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Research Article

HSP70 Gene Expression Analysis in *Dunaliella salina* Under Salt Stress

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ABSTRACT
Dunaliella salina a photosynthetic halotolerant microalgal strain was isolated during a screening program from Maharlu Salt Lake, Shiraz, Iran. After molecular identification and characterization, the influences of different salinity levels (0%, 5%, 12%, 20% and 30% NaCl) and different periods of salinity exposure (0, 1, 3, 6, 12, 18, 24, 48 h) on the expression of *hsp70* gene in *D. salina* was investigated. The highest expression levels of *hsp70* was seen under 12% NaCl concentration. Besides, When *D. salina* was stressed by NaCl, upregulation of *hsp70* specific mRNA occurred within 1 h and remained increased during a culture of up to 6 h, as compared with normal cells.

Keywords: *hsp70*, gene expression analysis, β-carotene, *Dunaliella salina*, salt stress.

INTRODUCTION

Dunaliella salina is a unicellular flagellate eukaryotic green alga with no cell walls[1]. *Dunaliella* have been used as a model system to study the molecular mechanisms of halotolerance in eukaryotes for more than 100 years[2]. *Dunaliella* is the most resistant halotolerant eukaryotic cell known, and can survive in media with wide range of chemical compositions and salt concentrations ranging from 0.05 M to 5.5 M saturation[3]. The significant intracellular components of *Dunaliella* are carotenoids, glycerol, protein and vitamins with antioxidant properties[4-6]. *D. salina* is one of the world's richest natural sources of β-carotene and glycerol, so it is a good choice for microbial cell factory applications and biofuel production[3,7].

While *D. salina* can proliferate in hypersaline medium, the intracellular concentrations of Na^+ and K^+ are maintained at a low level of 100–200 mM, means that this is a halotolerant but not halophilic algae[3]. The isolation of *Dunaliella* from hypersaline lakes like Maharlu Salt Lake in Shiraz, Iran, has been reported several times[7-11]. Cells have different strategies to deal with the environmental changes[12]. They 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)[13]. Some of these environmental stresses like low temperature, osmotic, salinity, oxidative, desiccation, and heavy metals are stressful for cells and these stresses were found to induce the synthesis of HSPs[14]. Their classification as stress or heat-shock proteins may be misunderstanding because these components are present in considerable amounts and fulfill essential functions under non-stressful conditions[15]. HSPs have multiple roles, including membrane translocation, protein degradation, protein folding, and repair misfolded proteins, in regulation of protein homeostasis in normal and stressed cells[16]. HSPs, in particular the 70 kDa (*hsp70*) family, first achieved fame as the genes whose expression are caused by heat and other stresses[17]. Later discoveries revealed their roles as molecular chaperones[15]. All known stresses, if sufficiently intense, induce HSPs expression. Some studies have reported the reversible increase in *hsp70* expression in response to salinity stress[18-20]. The primary objective of this research is to investigate the gene expression profile of *hsp70* in *D. salina* exposed as one of the osmotic response process to different salinities (5%, 12%, 20% and 30%).

MATERIALS AND METHODS

Strain and culture media

The green algae *D. salina* was isolated from Maharlu Salt Lake in south of Shiraz, Fars province. Identification and

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Table 1: Sequences of primers used for real-time PCR amplification and the resulting product size

Primer	Ta	Amplicon length (b)	Sequence
hsp70 F	*57.5*	137	CTGCTGTTGCTGGATGTCAC
hsp70 R	57.5	137	GGCTGGTTGTCGCTGTAGG
18S rRNA F	56.8	148	AGTGTTGGGCAAGTGGAC
18S rRNA R	56.8	148	TAGAAATAGCGAGCGATAAGC

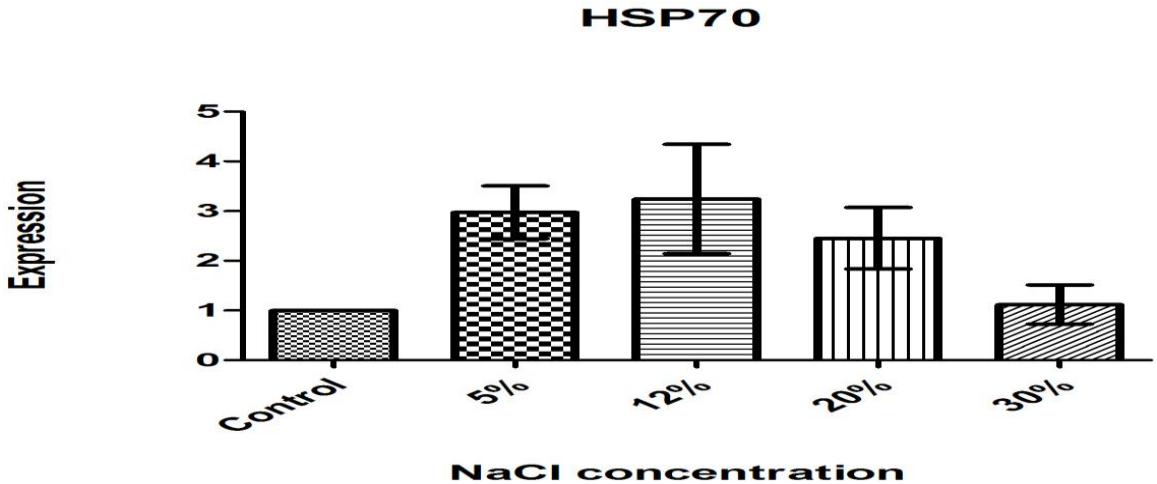

Figure 1: *hsp70* gene expression profile under different concentration of NaCl (control, 5%, 12%, 20% and 30% NaCl). SE indicated as vertical error bar for all the columns.

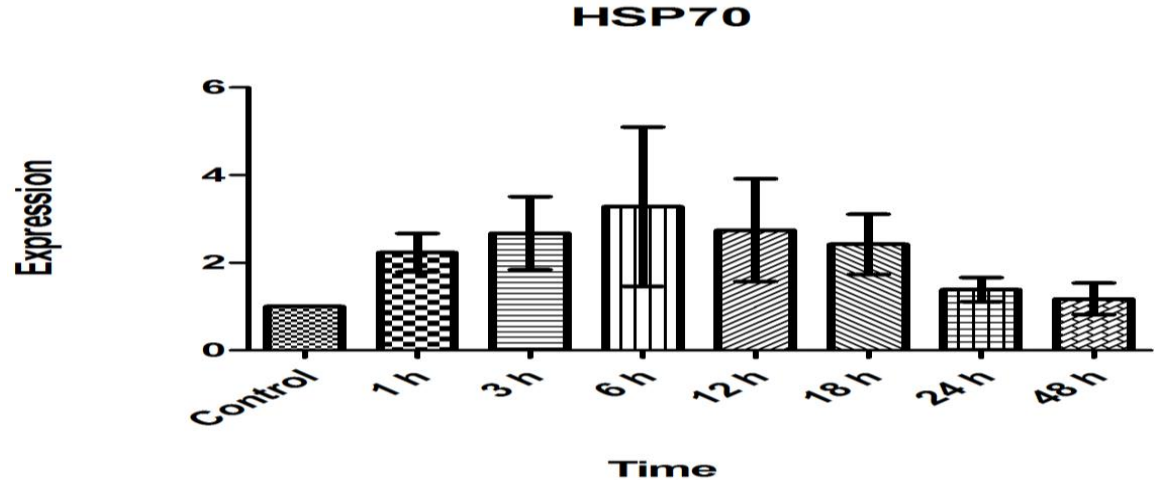

Figure 2: *hsp70* gene expression profile under different times after NaCl treatment (control (0), 1, 3, 6, 12, 18, 24 and 48 h). SE indicated as vertical error bar for all the columns.

characterization of the isolated strain was exploited as described before[21,22]. Cells of *D. salina* were cultured in a modified Johnson medium[23]. A laboratory experiment was conducted to investigate the effects of different salinity levels (0%, 5%, 12%, 20% and 30% NaCl) and different periods of salinity exposure (0, 1, 3, 6, 12, 18, 24, 48 h) on the expression of *hsp70* gene in *D. salina*. Cells were harvested by centrifugation and total RNA was isolated using RNX-Plus buffer (CINNAGEN, Iran) according to the manufacturer's protocol. The purified total RNA was quantified by Nano-Drop ND 1000 spectrophotometer (Wilmington, USA). To eliminate genomic DNA, the RNA preparations were treated with Fermentas DNase Kit (Fermentas, Hanover, MD) following the manufacturer's instructions. First-strand cDNA was synthesized from the total RNA by M-MuLV reverse transcriptase (Fermentas, Hanover, MD), oligo dT primers and random hexamer in a 20 µL final volume[24]. Primer design was carried out using Allele ID 7 software for *18S rRNA* (GQ337903) as an internal control gene and *hsp70* gene (AY078499) (Table 1). Relative real-time PCR was performed in a 20 µL volume containing 1 µL cDNA, 10 µL SYBR® Premix Ex Taq ™ II (TaKaRa, Japan) and 4 pmol of each primer. The amplification reactions were carried out in a lineGeneK thermal cycler (Bioer, China). The cycling program included 2 min at 94 °C for initial denaturing and followed by 40 cycles of 94 °C for 10 s alternating with annealing temperature (Ta) of each of the primer pairs for 15 and 30 s of extension at 72 °C. Two negative controls were added: one without reverse transcriptase, and one without cDNA template. After 40 cycles, the specificity of the amplifications was checked based on melting curves

resulted by heating the amplicons from 60 to 95 ^{o}C[24]. Data were expressed relative to the expression of the housekeeping gene *18S rRNA*. For quantitative real-time PCR data, relative expression for *hsp70* was calculated based on the threshold cycle (CT) method. The fold change of target mRNAs over the reference values were calculated by the equation $2^{-\Delta\Delta CT}$[25]. Statistical analysis was carried out using GraphPad Prism (CA, USA).

RESULTS AND DISCUSSION

As the success of the qRT-PCR experiment depends on the choice of PCR primers, in this experiment we designed primers using Allele ID 7 software with great sensitivity. Besides *hsp70* gene, the *18S rRNA* gene expression was calculated as the internal control (whose expression not influenced by salt stress) for data normalization. To date, several research has been conducted to demonstrate the effect of *hsp70* gene expression on salinity tolerance. In this experiment in order to aiming at identification of the mechanism of salinity tolerance, quantitative real-time PCR was employed to investigate the expression pattern of *hsp70* gene under different concentrations of NaCl. Figure 1 shows the quantitative expression profile of *hsp70* under different concentrations of NaCl (control, 5%, 12%, 20% and 30% NaCl). Kruskal-Wallis analysis showed significant impact of salinity on *hsp70* relative expression (*p*-value: 0.0204) but no significant differences was seen between groups using Dunn's Multiple Comparison Test ($p \leq 0.05$). The highest expression of *hsp70* was seen under 12% NaCl concentration, while control and 30% NaCl concentration showed lower levels of gene expression. In fact, a gradual decrease in gene expression was seen from 12% to 30% NaCl concentration. On the other hand, When *D. salina* was stressed by NaCl, upregulation of *hsp70* specific mRNA occurred within 1 h and remained increased during a culture of up to 6 h, as compared with cells not treated with NaCl, then slowly decrease up to 48 h (Figure 2). Kruskal-Wallis analysis for the investigation of differences in the *hsp70* gene expression under different times after NaCl treatment (control (0), 1, 3, 6, 12, 18, 24 and 48 h) showed no significant differences ($p \leq 0.05$). The highest level of *hsp70* gene expression was occurred at 12% NaCl concentration. It can be concluded that HSP70 protein is involved in the adaptation of *D. salina* to low salinity exposure and in the higher levels of salinity, other mechanisms of tolerance, like the regulation of intracellular concentrations of glycerol and expression of other genes for resistance to salinity, should be activated. In other hand the best growth rate of *Dunaliella* cell accrued in a 12% NaCl concentration that shows in this concentration cell function be better than other concentration. So cell hove to express *hsp70* gene for protein folding in addition of salinity stress. On the other hand, the highest level of *hsp70* gene expression was occurred 6 h after salinity treatment, which may indicate that *hsp70* is one of the early salt stress response genes. As more time passes from the NaCl treatment the HSP70 gene expression gradually decreases because of the activation of other mechanisms of tolerance or because of the stability of the produced protein. *Dunaliella* osmotic responses include three stages, which they divided according with the time. The first is immediate response, the second one is short-term and the last one is a long-term response, which long-term response include change and induce gene expression and also accumulation of some salt-induced proteins[26,27].

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REFERENCES

1. Borowitzka MA. *Dunaliella*: Biology, production, and markets. In: Richmond A, Hu Q, eds. Handbook of microalgal culture: Applied phycology and biotechnology. Second ed. Oxford, UK: John Wiley and Sons; 2013:359-368.
2. Oren A. A hundred years of *Dunaliella* research: 1905–2005. Saline Systems. 2005;1:2-2.
3. Chen XJ, Wu MJ, Jiang Y, Yang Y, Yan YB. *Dunaliella salina* Hsp90 is halotolerant. International Journal of Biological Macromolecules. 2015;75:418-425.
4. Gibbs N, Duffus CM. Natural protoplast *Dunaliella* as a source of protein. Applied and Environmental Microbiology. 1976;31(4):602-604.
5. Farouk K. El Baz , Ahmed M. Aboul-Enein, Gamal S. El-Baroty, M. YA, Abdel-Baky HH. Accumulation of antioxidant vitamins in *Dunaliella salina*. Journal of Biological Sciences. 2002;2(4):220-223.
6. Ghasemi Y, Rasoul-Amini S, Morowvat MH. Algae for the production of SCP. In: Liong MT, ed. Bioprocess Sciences and Technology: Nova Science Publishers, Inc.; 2011:163-184.
7. Rasoul-Amini S, Mousavi P, Montazeri-Najafabady N, et al. Biodiesel properties of native strain of *Dunaliella salina*. International Journal of Renewable Energy Research (IJRER). 2014;4(1):39-41.
8. Nikookar K, Moradshahi A, Kharati M. Influence of salinity on the growth, pigmentation and ascorbate peroxidase activity of *Dunaliella salina* isolated from Maharlu Salt Lake in Shiraz. Iranian Journal of Science And Technology Transaction A- Science. 2004;28(A1):117-125.
9. Marín N, Morales F, Lodeiros C, Tamigneaux E. Effect of nitrate concentration on growth and pigment synthesis of *Dunaliella salina* cultivated under low illumination and preadapted to different salinities. Journal of Applied Phycology. 1998;10(4):405-411.
10. Hosseinzadeh Gharajeh N, Hejazi M, Nazeri S, Barzegari A. Characterization of an indigenous isolate, *Dunaliella tertiolecta* ABRIINW-G3, from Gavkhooni Salt Marsh in Iran based on molecular and some morpho-physiological attributes. Journal of Agricultural Science and Technology. 2012;14:1579-1590.
11. Fazeli M, Tofighi H, Samadi N, Jamalifar H. Effects of salinity on β-carotene production by *Dunaliella tertiolecta* DCCBC26 isolated from the Urmia Salt

Lake, north of Iran. Bioresource Technology. 2006;97(18):2453-2456.
12. Welch WJ. Mammalian stress response: cell physiology, structure/function of stress proteins, and implications for medicine and disease. Physiological Reviews. 1992;72(4):1063-1081.
13. Sun M, Jiang K, Zhang F, et al. Effects of various salinities on Na(+)-K(+)-ATPase, Hsp70 and Hsp90 expression profiles in juvenile mitten crabs, *Eriocheir sinensis*. Genetics and Molecular Research : GMR. 2012;11(2):978-986.
14. Saito M, Tominaga L, Nanba E, Miyagawa I. Expression of heat shock protein 70 and its mRNAs during ischemia-reperfusion in the rat prostate. European Journal of Pharmacology. 2004;487(1-3):199-203.
15. Hartl FU. Molecular chaperones in cellular protein folding. Nature. 1996;381(6583):571-579.
16. Guo R, Youn SH, Ki J-S. Heat shock protein 70 and 90 genes in the harmful dinoflagellate *Cochlodinium polykrikoides*: Genomic structures and transcriptional responses to environmental stresses. International Journal of Genomics. 2015;2015:484626.
17. Ritossa F. Discovery of the heat shock response. Cell Stress and Chaperones. 1996;1(2):97-98.
18. Larsen P, Nielsen E, Koed A, Thomsen D, Olsvik P, Loeschcke V. Interpopulation differences in expression of candidate genes for salinity tolerance in winter migrating anadromous brown trout (*Salmo trutta* L.). BMC Genetics. 2008;9(1):12.
19. Smith TR, Tremblay GC, Bradley TM. Hsp70 and a 54 kDa protein (Osp54) are induced in salmon (*Salmo salar*) in response to hyperosmotic stress. Journal of Experimental Zoology. 1999;284(3):286-298.
20. Tine M, Bonhomme F, McKenzie D, Durand J-D. Differential expression of the heat shock protein Hsp70 in natural populations of the tilapia, *Sarotherodon melanotheron*, acclimatised to a range of environmental salinities. BMC Ecology. 2010;10(1):11.
21. Ghasemi Y, Rasoul-Amini S, Morowvat MH, et al. Bioconversion of hydrocortisone by unicellular microalga *Oocystis pusilla*. Biotechnology. 2008;7(2):293-298.
22. Rasoul-Amini S, Ghasemi Y, Morowvat MH, et al. Characterization of hydrocortisone bioconversion and 16S RNA gene in *Synechococcus nidulans* cultures. Applied Biochemistry and Microbiology. 2010;46(2):191-197.
23. Sathasivam R, Juntawong N. Modified medium for enhanced growth of *Dunaliella* strains. International Journal of Current Science. 2013;5:67-73.
24. Ramezani A, Niazi A, Abolimoghadam AA, et al. Quantitative expression analysis of TaSOS1 and TaSOS4 genes in cultivated and wild wheat plants under salt stress. Molecular Biotechnology. 2013;53(2):189-197.
25. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-} ΔΔCT method. Methods. 2001;25(4):402-408.
26. Chen H, Jiang JG. Osmotic responses of *Dunaliella* to the changes of salinity. Journal of cellular physiology. 2009;219(2):251-258.
27. Zhou L. *Role of calcium in osmotic stress regulation of D. salina* (Master Degree), Ocean University of China, Shandong, China; 2006.