ATP-independent Thermoprotective Activity of *Nicotiana tabacum* Heat Shock Protein 70 in *Escherichia coli*

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To study the functioning of HSP70 in *Escherichia coli*, we selected *NtHSP70-2* (AY372070) from among three genomic clones isolated in *Nicotiana tabacum*. Recombinant *NtHSP70-2*, containing a hexahistidine tag at the amino-terminus, was constructed, expressed in *E. coli*, and purified by Ni²⁺ affinity chromatography and Q Sepharose Fast Flow anion exchange chromatography. The expressed fusion protein, *H₆NtHSP70-2* (hexahistidine-tagged *Nicotiana tabacum* heat shock protein 70-2), maintained the stability of *E. coli* proteins up to 90°C. Measuring the light scattering of luciferase (*luc*) revealed that *NtHSP70-2* prevents the aggregation of *E. coli* proteins without ATP during high-temperature stress. In a functional bioassay (1 h at 50°C) for recombinant *H₆NtHSP70-2*, *E. coli* cells overexpressing *H₆NtHSP70-2* survived about seven times longer than those lacking *H₆NtHSP70-2*. After 2 h at 50°C, only the *E. coli* overexpressing *H₆NtHSP70-2* survived under such conditions. Our *NtHSP70-2* bioassays, as well as *in vitro* studies, strongly suggest that HSP70 confers thermo-tolerance to *E. coli*.

**Keywords:** *Escherichia coli*, Heat shock protein 70 (HSP70), *in vivo* and *in vitro* Functioning, *Nicotiana tabacum*, Thermotolerance

**Introduction**

Under stress conditions, molecular chaperones cooperate *in vitro* as part of a functional network in which the chaperones prevent the aggregation of misfolded proteins, while actively assist in their refolding (Veinger *et al.*, 1998; Bukau and Horwich, 1999; Mogk *et al.*, 1999; Mayer *et al.*, 2001; Lee and Tsai, 2005). The contributions of individual chaperones to this folding network and the identity of such stress-sensitive cellular proteins are not well known. Therefore, it is important that the extent of which chaperones can re-solubilize aggregates of proteins that escape the protection of holder chaperones is determined (Tatsuta *et al.*, 1998; Mayer *et al.*, 2001; Tomoyasu *et al.*, 2001).

Here, we investigated heat shock protein 70 (HSP70), a molecular chaperone that helps protect cellular proteins. The mechanistic aspects of the HSP70 system have been extensively studied with DnaK (a representative Hsp70), DnaJ (a member of the HSP40 family), and GrpE (a type of co-chaperone) from *Escherichia coli*. According to the study, DnaJ is believed to stabilize the binding of DnaK to the substrate by stimulating the hydrolysis of DnaK-bound ATP. GrpE plays a crucial role in releasing the folding substrates from DnaK (Mayer *et al.*, 2000; Groemping and Reinstein, 2001; Hartl and Hayer-Hartl, 2002; Tamaka *et al.*, 2002; Mayer and Bukau, 2005). Therefore, DnaJ, GrpE, and ATP are essential to this HSP70 chaperone action. However, this theory has been redefined in kinetic studies of HSP70 and substrates in the absence of co-chaperones such as DnaJ and GrpE. For example, the activity of HSP70 was unaffected by physiological levels of HSP40 and GrpE *in vivo* (Michels *et al.*, 1997; Nollen *et al.*, 1999; Silberg and Vicker, 2000; Mayer and Bukau, 2005). Although suggesting a new functional mode for HSP70 as mentioned above, HSP70 has still been reported as ATP-dependent chaperone (Haslbeck *et al.*, 2005; Tuttle, 2006). Therefore, investigation for action of ATP-independent HSP70 have a great value.

Although the functioning of HSP70 has been enhanced in plant systems (Luft and Dix, 1999; Lee and Vierling, 2000; Efremova *et al.*, 2002; Cho and Hong, 2004; Liu *et al.*, 2005), no reports have been made that this protein, in the absence of
co-chaperones or co-factor, can contribute to the protection of substrates from thermal aggregation in vitro. In the study presented here, we investigated the role of tobacco HSP70 isolated from *Nicotiana tabacum* to determine whether it acts as a molecular chaperone in vitro and in vivo and whether ATP and co-chaperones are necessary for HSP70 activity. The tobacco HSP70 *NtHSP70-2* has high homology with other HSP70s, and shows a typical heat shock-responsive expression pattern (Cho and Hong, 2004). This suggests that *NtHSP70-2* can function as a molecular chaperone under high temperatures. Therefore, to examine the functions of plant HSP70, we constructed a recombinant gene encoding *NtHSP70-2* and showed enhanced survival at high temperatures.

Materials and Methods

**Bacterial strains, chemicals, and plasmid.** *E. coli* strain DH15a was used in the construction of recombinant DNA, and the strain MC1061 was used for the expression of recombinant proteins. Recombinant *NtHSP70-2* was expressed in *E. coli* with pBADNH (Cho et al., 2005). Ni-NTA matrices from Qiagen and Q Sepharose Fast Flow from Amersham Biosciences were employed for the purification of 6XHis-tagged proteins. The dye reagent for protein quantitation was from Bio-Rad; DNA modifying enzymes, *Taq* DNA polymerase, and DNA ligase, from Promega; and all chemicals from Sigma, unless otherwise mentioned.

**Construction of an *E. coli* *NtHSP70-2* expression vector.** To prepare an *E. coli* *H6NtHSP70-2* expression construct, the *NtHSP70-2* open reading frame was amplified by PCR, using primers covering both termini of the *NtHSP70-2* coding region, which encodes a full-length polypeptide of 653 amino acids. The 5’ primer was 5’-AAAAAGATCCATGTGCTGGCATGCTGC-3’ and the 3’ primer was 5’-AAGGGCAGCTTATTAGTCGACGCTTCG3’. HindIII restriction sites (underlined) were included in the primers. The amplified product was digested with HindIII and ligated into the pBADNH expression vector between the HindIII sites to produce *H6NtHSP70-2*. The nucleotide sequence of the coding region fused into pBADNH was confirmed with an Applied Biosystems 3730 automatic sequencer. Nucleotide and deduced amino acid sequences were compared with contents in the GenBank and Swiss-Prot databases.

**Expression and purification of *H6NtHSP70-2*.** The above expression construct was transformed into *E. coli* strain MC1061, grown at 37°C to the mid-log phase (A600 = 0.5) in an LB medium containing ampicillin. Exponentially growing cells were induced by adding 0.2% (w/v) arabinose, grown for 1 h at 37°C, and harvested by centrifugation at 10,000 g for 30 min at 37°C. The proteins in the lysates were diluted to 3 mg ml⁻¹ in lysis buffer. Samples (100 µl) of the diluted lysates were layered with mineral oil and incubated for 15 min at 30°C to 100°C in a circulating water bath. The lysates were then allowed to cool to room temperature, the mineral oil was removed, and the samples were centrifuged at 15,000 g for 10 min to remove the denatured proteins. The supernatants were analyzed on 12% SDS-polyacrylamide gels (Sambrook et al., 1989).

**In vitro molecular chaperone activity of *NtHSP70-2*.** Thermally expressed aggregation of luc (Sigma) was examined to determine the molecular chaperone activity of *NtHSP70-2*. Luc (200 nM) was incubated under heat shock (42°C) either alone or in the presence of 20 to 120 mM purified *H6NtHSP70-2* in a buffer containing 25 mM Tris (pH 7.5), 1 mM EDTA, and 25 mM KCl (total volume 0.5 ml), and with or without 2 mM of ATP. Absorbance at 340 nm was monitored at 2.5-min intervals using the kinetics mode of a WinSpec spectrophotometer (Spectronic Instrument Inc.).

**Cell viability.** To measure cell viability under high temperatures, exponentially growing *E. coli* cells transformed with pBADNH *NtHSP70-2* or pBADNH were cultured with 0.2% (w/v) arabinose for 8 h at 28°C, then incubated at 37°C. Aliquots (100 µl) of the cultures were taken at 1, 2, 3, and 4 h after their transfer to 37°C, and serial dilutions were plated on LB agar containing ampicillin. Their viability was determined by counting the colony forming units (CFU) after the plates were incubated overnight at 37°C. These counts were plotted as the percentage of CFUs formed after heat treatment relative to the number of CFUs formed in equivalent, untreated cultures.

**Results**

**Expression in *E. coli* and purification of *NtHSP70-2*.** For our functional analysis, the *NtHSP70-2* coding region was inserted into the *E. coli* expression vector pBADNH to produce a *H6NtHSP70-2* (Fig. 1). *E. coli* cells containing pBADNH *NtHSP70-2* were treated with arabinose to induce production of the fusion protein, and expression was confirmed by SDS-PAGE. Overexpression of *H6NtHSP70-2* in *E. coli* at 37°C for 1 h 30 min resulted in the production of a soluble protein of about 72 kDa, which corresponded to the *NtHSP70-2*. The protein was purified by Ni-NTA affinity chromatography. To obtain more purified *H6NtHSP70-2*, Q Sepharose Fast Flow anion exchange chromatography was
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NtHSP70-2 was clarified in 320 to 360 mM NaCl solutions, achieving >90% purity (Fig. 2). After dialysis in TE buffer, this protein was used for further functional analysis.

**In vitro chaperone activity of NtHSP70-2.**

Recombinant NtHSP70-2 was assayed in vitro for molecular chaperone activity. Crude E. coli extracts were heated and centrifuged to remove heat-denatured and aggregated proteins.

Differences were observed in crude extracts from cells carrying the empty vector versus those that overproduced H$_6$NtHSP70-2 when both were grown at 50°C, i.e., the temperature that usually induces bacterial cell lysis (Fig. 3). Under such conditions, about 70% of the proteins in lysates from cells carrying the empty vector were not protected from aggregation (Fig. 3A), whereas the others were fully protected, remaining stable even at 70°C (Fig. 3B). However, H$_6$NtHSP70-2 was less stable at temperatures greater than 70°C, leading to the aggregation of the other cellular proteins.

**Thermal protection of aggregation of luc by NtHSP70-2.**

The molecular chaperone activity of NHSP70-2 was examined by monitoring the protection of aggregation of luc by the protein under thermal stress. A concentration-dependent effect was observed in vitro by measuring the light scattering of luc at 42°C. At 42°C and without any NHSP70-2, 200 nM of luc started to aggregate rapidly in the first 5 min, with saturation being nearly complete after 40 min. In the absence of 2 mM ATP, 20 nM (only 10% of the control level of Luc) NHSP70-2 was apparently able to reduce the aggregation of luc to 80% relative to the level aggregated when NHSP70-2 was lacking. As the molar ratio of luc to NHSP70-2 rose, relative light scattering decreased. When the ratio reached 1:0.4, the scattering percentage dropped to about 27%; at 1:0.5, the percentage was approximately 3%. For luc to NHSP70-2 ratios of more than 1:0.5, e.g., 1:1, no additional protective activity was observed. In the presence of 2 mM ATP, samples containing

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**Fig. 1.** Map of plasmid constructed to overexpress H$_6$NtHSP70-2 in E. coli.

**Fig. 2.** Overexpression and purification of H$_6$NtHSP70-2. Proteins were analyzed on 12% SDS-PAGE gel and stained with Coomassie Brilliant Blue. (A) Lysate of E. coli cells cultured with 0.2% arabinose. (B) H$_6$NtHSP70-2 purified by Ni$^{2+}$ affinity chromatography and Q Sepharose Fast Flow anion exchange chromatography.

**Fig. 3.** Effect of H$_6$NtHSP70-2 on the thermal stability of proteins in E. coli. Cell lysates lacking (A) or containing (B) H$_6$NtHSP70-2 were incubated at 30, 40, 50, 60, 70, 80, 90, or 100°C for 15 min, after which the soluble proteins were analyzed on 12% SDS-PAGE gels.
20 nM NtHSP70-2 reduced the aggregation of luc to 60% of the level calculated in the absence of NtHSP70-2. Likewise, as the molar ratio of luc to NtHSP70-2 increased, relative light scattering decreased, such that NtHSP70-2 showed stronger activity in the presence of ATP than in its absence. Therefore, these results support the theory that ATP is not necessary for NtHSP70-2 to prevent aggregation of luc. This protein strongly protected aggregation of luc, with its chaperone functioning being proportional to its concentration without ATP. The recombinant NtHSP70-2 also strongly suppressed aggregation of luc under high temperatures (Fig. 4).

Discussion

The possible functioning of HSP70s as chaperones has been suggested based on protein-folding studies as well as research with E. coli and mammalian cells. There, the modes of action have been elucidated mainly through the use of various model systems (Kelley, 1999; Mayer et al., 2001; Abdul et al., 2002; Mayer and Bukau, 2005) E. coli DnaK with the co-chaperones of DnaJ and GrpE (nucleotide exchange factor in bacteria); or via mammalian HSC70 with Hdj-1, the human DnaJ homologue, plus Bag-1 (nucleotide exchange factor in the eukaryotic cytosol). Nevertheless, it has been reported that co-chaperones are not necessary to the action of HSP70 chaperones (Silberg and Vickery, 2000; Kluck et al., 2002), suggesting a different explanation for the cellular functions of HSP70 (Hoff et al., 2000; Brehmer et al., 2001; Silberg et al., 2004). Here, we used E. coli to confirm the molecular chaperone activity of NtHSP70-2, a heat shock protein 70 (HSP70) gene isolated from Nicotiana tabacum. Because its mode of action and activities are not yet well known in plant species, our investigation now provides important evidence for the role of plant HSP70 during heat stress (Tanaka et al., 2002; Cho and Hong, 2004; Cho et al., 2005).

Our in vitro experiments first showed that NtHSP70-2 acts as a molecular chaperone (Fig. 3 and 4). Proteins in cell
lyses that lacked HNtHSP70-2 began to precipitate at 50°C, whereas those containing HNtHSP70-2 remained fully soluble up to 70°C, with over 60% of those continuing to be protected from aggregation to 90°C (Fig. 3). We also examined the thermal protection conferred by luc, a heat-labile protein that is the most commonly used model enzyme for in vitro chaperone activity assays. When HNtHSP70-2 was mixed with luc, light scattering of the latter was decreased in proportion to the concentration of protein tested (Fig. 4). This result suggests that HNtHSP70-2 acts as a molecular chaperone with little substrate specificity under high-temperature stress, while also demonstrating that co-chaperones and ATP are not necessary to the mode of action by HNtHSP70-2. Our conclusions agree with those from previous studies, in which the activity of HSP70 was unaffected by the physiological levels of HSP40 and GrpE (Nollen et al., 1999; Silberg and Vickery, 2000). However, more experiments are needed to confirm that only HNtHSP70-2 prevents the aggregation of denatured proteins by heat stress.

In our second experiment, E. coli cells overexpressing HNtHSP70-2 showed higher survival rates at 50°C than did the control cells (Fig. 5). These results, together with those from our in vitro functional analysis, indicate that HNtHSP70-2 alone acts to prevent the aggregation of denatured proteins by heat shock. However, the viabilities of E. coli cells carrying the empty vector and those overexpressing HNtHSP70-2 decreased abruptly after 1 h at 50°C, at which point the viability of pBADNH/HNtHSP70-2 containing cells was five-fold higher than that of the pBADNH-containing cells. This implies that HNtHSP70-2 only weakly assists in the refolding of denatured proteins following heat stress or requires ATP and other co-chaperones in protein refolding under stress conditions (Kluck et al., 2002; Silberg et al., 2004). Therefore, more experiments are needed to verify that the activity of HNtHSP70-2 is not affected by physiological levels of HSP40, GrpE, and ATP in vivo.

Although we did not demonstrate here the mechanism for a new mode of action by HSP70 in protein-refolding under heat stress, we can conclude that the overexpression of HSP70 alone increases both the level of cellular chaperone activity and the degree of thermo-resistance. In addition, this investigation shows the value of researching a possible new pathway for cellular chaperone activity by HSP70.

References


