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Tobacco mitochondrial small heat shock protein NtHSP24.6 adopts a dimeric configuration and has a broad range of substrates

Keun Pill Kim[1,2], Ji Hee Yu[1], Soo Min Park[1,3], Hyun Jo Koo[1] & Choo Bong Hong[1,]*

[1]School of Biological Sciences and Institute of Molecular Biology and Genetics, Seoul National University, Seoul 151-742, [2]School of Biomedical Sciences and Department of Food Science and Biotechnology, CHA University, Pocheon 463-836, [3]Experiment Research Institute of National Agricultural Products Quality Management Service, Ministry for Food, Agriculture, Forestry & Fisheries, Seoul 150-043, Korea

There is a broad range of different small heat shock proteins (sHSPs) that have diverse structural and functional characteristics. To better understand the functional role of mitochondrial sHSP, NtHSP24.6 was expressed in *Escherichia coli* with a hexahistidine tag and purified. The protein was analyzed by non-denaturing PAGE, chemical cross-linking and size exclusion chromatography and the H_6NtHSP24.6 protein was found to form a dimer in solution. The *in vitro* functional analysis of H_6NtHSP24.6 using firefly luciferase and citrate synthase demonstrated that this protein displays typical molecular chaperone activity. When cell lysates of *E. coli* were heated after the addition of H_6NtHSP24.6, a broad range of proteins from 10 to 160 kD in size remained in the soluble state. These results suggest that NtHSP24.6 forms a dimer and can function as a molecular chaperone to protect a diverse range of proteins from thermal aggregation. [BMB reports 2011; 44(12): 816-820]

INTRODUCTION

Small heat shock proteins (sHSPs) are a diverse family of proteins that contain a highly conserved α-crystalline domain at the N-terminal region of the protein (1). The independent evolution of the N- and C-terminal regions has been suggested to accompany an increase in variability to promote functional and structural differentiation of sHSPs (2). Monomeric sHSPs range in size from 12 to 42 kD, and are often assembled into oligomeric complexes of 9 to >24 subunits (3). Extensive studies on the function of sHSPs commonly depict them as ATP-independent molecular chaperones that prevent cellular proteins from irreversible aggregation and insolubilization (3). Heat-denatured client proteins bind to hydrophobic surfaces exposed on the sHSPs to form sHSP/substrate complexes and client proteins that bind to sHSPs in the folding-competent state can be reactivated by the ATP-dependent chaperone HSP70/DnaK and co-chaperones (3, 4). The functions of sHSPs can be very diverse and are often fundamental in cellular and organismal physiology; these functions include the protection of translation, transcription, secondary metabolism, cell signaling and the cell division cycle (4, 5).

In plants, six classes of sHSPs, i.e. CI, CII, CIII, plastids, endoplasmic reticulum, and mitochondria, have been identified with some possible additional classes based on DNA sequences, immunological cross-reactivity, and localization in cellular compartment. Although CI and CII were originally identified as cytosol-localized sHSPs, they are generally distributed in the nucleus as well (1, 4).

Since mitochondria are the fundamental energy producers of cells, it would be reasonable to expect that the mitochondria are involved in the responses to stress. It has been suggested that the plant mitochondrion plays a crucial role in conveying intracellular stress signals to the nucleus, leading to an altered expression of stress genes (6, 7). In this signal transduction pathway, reactive oxygen species that are over produced in the mitochondria under heat conditions have been shown to be the important signal initiation component, and the involvement of molecular chaperones including sHSP in this process also has been suggested (8). The amount of mitochondrial sHSPs accumulating under high-temperature stress is positively correlates with heat tolerance in plants (9), and mitochondrial sHSPs may also protect the mitochondrial electron transport chain under heat stress conditions (10). Based on these combined findings, it appears that sHSPs act as chaperones in mitochondria under stress conditions. However, there is little experimental evidence to support this hypothesis.

In an effort to identify the molecular chaperone activity and functional structure of mitochondrial sHSP, we evaluated a tobacco mitochondrial sHSP that was based on a previously cloned tobacco mitochondrial sHSP gene, *NtHSP24.6*, which is a single

*Corresponding author. Tel: 82-2-880-7526; Fax: 82-2-875-1157; E-mail: hcb@snu.ac.kr
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copy gene in the tobacco genome. This gene codes for a protein that is nuclear encoded and posttranslationally transported into the mitochondria. In RNA blot hybridization, the transcript of NtHSP24.6 was specifically induced upon heat shock treatment, and the coded protein contained a conserved alpha-cystallin domain with high homologies to other reported mitochondrial sHSPs. The level of deduced amino acid sequence homology between *NtHSP24.6* and the genes for cytoplasmic sHSPs in plants was rather low, i.e. between 31.8% and 38.4%, at the whole protein level and below 50% in the consensus region (11).

RESULTS AND DISCUSSION

When purified H_6NtHSP24.6 was analyzed on a 4-20% non-denaturating PAGE gel, the protein appeared as a smear between 48 and 60 kD (Fig. 1A), which indicates that a majority of NtHSP24.6 in the buffer was in dimeric form. The dimeric form of H_6NtHSP24.6 was again confirmed by chemical cross-linking the protein using glutaraldehyde. As the concentration of glutaradehyde was increased to 0.01%, a band with an apparent molecular mass of ~55 kD appeared, which corresponds to the size of a H_6NtHSP24.6 dimer, and its intensity increased with glutaradehyde concentration (Fig. 1B). Above this glutaldehyde concentration, tetramers, hexamers, aggregates of very high molecular mass were observed, which probably came from disorderly crosslinked H_6NtHSP24.6 proteins. Size exclusion chromatography (SEC), which was used to purify H_6NtHSP24.6, provided further evidence that H_6NtHSP24.6 existed primarily as a dimer, i.e. the single peak at the apparent molecular mass of ~55 kD was in good agreement with the non-denaturing PAGE and the chemical cross-linking results (Fig. 1C).

Luc and CS are heat-labile proteins that have been commonly used as model substrates for *in vitro* molecular chaperone assays under high temperature (6, 13). Luc and CS both showed a rapid increase in light scattering at 340 nm upon heat treatment at 42°C for Luc and 45°C for CS. However, when H_6NtHSP24.6 was added to the Luc or CS solution the increased light scattering upon heating decreased in a concentration-dependent manner. The purified H_6NtHSP24.6 alone did not exhibit any meaningful increase in light scattering upon heating, while 800 nM H_6NtHSP24.6 protected 200 nM Luc, and 2 μM H_6HSP24.6 protected 1 μM CS from heat-induced aggregation (Fig. 2).

To determine the substrate specificity of NtHSP24.6 in regards to thermal protection, *E. coli* cell lysate with or without H_6NtHSP24.6 was incubated at 44°C or 48°C for 2 hr, and then Ni-NTA resin was added. The resin-bound complexes of H_6NtHSP24.6 and *E. coli* cellular proteins were eluted from the Ni-NTA resin and analyzed on a 15% SDS-PAGE. Without the addition of H_6NtHSP24.6, no eluted protein from the Ni-NTA column resin could be detected (data not shown). However, when H_6NtHSP24.6 was added to the *E. coli* crude extract, many *E. coli* cellular proteins eluted from the Ni-NTA column resin ranging from 10 to 160 kD (Fig. 3). However, at

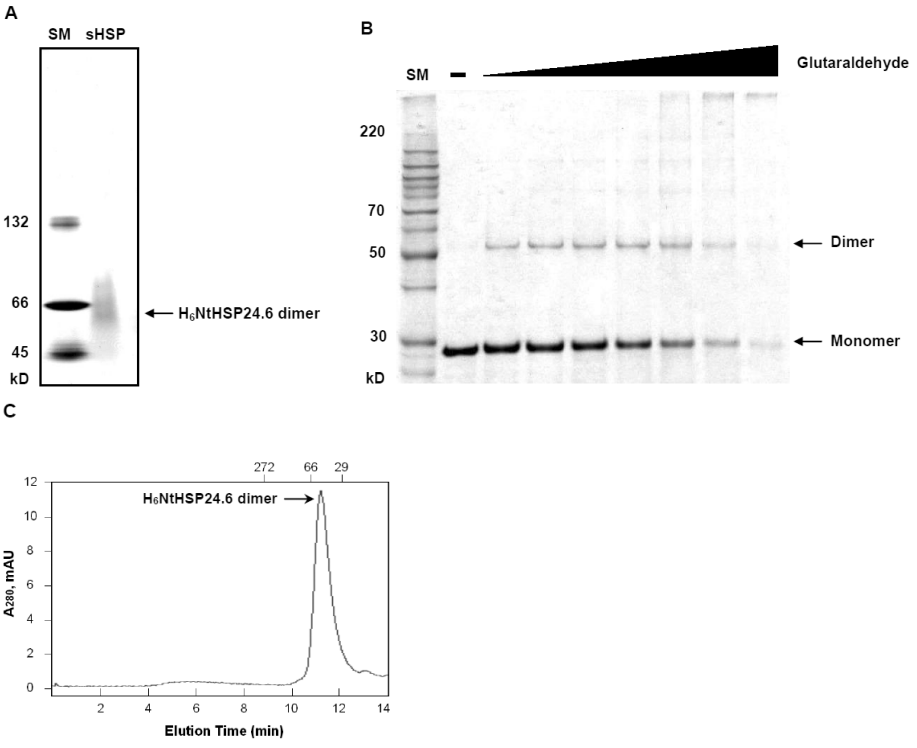

Fig. 1. Dimeric configuration of NtHSP24.6. (A) H_6NtHSP24.6 after 4-20% non-denaturing PAGE. Chicken egg albumin, 45 kD; bovine serum albumin (BSA) monomer, 66 kD; BSA dimer, 132 kD. (B) H_6NtHSP24.6 was incubated with increasing concentrations of glutaraldehyde (0, 0.003, 0.005, 0.008, 0.01, 0.03, 0.05, and 0.08%) and the cross-linked proteins were analyzed on 10% SDS-PAGE gel. (C) SEC chromatogram of H_6NtHSP24.6. Concentration of the H_6NtHSP24.6 was 1 mg/ml. Standard markers, carbonic anhydrase (29 kD), BSA (66 kD) and urease (272 kD), in kDa are shown at the top of the graph.

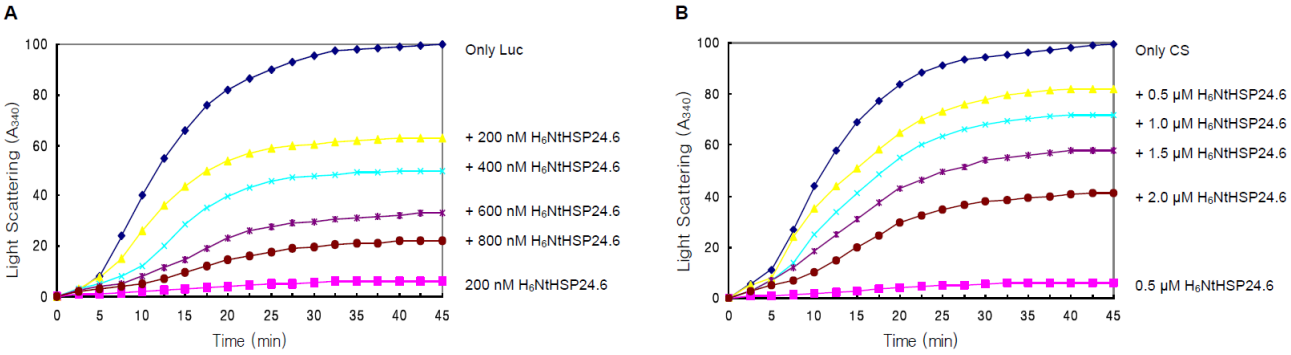

Fig. 2. NtHSP24.6 prevents thermal aggregation of Luc and CS. (A) Luc (200 nM) was incubated at 42°C in the absence and presence of increasing concentrations of H_6NtHSP24.6. (B) CS (1.0 μM) was incubated at 45°C in the absence and presence of increasing concentrations of H_6NtHSP24.6. Light scattering was monitored at a wavelength of 340 nm to measure thermally induced aggregation.

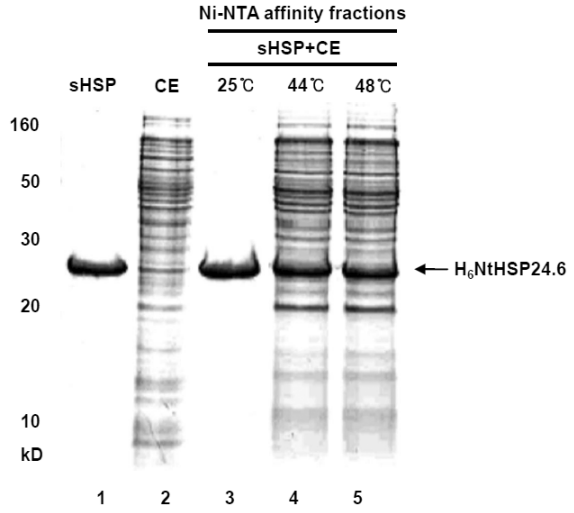

Fig. 3. NtHSP24.6 protects a variety of *E. coli* cellular proteins from thermal aggregation. Lane 1, purified H_6NtHSP24.6 (1 mg/ml); lane 2, the crude extract of *E. coli* (3 mg/ml); lanes 3, 4 and 5, *E. coli* cell lysate with H_6NtHSP24.6 was incubated at 25°C, 44°C, or 48°C for 2 hr and Ni-NTA column chromatography was performed. CE, crude extract.

25°C, the addition of H_6NtHSP24.6 to the crude extract apparently did not result in complex formation between *E. coli* cellular proteins and NtHSP24.6 since only the H_6NtHSP24.6 protein band was visible (Fig. 3).

Mitochondrial sHSP is expected to function as a molecular chaperone and prevents stress-induced aggregation of cellular proteins in an ATP-independent manner. The mitochondria-localized sHSPs are the most recently characterized member of the plant sHSPs family. A mitochondrial sHSP cDNA clone was reported from *Pisum sativum*, and the protein was shown to be encoded by the nuclear genome, heat-induced, and transported post-translationally into the mitochondria (14). NtHSP24.6 displayed the typical molecular chaperone activity on Luc and CS (Fig. 2), and thus the tobacco plant also has a typical sHSP in the mitochondria, which may be common to all plant species.

NtHSP24.6 was shown to protect various *E. coli* cellular proteins from heat-induced denaturation; however, this effect was temperature-dependent, since at 25°C no *E. coli* cellular proteins were protected from denaturation (Fig. 3). The specificity of molecular chaperone is an important and intriguing subject; however, most recent studies indicate that the sHSPs have a broad substrate specificity and can interact with a wide range of proteins (4, 15). Similarly, our results demonstrate that NtHSP24.6 protects a very wide range of *E. coli* cellular proteins from heat denaturation and aggregation. It seems that mitochondrial sHSP can protect various mitochondrial proteins in the matrix from stress-induced denaturation and aggregation, which is important in allowing mitochondria to function under high level stress including in the presence of reactive oxygen species (16-18). All sHsps investigated assemble into oligomeric complexes, mainly 12-42 subunits, with the exception of a few variant sHSPs (3). In their native state, they typically exist as multimeric complexes of 8 to 24 or more subunits (19-21) and each of these complexes is considered to act as a reservoir of sHSP rather than being an active chaperone. Once they are subjected to heat, these oligomeric complexes undergo a conformational changes to form either larger oligomers or dimers, which display chaperone activity (4, 15). In this work, a hexa-histidine tag was attached to the N-terminus, which likely hindered the formation of a higher order quaternary structure, i.e. formation of multiple complexes. However, NtHSP24.6 was clearly shown to form a dimeric complex and exhibit molecular chaperone activity as do most cytoplasmic sHSPs. As discussed above, NtHSP24.6 contained the characteristic sHSP domain, ACD, but also showed significant primary structural discrepancies relative to sHSPs localized in other subcellular compartments. Although much work still needs to be done to understand the importance of this structural difference, it is possible that this structural difference allows mitochondrial sHSP to function under the chemical environment

found in the mitochondrial matrix that can be significantly different from other subcellular compartments. For example, changes in the redox state in the mitochondria would be much more frequent than in the cytosol.

MATERIALS AND METHODS

NtHSP24.6 cDNA clone

A cDNA clone, *NtHSP24.6*, encoding a mitochondrial sHSP was isolated from a cDNA library prepared from a heat-shocked tobacco anther. *NtHSP24.6* codes for 216 amino acids with significant sequence homology to the previously reported mitochondrial sHSPs, and its transcript was quickly induced upon heat shock treatment (11).

Construction of His-tagged NtHSP24.6

To eliminate the mitochondria-target sequence of NtHSP24.6, a primer set flanking the open reading frame of the matured form of the protein was designed and a polymerase chain reaction (PCR) was performed. The primer set was 5′ATATGAGCTCGCC TCTGTTGCTCCC3′ and 5′ATATGCATGCTTAATCAACTTGAA CATGG3′ and the underlined sequences were used to introduce *Sac*I (5′ end) and *Sph*I (3′ end) restriction sites. The amplified PCR product was digested with *Sac*I and *Sph*I and then subcloned into a pBAD expression vector (22) to produce H_6NtHSP24.6, i.e. the mitochondrial target sequence-deleted NtHPS24.6 tagged with an N-terminal hexa-histidine. The construct was transformed into the competent *E. coli* strain DH5α cell.

Expression and purification of H_6NtHSP24.6 from *E. coli*

The H_6NtHSP24.6 expression construct was again transformed into the *E. coli* strain MC1061 to obtain higher levels of protein. The transformant was cultured with vigorous shaking to A_{600} = 0.5 at 37°C. Exponentially growing cells were induced with 0.125% L(+)arabinose (Lancaster, UK) for 4 hr and harvested by centrifugation at 5,000 g for 10 min. The cells were resuspended in cold lysis buffer (25 mM Tris·Cl, 100 mM NaCl, 10 mM imidazole, 1 mM EDTA, 10% glycerol, 1 mM benzamidine, and 5 mM aminocaproic acid, pH 7.5) and sonicated five times at 60 W for 30 sec each on ice using an ultrasonicator (Heat System, USA). Aliquots of 100 mM PMSF were then added to the suspension at a final concentration of 2 mM. The cell lysate was centrifuged at 17,500 g for 20 min at 4°C to remove cell debris. The soluble fraction was mixed with Ni-NTA resin (Qiagen, USA) and incubated for 1 hr 30 min at 4°C. The protein was eluted from the Ni-NTA column using a linear gradient of 20-200 mM imidazole in the same buffer. Relevant fractions confirmed by running SDS-PAGE were collected, pooled, and dialyzed overnight with dialysis buffer (25 mM Tris·Cl and 1 mM EDTA, pH 7.5). The dialyzed fraction was applied on a Q Sepharose fast flow column (Amersham Biosciences, UK). Protein was eluted from the column using a linear gradient of 40-400 mM NaCl in the same buffer. Fractions containing H_6NtHSP24.6 were pooled and dialyzed overnight in dialysis buffer, as described above. The dialyzed protein was mixed with urea to a final concentration of 3 M, incubated at 25°C for 15 min, and applied on a Q Sepharose fast flow column. The protein was eluted from the column using a linear gradient of 40-400 mM NaCl in the same buffer with 3 M Urea. Relevant fractions were collected, pooled, dialyzed overnight in buffer A (25 mM Tris·Cl, 25 mM KCl, 1 mM DTT, 10% glycerol, and 1 mM EDTA, pH 7.5), and concentrated using a Centriplus YM-10 filter (Millipore, USA) by centrifugation at 3,000 g at 4°C for 2 hr. After the purification steps, the homogeneity of H_6NtHSP24.6 was evaluated by SDS-PAGE. The protein appeared as a single band at ca. 28 kD, which corresponds to the molecular mass calculated from the deduced amino acid sequence of H_6NtHSP24.6, and its purity was greater than 96%.

Chemical cross-linking

The H_6NtHSP24.6 preparation (1 mg/ml) was reacted with various concentrations of glutaraldehyde (0.003, 0.005, 0.008, 0.01, 0.03, 0.05 and 0.08%) in buffer A at 25°C for 1 hr, and the cross-linked samples were analyzed on a 10% SDS-PAGE.

Size exclusion chromatography (SEC)

H_6NtHSP24.6 (1 mg/ml) was run on a TosoHaas TSK G4000SW column (TOSOH, Japan) equilibrated with buffer (10 mM Tris·Cl, 150 mM KCl, and 2 mM $MgCl_2$, pH 7.5) at a flow rate of 1.0 ml min^{-1} at room temperature. The proteins were quantified by measuring their absorbance at 280 nm using a Waters 2487 absorbance detector (Meadows, USA). The standards used for SEC were carbonic anhydrase (29 kD), BSA (66 kD), and urease (272 kD) (Amersham Biosciences).

Luc and CS protection assays

Measurements of thermal aggregation of luciferase (Luc) and citrate synthase (CS) were performed as described previously (12). Luc and CS were incubated both without and with varying molar concentrations of H_6NtHSP24.6 in 25 mM Tris buffer (pH 7.5), and thermal aggregation was induced at 42°C for Luc or at 45°C for CS. Light scattering was measured at 340 nm using a spectrophotometer (Win Spec, Spectronic Instrument, USA) to quantify the level of aggregation.

Interaction of H_6NtHSP24.6 with *E. coli* cellular proteins

Purified H_6NtHSP24.6 (1 mg/ml) and a crude extract of *E. coli* (3 mg/ml) were mixed and heated at 44°C or at 48°C for 2 hr. The mixture was incubated with Ni-NTA column resin at 4°C for 1 hr and centrifuged for 1 min at 12,000 rpm in a microcentrifuge. The precipitated resin was washed twice with buffer A containing 10 mM imidazole, and the proteins were eluted with 2× SDS sample buffer (60 mM Tris·Cl pH 6.8, 30% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, and 0.1% bromophenol blue) by heating the resin at 100°C for 5 min. The supernatant was analyzed on a 15% SDS-PAGE gel.

Polyacrylamide gel electrophoresis

SDS-PAGE was performed using a 10 or 15% acrylamide gel with

Coomassie brilliant blue staining as described by Sambrook *et al*. (23). Non-denaturing pore exclusion PAGE was performed using a 4-20% gradient acrylamide gel according to Anderson *et al*. (24). Standard markers for non-denaturing PAGE were chicken egg albumin (45 kD), BSA monomer (66 kD), and BSA dimer (132 kD) (Amersham Biosciences).

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