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Real-time monitoring of artemin *in vivo* chaperone activity using luciferase as an intracellular reporter

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ABSTRACT

Artemin is an abundant thermostable protein in *Artemia* encysted embryos and considered as a stress protein, as its highly regulated expression is associated with stress resistance. Artemin cDNA was previously isolated and cloned from *Artemia urmiana* and artemin was found as an efficient molecular chaperone *in vitro*. Here, co-transformation of *E. coli* was performed with two expression vectors containing artemin and firefly luciferase for *in vivo* studies. The time-course of luciferase inactivation at low and elevated temperatures showed that luciferase was rapidly inactivated in control cells, but it was found that luciferase was protected significantly in artemin expressing cells. More interestingly, luciferase activity was completely regained in heat treated artemin expressing cells at room temperature. In addition, in both stress conditions, similar to residual activity of luciferase, cell viability in induced cultures over-expressing artemin was significantly higher than non-expressed artemin cells. It can be suggested that artemin confers impressive resistance in stressful conditions when introduced into *E. coli* cells, which is due to that it protects proteins against aggregation. Such luciferase co-expression system can be used as a real-time reporter to investigate the activity of chaperone proteins *in vivo* and provide a rapid and simple test for molecular chaperones.

1. Introduction

The environmental changes, like temperature fluctuations can affect the native conformation of proteins. High temperatures lead to accelerated formation of protein aggregates. These effects include thermodynamic instability, hydrophobic interaction, protein diffusion and chemical reactions. Protein destabilization leads to the formation of unfolded protein intermediates and promotes aggregation process [1]. The hydrophobic residues are normally buried inside the proteins, whereas by increasing the temperature, the hydrophobic amino acids are mostly oriented to the surroundings which leads to their association to form large-scale precipitates or protein aggregates [2].

The influence of low temperature and cold shock responses were investigated in different organisms including eukaryotes, bacteria and archaea [3–5]. The effect of cold shock was observed at different levels: (1) fluidity of membrane was decreased and some membrane functions could be disturbed including active transport, protein secretion and cell signaling, (2) the secondary structure in RNA and DNA were stabilized, thereby the mRNA translation rate and the transcription efficiency were reduced, (3) some proteins folded more slowly and even inappropriate folding might occur and the ribosomal functions were disturbed [6,4] and (4) proteins could be destabilized at low temperatures, resulted in generating intermediates which promoted protein aggregation [1]. In microorganisms, it was observed that cold stress could induce the synthesis of several cold-shock proteins [7]. In some literature, the effects of chaperones were studied under cold stress conditions [8–12].

Small heat shock proteins (sHSPs) with low molecular weight (12–40 kD) are exclusively expressed in responding to various environmental changes and they can be found in all organisms including bacteria, plants and animals [13]. sHSPs have protective effects on cell viability at high temperatures or other stress conditions and it is suggested that they might have a role in forming or maintaining of the native conformation of proteins in the stress conditions. They bind to partially denatured proteins and prevent irreversible protein aggregation during stress conditions [14]. High temperature causes destabilization of proteins which leads to form

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folding intermediates that tend to associate with temperature-activated sHSPs. This phenomenon leads to intercellular complexes containing unstable proteins and sHSPs [15,16].

Artemin is a thermostable protein in cyst embryo of *Artemia* under stress conditions, and general stress resistance of this crustacea is relevant to high regulatory expression of artemin as a stress protein [17]. Artemin consists of 24 monomers with 27 kDa molecular weight that forms an oligomer with rosette-like structure and 600 kDa molecular weight [18]. Previous reports suggested different functions for artemin including promoting resistance of mammalian cells in stress conditions and increasing bacterial cell viability affected by high temperature stress *in vivo* [19,20], increasing protein refolding efficiency, the ability of binding to RNA at elevated temperature and its protecting heat-induced aggregation of different protein substrates *in vitro* [21–23]. All observations proposed that artemin acts as an efficient molecular chaperone. According to the previous studies, it was found that artemin showed significantly higher chaperone-assisted refolding and anti-aggregation efficiency compared to reported chaperones. In this study, the chaperoning behavior of artemin has been further investigated and the study has focused on the protective effects of artemin on proteins in living cells. For real-time monitoring of the artemin chaperone activity, a system was used to measure the protective potential of it in living cells (Scheme 1). The used real-time reporter system provided a rapid and simple test for molecular chaperone activity assays, in which luciferase was used as a reporter and the effect of artemin was considered under stress conditions. It should be mentioned that luciferase is a model protein used as a substrate for chaperones. Different experiments were designed by using cold and heat as stressors. In the present study, the real-time reporter system is introduced for *in vivo* measuring chaperone activity under the stress conditions, and we have investigated, for the first time, the protective effect for artemin under cold shock.

2. Materials and methods

2.1. Plasmids and co-transformation

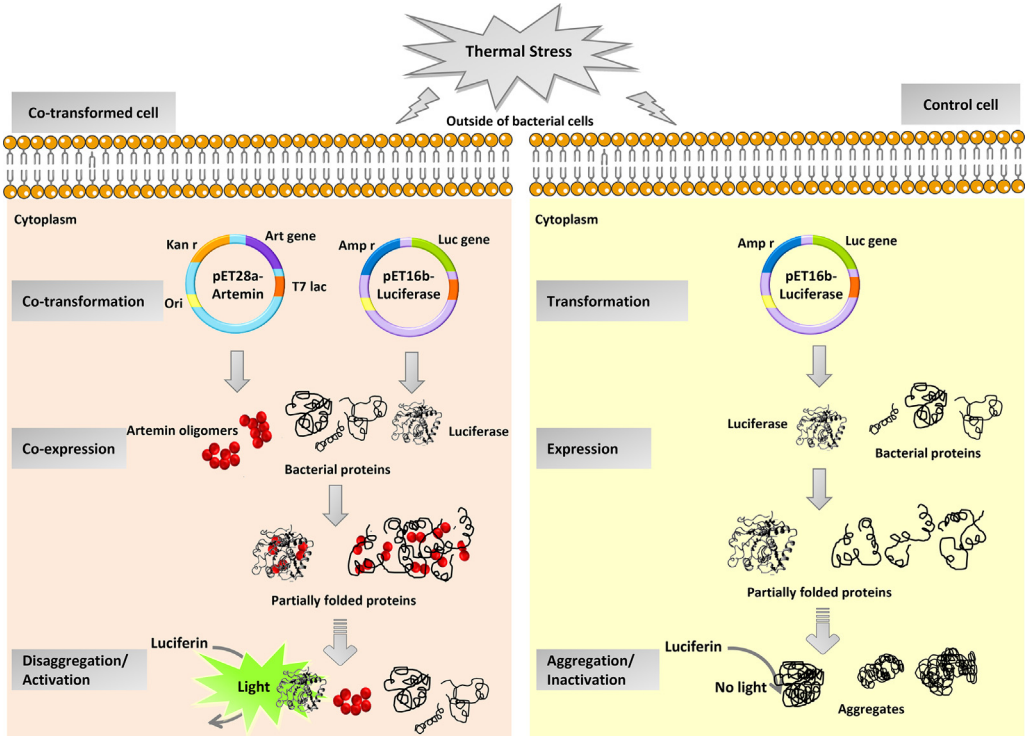
Artemin encoding gene from *Artemia urmiana* was sub-cloned into the expression vector pET28a as it was mentioned in our previous study (GenBank accession no: EU380315.1) [20]. The plasmid pET16b encoding luciferase gene from *Photinus pyralis* was also provided as described before [24]. The constructions were co-transformed into the *E. coli* strain, BL21 (DE3) for their simultaneous expression using standard technique [25]. The selected colonies on Luria–Bertani (LB)-agar medium contained 50 μg/ml ampicillin (Jaber Ebne Hayyan Pharmaceutical Co, Tehran, Iran) and 25 μg/ml kanamycin (Bio Basic INC, Canada). Then, more screening was performed *via* the polymerase chain reaction (PCR) by specific primers for artemin gene and also the presence of luciferase gene was detected by luciferase activity assay.

2.2. Co-expression conditions

Co-transformed cells were grown at 37 °C in LB medium with 50 μg/ml ampicillin and 25 μg/ml kanamycin. Isopropyl-β-D-thiogalactopyranoside (IPTG) (Invitrogen, Carlsbad, CA, USA) was added to a final concentration of 1 mM when the culture reached at OD_{600} of about 0.6 and incubation continued at 30 °C for 5 h. Also, each recombinant protein was separately expressed and purified as it was described previously [26,24]. The analysis of proteins expression was performed using 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.3. Luciferase assay

Co-expression of the co-transformed *E. coli* cells were done as mentioned. Then, tetracycline (Iran Daru Pharmaceutical Co,



Scheme 1. A schematic representation of co-expression of artemin with firefly luciferase, as an intracellular reporter, in *E. coli* for real-time monitoring of preventing heat and cold induced aggregation by artemin and its chaperone activity *in vivo*.

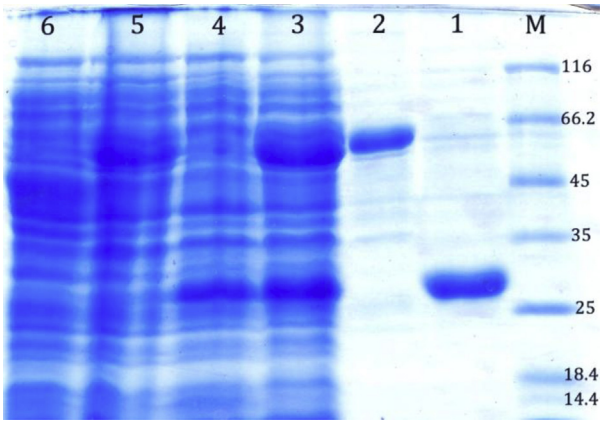


Fig. 1. Co-expression analysis of the co-transformed cells by SDS-PAGE. M: protein molecular mass marker; 1; purified artemin, 2; purified luciferase, 3; co-transformed cell supernatant with IPTG induction. 4; supernatant from induced cells producing artemin, 5; supernatant from induced cells producing luciferase, 6; *E. coli* cells without plasmid as a control.

Tehran, Iran) was added in a final concentration of 25 μg/ml to prevent *de novo* synthesis of proteins *in vivo* during the assay. Then, 5 μl of bacterial cells was added to the reaction mixture containing 0.25 mM D-luciferin (Resem BV, the Netherlands), 2 mM ATP (Roche, Switzerland), 5 mM $MgSO_4$, 25 mM Tris-HCl buffer (pH 7.8). The luciferase activity was measured using the luminometer (Berthold Detection System, Germany). In these experiments, the *E. coli* cells expressing luciferase were used as negative control.

2.4. Heat shock assays

As an inactivation period, the co-transformed and control cell suspensions were incubated at 40–46 °C for 10 and 30 min and this process was followed by incubating the suspensions at room temperature for recovery period. The samples were removed at the indicated times and assayed for luciferase activity. Luciferase activity was expressed as a percentage of the activity measured before the heat shock treatment. The data represent the mean of three independent experiments.

To determine the survival rate, the transformed *E. coli* cells (pET28a containing artemin) were induced by 1 mM IPTG at OD_{600} of 0.6 and incubated at 30 °C for 5 h. The cultures were diluted to OD_{600} of 0.5 and incubated at 48 °C for 60 min. The cultures were diluted serially to 10^6 and 10^7 -fold with LB medium and 50 μl of the samples were spread on LB-agar plates containing 50 μg/ml kanamycin in triplicates. The plates were incubated overnight at 37 °C and then were photographed. In these experiments, the *E. coli* cells carrying empty pET28a vector were used as negative control. Finally, the number of viable cells in each sample was compared to untreated samples (no exposure to heat).

2.5. Cold stress experiments

Cold stress analysis was performed by incubating appropriate dilutions of induced co-transformed and control cells at 0 °C in the presence of tetracycline (25 μg/ml) for different periods (0–10 days). At the indicated times, luciferase activity assay was done.

To determine the cell growth rate, the incubated cultures at 0 °C were inoculated to fresh LB medium with 1:100 ratios and the growing process of them were done at 37 °C. The samples were taken at different times and OD_{600} determined using microplate spectrophotometers (μQuant, BioTek, USA). Also, cell survival on the fifth day of stress treatment was estimated as mentioned. All experiments were repeated for three times.

2.6. ANS fluorescence measurements

Measurements were performed using a LS-55 fluorescence spectrometer (Perkin-Elmer, USA). Samples contained 0.1 mg/ml of artemin in 50 mM phosphate buffer, pH 7.2 were used. Emission spectra were taken from 400 to 600 nm by using an excitation wavelength of 390 nm. Excitation and emission slits were set at 10 nm. Artemin was incubated at temperatures in the 20–80 °C range for 15 min, then cool down to room temperature for 20 min. 8-anilino-*l*-naph-thalene sulfonic acid (ANS) (Merck, Darmstadt, Germany) was added at a final concentration of 13 μM and fluorescence intensity was measured.

3. Results

3.1. Preventing heat-induced aggregation of luciferase in E. coli cell by artemin

SDS-PAGE analysis showed the overproduction of recombinant artemin and luciferase (27 and 61 kDa) in extracts after the induction period (Fig. 1). Because luciferase was expressed alone under optimal conditions (22 °C for 16 h), the overexpression of luciferase in co-transformed cells under co-expression condition (30 °C for 5 h) could be related to the presence of artemin.

It was reported that a heat shock temperature as low as 40 °C was sufficient for inactivating luciferase. The obtained results showed that the luciferase activity in both samples decreased, but

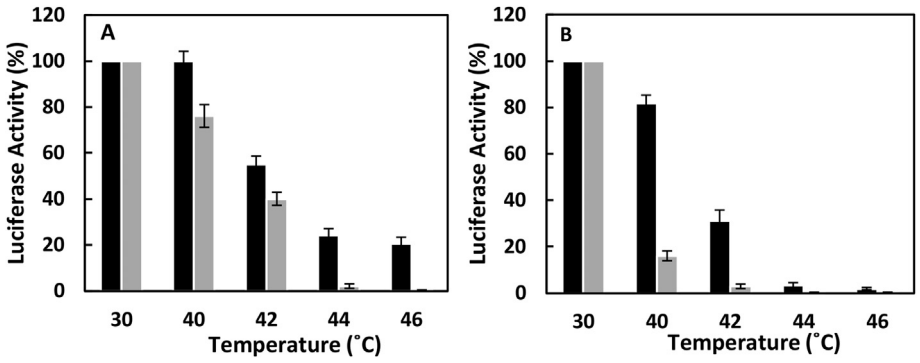


Fig. 2. Thermotolerance assays under heat stress. The co-transformed (black) and luciferase-producing (gray) cells were cultured and induced as described in Materials and Methods section. Then samples were exposed to 40–46 °C treatments in the presence of tetracycline (25 μg/ml) for 30 min. Samples were taken after 10 (A) and 30 (B) min of heat treatments and assayed for luciferase activity compared with activity before heating. Data points and associated error bars represent the means and SD from at least three replicates.

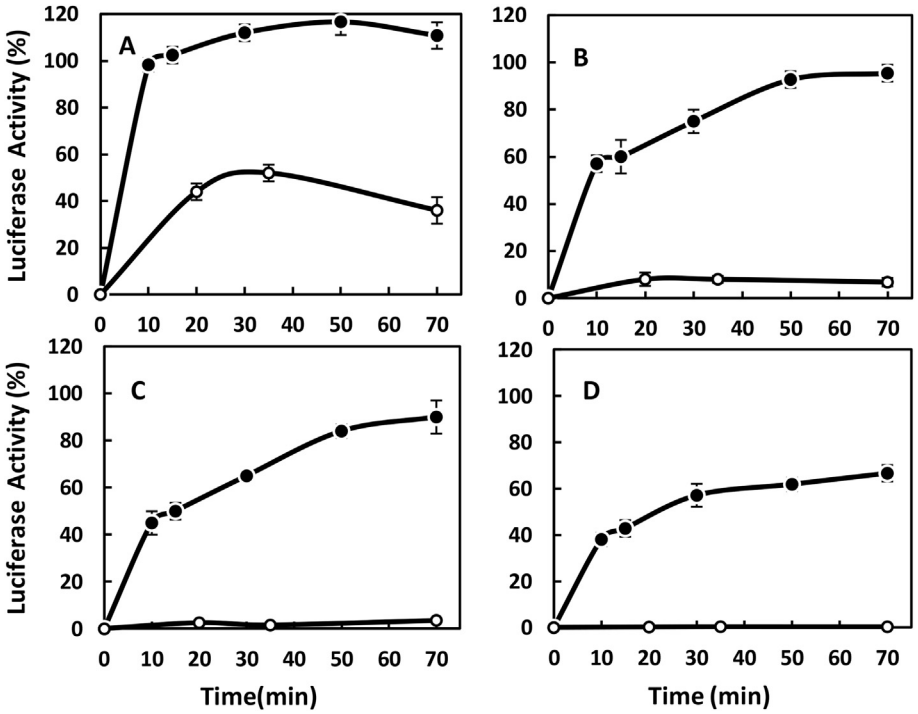


Fig. 3. Luciferase reactivation experiment. The co-transformed (●) and luciferase-producing (○) cells were exposed to heat stress (40–46 °C) as described, followed by further incubation at room temperature for luciferase activity recovery. Luciferase activity assays were measured during 60 min at different times after the incubation periods at A: 40, B: 42, C: 44 and D: 46 °C. Error bars represent the ±SD of three replicates.

the luciferase activity in co-transformed cells was higher than the one in control cells. As shown in Fig. 2, the luciferase activity at 42 °C was preserved at least 55 and 30% of its initial activity, after 10 and 15 min respectively, whereas the remaining activity was less than 40 and 5% for control cells. In addition, luciferase was relatively inactivated after incubation at 44 and 46 °C for 10 min in control cells, but it significantly protected in the artemin expressing cells by 20%. In fact, co-transformed cells were less influenced by thermal stress during inactivating periods (incubation at 40–46 °C). These results indicate that artemin plays an important role in suppressing luciferase inactivation at elevated temperatures.

3.2. *In vivo* reactivation of luciferase by artemin

The recovery experiments indicated that in all treatments of co-transformed cells, luciferase efficiently reactivated and regained more than 50% of its initial activity prior to heat inactivation after 60 min (Fig. 3). In more details, the co-transformed cells, which

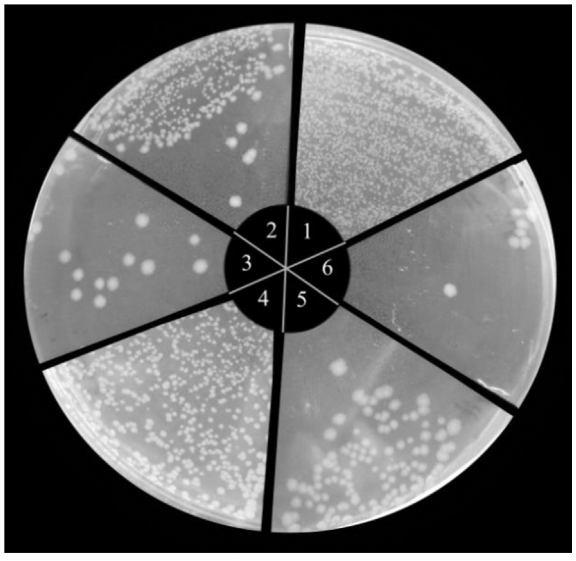


Fig. 4. Viability of transformed *E. coli* cells expressing artemin under heat stress. The bacterial cells were incubated at 48 °C for 60 min and spread on LB plates containing 50 μg/ml kanamycin. The plates were incubated overnight at 37 °C and then were photographed. 1 and 4: bacterial cells non-exposing to stress, 2 and 5: bacterial cells expressing artemin under heat stress, 3 and 6: bacterial cells carrying pET28a vector as negative control under heat stress, after 10^6 (1–3) and 10^7 (4–6) fold dilution.

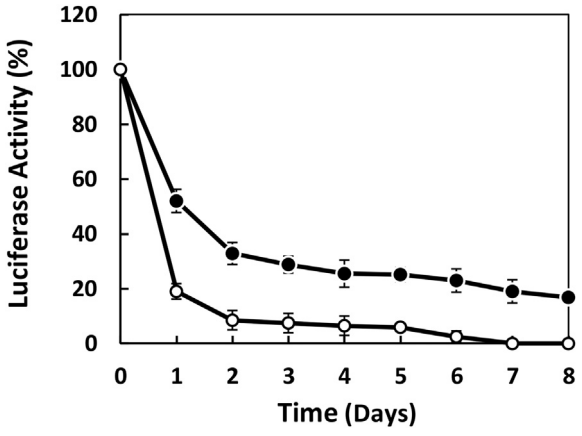


Fig. 5. The protection of luciferase against cold damage *in vivo* by artemin. The co-transformed (●) and luciferase-producing (○) cells were cultured as described in Materials and Methods section. Then samples were incubated on ice in the presence of tetracycline (25 μg/ml) for 8 days. During the cold treatment, the samples were taken in every day and assayed for luciferase activity. Error bars represent the ±SD of three replicates.

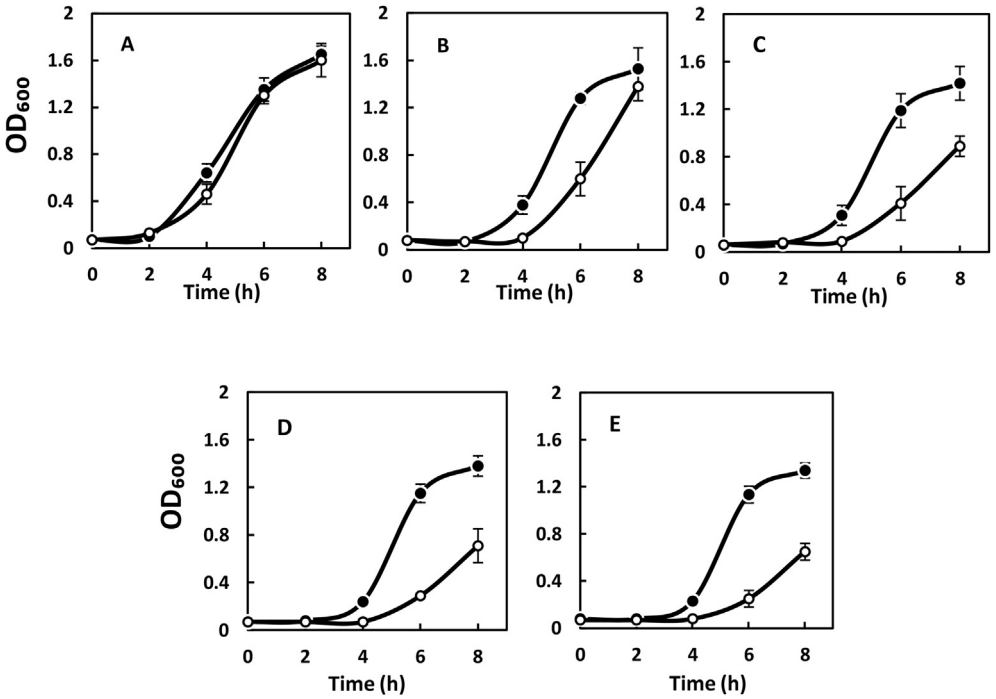


Fig. 6. The effect of artemin on cell growth during cold stress. The cells expressing artemin (●) and non-expressing cells (○) were cultured and followed by incubation at 0 °C for 10 days. In the 1st (A), 3th (B), 5th (C), 7th (D) and 10th (E) day after stress, samples were taken and optical density at OD_{600} was determined. Data points and associated error bars represent the means and SD from at least three replicates.

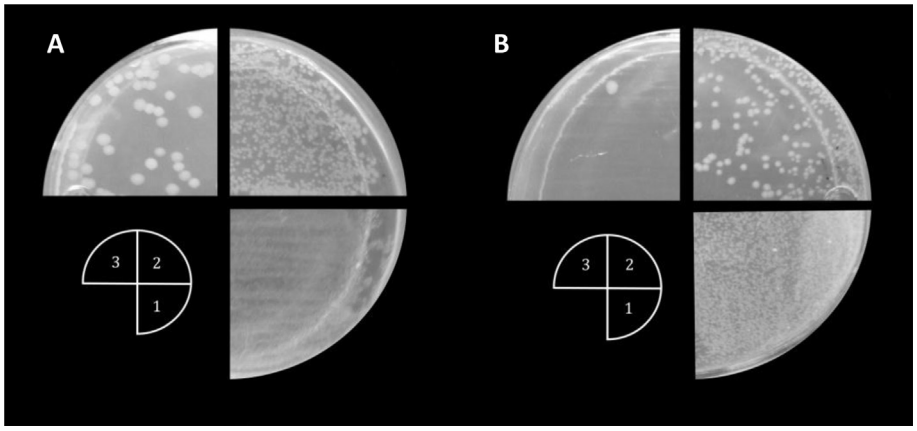


Fig. 7. The effect of artemin on cell survival during cold conditions. Cell viability of the artemin-expressing cells incubated at 0 °C on the 5th day was determined as mentioned in Materials and Methods section. The plates were photographed after overnight culture at 37 °C. 1: bacterial cells non-exposing to stress, 2: bacterial cells expressing artemin under cold stress, 3: bacterial cells containing pET28a vector without inserted gene under cold stress, after 10^6 (A) and 10^7 (B) fold dilution.

incubated at 42 °C, almost fully activated after incubating at room temperature for 60 min. Interestingly; only 10% of luciferase activity regained in the non-expressing artemin cells after this time (Fig. 3B). This lower extent of activity recovery for luciferase can probably be related to the formation of irreversible luciferase aggregates in the non-expressing artemin cells.

3.3. Thermotolerance of E. coli cells expressing artemin

According to cell viability experiments, upon incubation at 48 °C for 60 min, cell survival decreased in both cells producing and non-producing artemin after exposing to heat compared to non-exposed samples. The survival rates were significantly higher for the expressing artemin cells compared to non-expressing cells (Fig. 4).

3.4. Protective effect of artemin under cold stress

Real-time luciferase assay under cold stress condition showed that the reduction of enzyme activity in co-transformed cells were lower than the one in control cells. Fig. 5 illustrates that the co-transformed cells could keep their luciferase activity about 25% after 5 days, whereas the remaining activity reached to less than 6% for non-producing artemin cells. This result demonstrates that artemin can prevent cold inactivation of luciferase as a model protein.

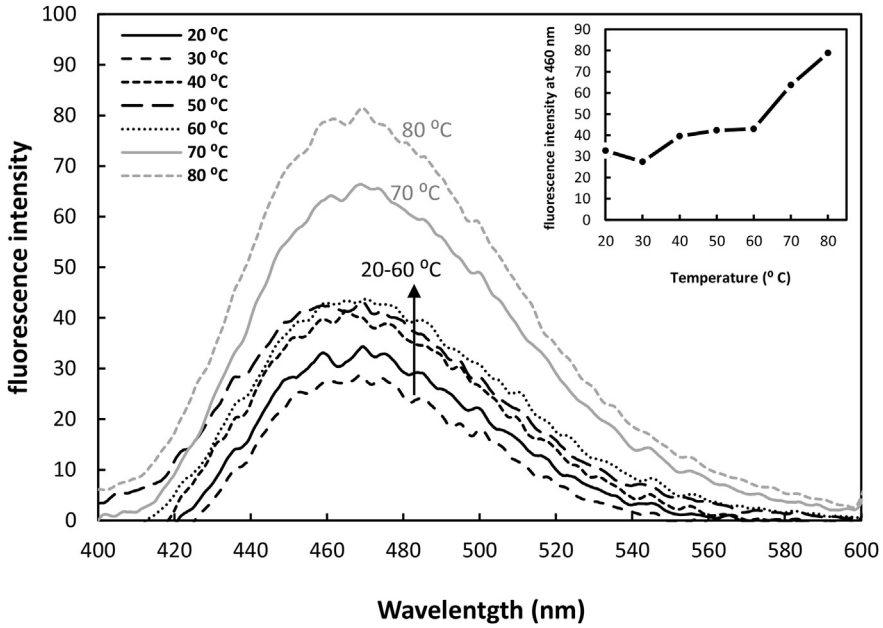


Fig. 8. Fluorescence emission intensity of ANS in the presence of artemin at different temperatures. Samples of artemin (0.1 mg/ml in 50 mM phosphate buffer) were pre-incubated at different temperatures for 15 min and cooled down to room temperature, ANS (13 μM at final concentration) was then added and fluorescence intensity measured with the exitation wavelength at 390 nm. The inset is temperature dependence of the fluorescence intensity of artemin at 460 nm.

3.5. Protective effect of artemin on cell viability against cold shock

Due to the data in Fig. 6, a significant difference was observed between the growth rate of the artemin expressing cells and the non-expressing ones and this difference got greater by passing days. The highest difference can be observed at 10th day after cold stress.

Besides, the results of cell survival analysis showed that, however the number of living cells was decreased in both samples after cold stress treatment within 5th day, the *E. coli* expressing artemin exhibited higher viable colonies than the non-producing cells (Fig. 7). These results indicates that artemin could enhance the *E. coli* resistance against cold shock.

3.6. Binding of ANS

ANS has been widely used as a probe to monitor conformational changes in proteins and to access surface exposure of hydrophobic sites. Fig. 8 shows the changes of the intensity of fluorescence of ANS in the presence of artemin, preincubated under 20–80°C. The result indicated that there was almost a linear increasing of the intensity from the point of 30–60°C. Interestingly, by increasing the temperature more than 60 °C, there was a sharp emission peak at 460 nm. The results confirmed that the conformational changes in artemin occur with increasing of temperature.

4. Discussion

Artemia species have been widely spread all around the world and many reports have been published about their significant adaptation to different extreme conditions [27]. The encysted dormant embryos can tolerate intense drought, long time anoxia, high temperatures and excessive UV radiation exposure or other forms of beam [28]. The unique compatibility of this organism is related to the presence of a special stress protein called artemin [29]. The ability of this protein to prevent heat-induced aggregation of citrate synthase *in vitro*, its role in giving thermostability to bacterial and mammalian cells and binding RNA at elevated temperatures make it a great candidate to be used as a chaperone on both proteins and RNA [22,21,30]. It can be suggested that artemin has protective effects on proteins or other cellular components under various stress conditions. In the present study, for the first time, it is claimed that artemin can protect proteins and bacterial cells against cold shock. Because of rapidity, sensitivity and inexpensive method of assay, the real-time reporter system using luciferase was utilized to investigate the *in vivo* protective role of artemin. Due to the obtained results, heat-induced denaturation of luciferase was prevented at the presence of artemin (Figs. 1 and 2). These results suggest that the critical role of artemin is protection of the substrates against irreversible damage which is consistent with our previous results [26,19]. Also, *in vivo* renaturation of the heat-denatured luciferase did not occur spontaneously and it strictly depended on the presence of artemin (Fig. 3). Recent models derived from *in vitro* analyses propose that sHSPs bind to heat denatured substrates and from this point of view, they prevent their irreversible aggregation and they can represent substrates to other cellular components for their ATP-dependent refolding [31,32]. It is suggested that artemin is able to bind to denatured luciferase and this is crucial for preventing its aggregation. This activity is essential for luciferase renaturation because the efficient reactivation of the aggregated luciferase can't be done alone.

It is important that prior to heat denaturation of luciferase, the presence of artemin is sufficient to suppress its partially unfolded states and consequently its efficient renaturation. It can be supposed that artemin binds to denatured luciferase and increases its stability in a folding intermediate before aggregation.

Thermal-induced protein unfolding is often followed by an immediate aggregation because of exposing the hydrophobic residues. Changing the relative composition of secondary structures due to high temperature can lead to protein aggregation indirectly, which results in different aggregation behavior [1]. Moreover, there is a direct correlation between chaperone activity and its surface

hydrophobicity [33]. Recent studies have shown that artemin has a high surface hydrophobicity compared to other chaperones [26]. It is also expected that by increasing temperature, artemin undergoes temperature-dependent structural changes which increase its surface hydrophobicity and enhance the potential of substrate binding. We proposed a mechanism in which at elevated temperatures, conformational changes occurred in both artemin and the substrate. These conformational changes can be led to an increase in the surface hydrophobicity of the proteins which finally results in hydrophobic interactions. Actually, hydrophobic interaction between denatured proteins and artemin can be led to substrate maintaining in a relatively stable state. The results from ANS fluorescence spectroscopy confirmed that upon increasing temperature, artemin undergoes the conformational changes that are associated with a marked increase in surface hydrophobicity leading to the enhanced chaperone activity of the protein (Fig. 8).

Heat treatment of bacterial cells at 45 °C, caused denaturation and aggregation of the majority of proteins by thermal unfolding [34]. Here, cell viability analysis showed that overexpression of artemin increased thermoresistance *in vivo* (Fig. 4). According to these results, it is assumed that artemin not only protects luciferase as a model substrate, but also preserves other heat-sensitive cytosolic proteins in *E. coli.* Also, the activity of this protein depends on its cellular concentration, but these ratios were not considered in this study.

It has been confirmed that under low-temperature condition, most of bacteria such as *E. coli* show a transient arrest of the cell growth. Also, the general protein synthesis is severely inhibited [4,35,36]. Moreover, low temperature causes protein denaturation as a general phenomenon which leads to cellular damages [7,37,38]. Based on previous investigations on the effect of low temperatures on protein destabilization, the simple reason for instability of proteins in aqueous solution is the ionization of some essential groups of protein molecules which is caused by decreasing temperature [39–41]. Since the protonation enthalpy of the ionizable groups is negative and for some groups it is rather high, it can be expected that at low temperatures, the protonation of these groups increases. Therefore, the protonation not only could switch off the active sites in enzymes, but also induce the dissociation of the subunits in multimeric proteins, or even unfold the compact structure of globular proteins. The temperature range at which these changes can occur depend on the ionization enthalpy [37].

Another explanation for cold-induced changes of proteins based on a very unusual feature of hydrophobic interactions. It is shown that the cold denaturation is a general phenomenon caused by the very specific and strongly temperature dependent interaction of non-polar groups of a protein with water molecules [42]. In contrast to expectations, hydration of these groups is thermodynamically acceptable. As a result, a polypeptide chain with a compact native structure unfolds at a sufficiently low temperature and it leads to exposure of internal non-polar groups to water [37]. Accordingly, under cold stress conditions, molecular chaperones can bind to unstable conformations of the proteins and make them stable. Therefore, the association of artemin with the exposed hydrophobic surfaces of the partially folded polypeptides keeps them in a stable intermediate state and thereby prevents them to be aggregated (Figs. 5–7).

In the other view, regarding to the RNA chaperone activity of artemin and the effect of low temperatures on stabilization of secondary structures of RNA (especially mRNA) and DNA, it seems that the presence of high levels of artemin in bacterial cells can prevent the secondary structure formation of RNAs at low temperatures. Consequently, the expression of chilling tolerance genes in cold stress conditions is increased.

5. Conclusion

In this study, it was confirmed that the expression of artemin in *E. coli* under heat and cold stress conditions provides significant resistance to the cells. The results from luciferase activity assay and viability experiments verify the protective role of artemin on various substrates. It is suggested that artemin can improve microbial tolerance to thermal stress through its protein and RNA chaperone activity. It is found that in co-transformed cells, the presence of artemin remarkably increased soluble luciferase levels at a temperature which is higher than its normal expressing condition. This result proposes that the presence of artemin can protect luciferase from aggregation and this feature can be utilized for a variety of recombinant proteins that are prone to aggregation in *E. coli*. As a suggestion, using artemin can improve the efficient storage of proteins and bacterial cells under long term storage. Future studies could develop an expression system in plant cells and estimate the ability of artemin to establish and protect cells from environmental stress for their agricultural and industrial applications.

Acknowledgments

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