expression level decreased significantly when CMCs were transferred from fresh water (S0, S2, S5) to salt water (S10, S15) and remained at a constant level for 24 h (Figure 1A-D). There was an increasing tendency in *Na^+-K^+-ATPase* expression level when the salinity was maintained at 10 and 15 ppt and the exposure time was extended from 24 to 48 and 72 h (Figures 1D and 2A). In addition, this increase fluctuated for 72 h in each treated group.

Hsp70 and *Hsp90*

Our findings showed that *Hsp70* and *Hsp90* levels under low-salinity conditions (S0,

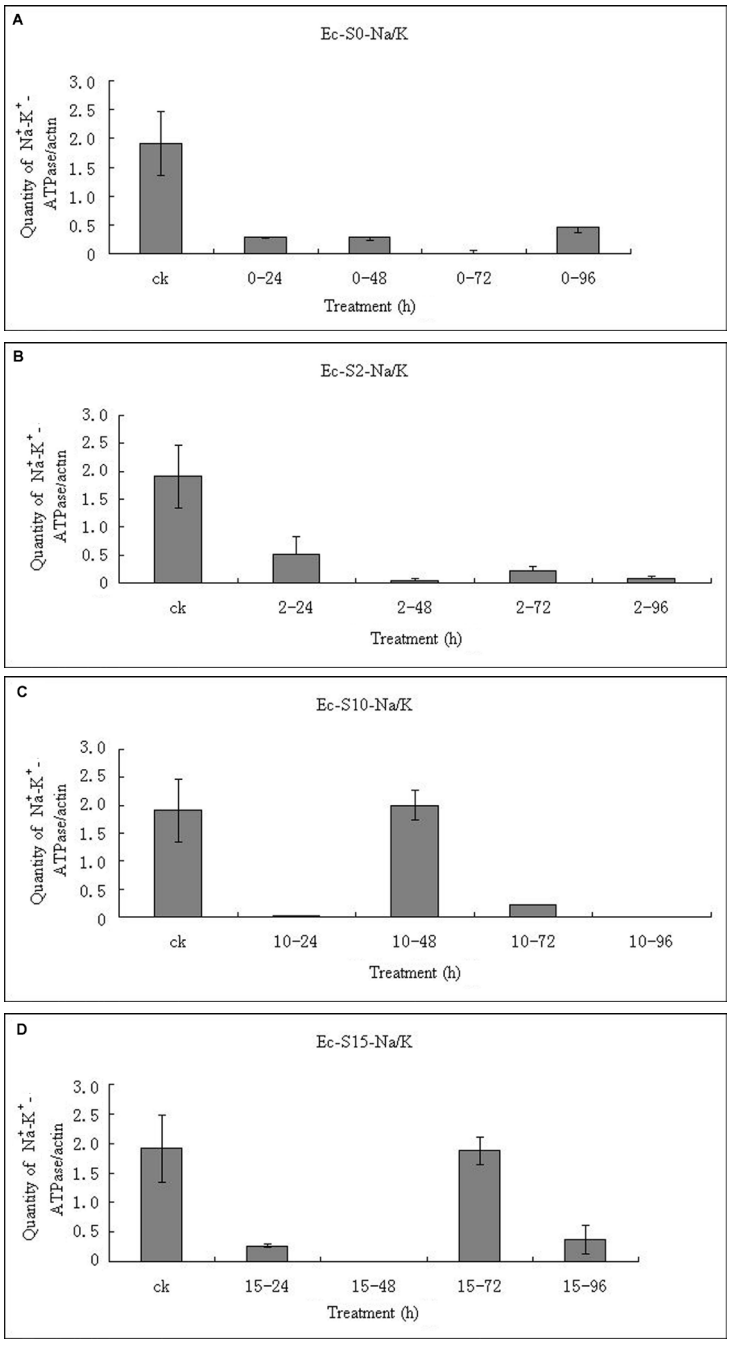

Figure 1. Expression profiles of the *Na⁺-K⁺-ATPase* gene at different treatment hours at salinity 0 (**A**), at salinity 2 (**B**), at salinity 10 (**C**), and at salinity 15 (**D**) revealed by qRT-PCR. The amount of *Na⁺-K⁺-ATPase* mRNA was normalized to the *actin* mRNA transcript level. Data are reported as means ± SD for three repeated experiments. The y-axis represents the relative ratio of mRNA expression levels of *Na⁺-K⁺-ATPase* to *actin*. ck = control.

S2) were lower than those of the control (S5) (Figures 2A,B and 3A,B). However, the *Hsp70* level under high-salinity conditions (S10, S15) was higher than that of the control (S5) (Figures 2C,D and 3C,D). The *Hsp70* expression level was the highest at 96 h (Figure 2D) and the *Hsp90* expression level was the highest at 72 h (Figure 3D).

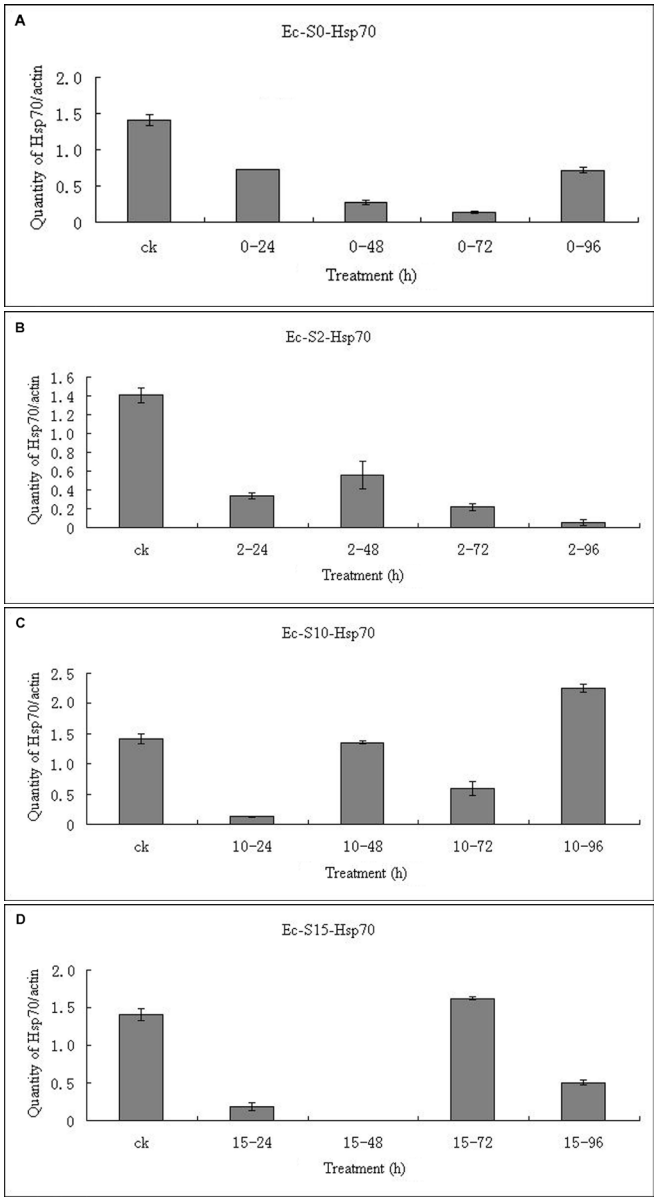

Figure 2. Expression profiles of the *Hsp70* gene at different treatment hours at salinity 0 (**A**), at salinity 2 (**B**), at salinity 10 (**C**), and at salinity 15 (**D**) revealed by qRT-PCR. The amount of *Hsp70* mRNA was normalized to the *actin* mRNA transcript level. Data are reported as means ± SD for three repeated experiments. The y-axis represents the relative ratio of mRNA expression levels of *Hsp70* to *actin*. ck = control.

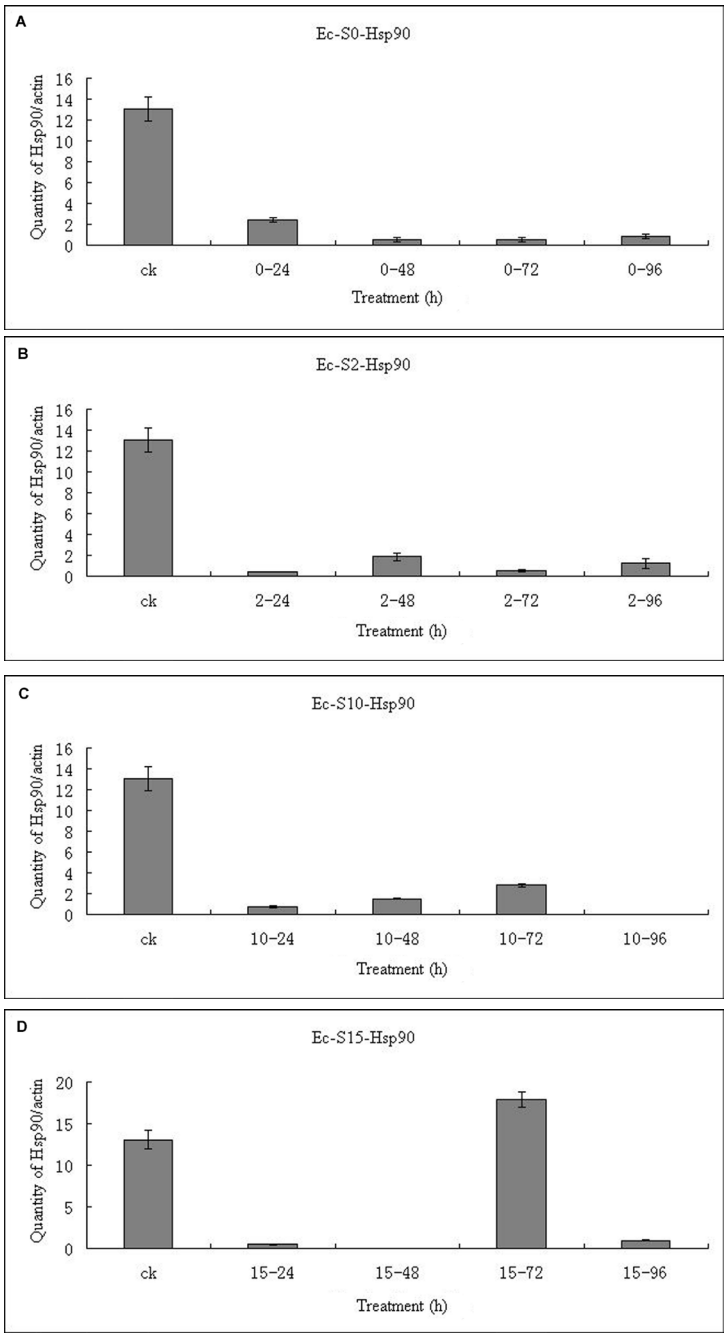

Figure 3. Expression profiles of the *Hsp90* gene at different treatment hours at salinity 0 (**A**), at salinity 2 (**B**), at salinity 10 (**C**), and at salinity 15 (**D**) revealed by qRT-PCR. The amount of *Hsp90* mRNA was normalized to the *actin* mRNA transcript level. Data are reported as means ± SD for three repeated experiments. The y-axis represents the relative ratio of mRNA expression levels of *Hsp90* to *actin*. ck = control.

DISCUSSION

Na⁺-K⁺-ATPase

In previous studies, adult crabs were tested by exposing them to various salinity conditions. While megalopa is the very stage when CMCs migrates from brackish water to limnetic water, there is still little knowledge about osmoregulation during this stage. Therefore, the variation in *Na⁺-K⁺-ATPase* expression level in megalopas of CMCs exposed to salinity stress was examined here.

Many fully aquatic species of euryhaline crabs, including *Callinectes sapidus*, *Carcinus maenas*, *Uca pugnax*, and *Ucides cordatus*, demonstrate enhanced *Na⁺-K⁺-ATPase* activity in gill homogenates from animals acclimated to low-salinities compared to animals acclimated to high salinities as well (Towle et al., 1976; Siebers et al., 1982; Holliday, 1985; Harris and Santos, 1993). The underlying cause may be that under low-salinity conditions, CMC hemolymph maintains an outwardly directed osmotic gradient of about 650 mOsmol/L (Mantel and Farmer, 1983; Péqueux et al., 1988). To maintain the osmotic pressure, the transcript level of *Na⁺-K⁺-ATPase* is raised to actively expel sodium ions from the gill epithelial cells into the hemolymph (Towle, 1997). According to a previous study (Whiteley et al., 2001), hemolymph acid-base changes in CMCs during exposure to low salinity are secondary to ion regulation and cell volume control. Therefore, *Na⁺-K⁺-ATPase* expression level is believed to be related to extracellular acid-base balance, although further investigation is needed to confirm this. Under high-salinity conditions, the high environmental osmotic pressure causes *Na⁺-K⁺-ATPase* expression to decrease. It could be argued that although CMCs has partial osmoregulatory capacity when it is in a high-salinity environment for a long time, the osmotic pressure of hemolymph is still slightly lower than the osmotic pressure of environment, so juvenile CMCs need to release inorganic ions by active transport, causing *Na⁺-K⁺-ATPase* expression level to increase. In addition, this increase fluctuated in each treated group over 72 h, which is consistent with the *Na⁺-K⁺-ATPase* expression level of *Marsupenaeus japonicus* postlarvae whose *Na⁺-K⁺-ATPase* expression level tended to be stable from 48 to 72 h (Pan and Luan, 2005).

Hsp70 and *Hsp90*

Osmotic shock is a physiological stress in which HSP induction plays a significant role. A broad spectrum of aquatic animals have been shown to react to osmotic stress through HSP production (Deane et al., 2002). Our results showed a variation in *Hsp70* and *Hsp90*, demonstrating that there is a relationship between *Hsp70* and *Hsp90* expression level and salinity.

The changes in *Hsp70* and *Hsp90* gene expression in CMCs observed in our experiment during the salinity fluctuation treatment indicate the occurrence of protein turnover. Although the salinity treatments used in this study may not be sufficient to denature proteins that have achieved their native state, it may be enough to affect the folding of nascent protein post-translation. Increased *Hsp70* and *Hsp90* expression level required for folding and refolding of most proteins may correlate with the addition of relatively membrane-impermeable substances (NaCl) (reviewed in Beck et al., 2000). On the contrary, the expression level of *Hsp70* and *Hsp90* decreased with low-salinity treatments.

The results of our study indicate that *Hsp70* and *Hsp90* act as monitors that maintain cellular homeostasis against environmental threats, and are closely related to the duration of stress and salinity.

ACKNOWLEDGMENTS

Research supported by the National Basic Research Program of China (#2010CB429005), the Basic Research Fund for State-Level Nonprofit Research Institutes of ESCFRI, CAFS (#2008M02, #2007Z01 and #2007M22), and the Open Project of the Key Laboratory of Freshwater Ecology and Healthy Aquaculture, Chinese Academy of Fishery Sciences (#2010FEA03009).

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