Appl Biochem Biotechnol (2010) 162:476–485
DOI 10.1007/s12010-009-8809-3

Isolation, Purification, and Properties of a Novel Small Heat Shock Protein from the Hyperthermophile *Sulfolobus solfataricus*

Yonghua Wang · Xun Xu · Zhenzhen Wen · Wencheng Li · Bo Yang · Chris Whiteley

Received: 10 June 2009 / Accepted: 1 October 2009 /
Published online: 31 October 2009

Abstract The isolation, purification, and properties of a putative small heat shock protein (sHsp), named SsHSP14.1, from the hyperthermophilic archaeon *Sulfolobus solfataricus* have been investigated. The sHsp was successfully expressed and purified from *Escherichia coli*. In vivo chaperone function of SsHSP14.1 for preventing aggregation of proteins during heating was investigated. It was found that recombinant SsHSP14.1 with a molecular mass of 17.8 kDa prevented *E. coli* proteins from aggregating in vivo at 50 °C. This result suggested that SsHSP14.1 confers a survival advantage on mesophilic bacteria by preventing protein aggregation at supraoptimal temperatures. In vitro, the purified SsHSP14.1 protein was able to prevent *Candida antarctica* lipase B from aggregation for up to 60 min at 80 °C. Moreover, the SsHSP14.1 enhanced thermostability of bromelain extending its half-life at 55 °C by 67%.

Keywords Small heat shock protein · Purification · Chaperone-like activity · Thermostability · Protein aggregation

Introduction

The small heat shock proteins (sHsps) are molecular chaperones of relatively small molecular masses (about 12–43 kDa, with the majority between 14 and 27 kDa), and they

Authors Yonghua Wang and Xun Xu contributed to the work equally.

Y. Wang · X. Xu
College of Light Industry and Food Sciences, Key Lab of Fermentation and Enzyamtic Engineering, South China University of Technology, Guangzhou 510640, China

Z. Wen · W. Li · B. Yang · C. Whiteley
School of Bioscience and Bioengineering, South China University of Technology, Guangzhou 510640, China

B. Yang (✉)
College of Bioscience and Biotechnlogy, South China University of Technology, Guangzhou 510640, People's Republic of China
e-mail: yonghw@scut.edu.cn

were found in almost all organisms, with the number of members varying from species to species [1]. Despite their small sizes, the active entities of sHsps are usually large oligomers consisting of multiple subunits. They are structurally subdivided into an N-terminal region of variable sequence and length, a conserved sequence of about 80 amino acids containing the α-crystallin domain which is known to function as molecular chaperones [2–4], and a short C-terminal region [5]. The multicomplex sHsps confer thermotolerance to cellular cultures and proteins in cellular extracts during prolonged incubations at elevated temperatures. Thus, they have the ability to protect cellular proteins and to maintain cellular viability under intensive stress conditions such as heat shock or chemical treatments. This discovery has boosted extensive research in this field. Structure and functional aspects of sHsps [6, 7] and their interacting clients, such as protein aggregates and membrane lipids [8], have been recently reviewed. Mechanisms of action, however, have not been defined.

The heat shock response in archaea has been extensively investigated for over a decade, and it has been established that at least 42 archaea species possess more than one sHsp gene, with four of them (~10%) having at least six sHsp genes [9]. The functions and regulation of archaean sHsps remain unclear. Although the two genes of the hyperthermophilic archaea *Sulfolobus solfataricus*, which grows optimally at about 80 °C, had been identified through annotation of its genome [10], however, there have no reports on the functions and properties of these two proteins. The smaller sHsp was composed of 124 amino acids encoded by an open reading frame of 375 nucleotides (GenBank accession no. NP-343935).

We now wish to report on this sHsp, whose function is yet to be confirmed. In order to study its characterization, the gene encoded this protein was cloned from its genome, and the recombinant protein, SsHSP14.1, was overexpressed and purified. To evaluate possible chaperone function of this putative sHsp, the bacteria host harboring the recombinant sHsp was heated, and the aggregation of proteins was analyzed. In addition, the chaperone function of SsHSP14.1 was also investigated by its preventing effect on the thermal aggregation of *Candida antarctica* lipase B (CALB) and heat-induced inactivation of bromelain. CALB and bromelain were chosen here because of their low stability at high temperature, and they are potential industrial enzymes. What is more, we considered about a potential industrial utilization of SsHSP14.1, given it could improve the thermal stability of some important industrial enzymes.

Materials and Methods

Materials

CALB and bromelain were obtained from our own laboratory. CALB was expressed in *Pcihia pastoris* and purified by ion exchange chromatography. The crude enzyme fluid was loaded on the carboxymethyldiethylaminoethyl (CM-DEAE) column in series, and the purified CALB protein penetrated from the columns in 20 mM potassium phosphate buffer (pH 6.0) was collected.

The pineapple juice was obtained by squeezing the cleaned pineapple stem and then clarified by centrifugation (4,000 r/min, 10 min).It was filtered through a 0.45-μm cellulose acetate filter and a 100-kDa molecular mass cutoff membrane (Pall, USA) and then concentrated by ultrafiltration through a 10-kDa molecular mass cutoff membrane (Pall, USA). The obtained crude bromelain was kept at 4 °C.

Plasmids and Bacterial Strains

Escherichia coli strains BL21(DE3) was used as host cells. Plasmid pET-28a (Novagen, Madison, WI) was used as an expression vector which has a His-tag. DNA polymerase and restriction enzymes were purchased from Takara. All molecular techniques were performed as described previously [11].

Construction of Expression Plasmid

The gene of SsHSP14.1 was amplified from the genome of *S. solfataricus* P2 by polymerase chain reaction (PCR) using the sense primer, P1(5′-TCTAGGATCCATGAT GAATGTGATAATGAG-3′) and antisense primer, P2(5′-AAAAGAATTCATTATTC TATTCTGATTGAG-3′). The PCR profile included 94 °C 2 min, 30 cycles of 94 °C 30 s, 53 °C 30 s, and 72 °C 30 s. The purified PCR product was digested with E*coR* I and B*amH* I and inserted into pET-28a (+) expression vector, resulting in pET28a-SsHSP14.1 plasmid. The extracted plasmid designated PHLK1 was subjected to DNA sequencing by Sangon, Shanghai, China.

Expression of the Recombinant Protein

The PHLK1 plasmid was transformed into *E. coli* BL21(DE3) cells. To express the protein of interest, transformants were grown in Luria–Bertani (LB) medium containing kanamycin (0.05 mg/ml) overnight at 37 °C for small scale culture. The overnight culture (7.5 ml) was then inoculated into 150 ml of the fresh LB medium at 37 °C with shaking until the optical density of the culture at 600 nm reached 0.5. Isopropyl β-D-thiogalactoside (IPTG) was then added to a final concentration of 0.1, 0.5, 1, 1.5, and 2.0 mM to promote induction of expression. After the optimization of the concentration of IPTG, the induction was carried out at 25, 30, and 37 °C for 4 h. The PHLK transformed culture without induction was used as control. The bacteria were centrifuged (10,000×*g*, 5 min, 4 °C), and then, a sample was lysed and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The pellet was stored at −80 °C before being subjected to cell disruption.

To monitor the solubility of the expressed protein, the pellet was washed twice with buffer (10 mM Tris-HCl, 1 mM EDTA, pH=8.0) then resuspended in Tris-HCl buffer (20 mM, pH=8.0). The cell suspension was sonicated on ice (60×5 s, 5 s intervals), and the lysate was centrifuged (11,000×*g*, 4 °C, 60 min). The pellets were then resuspended in Tris-HCl buffer (20 mM, pH 8.0), and both soluble and insoluble fractions were electrophoresed in 12% SDS-PAGE and viewed with Coomassie brilliant blue R250 staining.

Purification of the Recombinant Protein

Sodium chloride and imidazole, at final concentrations of 500 and 50 mM, respectively, were added to the lysate after centrifugation ("Expression of the Recombinant Protein" section). The supernatant was then applied to the Hightrap chelating HP (Hightrap chelating HP, GE) Ni^{2+} column and pre-equilibrated with five column volumes of buffer A (0.5 mM NaCl, 20 mM Tris-HCl, pH 8.0). The column was washed with the same binding buffer until a stable absorbance baseline was reached. The fusion protein was eluted with a four bed volume of elution buffer (0.5 mM NaCl, 20 mM Tris-HCl, pH 8.0) containing 50, 100, 300, and 500 mM imadazole, respectively, at a rate of 5 ml min^{-1}, and the fractions were monitored by SDS-PAGE.

The elution fraction containing SsHSP14.1 were combined and applied onto a Sephadex G-75 column (1.6 cm×100 cm, GE Healthcare) equilibrated with Tris-HCl buffer (20 mM, pH 8.0) containing 0.5‰ Triton X-100. Fractions (5 ml) were collected at a flow rate of 30 ml/h with the same buffer, and the protein content was measured at A_{280}nm. The gel filtered SsHSP14.1 was subjected to SDS-PAGE.

Protein Thermal Stability Assays

Cell cultures were grown at 37 °C to an optical density$_{600}$ of 0.6. Expression of SsHSP14.1 was induced by 0.5 mM IPTG for 4 h. Then, the culture was transferred to 50 °C. Four hours later, the cell pellet was lysed by sonication. The crude lysates were centrifuged at 15,000×*g* for 60 min, and the supernatant were analyzed by 12% SDS-PAGE. The samples for analyzing were prepared in Tris-HCl buffer (20 Mm, pH 8.0) to a volume of 10 ml per 1 g of cell pellet. For each analysis, 10 μl of samples were loaded.

Measurement of the Total Protein Concentration

The total protein concentration was measured by the bicinchoninic acid protein assay Kit (Sangon, Shanghai).

Thermal Protection of CALB by Purified SsHSP14.1

The purified CALB was incubated at a concentration of 100 μM in the presence of SsHSP14.1 at equimolar ratios in Tris-HCl buffer (20 mM, pH 8.0) in a final volume of 350 μl. The samples were covered with mineral oil and heated at 50 °C for 20 min and at 60 °C for 10 and 20 min, respectively. After cooling to room temperature, the mineral oil was removed, and the samples were centrifuged (11,000×*g*, 20 min, 4 °C). The supernatants were collected and analyzed by SDS-PAGE.

Bromelain Thermostability Assay

Purified SsHSP14.1 and bromelain (molar ratio 3:1) were coincubated at 55 °C. The bromelain activity was assayed at time intervals (0, 20, 40, 60, 80, and 100 min) by monitoring casein reduction at 280 nm [12].

Results

Expression and Purification of Recombinant Protein SsHSP14.1

Results of 12% SDS-PAGE showed that the protein was expressed after 4 h postinduction, and the expression of the protein peaked when the final concentration of IPTG was 1 mM (Fig. 1a). After the IPTG concentration was optimized, the optimal expression condition was investigated by varying the induction temperature. Since the cell free extract of *E. coli* BL21 contained a large proportion of SsHSP14.1, the recombinant SsHSP14.1 produced in *E. coli* BL21 was mainly in the soluble form. As shown in Fig. 1b, the amount of expected protein and of the soluble fraction was higher when induced at 37 °C, compared to the induction at 30 °C or at 25 °C.

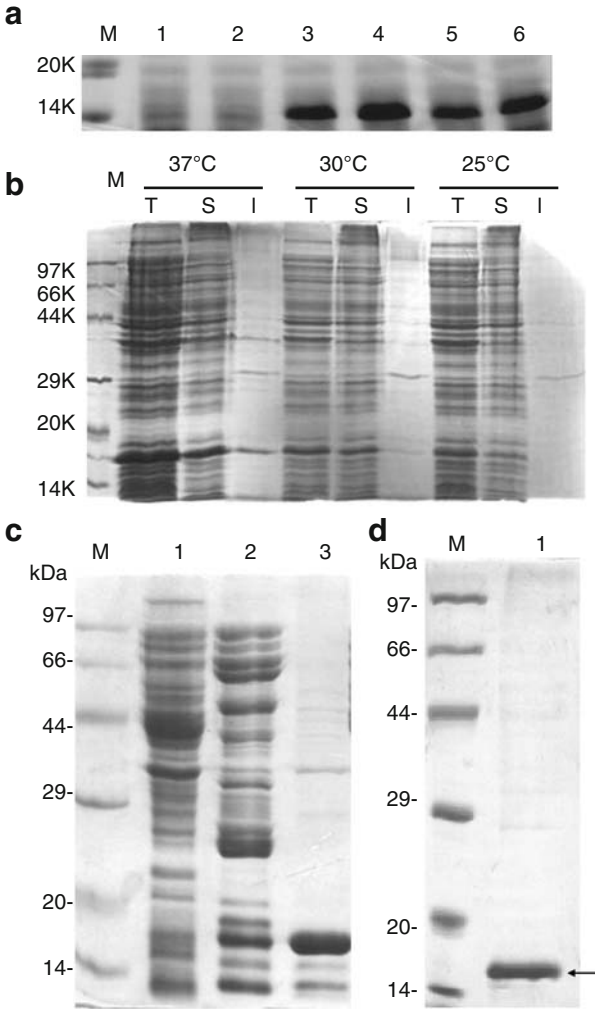

Fig. 1 SDS-PAGE analysis of expression and purification of recombinant SsHSP14.1. **a** Expression of SsHSP14.1 induced by IPTG of different concentration at 37 °C. *Lane 1* total protein of *E. coli* BL21(DE3) containing PHLK1 before induced with IPTG, *lanes 2–6* total protein of *E. coli* BL21(DE3) containing PHLK1 after induced with IPTG whose final concentration was 0.1, 0.5, 1.0, 1.5, and 2.0 mM, respectively. **b** Solubility of SsHSP14.1 expressed at different temperature. The letters *T*, *S*, and *I* represented the total, soluble, and insoluble proteins of BL21 harboring PHLK1 after induction with IPTG for 4 h, respectively. **c** Purified SsHSP14.1 by Ni^{2+} column. *Lane 1* penetrable apex, lanes 2–*3* purified SsHSP14.1 from soluble protein washed at 100 and 300 mM imidazole on Ni-NTA His Bind resin. **d** Gel-filtered SsHSP14.1 on a Sephadex G-75 column (the corresponding band of SsHSP14.1 was shown by *arrow*)

The result of the affinity chromatography showed that the contaminating proteins were almost fully removed in the wash buffer containing 100 mM imidazole while the fusion protein SsHSP14.1 was successfully purified to near homogeneity with buffer containing 300 mM imidazole (Fig. 1c). This fraction was then subjected to gel filtration on a Sephadex G-75 column. Fractions were collected and analyzed for protein purity (Fig. 1d).

SsHSP14.1 Improved the Thermotolerance of *E. coli* Cells at 50 °C

sHsps are proved to be able to confer thermotolerance to cells expressing sHsps in vivo [13]. To evaluate the liability of *E. coli* cells, the degree of proteins' aggregation was considered as a criterion [14]. The effects of expressing of SsHSP14.1 on soluble proteins of cell extracts of *E. coli* were investigated here. Results showed that after heating treatment at 50 °C for 4 h, the concentration (3.26 mg/ml) of the soluble proteins in cell extracts of *E. coli* expressing SsHSP14.1 was much higher than that of *E. coli* without SsHSP14.1 expressed (0.43 mg/ml; Fig. 2). These suggested that the expression of SsHSP14.1 was associated with an increase in the thermostability of soluble proteins, and it is reasonable to say that SsHSP14.1 can prevent heat-induced protein aggregation in vivo.

CALB is Protected from Aggregation by Purified SsHSP14.1 at High Temperature

To show that thermal protection was caused solely by SsHSP14.1 and not by induction of other *E. coli* proteins in response to SsHSP14.1 overexpression, SsHSP14.1 was purified to 95% homogeneity and added to purified CALB. As shown in Fig. 3, the incubation of CALB and SsHSP14.1 at a 1:1 molar monomer ratio at 50 °C for 20 min resulted in both SsHSP14.1, and a moiety of CALB remained soluble (Fig. 3, lane 4). When they were incubated at 60 °C, both SsHSP14.1 and a little of CALB remained soluble after 20 min (Fig. 3, lane 10).

As controls, the incubation at 60 °C of CALB alone resulted in the protein precipitation (Fig. 3, lane 7) while most of the protein precipitated when incubation was at 50 °C (Fig. 3, lane 2).

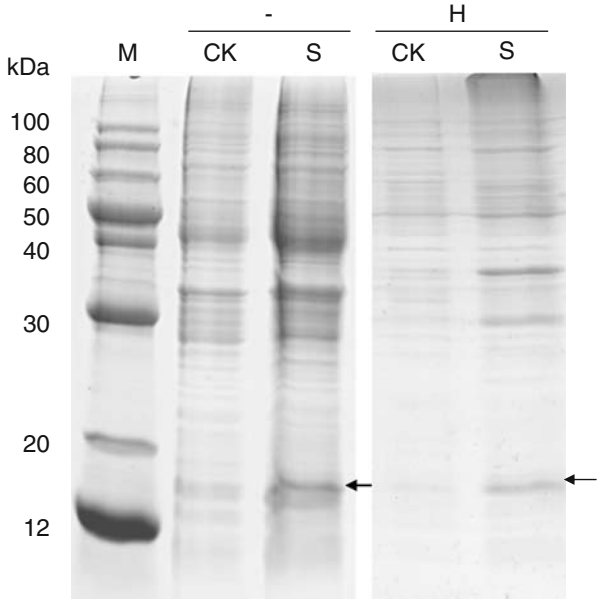

Fig. 2 Thermal stability of soluble protein of *E. coli* crude extract expressing SsHSP14.1. Soluble fraction of the crude extract of BL21 cells with (*S*) or without (*CK*) SsHSP14.1 overexpressed was analyzed, and the band of SsHSP14.1 was shown by an *arrow*. *Dash* stands for the samples before heat treatment, and *H* stands for those after being heated

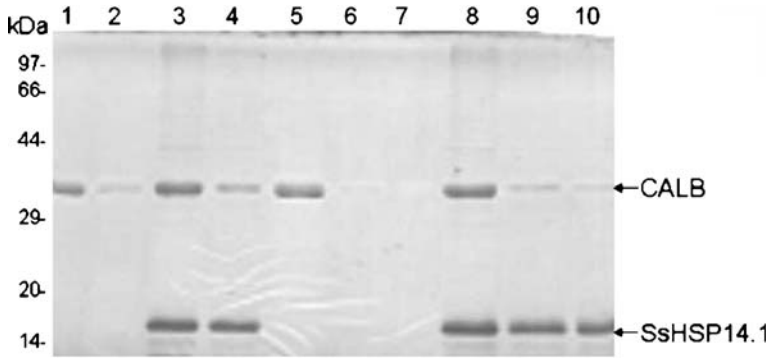

Fig. 3 Thermal protection of CALB by purified SsHSP14.1. *Lanes*: *1* and *5* CALB, unheated; *2* CALB, heated at 50 °C for 20 min, supernatant; *3* and *8* CALB:SsHSP14.1 (1:1 molar ratio), unheated; *4* CALB: SsHSP14.1 (1:1 molar ratio), heated at 50 °C for 20 min, supernatant; *6–7* CALB, heated at 60 °C for 10, 20 min, respectively, supernatant; *9–10* CALB:SsHSP14.1 (1:1 molar ratio), heated at 60 °C for 10 and 20 min, respectively, supernatant

Improving Bromelain Thermostability by SsHSP14.1

Since SsHSP14.1 can prevent aggregation of other proteins in response to heat stress, we addressed the question of whether or not it would extend the half-life ($t_{1/2}$) of a purified enzyme in vitro. Bromelain [15] was used as a model. Bromelain is a mesophilic enzyme with an optimal assay temperature of 37 °C and is inactivated rapidly at 55 °C. As shown in Fig. 4, bromelain rapidly lost 70% of its activity upon incubation at 55 °C for 20 min. In the presence of SsHSP14.1, however, the loss of enzyme activity was significantly diminished with nearly 80% of the bromelain activity being retained. This behavior was not observed

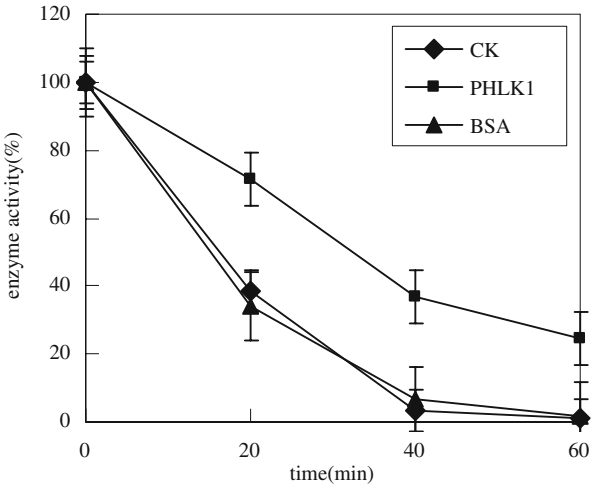

Fig. 4 Effects of SsHSP14.1 on bromelain incubated at 55 °C for up to 60 min. Activity of bromelain is shown in the presence (*square*) and absence (*diamond*) of SsHSP14.1 and in the presence (*triangle*) of BSA

for bovine serum albumin (BSA). It shows that the half-life of bromelain is lengthened, and its thermostability is improved.

Discussion

Several chaperones of the hyperthermophilic archaeon *S. solfataricus* exhibit activity in helping folding and preventing the aggregation of misfolded or partially denatured proteins [16–18]. The functions and regulation of archaeal sHsps remain unclear, although the sHsp from the hyperthermophilic methanogenic archaeon *Methanococcus jannaschii* has been cloned and expressed, and its crystal structure has been reported [19]. To date, there are few reports on the sHsp from *S. solfataricus*. The detail about the function of these sHsp is unknown. We assessed the chaperone-like activity of SsHSP14.1 by an in vivo and in vitro thermal aggregation assay, respectively, and by determining the thermostability of bromelain conferred by SsHSP14.1.

Under our experimental conditions, SsHSP14.1 decreased thermal aggregation of *E. coli* cell extract in vivo. SDS-PAGE analysis of these extracts showed that although many soluble proteins precipitated or were rapidly degraded in control cells during the heat shock, this effect was delayed and quantitatively less pronounced in *E. coli* expressing SsHSP14.1. Whether such stabilization was due directly to the chaperone function of sHsp or that the process of overexpressing SsHSP14.1 triggered the expression of unknown *E. coli* proteins (e.g., heat-induced chaperones) that were capable of protecting other *E. coli* proteins from heat denaturation remained to be determined.

Since purified CALB is denatured when heated at 60 °C for 20 min, it is a good substrate for in vitro studies of the role of SsHSP14.1 in preventing thermal aggregation. Consequently, we have shown that SsHSP14.1 can interact with purified CALB and prevent aggregation of this lipase at 60 °C. The result proves that SsHSP14.1 alone provides thermal protection to aggregation-prone proteins without the need for auxiliary proteins. This confirms that SsHSP14.1 has the same chaperone-like activity that has been shown for other sHsps from bacteria and eukaryotes [20]. SsHSP14.1 exhibited broad substrate specificity as shown by protection of the *E coli* cell extracts, in a manner similar to that of murine Hsp25 [21].

It has been proposed that sHsps associated with aggregated proteins change their physical properties in such a way that chaperone-mediated disaggregation and refolding become much more efficient [22, 23]. The genome analysis showed that there were two sHsps in *S. solfataricus*, and so, the influence of these two sHsps on the properties of aggregations formed under heat shock conditions, and the susceptibility of these aggregates to chaperone-dependent reactivation remained to be investigated.

The ability of SsHSP14.1 to suppress aggregation might be used to an advantage in the commercial production of recombinant proteins expressed in *E coli*, especially for the proteins which frequently aggregate into inclusion bodies. The overexpression of the bacterial chaperones GroEL and GroES has already been shown to be of some help in preventing inclusions [24, 25], but the general promise that sHsps were thought to hold has not been realized. The production of functional recombinant proteins may instead require the overproduction of SsHSP14.1 together with chaperones. Even if substrate proteins were only prevented from aggregating, it may be possible to induce them then to fold properly through the careful adjustment of the conditions following lysis.

SHsps are very stable in extreme conditions such as high temperatures. Here, we have investigated whether SsHSP14.1 could act as a protective molecule against thermal stress,

and we have indicated that SsHSP14.1 can protect the catalytic activity of enzymes against thermal stress in vitro. This effect was in striking contrast to the lack of effect observed for BSA. Whether SsHSP14.1 could improve thermostability of other enzymes in vitro remains unknown. At present, the improvement of thermostability of enzyme for industry application to enhance its rate of usage is desirable. Future studies will focus on the relationship between enzyme thermostability and SsHSP14.1.

The in vivo and in vitro demonstration that SsHSP14.1 from the hyperthermophilic archaeon *S. solfataricus* prevented thermal aggregation and improved the thermostability of enzymes in vitro were consistent with the roles played by other sHsps [26]. Some of the most fundamental aspects of SsHSP14.1, such as its structure and regulation, still remain to be elucidated. Trent et al. found that the chaperone TF55 from *Sulfolobus shibatae* could bind a denatured protein at 25 °C as efficiently as at 70 °C [27]. Nevertheless, as our understanding of these concrete aspects continue to grow, and the prospects for harnessing its properties for promising biological and commercial applications become ever brighter.

Acknowledgment This work was made possible with funding provided by National Natural Science Foundation of China (20706021) and the Research Fund for the Doctoral Program of Higher Education (20070561073).

References

1. Narberhaus, F. (2002). α-Crystallin-type heat shock proteins: socializing minichaperones in the context of a multichaperone network. *Microbiology and Molecular Biology Reviews, 66*, 64–93. doi:10.1128/MMBR.66.1.64-93.2002.
2. Horwitz, J. (1992). α-Crystallin can function as a molecular chaperone. *Proceedings of the National Academy of Sciences, 89*, 10449–10453.
3. Rosalind, K., Kyeong, K. K., & Hisao, Y. (1998). Small heat shock protein of *Methanococcus jannaschii*, a hyperthermophile. *Proceedings of the National Academy of Sciences, 95*, 9129–9133.
4. Alvaro, S., Isabel, A., & Carmen, C. (1999). Heterologous expression of a plant small heat-shock protein enhances *Escherichia coli* viability under heat and cold stress. *Plant Physiology, 120*, 521–528. doi:10.1104/pp.120.2.521.
5. Caspers, G., Leunissen, J., & De, J. (1995). The expanding small heat-shock protein family, and structure predictions of the conserved "α-crystallin domain". *Journal of Molecular Evolution, 40*, 238–248. doi:10.1007/BF00163229.
6. Sun, Y., & Macrae, T. (2005). Small heat shock proteins: molecular structure and chaperone function. *Cellular and Molecular Life Sciences, 62*, 2460–2476. doi:10.1007/s00018-005-5190-4.
7. Haslbeck, M., Franzmann, T., & Weinfurtner, D. (2005). Some like it hot: the structure and function of small heat-shock proteins. *Nature Structural & Molecular Biology, 12*, 842–846. doi:10.1038/nsmb993 Perspective.
8. Nakamoto, H., & Vigh, L. (2007). The small heat shock proteins and their clients. *Cellular and Molecular Life Sciences, 64*, 294–306. doi:10.1007/s00018-006-6321-2.
9. Mee-Jung, H., Hongseok, Y., & Sang, Y. L. (2008). Microbial small heat shock proteins and their use in biotechnology. *Biotechnology Advances, 26*, 591–609. doi:10.1016/j.biotechadv.2008.08.004.
10. Qunxin, S., Ramak, S., & Fabrice, C. (2001). The complete genome of the crenarchaeon *sulfolobus solfataricus* P2. *Proceedings of the National Academy of Sciences, 98*, 7835–7840. doi:10.1073/pnas.141222098.
11. Sambrook, J., Fritsch, E. F., & Maniatis, T. (2002). *Molecular cloning: A laboratory manual* (3rd ed.). Peking: Science press.
12. Takashi, M., & Hans, N. (1960). Fractionation and specificity studies on stem bromelain. *Journal of Biological Chemistry, 235*, 99–107.
13. Muchowski, P. J., & Clark, J. I. (1998). ATP-enhanced molecular chaperone functions of the small heat shock protein human αB-crystallin. *Proceedings of the National Academy of Science, 95*, 1004–1009.

14. Satoru, T. (2006). Analytical assays of human HSP27 and thermal-stress survival of *Escherichia coli* cells that overexpress it. *Biochemical and Biophysical Research Communication, 341*, 1252–1256. doi:10.1016/j.bbrc.2006.01.090.
15. Nakamoto, H., Suzuki, N., & Roy, S. K. (2002). Constitutive expression of a small heat-shock protein confers cellular thermotolerance and thermal protection to the photosynthetic apparatus in cyanobacteria. *FEBS Letters, 483*, 169–174. doi:10.1016/S0014-5793(00)02097-4.
16. Ivano, C., Davide, R., & Richard, R. (1998). A novel aminopeptidase associated with the 60 kDa chaperonin in the thermophilic archaeon *sulfolobus solfataricus*. *Molecular Microbiology, 29*, 775–785. doi:10.1046/j.1365-2958.1998.00971.
17. Annamaria, G., Laura, C., & Mose, R. (1995). Prevention of in vitro protein thermal aggregation by the *sulfolobus solfataricus* chaperonin. *Journal of Biological Chemistry, 270*, 28126–28132. doi:10.1074/jbc.270.47.28126.
18. Annamaria, G., Laura, C., & Simonetta, B. (1994). The chaperonin from the archaeon *sulfolobus sofataricus* promotes correct refolding and prevents thermal denaturation in vitro. *Protein Science, 3*, 1436–1443. doi:10.1002/pro.5560030910.
19. Kyeong, K. K., Rosalind, K., & Sung-Hou, K. (1998). Crystal structure of a small heat shock protein. *Nature, 394*, 595–599. doi:10.1038/29106.
20. Zhen, Y., Shinesuke, F., & Katsunori, K. (1997). In vitro stabilization and in vivo solubilization of foreign proteins by the subunit of a chaperonin from the hyperthermophilic archaeon pyrococcus sp. Strain KOD1. *Applied and Environment Microbiology, 63*, 785–789.
21. Ehrnsperger, M., Graber, S., & Gaestel, M. (1997). Binding of non-native protein to Hsp25 during heat shock creates a reservoir of folding intermediates for reactivation. *The Embo Journal, 16*, 221–229. doi:10.1093/emboj/16.2.221 Article.
22. Laskowska, E., Kuczynska-Wisnik, D., & Taylor, A. (1996). Degradation by proteases Lon, Clp and HtrA, of *Escherichia coli* proteins aggregated in vivo by heat shock, HtrA protease action in vivo and in vitro. *Molecular Microbiology, 22*, 555–571. doi:10.1046/j.1365-2958.1996.1231493.
23. Zolkiewski, M. (1999). ClpB cooperates with DnaK, DnaJ, and GrpE in suppressing protein aggregation. A novel multi-chaperone system from *Escherichia coli*. *Journal of Biological Chemistry, 274*, 28083–28086. doi:10.1074/jbc.274.40.28083.
24. Philip, A. C. (1996). Chaperone-assisted protein expression. *Structure, 4*, 239–242. doi:10.1016/S0969-2126(96)00028-7.
25. Chin-Kai, C., Yu-Show, S., & Chiu-Tin, F. (2009). A dual-functional *E. coli* vector for expressing recombinant protein with high solubility and antigen presentation ability. *Protein Expression and Purification*, 51-56. doi:10.1016/j.pep.2008.12.011.
26. Pongpan, L., Dennis, L. M., & Frank, T. R. (2001). Regulation and mechanism of action of the small heat shock protein from the hyperthermophilic archaeon *Pyrococcus furiosus*. *The Journal of Bacteriology, 183*, 5198–5202. doi:10.1128/JB.183.17.5198-5202.2001.
27. Trent, J. D., Nimmesgern, E., & Wall, J. S. (1991). A molecular chaperone from a thermophilic archaebacterium is related to the eukaryotic protein-complex polypeptide-1. *Nature, 354*, 490–493. doi:10.1038/354490a0.