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Original article

Peranan protein kejut panas HSP70 dan HSP90 di dalam osmoregulasi dan metabolisme jaringan pada ikan bandeng (*Chanos chanos*) di bawah tekanan osmotik

The roles of heat shock protein 70 (HSP70) and heat shock protein 90 (HSP90) in osmoregulatory and metabolic tissues of milkfish (Chanos chanos) *under osmotic stress*

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Abstract

As an euryhaline fish, milkfish was able to adapt to a wide range of environmental salinity. However, to improve the survival and health of the stressed milkfish, the roles of HSPs are needed to prevent some damages caused by salinity effects. Therefore, maintenance of osmotic homeostasis is very important for milkfish. The present study investigated the HSPs expression induced by hypotonic stress in gills, kidney (osmoregulatory organ) and liver (metabolic organ) of euryhaline milkfish. Since heat shock response is a predominant cellular stress response, two of its major components, heat shock protein 70 (HSP70) and 90 (HSP90) were examined in this study. Two major hsp genes were first identified by cloned from the transcriptome database. In addition, the protein expression were examined through seawater (SW; 35‰) and freshwater (FW) acclimation experiments (> 2 weeks). At the osmoregulatory organ (gill and kidney), the expression of gill HSP90 and 70 revealed upregulation in the FW group. While, kidney HSP90 and 70 was showed no significant different between FW and SW. On the other hand, the expression of HSP90 and 70 based on relative intensities of immunoreactive bands at liver organ was similar between the FW and SW. These findings demonstrated that compared to SW environment, the FW was more stressful to milkfish osmoregulation and effected significantly to osmoregulatory organ.

Keywords ***:*** *heat shock protein, milkfish, osmotic stress*

Introduction

Osmotic stress is one of the principal challenges that can fluctuate greatly in aquatic environments. When osmotic stresses occur, protein synthesis, stabilization, and biological functions become disrupted, eventually causing protein damage harmful to cellular functions and viability (Deane and Woo, 2004; Choe and Strange, 2008).This adaptation requires an adjustment of the wholebranchial epithelium, of which the three main cellular types are the pavement, chloride (mitochondria-rich) and mucous cells (Choe and Strange, 2008; Choi and An, 2008). Hence, upon salinity challenge, to repair such damage or eliminate damaged components, organisms have evolved the cellular stress responses including the induction of a highly conserved set of cytoprotective proteins called stress proteins or heat shock proteins (HSPs) (Schlesinger et al., 1982).

HSPs are highly conserved cellular proteins observed in all organisms including fish (Iwama et al., 1998). Extensive studies on HSPs reported that HSPsserve to protect the organism against cellular damage when exposure of organisms to such diverse stressors (Fishelson et al., 2001; Tine et al., 2010). The primary physiological functions for all of the HSPsare to fulfill chaperoning activities, although each HSPs family plays its own specific roles (Padmini, 2010). The family of HSP70 was known to assist the folding and degradation of altered or denatured proteins. On the other hand, HSP90 family played a specific role in protein folding, degredation, signal transduction (Wu et al., 2012) cell cycle regulation, stress-induced cytoprotection, with long-term cellular adaptation and more specifically responsible for germ cell maturation (Sreedhar and Csermely, 2004; Iwama et al., 2004).

Fish, like the other vertebrates, respond to stressors by eliciting a generalized physiological stress response, which is characterized by an increase in stress hormones and consequent changes helping maintain the normal or homeostatic state of the animals (Barton, 2002). Euryhaline fish are able to adapt to a wide range of salinities, which can live in the fresh water, brackish water, and seawater. Therefore, euryhaline fishes are mostly common to be used in the experiments to investigate their physiological functions as an indicator of ambient salinity tolerance. The milkfish (*Chanoschanos*) is an important aquaculture species with great euryhalinity leading to be a good model for studies on the molecular mechanisms of osmotic stress resistance. In the Southeast Asia, milkfish was very popular as an aquaculture species reared in fresh, brackish and seawater. Moreover, this species could survive in environments with salinity ranging from FW to 70% SW (Bagarinao, 1994). Thus, milkfish became a well-suited subject for studying the mechanisms of salinity adaptation (Ferraris et al., 1988).

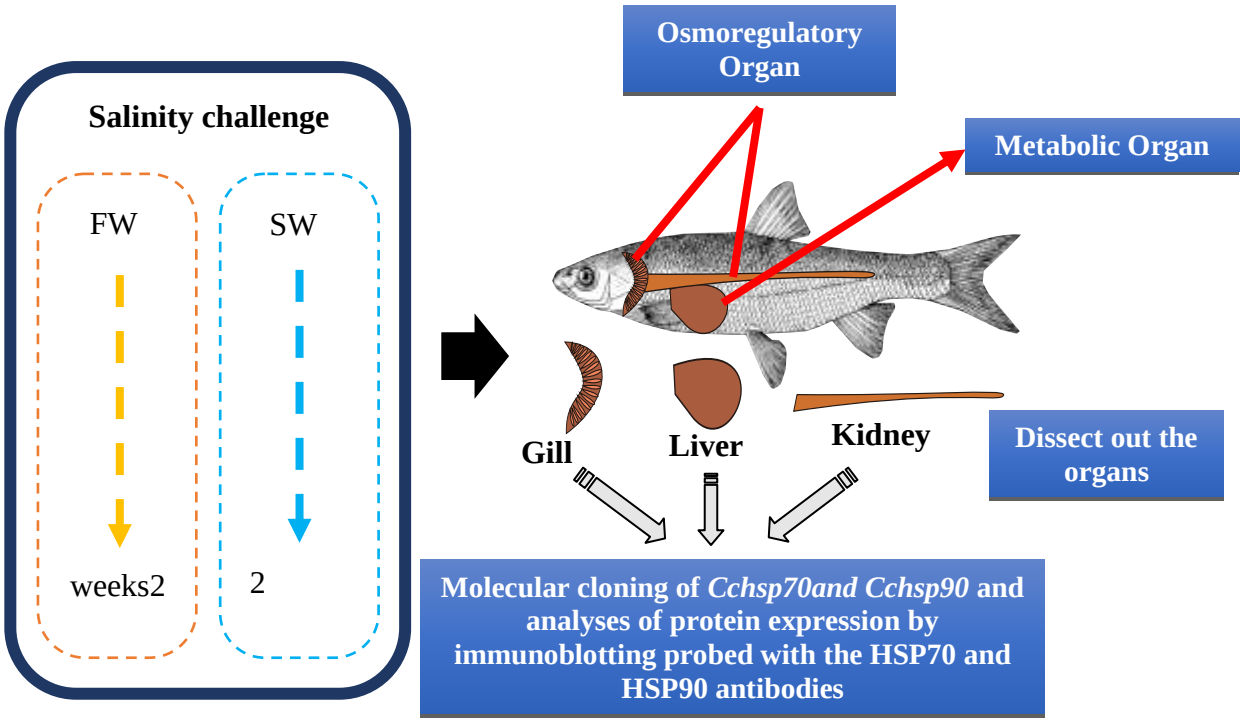
Since alterations of HSPs expression were supposed to be a crucial mechanism of euryhalineteleosts to adapt to environments of different salinities, it was intriguing to investigate the role of heat shock proteins in milkfish osmoregulatory and metabolic organ, to compared their respons upon salinity stress. In order to reveal the HSPs roles, we addressed to (1) identify *hsp*s genes in milkfish; (2) investigate the expression of milkfish *hsps* through translational levels in SW/FW acclimation experiments upon salinity challenge.

Material and Methods

Experimental fish and environments

Juvenile milkfish (*C. chanos*) with 31.0±11.8 g body weight and 12.5±1.3 cm standard length were obtained from a local fish farm. Experimental environments of different salinities were made fromdechlorinated local tap water (fresh water, FW) and seawater (SW; 35‰) with proper amounts of RealOcean synthetic sea salt (Camarillo, CA, USA). The milkfish were maintained for one month in 15‰ brackish water (BW) at 27±1°C as the holding period, then moved to either FW or SW with a daily photoperiod cycle of 12 hrs light: 12 hrs dark. The water was continuously circulated through fabric-floss filters and partially refreshed every 3 days. Fish were fed a daily diet of commercial pellets *ad libitum*. For the acclimation experiments, the milkfish were kept in SW or FW for over than two weeks and the target organ; gill, kidney and liver were sampled and frozen at -80°C for subsequent analyses.

Molecular cloning of *hsps*



Gene cloning was perform by polymerase chain reaction (PCR) amplification, 3 µl of cDNA was used as a template in 25 µl reaction containing 2.5 µl 10X Ex Taq Buffer (Takara, Shiga, Japan), 2 µl dNTP mixture, 0.25 µl EX-Taq polymerase (Takara) and 0.6µM of each primer (forward and reverse primers). The primers for PCR amplification were designed to identify specific regions of HSP according to the cDNA library in the milkfish transcriptome database (http://140.120.209.83/CCD/index/C.chanos_home.htm). Primers designs were supported using primer online tool primer3plus (http://primer3plus.com) with primers list that used in this study was shown in Table 1. PCR products were sent to the company for sequencing. BLAST analysis was used to ensure the sequence are belonging to the gene target. BLAST resources available from the national center for biotechnology information (NCBI; http://ncbi.nlm.nih.gov).

Antibodies

The primary antibodies used in the present study included: (1) anti-HSP70, a mouse monoclonal antibody (H5147; Sigma, St. Louis, MO, USA) generated by immunization with purifiedbovine brain HSP70, diluted 1:10000 in immunoblotting and 1:2000 in co-immunoprecipitation (Co-IP); (2) anti-HSP90, a rabbit polyclonal antibody (#4874; Cell Signaling Technology, Beverly, MA, USA) corresponding to human HSP90, diluted 1:4000 in immunoblotting and 1:2000 in Co-IP; (3) anti-β-actin, a mouse monoclonal antibody (JC1629852; Millipore, Bedford, MA, USA) diluted 1:5000 in immunoblotting.

Gill homogenates

The first pairs of gills from the milkfish were excised and directly kept into liquid nitrogen. The tissues were rapidly homogenized in 500 µl SEID buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, 0.1% sodium deoxycholate, pH 7.5) and protease inhibitor (Roche; v/v: 25/1). Homogenization was performed in 2 mL tube for homogenizer with a motorized POLYTRON PT1200E (kinematica) at maximal speed for 10 s per each tube. The homogenates were then centrifuged at 5500 ×*g*, 4°C for 15 min to remove pellet with consisted of unbroken tissue, cell nuclei, large debris and mitochondria (Chu, unpublished data). The supernatants were used for determination of protein concentrations. The reagents of BCA protein assay (Piece, Herclus, CA, USA) were measured the protein concentrations using bovine

serum albumin (BSA) as the standard.

Immunoblotting

The procedures of immunoblottingwere performed as described byLee et al., (2000) with some modification. Briefly, the sample mixture of protein lysate and sample buffer (v/v: 1/5) were heated to denature protein at 95 °C for 5 min and then fractionated by electrophoresis on sodium dodecyl sulfate (SDS)-containing 7.5% polyacrylamide gels (15 µg of protein lane) on a mini protein II electrophoresis cell (Bio-Rad). Pre-stained protein molecular weight standard marker was purchased from Thermo scientific (#26616). Separated proteins were transferred from unstained gels to PVDF (Millipore) using a transfer system (Bio-Rad Mini Protean 3) by electroblotting at 100V for 70 min. Blots were pre-incubated for 2 hrs in PBST buffer (137 mMNaCl, 3 mMKCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.2% (v/v) Tween 20, pH 7.4) containing 5% (w/v) non-fat dried milk to minimize non-specific binding, then incubated overnight at 4°C with primary antibody diluted in 1% BSA and 0.05 sodium azide in PBST. The blot washed in PBST, incubated at room temperature for 1 hrwith diluted secondary antibody (1:20000). The bound immunoproteins were detected using enhanced chemiluminescent assay (ECL; Millipore). The luminescent signals were assessed using a cooling-CCD (charge-couple device) camera (ChemiDoc XRS+; Bio-Rad) and associated software (Quantity One version 4.6.8; Bio-Rad). The results were converted to numerical values in order to compare the relative protein abundance of the immunoreactive bands by using Image Lab software version 3.0 (Bio-Rad).

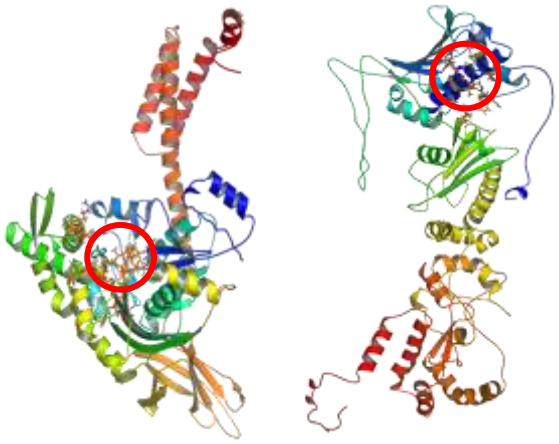


Fig 1.Architectures of deduced HSPs of the milkfish. Molecular models were CcHSP70 (A) and CcHSP90 (B). Different colors indicated the regions of the proteins: blue - N- terminal; green to orange - transmembrane regions; red - C-terminal,. The red circle indicated the ATP-binding site. The structures were constructed according to the nucleotide sequences of*PDB ID* from others species.

Data analysis

The data were analyzed via T test ANOVA statistics of SPSS 20.0 (SPSS, Chicago, IL, USA). Analyze the significance difference between study and control groups of experiments. The number of sample were N=6 with Pvalue<0.05 was set as the significance level and values were expressed as mean ± SEM (standard error of the mean).

Results and discussions

In fish, HSP70 isoforms have been sequenced in different species including zebrafish(*hsc70*: Graser et al.,1996), tilapia (*hsp70*: Molina et al., 2000), medaka (*hsp70* and *hsc70*: Arai et al., 1995), and rainbow trout (*hsp70* partial sequence: Zafarullah et al., 1992). However, only a relatively small number of genes of *hsp70* family has been clear characterized (Basu et al., 2002). This study has identified the two members of *hsp*family, the *hsp70* and *hsp90* in milkfish. The amino acid homology between mikfish*hsp70* and *hsp90* was over than 70% which was in the expected range since both genes belonged to the same family. After sequence comparisons using the Basic Local Alignment Search Tool Program (Altschul et al., 1990), it was found that mikfish*hsp70* shared 77-83% amino acid homology with the *hsp70* of many other species of animals, while milkfish *hsp90* shared about 91-97% amino acid homology with the *hsp90* of other animals.

Previous studies revealed that in the sequences of *mfhsp70*, the three signature sites which characterized all *hsp70* family members were present and showed the same sites as the other teleosts, because multiple sequence alignment suggested that they were highly conserved, especially in the regions of three classical *hsp70* family signatures (Deane and Woo, 2005).

The prediction models of *hsps* was built according to the template *E.coli*and *S. cerevisae* for the hsp70 and 90 family, respectively, showed the higher similarity of templates with milkfish hsps (Fig 1A and 1B). The construction models demonstrated similar structure in the HSP family. It is reasonable since the presence of specific amino acid domains as well as overall nucleotide/amino acid structure homology in fish *hsp*

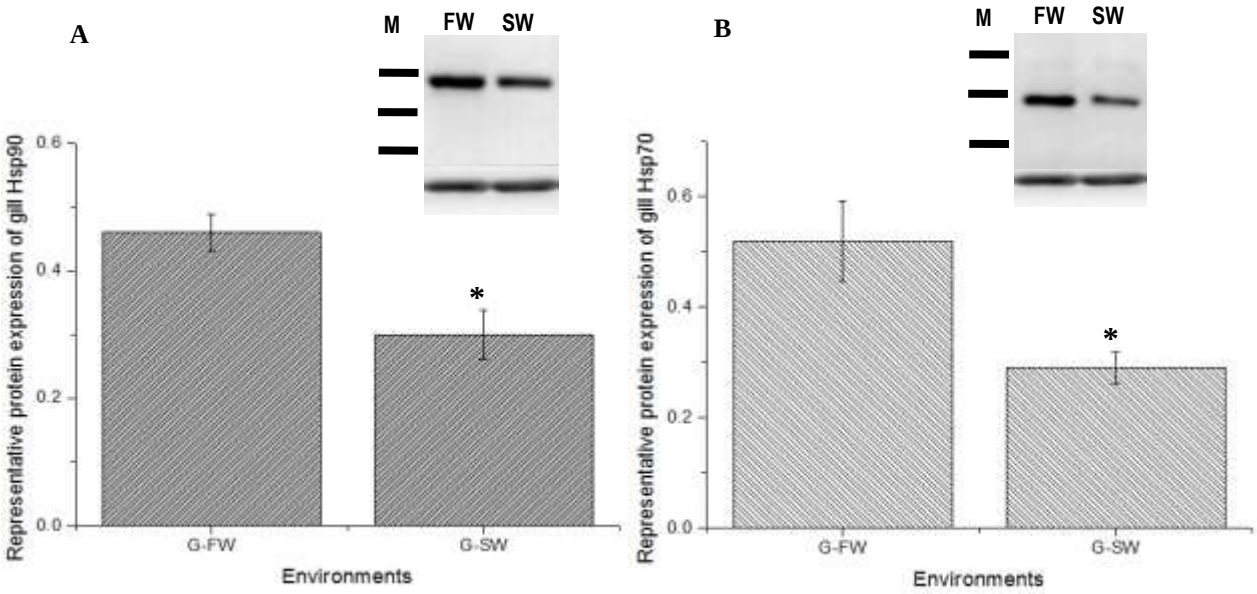


Fig 2.Comparisons in protein abundance of branchial HSP90 (A) and HSP70 (B) between FW and SW milkfish. The representative immunoblots showed the immunoreactive bands at 90 and 70 kDa, respectively. Relative intensities of immunoreactive bands of HSP90 and 70 revealed upregulation in the FW group. N=6 for SW and FW groups. The asterisk indicated a significant difference ($P < 0.05$, by Student's t-test). Values are means ± S.E.M.

genes (Deane and Woo, 2010). Taken together, based on gene length, amino acid sequence and compared to the others species of teleosts, as well as the homology of structure, allowed us to made a classification genes between these two genes, *mfhsp70* and *mfhsp90.*

In the natural environments, salinity is one of the major abiotic factors thatstrongly influence cellular functionsand homeostasis in organisms (Evan et al., 2009).According to

our findings, HSPs in milkfish were separated into *HSP70* and *HSP90.* Accliamation of milkfish to FW and SW for two weeks increased the branchial protein abundance of *HSP70* and *HSP90* significantly, while slightly but not significantly increase of renal *HSP70* and *HSP90* expressionwere found, in contrast compared to result as shown as branchial (Fig.2 and Fig.3). Previous studies has demonstrated that higher or lower salinity disrupted the mechanisms of protein synthesis and reduced the rate of protein synthesis *in vivo* (Smith et al., 1999). The induction of mRNA abundance of *hsp*s might have a role as the molecular chaperones. Compared with the SW group, the higher expression of *hsp*s in the FW group obviously indicated HSPs response effectively in gills of the milkfish.

The HSP70is considered to be one the most important biomarkers because of its rapid response to stressors. It is well known that gill is osmoregulatory organ that located externally. The first organ which is coped directly to the environment exposure (Deane and Woo, 2010). However, kidney located appears to be more inside of fish body, that response lately compared to the gill (Kelly and Woo, 1999). Kang et al. (2015) revealed that compared to hyper-salinity, hypo-salinity was more stressful for euryhaline milkfish by the significant expression of HSP70. The response of HSP70 was to protect the cell and maintain native functions in milkfish gills because HSP70 is a chaperone with the capacity to refold proteins and bind to abnormal proteins (Kang et al., 2015). Deane and Woo (2004) examined the expression of HSP70 in the silver seabream for one month demonstrated that HSP70 was found to be highly upregulated in gills of seawater anhypersaline adapted fish. Similar to our findings, the protein abundance of HSP70 was significantly increased in the FW-acclimated group, but only slightly increased in HSP90. Yang et al. (2009) reported that HSP90 was increased significantly in long-term salinity challenge to the sailfin molly. It indicated the HSP90 expression in SW-acclimated sailfin molly might play important roles for reaching homeostasis upon salinity challenge.

Increased expression of certain proteins suggests the presence of stress indicators in the fish attempting to adjust to the homeostatic situation (Iwama et al., 2004). Therefore, these findings demonstrated the upregulation of HSPs in the milkfish during hypotonic stress could be indicative of a condition of both reduced stress and enhanced osmoprotective response for the fish. Gills have to contend with environmental perturbations (Deane and Woo, 2004, 2005). The external position and the functioning of gills in fish may explain why this tissue displayed such a high induction HSP expression even in long-term exposure as a consequence of the regular passage of ambient water.

Furthermore, during acclimation in hepatic expression, the HSP70 and HSP90 were showed similar pattern between SW and FW group (Fig. 4). Stress responses are energy demanding processes and animals must regulate energy substrates to metabolically resist the stresses by metabolic organ. Changes in plasma glucose concentrations have commonly been used as a stress bioindicator at the organismal level (Iwama et al., 2006). Plasma glucose could be used as an osmolyte for plasma osmolality or as an important energy source for metabolism to resist the stress (Kelly et al., 1999). Lin et al. (2006) revealed that decrease in plasma osmolality in milkfish stabilized in 3hrs after hyposmotic shock. Thus, sustained lower levels of plasma ions to a FW level after a rapid drop following FW transfer indicated an early and

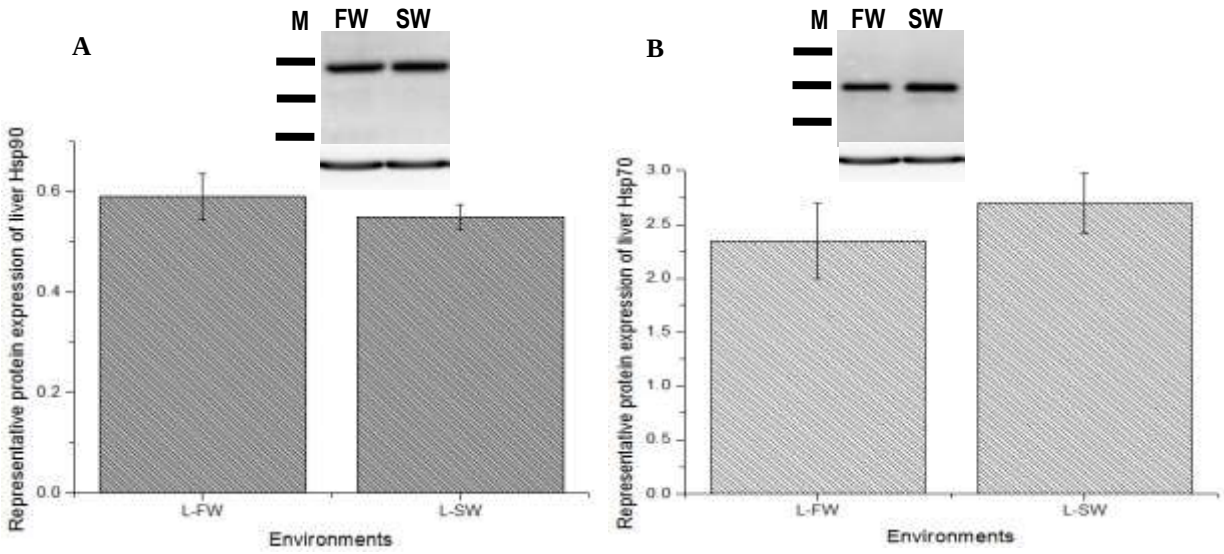


Fig 5.Comparisons in protein abundance of hepatic HSP90 (A) and HSP70 (B) between FW and SW milkfish. The representative immunoblots showed the immunoreactive bands at 90 and 70 kDa, respectively. Relative intensities of immunoreactive bands of HSP90 and 70 were similar. N=6 for SW and FW groups. ($P < 0.05$, by Student's t-test). Values are means ± S.E.M.

efficient modification of regulatory mechanisms of milkfish. This evidence supported the result by HSPs expression to hepatic organ might be as later as response since the acclimated in long-term exposure, thus the metabolic organs has returned to normal condition after two week experiment. Fish also increased the HSPs expression substantially (Basu et al., 2002),However, the cellular stress response can vary according to tissues and HSP families.

The present study showed that theHSP70 and HSP 90 family, both genes were expressed with hypotonic stress, while different pattern was showed in the different organ.These results of these studies indicated that reduced salinity, induced osmotic stress, or stressful environments interfered cellular protein stabilization in the organisms, while activation of HSP expression is essential for cytoprotection (Getting and Sambrook,1992; Tang et al 2015).

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