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Molecular analysis of inducible Heat shock protein 70 of *Pelodiscus sinensis* and its effects during pathogen (*Aeromonas hydrophila*) infection

DangWei *, LuHongliang, GaoYuan, XuNing, QuTingting, LiuYahong

Hangzhou Key Laboratory of Animal Adaptation and Evolution, Hangzhou Normal University, Hangzhou 310036, China

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ABSTRACT

The physiological characteristics and survival of ectotherms are significantly influenced by the environmental temperature. Heat shock proteins (Hsps) are the primary molecular factors that are associated with ectotherm thermal adaption. Heat shock protein 70 (Hsp70) is an important Hsp family member that is involved in many aspects of protein homeostasis and in the immune response. In this study, an inducible Hsp70 homologue (*PsHsp70*) from the soft-shelled turtle *Pelodiscus sinensis* was analysed at the molecular level. The recombinant PsHsp70 was purified from *Escherichia coli* and showed apparent ATPase activity by spectrophotometric assay. The constitutive expression of *PsHsp70* was detected in the muscle, liver, kidney, brain, heart and lung by expression assay. The *PsHsp70* expression pattern slightly differed in hatchlings incubated at 26 °C, 28 °C, and 30 °C. However, bacterial pathogens induced different levels of *PsHsp70* expression in the livers of hatchlings incubated at 26 °C, 28 °C, and 30 °C. Under the harsh temperature of 34 °C, the overexpression of *PsHsp70* in the pIRES2-EGFP plasmid in turtle embryos did not significantly affect the survival rate of either hatchlings or two-month-old turtles; however, the lysozyme activity significantly differed between hatchlings and two-month-old turtles before bacterial challenge, and the lysozyme activity of both hatchlings and two-month-old turtles changed significantly after bacterial challenge. Taken together, these results suggest that PsHsp70 may play a role in coping with environmental and biological stresses and may be involved in the immune response.

1. Introduction

The soft-shelled turtle *Pelodiscus sinensis* is representative of the ectotherms broadly distributed in China and is an important economic aquaculture species. Currently, the survival of these turtles is largely affected by the culture environment, particularly temperature and water quality. Disease outbreaks are the worst outcome during culture. The immune response and survival of turtle hatchlings are affected by the incubation temperature (Freedberg et al., 2008). The body temperature of turtles changes according to the environmental temperature, similar to other ectotherms, resulting in corresponding alterations in the rates of all physiological and biochemical reactions and in the stability of biological molecules (da Silva et al., 2013; Dillon et al., 2009; James, 2013; Ohlberger, 2013; Peck et al., 2014; Poertner et al., 2007). Global warming is a general trend according to data reported by the Intergovernmental Panel on Climate Change (IPCC) in 2013 (IPCC, 2013). In the present study, we focused on the underlying mechanisms of thermal adaptation because environmental temperatures affect the growth, development and reproduction of ectotherms. Previous controlled laboratory-based physiological experiments showed that heat shock proteins are induced and constitute steps towards field interactions between global warming and animal survival (Bergmann et al., 2010; Colson–Proch et al., 2010; Folguera et al., 2011).

Heat shock proteins (Hsps), which were first reported in the fruit fly, are related to temperature fluctuations in bacteria, plants, and animals. Previous studies showed that Hsps not only respond to temperature changes but also reflect other changes in the surrounding environment, such as heavy metal contents, infection, and organic contamination, for survival (Silver and Noble, 2012). Heat shock protein 70 belongs to the Hsp family, the members of which are marvellously well conserved throughout evolution and work as evident stress markers and molecular chaperons that protect cells from harmful conditions. Hsp70s are induced to protect cells under lethal conditions; this induction involves protein translocation across membranes to regulate apoptosis. Structurally, Hsp70s contain an ATP-binding domain, a substrate-binding domain, and a carboxy-terminal domain. The functions of Hsp70s are implemented by fundamental structure-based mechanisms of substrate binding and release. The expression of Hsp70s is regulated by heat shock factors (HSFs) that recognise heat shock elements (HSEs) (Bolhassani and Rafati, 2008).

Hsp70s, which function as biomarkers, respond to the surrounding environment, similar to the other members of the Hsp family. Hsp70s are also related to the immune system in animals. In aquaculture animals, such as fish, high expression of Hsp70 is induced by vaccination or dietary supplementation, which indicated the functional role of

* Corresponding author.
E-mail address: dang.wei@hotmail.com (W. Dang).

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Hsp70 in resistance against pathogenic infections (Mosca et al., 2013; Liu et al., 2013). Recent research showed that Hsp70s were related to innate immunity in humans as a positive alarmin (Horn et al., 2007; Jean–Pierre et al., 2006; Joly et al., 2010; Lee et al., 2013). A correlation between Hsp70 and IL-8 transcriptional expression is observed in fish (Polinski et al., 2013). Hsp70s also participate in antigen processing and presentation partly by increasing the stability and expression of major histocompatibility complex molecules, and bind to TLR4 thus activating the My88 pathway (Nair et al., 2013; Polla et al., 2007; Tsan and Gao, 2009). Thus Hsp70 acts as immune adjuvant in some aquaculture animals, or the conserved domain of Hsp70 is used as protective antigen against pathogen in others (Baruah et al., 2013; Hu et al., 2012a). In cancers, tumour-associated peptides that are chaperoned by Hsp70s can lead to the activation of anti-tumour T-cell immunity. Hsp70 levels may constitute a biomarker for a favourable clinical outcome and have implications for tumour treatment (Cui et al., 2013; Haen et al., 2011) and for a promising cancer vaccine (Binder, 2008).

Constitutive Hsp70s, such as Hsc70 (heat shock cognate protein 70), play important chaperon roles in unstressed cells, and inducible Hsp70s are expressed in detectable levels after acute stressor insults. In this report, we described the cloning and expression of an inducible Hsp70 from soft-shelled turtles, *PsHsp70*. We found that the expression levels of *PsHsp70* changed in different tissues. The expression levels were slightly influenced by incubation temperature and significantly infected by bacterial challenge. When *PsHsp70* was overexpressed in soft-shelled turtle embryos, the hatchlings showed no significant immunity difference compared to sham control hatchlings: however, the serum lysozyme expression changed significantly.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Aeromonas hydrophila TL1, which is a pathogen strain, was isolated from the liver of a diseased soft-shelled turtle at a turtle farm in the Zhejiang Province of China. The TL1 strain was cultured in Luria-Bertani (LB) lysis broth at 28 °C. *Escherichia coli* BL21 (DE3) pLysS was purchased from TransGen (Beijing, China) and cultured in LB broth at 37 °C.

2.2. Eggs

The fertilised eggs of soft-shelled turtle (*P. sinensis*) were purchased from a private hatchery (Hangzhou, Zhejiang, China) and weighed to ±1 mg using an electronic balance (AB135-S; Mettler Toledo, Greifensee, Switzerland). Then, the eggs were randomly allocated into plastic containers (25 × 20 × 10 mm) filled with moist vermiculite with a water potential of −220 kPa (1 g water/1 g vermiculite) (Du and Zheng, 2004); each container held eight eggs. The containers were put into incubators set at different temperatures (Ningbo Life Science and Technology, Ltd., China). The containers were moved among the shelves inside the incubators every week according to a predetermined schedule to minimise any effects of thermal gradients inside the incubators. We examined the eggs twice per week and added an appropriate amount of water to keep the vermiculite moist.

2.3. Sequence analysis

The *PsHsp70* sequences were obtained by PCR from soft-shelled turtle liver cDNA using the primers F1 and R1 (Table 1) based on the sequence (GenBank accession no. JN582024) reported by Li et al. (2011). The sequence was analysed using the Basic Local Alignment Search Tool (BLAST) programme at the National Centre for Biotechnology Information (NCBI) and the Expert Protein Analysis System. A domain search was performed using the Simple Modular Architecture Research Tool (SMART) version 4.0 and the conserved domain search programme of NCBI. The molecular mass and theoretical isoelectric point were predicted using DNAMAN software.

Table 1
Primers used for PCR and qRT-PCR.

Primers	Sequences (5′-3′)[a]
PsHsp70 F1	CATATGATATCGCCACCATGTCCGGCAAAGCGCCT
PsHsp70 R1	CTCGAGATATCGTCGACTTCTTCAATGGTTGGT
PsHsp70 RTF1	GCTTGACAAATGCCAGGAGGT
PsHsp70 RTR1	TGTGACGATAGGGTTGCAGAGT
β-actin RTF1	TGTTACCCATACTGTGCCCATC
β-actin RTR1	TAGCCATCTCCTGTTCAAAATCC
IRG F1	CTACTTGGCAGTACATCTACGT
IRG R1	GACGGCAATATGGTGGAAAAT

[a] Underlined nucleotides are the restriction sites of the enzymes.

2.4. Plasmid construction

The coding region of *PsHsp70*, which was amplified using PCR primers F1 and R1, was ligated into the T-A cloning vector named *pEASY*-T1 Simple Cloning Vector (TransGen, Beijing, China). The recombinant plasmids were digested with EcoRV to retrieve the 1.9 kb fragment. The fragment was inserted into pEt259 (Dang et al., 2010; Hu et al., 2012b) at the ScaI site to construct pEtPsHsp70, which can express a His-tagged PsHsp70 in *E. coli* BL21 (DE3) pLysS. The fragment was also inserted into pIRES2-EGFP (Clontech, Mountain View, CA, USA) at the SmaI site to construct pIRESPsHsp70, which can express an EGFP-tagged PsHsp70 in the soft-shelled turtle and its embryos.

2.5. Protein purification

E. coli BL21 (DE3) pLysS was transformed with pEtPsHsp70. The transformants were cultured in LB medium at 37 °C to OD_{600} = 0.5, and the expression of the exogenous protein was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. After growing at 20 °C for an additional 11 h, the cells were harvested by centrifugation, and the His-tagged protein was purified under native conditions using nickel-nitrilotriacetic acid columns (GE Healthcare, Piscataway, NJ, USA) as recommended by the manufacturer. The purified protein was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualised after staining with Coomassie Brilliant Blue R-250. Then, the protein was concentrated using an Amicon Ultra Centrifugal Filter Device (Millipore, Billerica, MA, USA) and stored at −20 °C with 50% highly pure glycerin for protection. The concentration of the protein was determined using the Bradford method. Briefly, Bovine serum albumin (Sangon, Shanghai, China) was used as standard. Bradford reagent was added to the standard solutions and rPsHsp70. The absorbance of standard solutions and rPsHsp70 was recorded at 595 nm after 10 min incubation at room temperature. A standard curve was prepared using the standard solutions absorbance and the concentrations of rPsHsp70 was estimated (Bradford, 1976).

2.6. ATPase assay

The ATPase activity of recombinant PsHsp70 was determined as described previously (Dang et al., 2010). Briefly, 0.7 μg rPsHsp70 was added to Buffer I (10 mM HEPES, 10 mM $MgCl_2$, 20 mM KCl, 0.5 mM dithiothreitol, and 1 mM ATP) at 37 °C for 10 min. The reaction was stopped by adding trichloroacetic acid to 15%. An equal volume of Buffer II (1% ammonium molybdate, 6% ascorbic acid, 2% sodium citrate, and 2% acetic acid) was added to the assay mixture, followed by incubation at 45 °C for 25 min. The release of inorganic phosphate was quantified by measuring the absorbance at 660 nm. ATPase activity was presented