Journal of Biochemistry and Molecular Biology, Vol. 40, No. 1, January 2007, pp. 107-112

JOURNAL OF Biochemistry and Molecular Biology

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

Eun Kyung Cho[1,*] and Song-Ja Bae[2,3]

[1]Department of Bio-Food Materials, College of Medical Life Science, Silla University, Busan 617-736, Korea
[2]Department of Food and Nutrition, College of Medical Life Science, Silla University, Busan 617-736, Korea
[3]Marine Biotechnology Center for Bio-Functional Material Industries, Silla University, Busan 617-736, Korea

Received 21 July 2006, Accepted 22 September 2006

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 Ni^{2+} affinity chromatography and Q Sepharose Fast Flow anion exchange chromatography. The expressed fusion protein, H_6NtHSP70-2 (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 luc without ATP during high-temperature stress. In a functional bioassay (1 h at 50°C) for recombinant H_6NtHSP70-2, *E. coli* cells overexpressing H_6NtHSP70-2 survived about seven times longer than those lacking H_6NtHSP70-2. After 2 h at 50°C, only the *E. coli* overexpressing H_6NtHSP70-2 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*, Thermo-tolerance

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; Tanaka *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 Vickery, 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

Abbreviations: H_6NtHSP70-2, hexahistidine-tagged *Nicotiana tabacum* heat shock protein 70-2; luc, luciferase

*To whom correspondence should be addressed.
Tel: 82-51-999-6242, Fax: 82-51-999-5176
E-mail: choeun@silla.ac.kr

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* with a hexahistidine tag at the amino terminus, and then expressed it in *E. coli*. Cells in which NtHSP70-2 was over-expressed showed enhanced survival at high temperatures.

Materials and Methods

Bacterial strains, chemicals, and plasmid. *E. coli* strain DH5α 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 6 X His-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* H_6NtHSP70-2 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' AAAAAAAAGCTTATGGGTCCCGCCGTC3' and the 3' primer was 5'AAAGGGAAGCTTTTAGTCGACCTCCTC3'. *Hin*dIII restriction sites (underlined) were included in the primers. The amplified product was digested with *Hin*dIII and ligated into the pBADNH expression vector between the *Hin*dIII sites to produce H_6NtHSP70-2. 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 H_6NtHSP70-2. 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 30 min at 37°C, and harvested by centrifugation at 10,000 *g* for 5 min. The pellet was re-suspended in protein extraction lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole), then sonicated (total processing time 10 min, pulse-on time 3 s, pulse-off time 12 s), and centrifuged at 15,000 *g* for 25 min. NtHSP70-2 expressing cells were separated into soluble and insoluble proteins by centrifugation, and suspended in loading buffer for electrophoresis on 12% SDS-PAGE gels. Soluble H_6NtHSP70-2 was partially purified on a Ni^{2+} affinity column using washes with 20 and 40 mM imidazole and elution with 250 mM imidazole. Fractions containing the eluted H_6NtHSP70-2 were applied to a Q Sepharose Fast Flow anion exchange chromatography column and eluted at a linear gradient of 40 to 400 mM NaCl in protein buffer (25 mM Tris-Cl and 1 mM EDTA).

Thermal stability of proteins in *E. coli* cell lysates. *E. coli* cell lysates either containing or lacking H_6NtHSP70-2 were prepared as described above, with exponentially growing cells induced for 1 h 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 to 100°C in a circulating water bath (Polyscience Co.). 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 induced 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 nM purified H_6NtHSP70-2 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 50°C. Aliquots (100 μl) of the cultures were taken at 1, 2, 3, and 4 h after their transfer to 50°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 H_6NtHSP70-2 (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 H_6NtHSP70-2 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 H_6NtHSP70-2. The protein was purified by Ni-NTA affinity chromatography. To obtain more purified H_6NtHSP70-2, Q Sepharose Fast Flow anion exchange chromatography was

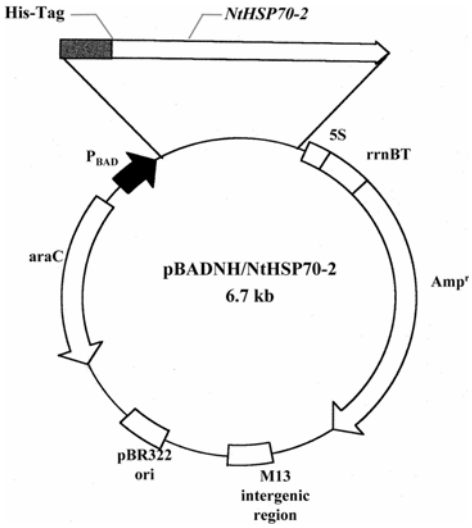

Fig. 1. Map of plasmid constructed to overexpress H_6NtHSP70-2 in *E. coli*.

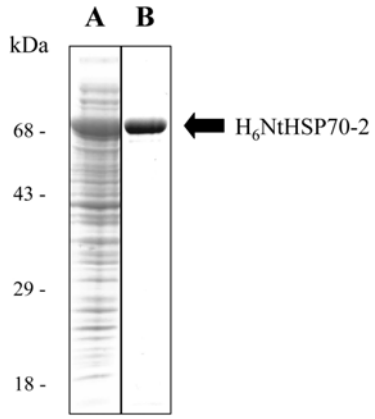

Fig. 2. Overexpression and purification of H_6NtHSP70-2. Proteins were analyzed on 12% SDS-PAGE gel and stained with Coomassie Brilliant Blue. (A) Lysate of *E. coli* cells cultured with 0.2% arabinose. (B) H_6NtHSP70-2 purified by Ni^{2+} affinity chromatography and Q Sepharose Fast Flow anion exchange chromatography.

performed. H_6NtHSP70-2 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_6NtHSP70-2 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

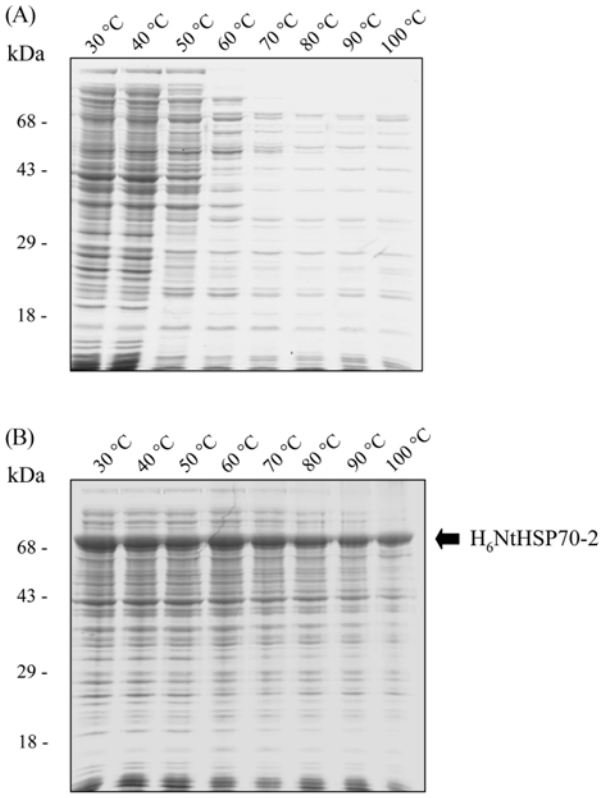

Fig. 3. Effect of H_6NtHSP70-2 on the thermal stability of proteins in *E. coli*. Cell lysates lacking (A) or containing (B) H_6NtHSP70-2 were incubated at 30, 40, 50, 60, 70, 80, 90, or 100°C for 15 min, after which the soluble proteins were analyzed on 12% SDS-PAGE gels.

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_6NtHSP70-2 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 NtHSP70-2 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 NtHSP70-2, 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) NtHSP70-2 was apparently able to reduce the aggregation of luc to 80% relative to the level aggregated when NtHSP70-2 was lacking. As the molar ratio of luc to NtHSP70-2 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 NtHSP70-2 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

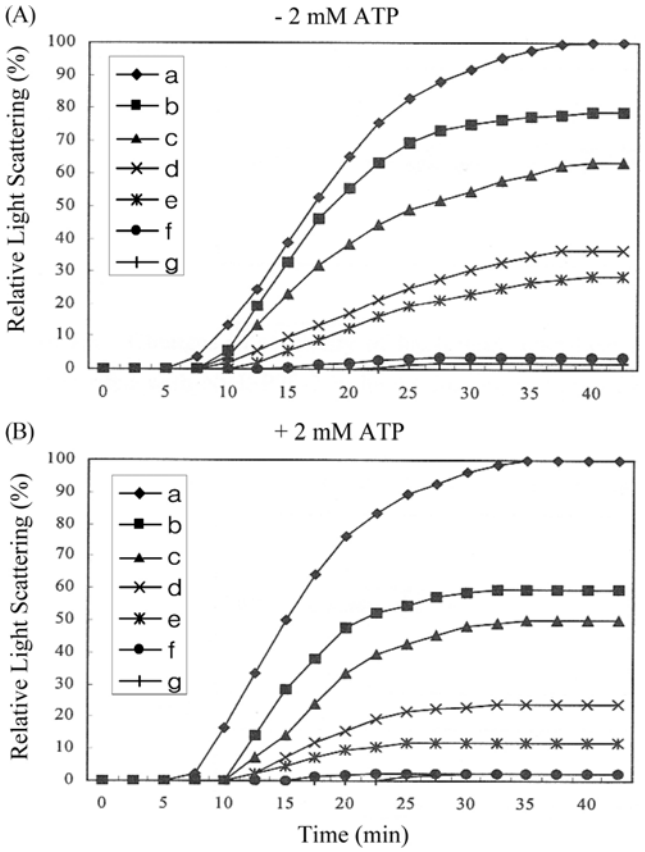

Fig. 4. Suppression of thermal aggregaion of luciferase by H_6NtHSP70-2. Light scattering of aggregated Luc was defined according to absorbance at 340 nm. Luc (200 nM) was heated at 42°C with 20 to 120 nM H_6NtHSP70-2, without ATP (A) or with ATP (B): a, 200 nM Luc; b, 200 nM Luc + 20 nM H_6NtHSP70-2; c, 200 nM Luc + 40 nM H_6NtHSP70-2; d, 200 nM Luc + 60 nM H_6NtHSP70-2; e, 200 nM Luc + 80 nM H_6NtHSP70-2; f, 200 nM Luc + 100 nM H_6NtHSP70-2; g, 200 nM Luc + 120 nM H_6NtHSP70-2.

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).

***In vivo* chaperone activity of NtHSP70-2.** To investigate the molecular chaperone function of H_6NtHSP70-2 *in vivo*, the viabilities of *E. coli* cells transformed with pBADNH/NtHSP70-2 or pBADNH were determined at 50°C. Cells carrying the empty vector (control) were less viable than those overexpressing H_6NtHSP70-2 (Fig. 5). Heating the pBADNH-containing cells

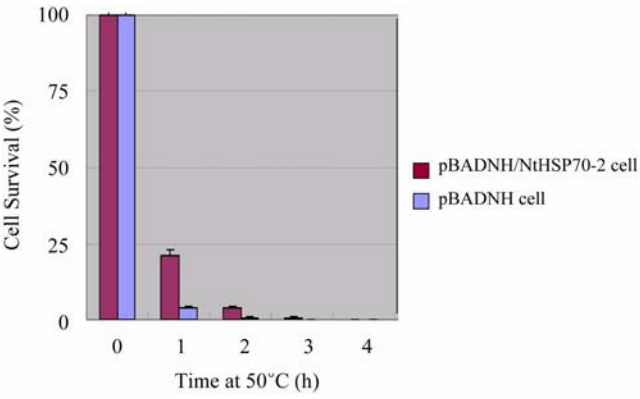

Fig. 5. Influence of H_6NtHSP70-2 on viability of *E. coli* at 50°C. Cells carrying pBADNH/NtHSP70-2 or pBADNH were incubated at 50°C for 1, 2, 3, or 4 h. Viabilities were plotted as percentage of heat-treated colony forming units relative to number of untreated colonies. All data represent means of four independent experiments; error bars indicate standard error.

at 50°C for 1 h resulted in a survival rate of only 3.7% compared with 21.2% survival (7-fold higher) for the pBADNH/NtHSP70-2 cells. However, the viabilities of both types of *E. coli* cells, either empty or overproducers, decreased abruptly after 1 h at 50°C, so that the viability of our pBADNH/NtHSP70-2 containing cells was 5-fold higher than that of the pBADNH-containing cells. After 2 h, only the over-expressing cells could survive at that high temperature.

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

lysates that lacked H_6NtHSP70-2 began to precipitate at 50°C, whereas those containing H_6NtHSP70-2 remained fully soluble up to 70°C, with over 60% of those continuing to be protected from aggregation to 90 (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 NtHSP70-2 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 NtHSP70-2 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 NtHSP70-2. 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 NtHSP70-2 prevents the aggregation of denatured proteins by heat stress.

In our second experiment, *E. coli* cells overexpressing NtHSP70-2 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 NtHSP70-2 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 overproducing H_6NtHSP70-2 decreased abruptly after 1 h at 50°C, at which point the viability of pBADNH/NtHSP70-2 containing cells was five-fold higher than that of the pBADNH-containing cells. This implies that NtHSP70-2 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 NtHSP70-2 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

Abdul, K., Terada, K., Gotoh, T., Hafizur, R. and Mori, M. (2002) Characterization and functional analysis of a heart-enriched DnaJ/ Hsp40 homolog dj4/DjA4. *Cell Stress Chap.* **7**, 156-166.

Brehmer, D., Rüdiger, S., Gässler, C. S., Klostermeier, D., Packschies, L., Reinstein, J., Mayer, M. P. and Bukau, B. (2001) Tuning of chaperone activity of Hsp70 proteins by modulation of nucleotide exchange. *Nat. Struct. Biol.* **8**, 427-432.

Bukau, B. and Horwich, A. L. (1999) The Hsp70 and Hsp60 chaperone machines. *Cell* **692**, 351-366.

Cho, E. K. and Hong, C. B. (2004) Molecular cloning and expression pattern analyses of heat shock protein 70 genes from *Nicotiana tabacum*. *J. Plant Biol.* **47**, 149-159.

Cho, E. K., Lee, Y. K. and Hong, C. B. (2005) A Cyclophilin from *Griffithsia japonica* has thermoprotective activity and is affected by CsA. *Mol. Cells* **20**, 142-150.

Efremova, S. M., Margulis, B. A., Guzhova, I. V., Itskovich, V. B., Lauenroth, S., Muller, W. E. and Schroder, H. C. (2002) Heat shock protein hsp70 expression and DNA damage in Baikalian sponges exposed to model pollutants and wastewater from Baikalsk Pulp and Paper Plant. *Aquat. Toxicol.* **57**, 267-280.

Groemping, Y. and Reinstein, J. (2001) Folding properties of the nucleotide exchanger factor GrpE from *Thermus thermophilus*: GrpE is a thermosensor that mediates heat shock response. *J. Mol. Biol.* **314**, 167-178.

Hartl, F. U. and Hayer-Hartl, M. (2002) Molecular chaperones in the cytosol: From nascent chain to folded protein. *Science* **295**, 1852-1858.

Haslbeck, M., Miess, A., Stromer, T., Walter, S. and Buchner, J (2005) Disassembling protein aggregates in the yeast cytosol; The cooperation of HSP26 with SSA1 and HSP104. *J. Biol. Chem.* **280**, 23861-23868.

Hoff, K. G., Silberg, J. J. and Vickery, L. E. (2000) Interaction of the iron-sulfur cluster assembly protein IscU with the Hsc66/ Hsc20 molecular chaperone system of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **97**, 7790-7795.

Kelley, W. L. (1999) Molecular chaperones: How J domains turn on Hsp70s. *Curr. Biol.* **22**, 305-308.

Kluck, C. J., Patzelt, H., Genevaux, P., Brehmer, D., Rist, W., Schneider-Mergener, J., Bukau, B. and Mayer, B. P. (2002) Structure-function analysis of HscC, the *Escherichia coli* member of a novel subfamily of specialized Hsp70 chaperones. *J. Biol. Chem.* **277**, 41060-41069.

Lee, G. J. and Vierling, E. (2000) A small heat shock protein cooperates with heat shock protein 70 systems to reactivate a heat-denatured protein. *Plant Physiol.* **122**, 189-198.

Lee, S. Y. and Tsai, F. T. F. (2005) Molecular chaperones in protein quality control. *J. Biochem. Mol. Biol.* **38**, 259-265.

Liu, J. G., Yao, Q. H., Zhang, Z., Peng, R. H., Xiong, A. S., Xu, F. and Zhu, H. (2005) Isolation and characterization of a cDNA encoding two novel heat-shock factor *OsHSF6* and *OsHSF12* in *Oryza Sativa L. J. Biochem. Mol. Biol.* **38**, 602-608.

Luft, J. C. and Dix, D. J. (1999) HSP70 expression and function during embryogenesis. *Cell Stress Chap.* **4**, 162-170.

Mayer, M. P., Brehmer, D., Gassler, C. S. and Bukau, B. (2001) Advances in protein chemistry: Protein folding in the cell. *Acad. Press* **59**, 1-44.

Mayer, M. P. and Bukau, B. (2005) Hsp70 chaperones: Cellular functions and molecular mechanism. *Cell. Mol. Life Sci.* **62**, 670-684.

Mayer, M. P., Rudiger, S. and Bukau, B. (2000) Molecular basis of interactions of the DnaK chaperone with substrates. *Biol. Chem.* **381**, 877-885.

Michels, A. A., Kanon, B., Bensaude, O., Konings, A. W. T. and Kampinga, H. H. (1997) HSP70 and HSP40 chaperone activities in the cytoplasm and the nucleus of mammalian cells. *J. Biol. Chem.* **272**, 33283-33289.

Mogk, A., Tomoyasu, T., Goloubinoff, P., Rudiger, S., Roder, D.,

Langen, H. and Bukau, B. (1999) Identification of thermolabile *Escherichia coli* proteins: Prevention and reversion of aggregation by DnaK and ClpB. *EMBO J.* **18**, 6934-6949.

Nollen, E. A. A., Brunsting, J. F., Roelofsen, H., Weber, L. A. and Kampinga, H. H. (1999) *In vivo* chaperone activity of heat shock protein 70 and thermotolerance. *Mol. Cell. Biol.* **19**, 2069-2079.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, New York.

Silberg, J. J. and Vickery, L. E. (2000) Kinetic characterization of the ATPase cycle of the molecular chaperone Hsc66 from *Escherichia coli*. *J. Biol. Chem.* **275**, 7779-7786.

Silberg, J. J., Tapley, T. L., Hoff. K. G. and Vickery, L. E. (2004) Regulation of the HscA ATPase reaction cycle by the cochaperone HscB and the iron-sulfur cluster assembly protein IscU. *J. Biol. Chem.* **279**, 53924-53931.

Tanaka, N., Nakao, S., Wadai, H., Ikeda, S., Chatellier, J. and Kunugi, S. (2002) The substrate binding domain of DnaK facilitates slow protein refolding. *Proc. Natl. Acad. Sci. USA* **99**, 15398-15403.

Tatsuta, T., Tomoyasu, T., Bukau, B., Kitagawa, M., Mori, H., Karata, K. and Ogura, T. (1998) Heat shock regulation in the *ftsH* null mutant of *Escherichia coli*: Dissection of stability and activity control mechanisms of σ^{32} *in vivo*. *Mol. Microbiol.* **30**, 583-593.

Tomoyasu, T., Mogk, A., Langen, H., Goloubinoff, P. and Bukau, B. (2001) Genetic dissection of the roles of chaperones and proteases in protein folding and degradation in the *Escherichia coli* cytosol. *Mol. Microbiol.* **40**, 397-413.

Tuttle, A. M. (2006) Characterization of the expression and function of *Rana catesbeiana* HSP30 and *Xenopus laevis* HSP27. M.S. thesis. University of Waterloo, Ontario.

Veinger, L., Diamant, S., Buchner, J. and Goloubinoff, P. (1998) The small heat-shock protein IbpB from *Escherichia coli* stabilizes stress-denatured proteins for subsequent refolding by a multichaperone network. *J. Biol. Chem.* **273**, 11032-11037.