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Heterologously expressed carrot Hsp17.7 was denatured by ATP treatment under abiotic stress

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ABSTRACT

Heat shock proteins (HSPs) play a key role to maintain the solubility of cellular proteins under various stress conditions. Previous studies showed that Hsp17.7 from carrot (*Daucus carota* L.) increased the amount of soluble proteins under the various stress of heat, lead (Pb), and arsenic (As). We studied the molecular chaperon function of the heterologously expressed Hsp17.7 in *E. coli* with the supplement of ATP. In the Hsp17.7 expressing cells, the ATP supplement under the stress of heat and Pb decreased the amount of soluble proteins, in which the expression level of carrot Hsp17.7 also decreased. The same ATP supplement under the stresses of As maintained the amount of soluble proteins, in which the expression level of Hsp17.7 was also maintained. Our results suggest that the decreased amount of soluble protein under the stress conditions of heat and Pb could be due to the decreased expression level of Hsp17.7 and the influence of ATP on the stressed Hsp17.7 transformed *E. coli* depends on the type of stress presented. Heterologously expressed Hsp17.7 was purified by affinity chromatography. Under heat stress, purified Hsp17.7 was stable, mostly remained in a soluble fraction, while citrate synthase, a control model substrate, was denatured into the pellet fraction. The ATP supplement under both normal and heat conditions denatured the Hsp17.7 protein into the pellet fraction. Our results suggest that the decreased molecular chaperone function under heat and Pb stress conditions with the ATP supplement could be due to the instability of Hsp17.7.

1. Introduction

Heat shock proteins (Hsps) are a group of molecular chaperones, which prevent cellular proteins from stress-induced denaturation and/or renature partially denatured proteins to restore their activity (reviewed in Wang et al., 2004). They are found in all organisms, from prokaryotic bacteria to eukaryotic plants and animals, suggesting their fundamental importance in sustaining lives under stress conditions. Hsps are divided into five classes, Hsp100, Hsp90, Hsp70, Hsp60, and small(s) Hsps (12–42 kDa), based on their molecular weight (Richter et al., 2010). sHsps are characterized by a conserved α-crystallin domain in the C-terminal region and a very diverse N-terminal region (Basha et al., 2012). α-crystallin is a major protein in vertebrate eye lens, which prevents proteins from light-scattering aggregation (Slingsby et al., 2013). The diverse N-terminal region functioned as a platform for binding to many substrate proteins (Basha et al., 2006).

Heterologous expression of Hsps enhanced thermotolerance and cell viability under heat and other abiotic stress (chestnut, *Castanea sativa* Hsp17.5, Soto et al., 1999; rose, *Rosa chinensis* Hsp17.8, Jiang et al., 2009; nematode, *Caenorhabditis elegans* Hsp17, Ezemaduka et al., 2014). Bacterial proteins extracted from transformed *E. coli* heterologously expressing Hsp16.9 from rice (*Oryza sativa*) showed higher soluble protein levels (68%), compared to the proteins extracted from pGST vector-only control cells (37%), after heat treatment at 55 °C for 30 min (Yeh et al., 1997), in which most Hsp16.9 existed in the soluble fraction.

Previous studies have shown that functions of some Hsps are affected by ATP. Hsp70s contain an ATPase domain in the N-terminal region and refold denatured proteins through ATP hydrolysis (reviewed in Young, 2010). ATP binding causes conformational changes in Hsp70s, which facilitate substrate binding and release. Molecular chaperone activities of human α-crystallin (Biswas and Das, 2004) and *Mycobacterium tuberculosis* Hsp16.3 (Valdez et al., 2002) were enhanced by ATP via facilitated binding between sHsps and substrates. On the other hand, the molecular chaperon function of human Hsp27 (Wang and Spector, 2001) and tobacco Hsp18 (Smýkal et al., 2000) was inhibited in the presence of ATP. These sHsps have lower affinity for denatured protein substrates in the presence of ATP, thus showing decreased chaperone activities. Considering the decreased level of ATP in stressed cells, the molecular chaperone machinery, including large and

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small Hsps, needs to adjust their functional activity according to the available ATP amount.

In this study, carrot Hsp17.7 (*Daucus carota* L.; Malik et al., 1999) was heterologously expressed in *E. coli*. The solubility of total bacterial proteins in the Hsp17.7 transformed cells and Hsp17.7 stability were examined either in the presence or absence of ATP under various stress conditions. Hsp17.7 was then purified by affinity chromatography and treated with ATP to examine a possible effect of ATP on the stability of Hsp17.7.

2. Materials and methods

2.1. Cloning of carrot Hsp17.7 gene

Carrot (*Daucus carota* L. 'Mussangochon') plants were grown in a mixture of peat: vermiculite: perlite: zeolite [75:10:8:7 (v/v/v/v), Minong, Namyangju, Korea] under a controlled environment [a 16 h photoperiod (19–21 °C, night-day), 2 μmol photons m^{-2} s^{-1} light intensity and 60% relative humidity; Jeio Tech, Seoul, Korea]. Genomic DNA was extracted from 0.3 g of leaf tissue from 3 to 4-month-old plants by grinding them with a mortar and pestle in liquid nitrogen into a fine powder. DNA extraction buffer (2 ml; 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM EDTA, pH 8.0, and 0.3% β-mercaptoethanol) was added, followed by RNase A (10 mg ml^{-1}) treatment at 37 °C for 30 min to remove RNA. SDS (sodium dodecyl sulfate, 20%) was then added to incubate the reaction mixture at 65 °C for 10 min, followed by addition of 5 M potassium acetate on ice for 20 min to denature proteins. Samples were then centrifuged at 15,700 × *g* at 4 °C for 15 min and the pellet was washed in 70% ethanol, followed by another round of centrifugation. The pellet was air-dried and dissolved in autoclaved double-distilled water. The DNA quality was confirmed by gel electrophoresis.

Carrot *Hsp17.7* gene was amplified using a set of primers (Forward: 5'-GGGGGGCATATGTCGATCATTCCAAGC-3', Reverse: 5'-GGGGGGGC TAGCTTAACCAGAAATATCAATGGC-3') via PCR (1 cycle at 94 °C for 5 min followed by 35 cycles of 94 °C for 40 s, 58 °C for 50 s, and 72 °C for 50 s). The amplicon was inserted into a modified pET11a expression vector (Novagen, Merck Millipore, Darmstadt, Germany), containing a 6× Histidine tag before the *Nde*I and *Nhe*I restriction enzyme sites. After confirming the sequence (Macrogen, Seoul, Korea), the recombinant plasmid containing the carrot *Hsp17.7* gene was transformed into *E. coli* (BL21-DE3) to express the protein.

2.2. Heterologous expression of carrot Hsp17.7

Transformed *E. coli* cells were grown in Luria-Bertani (LB) medium containing 50 μg ml^{-1} ampicillin at 37 °C with shaking (130 rpm) overnight. After 1:10^3 dilution with LB medium, bacterial cells were continuously cultured at 37 °C with shaking (225 rpm) until the optical density (O.D.) reached 0.6 at 600 nm. Isopropyl-β-D-1-thiogalactopyranoside (IPTG; 1 mM) was added to induce the transgene expression for 2 h, followed by centrifugation at 1360 × *g* (4 °C) for 30 min. The resulting pellet was dissolved in lysis buffer (25 mM Tris-HCl pH 7.5, 150 mM KCl, 5 mM Imidazole, 10% glycerol, and 3 mM β-mercaptoethanol) and subjected to ultra-sonication (Sonosmasher, S & T Science, Seoul, Korea; 420 W, 20 kHz, for a total of 30 min; repetitions of 10 s ultra-sonication with 30 s pauses). After centrifugation at 20,900 × *g* for 30 min, the total protein content in the supernatant was quantified by the Bradford assay (Bradford, 1976).

2.3. Molecular chaperone assay, SDS-PAGE, and immunoblot analysis

Proteins (2 mg per sample) were exposed to heat (50 °C, up to 6 h), Pb [lead(II) nitrate; N_2O_6Pb, Sigma-Aldrich, Darmstadt, Germany; up to 20 mM, 25 °C, 1 h], and As (sodium arsenate dibasic heptahydrate; $HAsNa_2O_4{\cdot}7H_2O$; Sigma-Aldrich; up to 500 mM, 25 °C, 2 h) in the presence or the absence of adenosine triphosphate (ATP; 3.5 mM), followed by ultra-centrifugation at 20,900 × *g* at 4 °C for 1 h. Soluble protein in the supernatant was quantified by the Bradford assay (Bradford, 1976).

Soluble proteins in the supernatant (20 μl per sample) were separated in a 17% SDS-PAGE gel and either stained with Coomassie blue dye (BioRad, Hercules, US) or electroblotted to polyvinylidene difluoride (PVDF) membrane (GE Healthcare Life Science, Buckinghamshire, UK), followed by blocking in 5% non-fat milk in PBS-T buffer (phosphate buffered saline buffer containing 0.1% Tween-20) with shaking. The membrane was incubated with a polyclonal antibody raised against carrot Hsp17.7 and secondary antibody (anti-rabbit conjugated HRP, Amersham Biosciences, Pittsburgh, PA; 1:30,000 dilution). Chemiluminescent signal was detected by the ECL Prime System (GE Healthcare Life Science) and quantified by the image analysis system (ImagerIII-ID Main Software, Bioneer, Korea).

2.4. Purification of recombinant Hsp17.7

Heterologous IPTG-induced expression of carrot Hsp17.7 and protein extraction by ultrasonication were performed as described above, followed by incubation with the Ni^{2+}-NTA His-Bind resin (Novagen, Madison, US) for 1 h for 6 His-Hsp17.7 binding to the resin. To eliminate non-specific protein binding, the resin was washed with Washing buffer 1 (25 mM Tris-HCl pH 7.5, 200 mM KCl, 10 mM Imidazole, 10% glycerol, and 3 mM β-mercaptoethanol) and Washing buffer 2 (25 mM Tris-HCl pH 7.5, 500 mM KCl, 50 mM Imidazole, 10% glycerol, and 3 mM β-mercaptoethanol). Elution buffer (25 mM Tris-HCl pH 7.5, 200 mM KCl, 250 mM Imidazole, 10% glycerol, and 3 mM β-mercaptoethanol) was then applied and the eluate was collected in 1-ml fractions. Protein was quantified by the Bradford assay (Bradford, 1976), and fractions with higher than 1 μg $μl^{-1}$ protein concentration were subjected to SDS-PAGE and Coomassie blue staining to confirm the presence of Hsp17.7 and low levels of non-specific proteins.

Eluate fractions were placed in Cellu-Sep T2 membrane (Ø Dry 14.6 nm, Uptima, Montlucon, France) and placed in the dialysis buffer (25 mM Tris pH 7.5, 200 mM KCl, 3 mM β-mercaptoethanol, 10% glycerol, and 200–0 mM imidazole) with gentle stirring at 4 °C. Dialysis buffer was exchanged every hour with decreasing imidazole concentration. Protein samples were then centrifuged at 4 °C, 20,900 × *g* for 30 min, resolved on a 17% SDS-PAGE gel, and stained with Coomassie blue dye to confirm the quality and quantity of the protein samples.

2.5. Thermostability of recombinant Hsp17.7

Purified recombinant 6× His-Hsp17.7 and citrate synthase (2 μM, Sigma-Aldrich) were incubated at elevated temperatures (37–52 °C) for 1 h and centrifuged at 4 °C 20,900 × *g* for 30 min. Soluble and insoluble fractions were obtained from the supernatant and the pellet, respectively, and visualized in SDS-PAGE and Coomassie blue staining.

To examine the effect of ATP on the stability of recombinant Hsp17.7, purified 6 His-Hsp17.7 (2 μM) was incubated at a normal (25 °C) and elevated temperatures (50 and 60 °C) in the presence or the absence of ATP for 1 h. Samples were then centrifuged at 4 °C, 20,900 × *g* for 30 min. Soluble and insoluble fractions in the supernatant and the pellet, respectively, were visualized with SDS-PAGE and Coomassie blue staining.

3. Results and discussion

3.1. Bacterial protein solubility in the presence of ATP

Previous studies have shown that heterologously expressed Hsp17.7 increased protein solubility in transformed *E. coli* under various abiotic stress conditions, including heat (Kim and Ahn, 2009) and heavy metals