Artemin protects cells and proteins against oxidative and salt stress

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Abstract

Encysted embryos of the crustacean *Artemia* have acquired unique environmental adaptations that enable them to persist under a wide variety of extreme conditions including desiccation, repeated hydration–dehydration, ultraviolet and ionizing radiations and prolonged anoxia. Artemin is an abundant thermostable protein in *Artemia* encysted embryos under environmental stresses. It is confirmed that high regulatory expression of artemin is relevant to stress resistance in this crustacean. Previous studies on artemin showed that artemin is an efficient molecular chaperone *in vitro* with the thermotolerance feature *in vivo*. Here, the protective role of artemin from *Artemia urmiana* has been investigated on survival of bacterial cells under salt and oxidative shocks. Also, for continuous monitoring of the effect of artemin in prevention of proteins aggregation/ inactivation, co-expression of artemin and luciferase, as an intracellular reporter, in bacterial cells was also performed. Residual activity of luciferase in artemin-expressing *E. coli* cells exposing to different concentrations of H₂O₂ and NaCl was significantly higher than non-expressing cells. In the present study, we document for the first time that artemin can protect proteins and bacterial cells against oxidative and salt stress conditions. These results can declare the resistance property of this crustacean against harsh environmental conditions.

Keywords: Artemin; luciferase reporter; oxidative and salinity stress; co-expression; *in vivo* assay

1. Introduction

Species of *Artemia* are found in a wide variety of extreme environments world wide and exhibit an impressive diversity of adaptations enabling them to deal with such unfavorable conditions[1]. In response to unfavourable environmental conditions, gastrula-stage embryos of *Artemia* are able to enter a quiescent state (cysts) in which metabolism and development are reversibly downregulated to a profound degree, a state that can last for up to several years. Cysts tolerate temperature fluctuation, desiccation, and hypoxia/ anoxia with low mortality[2].

Biological stress is the impact of incompatible condition or mechanical tension on biological systems of living organisms[3]. Actually, any form of pressure that gets an organism's defences up and threatens its existence can be called a biological stress. Environmental stress is one of the main categories of the biological stresses including abiotic (drought, high temperature, chilling and freezing, UV radiation, salinity and heavy metals) and biotic (herbivory, pathogen and parasite and allelopathy) stresses [4]. Abiotic stresses refer to non-living factors or suboptimal environmental conditions leading to disturbing of protein functions and stress-inducible cellular responses that adversely affect cellular homeostasis[5],[6], [7].

Reactive Oxygen Species (ROS) are highly reactive molecules and free radicals derived from molecular oxygen. ROS production in cells results in oxidative stress leading to severe damage to DNA, protein, and lipids in living cells via the Fenton reaction. Oxygen radicals and other activated oxygen species cause modifications of amino acid residues on protein backbones that generally result in loss of protein function[8]. Numerous stress response mechanisms have evolved for suppressing oxidative insults. Some of these mechanisms are preferentially linked to enhanced survival, while others are more frequently associated with

cell death [9]. Expression of heat shock proteins is one of the essential defence mechanisms for protection of cells against oxidative stress [10], [11], [12]. Besides, salinity is an important physical factor in the life of marine and brackish water organisms [13]. The brine shrimp *Artemia* inhabit hypersaline lakes and ponds, which differ in water chemistry, seasonality, species composition, and productivity [14], [15]. Numerous studies have estimated the effects of salinity on life-history traits of *Artemia* populations [13]. Salt stress negatively changes many physiological processes in cells. Some of the effects may involve the oxidative damage of cellular components, which can be promoted by reactive oxygen species and prevented by antioxidants [16]. Like other environmental stresses, salinity leads to changes in transcription levels and it has been reported that, the protein synthesis machinery is sensitive to NaCl and ability of protein synthesis affected by salinity stress that closely correlates with growth [17]. Totally, sodium toxicity affects metabolism, protein synthesis, and the energy household [18], [19].

Small heat shock proteins (sHSPs) are induced by various stress conditions including heat, chilling, toxic metals and oxidative stimuli. The correlation between sHSP over-expression and the stress response led to the hypothesis that sHSPs protect cells from the damaging effects of abiotic stress [20]. Artemin is a stress protein with high thermal stability and potential protein and RNA chaperone activity in *Artemia* encysted embryos. It's confirmed that high regulatory expression of artemin is relevant to stress resistance in this crustacean [21]. Artemin is a globular, 600 kDa Rosset-like oligomeric protein composed of 24 subunits [22] and also enriched in cysteine residues [23]. Artemin from *Artemia franciscana* has been extensively characterized in previous studies [24], [25]. This protein prevents heat-induced aggregation of different proteins *in vitro*. Previous studies indicated that *E. coli* cells containing artemin showed resistance enhancement to heat stress. Also, presence of artemin in transfected mammalian cells promoted cell viability under thermal and oxidative stress [26]. We have

1 previously isolated and cloned artemin cDNA from encysted embryos of *A. urmiana*,
2 inhabiting hypersaline water from Urmia Lake[27]. We found that artemin acts as a highly
3 efficient molecular chaperone through preventing heat-induced protein denaturation *in vitro*
4 and *in vivo*[27], [28]. The purpose of the present study is the investigation of chaperone
5 activity of artemin under oxidative and salinity stress conditions *in vivo*. To characterize the
6 roles of artemin in oxidative and salinity stress responses, three main experiments were
7 performed; luciferase activity assay, survival and growth rate analyses.

8 Luciferase co-expression system can be used as a real-time reporter system to investigate the
9 activity of chaperone proteins *in vivo* and provide a rapid and simple testing of molecular
10 chaperones. Although luciferase is not a bacterial protein, because of high sensitivity for
11 luciferase activity, it has been used extensively as a model substrate for protein folding *in*
12 *vitro*[29] and has also been studied *in vivo* in prokaryotes [30]. Therefore, co-transformation
13 of *E. coli* BL21 (DE3) was done with two expression vectors containing *A. urmiana* artemin
14 and *Photinus pyralis* firefly luciferase for *in vivo* studies of oxidation and salt induced
15 inactivation of the test substrate. The cells were subjected to stress conditions and luciferase
16 activity was monitored as an intracellular reporter. Further, effects of H₂O₂ and NaCl
17 treatments on viability of artemin-carrier strains were determined. Results from this study
18 introduce artemin as a highly efficient protective protein to protect and maintain cells against
19 environmental stress conditions for their medical and industrial applications.

21 2. Materials and Methods

22 2.1. Chemicals

Tris, glycine and sodium dodecyl sulfate (SDS) were obtained from Acros organics (New Jersey, United States). Imidazole and agarose were obtained from Bio Basic Inc. (Markham, ON, Canada). Isopropyl- β -D-thiogalactopyranoside (IPTG) was purchased from Invitrogen (Carlsbad, CA, USA). Kanamycin, ampicillin and tetracycline were obtained from Bio Basic Inc. (Markham, ON, Canada), JaberEbneHayyan Pharmaceutical Co (Tehran, Iran) and Iran Daru Pharmaceutical Co (Tehran, Iran) respectively. Soy peptone and yeast extract were provided by Micromedia Trading House Ltd (Hungary). Acrylamide, hydrogen peroxide, sodium chloride and all other chemicals were purchased from Merck Company (Darmstadt, Germany). Nickel-nitrilotriacetic acid(Ni-NTA) affinity chromatography was provided by Qiagen (Hilden, Germany). Luciferin and ATP were purchased from Resem BV (Lijnden, Netherlands) and Roche (Basel, Switzerland) respectively. Aqueous solutions were prepared in doubly distilled deionized water.

2.2. Plasmids and constructs

The plasmids pET28a containing artemin gene from *A.urmiana*, (GenBank accession no: [EU380315.1](#))[31] and the pET16b encodes *P. pyralis* luciferase gene[32] was provided as described previously.

2.3. Co-transformation and bacterial cell culture conditions

E. coli BL21 (DE3) cells were co-transformed with the plasmids of pET28a and pET16b carrying artemin and luciferase genes respectively using standard technique. Colonies were selected using colony PCR method by specific primers for the artemin and luciferase genes. For co-expression of the proteins, co-transformed cells were cultured at 37 °C in Luria–Bertani (LB) medium supplemented with 50 μ g/ml ampicillin and 25 μ g/ml kanamycin. Production of the recombinant proteins was induced at OD₆₀₀ of 0.6 by adding IPTG to a

final concentration of 1 mM and then incubation continued at 30 °C for 5 h. Also, artemin and luciferase co-expression was evaluated by electrophoresis in SDS-PAGE followed by Coomassie Brilliant Blue staining [33].

2.4. *In vivo* oxidative stress tolerance assay

Cell growth rate (optical density at 600 nm) of strains carrying artemin at highly oxidizing environment was determined after H₂O₂ treatment. To test oxidative stress resistance, *E. coli* cells containing artemin were induced with 1 mM IPTG (at OD₆₀₀ of 0.5) and the cells were treated with different concentrations of H₂O₂ (0, 2.5, 5, 7.5 and 10 mM) at OD₆₀₀ of 0.7 and then were grown at 37 °C for 5 hours in dark conditions. The samples were taken at various times and optical density at wavelength of 600 nm was determined in triplicate by microplate spectrophotometers (μQuant, BioTek, USA).

To determine the H₂O₂ stress resistance, the cells were cultivated in LB media at 37 °C until OD₆₀₀ of 0.5–0.6. Then IPTG (1 mM) was added and the cells were incubated at 30 °C for 5 h. The cells were diluted to OD₆₀₀ of 0.5 and treated with 15 mM H₂O₂ in the dark. After incubation for 60 min at room temperature, the cultures were diluted 10⁶ and 10⁷-fold with LB medium to quench the remaining H₂O₂ and the samples spread onto LB-agar plates containing 50 μg/ml kanamycin. The plates were incubated at 37 °C overnight and the colony-forming units were photographed. In these experiments (growth and survival rate determination), the *E. coli* cells carrying pET28a vector were used as negative control. Finally, the number of viable cells in each sample was compared against untreated samples (no exposure to H₂O₂).

2.5. Real-time monitoring luciferase activity during oxidative stress

For *in vivo* luciferase activity assay, in order to prevent *de novo* synthesis of proteins during assay, tetracycline was added to co-transformed *E. coli* cells in a final concentration of 25 µg/ml and then samples were treated with H₂O₂ (5, 7.5, 10, 12 and 15 mM). Samples were withdrawn at various times and assayed for luciferase activity by adding to the reaction mixture including 0.25 mM D-luciferin, 2 mM ATP, 5 mM MgSO₄, 25 mM Tris-HCl buffer (pH 7.8). After initiating the reaction, the luciferase activity was measured with the *Sirius L Tube Luminometer* (Berthold Detection System, Germany). In this experiment, the *E. coli* cells containing only luciferase gene were used as negative control. Luciferase activity was expressed as a percentage of the activity measured before the oxidative stress treatment. The data represent the mean of three independent experiments.

2.6. Viability assay of transformed *E. coli* under salt stress

For cell growth rate determination under salinity condition, transformed *E. coli* cells containing artemin were cultured at 37 °C in Luria-Bertani (LB) containing 50 µg/ml kanamycin. The overnight cultures were diluted 1:1000 with fresh LB and then continuously incubated at 37 °C and then induced (at OD₆₀₀ of 0.5) at a final concentration of 1 mM. Next, NaCl stock solution was added to a final concentration of 400, 500 and 600 mM at OD₆₀₀ of 0.7 and the growth was continued at 37 °C for 3 hours. The samples were taken at various times and optical density at wavelength of 600 nm was determined by the microplate spectrophotometers.

For survival rate determination, the cell culture and protein expression was performed as described above. The artemin expressing cells were incubated in LB medium containing NaCl (final concentrations of 400, 500, 600 mM) for 1 hour. Then the samples were taken and diluted serially to 10⁷ and 10⁸ -fold and streaked on LB plates containing kanamycin (50 µg/ml). Photos were taken after 17 h of cultivating at 37 °C. The bacterial cells containing

pET28a vector were used as negative control. Finally, the number of viable cells in each treated sample was compared with the untreated control.

2.7. Real-time monitoring luciferase activity during salt stress

Luciferase activity assay of the NaCl-treatment cells was also performed. Co-transformed bacterial cells expressing artemin were grown as mentioned before. To induce salinity stress, after adding tetracycline in high concentration, NaCl was added to final concentrations of 400, 500 and 600 mM to the induced cells. The culture was taken at 0, 15, 30, 45 min and assayed for luciferase activity in the luminometer. In this experiment, the *E. coli* cells containing only luciferase gene were used as negative control. The results represent the means of triplicate determinations. In general, the mean values of luciferase activities of three independent experiments were presented as percent of luciferase activity measured before the stress treatment.

3. Results

3.1. Co-expression of artemin and luciferase in bacterial cells

Co-transformation of the plasmids in bacterial cells was confirmed using colony PCR (Fig. 1a). The expected bands of artemin and luciferase proteins (27 and 61 kDa) were also observed in SDS-PAGE (Fig. 1b).

Suggested place for Fig. 1

3.2. Artemin confers resistance to H₂O₂-treated cells

The effect of H₂O₂ on bacterial cell viability was evaluated by measuring the growth rate during oxidative stress. According to data in Fig. 2, in control cells, growth deficiency was

observed immediately after H_2O_2 treatment. In contrast, treated transformed cell showed a more efficient growth in concentrations of 2.5 and 5 mM H_2O_2 . This protective effect was decreased by increasing of the oxidant concentration in both samples until the difference was abolished completely in 10 mM H_2O_2 .

Suggested place for Fig. 2

Although cell viability decreased in both artemin-expressing and non-expressing cells compared to non-treated samples, survival rates were significantly higher for the artemin-over-expressing cells compared to the non-expressing cells (Fig. 3).

Suggested place for Fig. 3

3.3. Preventing H_2O_2 -induced inactivation of luciferase by artemin

Co-transformed bacterial cells treated with high concentration of tetracycline were kept at lethal concentrations of H_2O_2 followed by luciferase activity determination. Results in Fig. 4 showed that, however decrease in luciferase activity was observed in both samples, co-transformed cells showed a slight decrease compared to control cells, especially at concentrations of 5 and 7.5 mM H_2O_2 . The results showed that, luciferase activity was preserved at least 90 and 70% of the initial activity at 5 and 7.5 mM H_2O_2 respectively, after 120 min, whereas the remaining activity was less than 40% for control cells.

Suggested place for Fig. 4

The results showed that with increasing H_2O_2 exposing time, luciferase activity declined in both samples, but co-transformed cells were less influenced by oxidative stress during incubation period. These results indicated that artemin plays a key role in luciferase protection under oxidation.

3.4. Artemin confers tolerance to salinity stress-induced cells

To evaluate whether artemin expression enhanced the salinity stress tolerance of recombinant microorganisms, the effects of artemin heterologous expression on the salinity tolerance of *E. coli* cells was determined *via* growth and survival rate assay.

As shown in Fig.5, artemin expression in *E. coli* confers salt resistance to the cells and improves cell growth especially in the first 60min after the start of stress treatment in comparison to control cells. In addition, increasing concentration of the salt, showed a more decreases in the tolerance of control cells compared to the transformed cells.

Suggested place for Fig. 5

Similar to cell growth, survival rate of artemin-expressing cells under a high level of NaCl was significantly higher than artemin non-expressed cells. As shown in Fig.6, there were very few colonies in the control cells in 10^8 -fold dilution. All of these results reveal that artemin confers tolerance to salinity stress to the cells.

Suggested place for Fig. 6

3.5. Preventing NaCl-induced inactivation of luciferase by artemin

Luciferase activity assay under salinity stress showed that, decrease in enzyme activity in the co-transformed cells was lower compared to control cells. As shown in Fig.7, in co-transformed cells, about 80% of the luciferase activity was remain after 15 min in 400 mM NaCl, whereas the activity reached to less than 30% for artemin non-expressing cells. Also, luciferase was relatively inactivated after 15 min in 500 and 600 mM NaCl in control cells, but it significantly protected in the artemin-expressing cells. This result demonstrates that the presence of artemin in cells can preserve the activity of this enzyme.

Suggested place for Fig. 7

1

2 **4. Discussion**

3 The role of heat shock proteins (HSPs) in protecting cells against damage associated with
4 environmental stresses has been well documented in a wide range of organisms, ranging from
5 humans to bacteria[34].Molecular chaperones are able to temporarily stabilize unfolded or
6 partially folded proteins and prevent inappropriate inter and intra-molecular interactions.
7 They reduce the concentration of aggregation-sensitive folding intermediates and effectively
8 prevent aggregation processes both *in vivo* and *in vitro*[35].HSPs are induced upon exposure
9 to abiotic stresses, such as cold, salinity, drought, oxidation andheavy metals, which suggests
10 that HSPs are likely to be involved in a broad range of abiotic stress tolerances[36].Small
11 heat shock proteins stably bind stress-denatured substrates and can maintain a substrate in a
12 folding-competent state [37].It appears that sHSP-related proteins selectively recognize and
13 stabilize a variety of non-native proteins. Unfortunately, there is no enough information
14 regarding the mechanism of interaction with substrates and in the absence of detailed
15 structural information, it remains unclear what structural characteristics make sHSPs suited
16 for their *in vivo* chaperone activity[37]. Some studies showed it is likely that non-native
17 substrates interact withHSPs through hydrophobic interactions[38]. We describe here
18 artemin, a member of a novel heat shock protein family, which acts as a very efficient
19 molecular chaperone *in vitro* and *in vivo*.

20 Artemin is animportant protein in*Artemia*that acts as a molecular chaperone by protecting
21 encysted*Artemia* embryos from damage, caused by environmental stress. This protein is
22 produced only in diapause-destined *Artemia* embryos, preventing heat induced precipitation
23 of citrate synthase *in vitro* and protecting transfected mammalian cells against heat and
24 oxidative damage [26].Previous studies on artemin from *A.urmiana* showed thatarteminisan

1 efficient molecular chaperone *in vitro* with the thermotolerance feature *in vivo* [28]. The
2 purpose of this study is to investigate the protective role of artemin on oxidative and salinity-
3 tolerance of expressing artemin *E. coli* cells.

4 Oxidative stress-induced aggregation of certain cellular proteins leads to the induction of the
5 heat shock response both in pro- and eukaryotes [39], [40]. To elucidate whether damaged
6 proteins under oxidative stress serve as substrates for artemin or not, we used luciferase as a
7 sensitive model protein. To analyze the function of this heat shock protein, the expressing
8 artemin cells were exposed to oxidative stress. The potential role of artemin was analyzed
9 using luciferase as a model substrate by luciferase activity assay. In this study, the
10 transformed bacterial cells exposing to H₂O₂ provide an *in vivo* model for the damaging
11 effects of ROS. Transient fluctuations in ROS serve some regulatory functions, whereas high
12 and sustained levels can cause long-term and serious damage to DNA, protein, and
13 lipids [41]. Many organisms have evolved highly complex antioxidant systems include various
14 non-enzymatic molecule as well as enzymatic scavengers of ROS (e.g., superoxide
15 dismutases, catalase, and glutathione peroxidase). Nevertheless, sometimes these defense
16 mechanisms are not sufficient to counteract the production of ROS and this phenomenon
17 referred to oxidative stress [9]. In the case of proteins, and particularly when the oxidation
18 occurs at the level of the carbonyl groups on the side chains, most oxidized proteins undergo
19 selective proteolysis because cells exhibit only limited direct repair mechanisms [42].

20 The oxidative modification of proteins by ROS can cause a multitude of problems including
21 dissociation of subunits, local or global unfolding, exposure of hydrophobic residues,
22 aggregation, oxidation of side-chain groups, backbone fragmentation and loss of functional
23 properties of proteins [43]. Apart from direct modification of proteins with ROS, lipid,
24 carbohydrate and nucleic acid radicals are all able to react with intracellular proteins to

1 oxidize them and also form a wide array of adducts [8]. During oxidative conditions, ROS can
2 inflict direct chemical fragmentation to proteins and also modify them to form large
3 aggregates because of covalent cross-linking reactions and increased surface
4 hydrophobicity [44].

5 Artemin is a cysteine-rich protein [45]. The oxidative state of cysteines moderates the activity
6 of redox-regulated chaperones [46], and the cysteine residues may form disulfide bridges or
7 interact with other residues, thereby stabilizing monomer and/or oligomer structure [45]. The
8 high content of cysteine and its distribution proposes artemin as a redox-dependent molecular
9 chaperone [23]. As a remarkable point, since the cytoplasm of cells in *Artemia* embryos is
10 reduced, cysteines of artemin are in their reduced form because the location of artemin in the
11 cytoplasm [23]. Furthermore, the ability of cysteines to undergo oxidation/reduction reactions
12 is considered to be a key factor contributing to the physiological properties of artemin [45]. It
13 seems that artemin can act as a reducing reservoir to protect the cells from oxidative
14 damage. These findings explain the likely cytoprotective effects of artemin under oxidative
15 stress conditions in *Artemia* cysts [23]. Totally, artemin may also be a molecular chaperone
16 that its activity, like HSP33, is redox controlled [47]. For support of this, large amounts of
17 artemin are observed during encystation of *Artemia* embryos, but not present in the
18 developing ones, and it is not degraded in cysts during long-term anoxia [48]. Also, artemin
19 probably can protect numerous essential bacterial proteins against H₂O₂-mediated
20 aggregation (Fig. 2, 3).

21 Also, we investigated the putative role of artemin under salinity stress condition *in*
22 *vivo*. Salinity is one of the serious challenges that *Artemia* cysts are faced during their life
23 period [49], [50]. The brine shrimp *Artemia* is well adapted to the harsh conditions and survive
24 in severely hypersaline environments. Such conditions are experienced by very few

1 equivalent macro-planktonic organisms [49]. Some studies have previously documented a
2 correlation between sHSPs induction and adaptation to salt stress[51], [52]. DcHSP17.7 is a
3 small heat shock protein from carrot which confers the salinity tolerance to plant cells and
4 transgenic *E. coli* cells[53]. Here, *in vivo* experiments showed that artemin plays an important
5 role in the defense of bacteria against salinity stress (Fig. 5, 6). Also, artemin is able to
6 protect luciferase inactivation during salt stress (Fig.7). These results lead us to conclude that
7 artemin plays an important role in the salt stress tolerance of bacterial cells.

8 Salt stress negatively affects many physiological processes in cells [18], [54]. It is known that
9 osmotic stress induces oxidative damage [19]. Some of these effects may involve the
10 oxidative damage of cellular components, which can be promoted by reactive oxygen species
11 [16]. So it is suggested that the protective role of artemin could be attributed mainly to its
12 antioxidant property. An important aspect in understanding how artemin performs this
13 protective task is to elucidate the fate of substrates that interact with artemin. Molecular
14 chaperones have the ability to bind folding intermediates and to prevent their nonspecific
15 aggregation [55]. The fate of the substrate proteins once bound to the chaperones depends
16 largely on the mechanism of the individual chaperone system. So, it is possible that artemin
17 functions to enhance salt tolerance of bacterial cells through a different, yet unknown,
18 mechanism.

19 In conclusion, according to cell viability and real time monitoring experiments, we showed
20 here that artemin protects proteins and cells against oxidative and salinity damage. We expect
21 that artemin like many other sHSPs helps to protect cell against stress by controlling the
22 proper folding and conformation of both structural (i.e. cell membrane) and functional (i.e.
23 enzymes) proteins. The results leading us to conclude that we have found a protein with a
24 significant protective effect under a wide variety of unfavourable environmental conditions.
25 Furthermore, it is suggested that artemin has an ability to confer stress resistance to other

1 cells like plants. Artemin appears to be a promising candidate for the generation of transgenic
2 plants with increased abiotic stress tolerance to stressors, such as salt and oxidative stress.

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2 Reference

- 3 [1] J.S. Clegg, Protein stability in *Artemia* embryos during prolonged anoxia, *Biological*
4 *Bulletin*. 212 (2007) 74–81.
- 5 [2] T.H. MacRae, Stress tolerance during diapause and quiescence of the brine shrimp,
6 *Artemia*, *Cell Stress Chaperones*. 21 (2016) 9–18.
- 7 [3] S. Mahajan, N. Tuteja, Cold, salinity and drought stresses: an overview, *Archives of*
8 *biochemistry and biophysics*. 444 (2005) 139–158.
- 9 [4] S. Redondo-Gómez, Abiotic and biotic stress tolerance in plants. In *Molecular Stress*
10 *Physiology of Plants*, India: Springer India, 2013, pp.1–20.
- 11 [5] M.V. Mickelbart, P.M. Hasegawa, J. Bailey-Serres, Genetic mechanisms of abiotic stress
12 tolerance that translate to crop yield stability, *Nature Reviews Genetics*. 16 (2015) 237-
13 251.
- 14 [6] M.E. Feder, G.E. Hofmann, Heat-shock proteins, molecular chaperones, and the stress
15 response: evolutionary and ecological physiology, *Annual Review of Physiology*. 61
16 (1999) 243–282.
- 17 [7] U. Feige, R.I. Morimoto, B. Polla, *Stress-inducible cellular responses*, Birkhäuser
18 publication, 2013.
- 19 [8] T. Grune, T. Reinheckel, K.J. Davies, Degradation of oxidized proteins in mammalian
20 cells, *The FASEB Journal*. 11 (1997) 526–534.
- 21 [9] J.L. Martindale, N.J. Holbrook, Cellular response to oxidative stress: Signaling for suicide
22 and survival, *Journal of cellular physiology*. 192 (2002) 1–15.
- 23 [10] I.J. Benjamin, D.R. McMillan, Stress (heat shock) proteins molecular chaperones in
24 cardiovascular biology and disease, *Circulation research*. 83 (1998) 117–132.
- 25 [11] A.T. McDuffee, G. Senisterra, S. Huntley, J.R. Lepock, K.R. Sekhar, M.J. Meredith,
26 M.J. Borrelli, J.D. Morrow, M.L. Freeman, Proteins containing non-native disulfide
27 bonds generated by oxidative stress can act as signals for the induction of the heat shock
28 response, *Journal of cellular physiology*. 171 (1997) 143–151.
- 29 [12] C. Jolly, R.I. Morimoto, Role of the heat shock response and molecular chaperones in
30 oncogenesis and cell death, *Journal of the National Cancer Institute*. 92 (2000) 1564–
31 1572.
- 32 [13] R.A. Browne, G. Wanigasekera, Combined effects of salinity and temperature on
33 survival and reproduction of five species of *Artemia*, *Journal of Experimental Marine*
34 *Biology and Ecology*. 244 (2000) 29–44.
- 35 [14] A. Eimanifar, S. Rezvani, J. Carapetian, Genetic differentiation of *Artemia urmiana* from
36 various ecological populations of Urmia Lake assessed by PCR amplified RFLP
37 analysis, *Journal of Experimental Marine Biology and Ecology*. 333 (2006) 275–285.

- [15] R.A. Browne, P. Sorgeloos, C.N.A. Trotman (eds), *Artemia Biology*, CRC Press, Boca Raton, Florida, U.S.A. 10 (1991) 237-253.
- [16] M.C. Rubio, P. Bustos-Sanmamed, M.R. Clemente, M. Becana, Effects of salt stress on the expression of antioxidant genes and proteins in the model legume *Lotus japonicus*, *New Phytologist*. 181 (2009) 851–859.
- [17] M.A. Omidbakhshfard, N. Omranian, F. Shahriari Ahmadi, Z. Nikoloski, B. Mueller-Roeber, Effect of salt stress on genes encoding translation-associated proteins in *Arabidopsis thaliana*, *Plant signaling & behavior*. 7 (2012) 1095–1102.
- [18] Ö. Çelik, C. Atak, The effect of salt stress on antioxidative enzymes and proline content of two Turkish tobacco varieties, *Turkish Journal of Biology*. 36 (2012) 339–356.
- [19] G.I. Ayala-Astorga, L. Alcaraz-Meléndez, Salinity effects on protein content, lipid peroxidation, pigments, and proline in *Paulownia imperialis* (Siebold & Zuccarini) and *Paulownia fortunei* (Seemann & Hemsley) grown *in vitro*, *Electronic Journal of Biotechnology*. 13 (2010) 13–14.
- [29] K-W. Lee, J-Y. Cha, K-H. Kim, Y-G. Kim, B-H. Lee, S-H. Lee, Overexpression of alfalfa mitochondrial HSP23 in prokaryotic and eukaryotic model systems confers enhanced tolerance to salinity and arsenic stress, *Biotechnology letters*. 34 (2012) 167–174.
- [21] A.H. Warner, R.T. Brunet, T.H. Macrae, J.S. Clegg, Artemin is an RNA-binding protein with high thermal stability and potential RNA chaperone activity, *Archives of Biochemistry and Biophysics*. 424 (2004) 189–200.
- [22] J. Graaf, R. Amons, W. Möller, J. De Graaf, The primary structure of artemin from *Artemia* cysts, *European journal of biochemistry*. 193 (1990) 737–741.
- [23] T. Chen, R. Amons, J.S. Clegg, A.H. Warner, T.H. MacRae, Molecular characterization of artemin and ferritin from *Artemia franciscana*, *European journal of biochemistry*, 270 (2003) 137–145.
- [24] J.S. Clegg, V. Campagna, Comparisons of stress proteins and soluble carbohydrate in encysted embryos of *Artemia franciscana* and two species of *Parartemia*, *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*. 145 (2006) 119–125.
- [25] T.H. MacRae, Molecular chaperones, stress resistance and development in *Artemia franciscana*, In *Seminars in cell & developmental biology*. 14 (2003) 251–258.
- [26] T. Chen, T.S. Villeneuve, K.A. Garant, R. Amons, T.H. MacRae, Functional characterization of artemin, a ferritin homolog synthesized in *Artemia* embryos during encystment and diapause, *FEBS Journal*. 274 (2007) 1093–1101.
- [27] S.S. Shahangian, B. Rasti, R.H. Sajedi, R. Khodarahmi, M. Taghdir, B. Ranjbar, Artemin as an efficient molecular chaperone, *The protein journal*. 30 (2011) 549–557.
- [28] F. Shirzad, R.H. Sajedi, S.S. Shahangian, B. Rasti, B. Mosadegh, M. Taghdir, S. Hosseinkhani, Deletion of extra C-terminal segment and its effect on the function and structure of artemin, *International Journal of Biological Macromolecules*. 49 (2011) 311–316.

- 1 [29] G.J. Lee, E. Vierling, A small heat shock protein cooperates with heat shock protein 70
2 systems to reactivate a heat-denatured protein,*Plant Physiology*. 122 (2000)189–198.
- 3 [30] H. Schröder, T.H. Langer, F.U. Hartl, B. Bukau, DnaK, DnaJ and GrpE form a cellular
4 chaperone machinery capable of repairing heat-induced protein damage,*The EMBO*
5 *journal*. 12 (1993) 4137-4144.
- 6 [31] M. Imani, S. Hosseinkhani, S. Ahmadian, M. Nazari, Design and introduction of a
7 disulfide bridge in firefly luciferase: increase of thermostability and decrease of pH
8 sensitivity,*Photochemical & Photobiological Sciences*. 9 (2010) 1167–1177.
- 9 [32] B. Rasti, S.S. Shahangian, R.H. Sajedi, M. Taghdir, S. Hasannia, B. Ranjbar, Sequence
10 and structural analysis of artemin based on ferritin: A comparative study,*Biochimica et*
11 *Biophysica Acta - Proteins and Proteomics*. 1794 (2009) 1407–1413.
- 12 [33] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of
13 bacteriophage T4,*Nature*. 227 (1970) 680–685.
- 14 [34] K.C. Kregel, Invited review: heat shock proteins: modifying factors in physiological
15 stress responses and acquired thermotolerance,*Journal of applied physiology*. 92 (2002)
16 2177–2186.
- 17 [35] U. Jakob, J. Buchner, Assisting spontaneity: the role of Hsp90 and small Hsps as
18 molecular chaperones,*Trends in biochemical sciences*. 19 (1994) 205–211.
- 19 [36] B. Vinocur, A. Altman, Recent advances in engineering plant tolerance to abiotic stress:
20 achievements and limitations,*Current opinion in biotechnology*. 16 (2005) 123–132.
- 21 [37] G.J. Lee, A.M. Roseman, H.R. Saibil, E. Vierling, A small heat shock protein stably
22 binds heat-denatured model substrates and can maintain a substrate in a folding-
23 competent state,*The EMBO Journal*. 16 (1997) 659–671.
- 24 [38] X. Fu, Chaperone function and mechanism of small heat-shock proteins,*Acta Biochimica*
25 *et Biophysica Sinica*. 46 (2014) 347–356.
- 26 [39] M.F. Christman, R.W. Morgan, F.S. Jacobson, B.N. Ames, Positive control of a regulon
27 for defenses against oxidative stress and some heat-shock proteins in *Salmonella*
28 *typhimurium*,*Cell*. 41 (1985) 753–762.
- 29 [40] T.E. Cullingford, R. Wait, A. Clerk, P.H. Sugden, Effects of oxidative stress on the
30 cardiac myocyte proteome: modifications to peroxiredoxins and small heat shock
31 proteins,*Journal of molecular and cellular cardiology*. 40 (2006) 157–172.
- 32 [41] E. Cabiscol, J. Tamarit, J. Ros, Oxidative stress in bacteria and protein damage by
33 reactive oxygen species,*International Microbiology*. 3 (2010) 3–8.
- 34 [42] A-P. Arrigo, Hsp27: novel regulator of intracellular redox state,*IUBMB life*. 52 (2001)
35 303–307.
- 36 [43] R.A. Dunlop, U.T. Brunk, K.J. Rodgers, Oxidized proteins: mechanisms of removal and
37 consequences of accumulation,*IUBMB life*. 61 (2009) 522–527.
- 38 [44] J. Cervera, R.L. Levine, Modulation of the hydrophobicity of glutamine synthetase by
39 mixed-function oxidation,*The FASEB journal*. 2 (1988) 2591–2595.

- 1 [45] Y. Hu, S. Bojikova-Fournier, A.M. King, T.H. MacRae, The structural stability and
2 chaperone activity of artemin, a ferritin homologue from diapause-destined *Artemia* embryos,
3 depend on different cysteine residues,*Cell Stress and Chaperones*. 16 (2011) 133–141.
- 4 [46] C. Kumsta, U. Jakob, Redox-regulated chaperones,*Biochemistry*. 48 (2009) 4666–4676.
- 5 [47] J. Winter, K. Linke, A. Jatzek, U. Jakob, Severe oxidative stress causes inactivation of
6 DnaK and activation of the redox-regulated chaperone Hsp33,*Molecular cell*. 17 (2005)
7 381–392.
- 8 [48] J.S. Clegg, S.A. Jackson, V.I. Popov, Long-term anoxia in encysted embryos of the
9 crustacean, *Artemia franciscana*: viability, ultrastructure, and stress proteins,*Cell and*
10 *tissue research*. 301 (2000) 433–446.
- 11 [49] L. Litvinenko, A. Litvinenko, E. Boyko, K. Kuzanov, *Artemia* in the Salt Lakes of
12 Russia: the Productivity of Populations, the Reserves of the Cysts and the Fisheries,*Acta*
13 *Geologica Sinica - English Edition*. 88 (2014) 87–88.
- 14
- 15 [50] G.M. Gajardo, J.A. Beardmore, The brine shrimp *Artemia*: adapted to critical life
16 conditions,*Frontiers in physiology*. 3 (2012) 1–8.
- 17 [51] C. Mu, S. Zhang, G. Yu, N. Chen, X. Li, H. Liu, Overexpression of small heat shock
18 protein LimHSP16. 45 in *Arabidopsis* enhances tolerance to abiotic stresses,*PloS one*. 8
19 (2013) e82264.
- 20 [52] G.B. Khaskheli, F. Zuo, R. Yu, S. Chen, Overexpression of small heat shock protein
21 enhances heat-and salt-stress tolerance of *Bifidobacterium longum* NCC2705,*Current*
22 *microbiology*. 71 (2015) 8–15.
- 23 [53] N.H. Song, Y.J. Ahn, DcHsp17. 7, a small heat shock protein in carrot, is tissue-
24 specifically expressed under salt stress and confers tolerance to salinity,*New*
25 *biotechnology*. 28 (2011) 698–704.
- 26 [54] A. Qados, Effect of salt stress on plant growth and metabolism of bean plant *Vicia faba*
27 (L.),*Journal of the Saudi Society of Agricultural Sciences*. 10 (2011) 7–15.
- 28 [55] R.U.H. Mattoo, P. Goloubinoff, Molecular chaperones are nanomachines that catalytically
29 unfold misfolded and alternatively folded proteins,*Cellular and Molecular Life Sciences*.
30 71 (2014) 3311–3325.

Figure legends

Fig.1a) Colony screening of co-transformed cells Lane 1-4, artemin and luciferase genes in co-transformed cells, lane 5 and 6, pET28a and pET16b plasmids containing artemin and luciferase genes respectively as positive controls. M, DNA molecular mass markers. **b)** Co-expression analysis of the co-transformed cells by SDS-PAGE. Protein profiles of co-transformed cells (1), supernatant from induced cells producing artemin (2), supernatant from induced cells producing luciferase (3), *E.coli* cells containing pET28a and pET16b vectors without any gene as control cells (4) and purified luciferase (5), purified artemin (6) and protein molecular mass markers (M).

Fig. 2. Cell growth assay during oxidative stress. The cells expressing artemin (●) and non-expressing cells (○) were cultured and followed incubation at 2.5 (a), 5 (b), 7.5 (c) and 10 (d) mM H₂O₂. The cells non-exposed to oxidation (●) were used as control. Data are representative of three trials and were expressed as the mean ± sd.

Fig. 3. Viability of transformed *E. coli* cells expressing artemin under oxidative stress. The bacterial cells were incubated at 15 mM H₂O₂ and spread on LB plates containing 50 µg/ml kanamycin. 1 and 4: bacterial cells non-exposed to stress, 2 and 5: bacterial cells expressing artemin under oxidative stress, 3 and 6: bacterial cells carrying empty pET28a vector under stress, after 10⁶ (1-3) and 10⁷ (4-6) fold dilutions.

Fig. 4. Luciferase activity assay under oxidative stress conditions; co-transformed (■) and luciferase-expressing (■) cells after incubation at 5 (a), 7.5 (b), 10 (c), 12 (d) and 15 mM H₂O₂. The activities were compared with initial luciferase activity before treatment. Data

points represent the average of three independent experiments. Error bars represent the $\pm$ sd of three replicates.

Fig. 5. The effect of artemin on cell growth during salinity stress. Growth rate of the artemin expressing cells (●) and non-expressing cells (○) was followed after incubation at 400 (a), 500 (b) and 600 (c) mM NaCl. Bacterial cells non-exposing to stress were used as control (●). Data are representative of three trials and were expressed as the mean $\pm$ sd.

Fig. 6. Cell viability of the artemin-expressing cells incubated at 600 mM NaCl for 60 min. 1 and 4: bacterial cells non-exposing to stress, 2 and 5: bacterial cells expressing artemin under NaCl treatment, 3 and 6: bacterial cells containing pET28a under stress, after 10^7 (1-3) and 10^8 (4-6) fold dilution.

Fig. 7. The protective effect of artemin against luciferase inactivation under salt shock. The co-transformed (■) and luciferase-producing (■) cells were incubated at 400 (a), 500 (b) and 600 (c) mM NaCl for 45 min. During treatment, the samples were taken in every 15 min and assayed for luciferase activity. Data points represent the average of three independent experiments. Error bars represent the $\pm$ sd of three replicates.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Graphical Abstract

Environmental stresses such as
salt and oxidative stress

Co-transformed cells

Control cells

Unfolded/Inactivated proteins
Modified lipids, nucleic acids, ...