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Inhibition of Citrate Synthase Thermal Aggregation *In Vitro* by Recombinant Small Heat Shock Proteins

Gong, Weina[1,2], Ming Yue[3], Bingyan Xie[2], Fanghao Wan[1]*, and Jianying Guo[1]

[1]*State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100081, China*
[2]*Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing 100081, China*
[3]*Laboratory of Feed Ecology and Biotechnology, Feed Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, China*

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Small heat shock proteins (sHSPs) function as molecular chaperones that protect cells against environmental stresses. In the present study, the genes of *hsp*17.6 and *hsp*17.7, cytosolic class I sHSPs, were cloned from a tropical plant, *Ageratina adenophorum*. Their C-terminal domains were highly conserved with those of sHSPs from other plants, indicating the importance of the C-terminal domains for the structure and activity of sHSPs. The recombinant HSP17.6 and HSP17.7 were applied to determine their chaperone function. *In vitro*, HSP17.6 and HSP17.7 actively participated in the refolding of the model substrate citrate synthase (CS) and effectively prevented the thermal aggregation of CS at 45°C and the irreversible inactivation of CS at 38°C at stoichiometric levels. The prior presence of HSP17.7 was assumed to suppress the thermal aggregation of the model substrate CS. Therefore, this report confirms the chaperone activity of HSP17.6 and HSP17.7 and their potential as a protectant for active proteins.

Keywords: Citrate synthase, *Escherichia coli*, small heat shock proteins, thermal aggregation

Small heat shock proteins (sHSPs) are molecular chaperones that are readily induced by a ubiquitous heat shock response in both prokaryotic and eukaryotic cells [21, 30]. sHSP monomers range from 15 to 30 kDa and assemble into large multimers [10, 33]. sHSPs also form large homooligomers both *in vitro* and *in vivo* [12, 17, 33]. The proteins encoded by the different sHSP gene families are found in all the major cellular compartments, with two distinct sHSP families in the cytosol (cytosolic I and II) and other sHSPs found in the mitochondrion (MT), chloroplast (CP), and endomembrane system (ER) [5, 27].

sHSPs are the most prominent component of heat shock response [13, 18, 22] and play a particularly important role, as they maintain a state of solubility and prevent unwanted proteins from aggregating within cells [20, 28]. Consistent with their chaperone function, sHSPs are associated with protein aggregates in a number of human diseases, including cataracts, neurogenerative diseases, and myopathies [3, 29]. Native plant sHSPs tend to be stored as oligomeric complexes with molecular masses between 200 and 240 kDa, such as At-HSP17.6A [26, 30], and probably composed of 12 subunits [17]. Upon heat shock, the oligomeric storage form of sHSPs dissociates into smaller complexes to bind with non-native proteins, thereby preventing their aggregation [9, 19].

sHSPs are believed to play a role in thermal protection, as their overexpression has been shown to be correlated with the acquisition of thermotolerance by their host cells [15, 24, 34]. For example, the thermostabilization of soluble proteins has been demonstrated *in vitro* with an sHSP-enriched fraction [15]. Moreover, these sHSP-enriched fractions are exchangeable in providing protein thermostability [14], and increased expression of these ubiquitous stress proteins have been demonstrated to enhance cellular tolerance to heat stress [8].

To understand how these chaperones participate in diverse cellular processes, a model substrate, pig heart citrate synthase (CS), a dimer with identical 43.5 kDa subunits, has recently been widely used in chaperone assays [23]. Well characterized in terms of its thermostability and unfolding, the atomic structure of CS has also been determined for both thermosensitive and thermostable forms [1, 16, 31]. *In vitro*, the aggregation of CS during

*Corresponding author
Phone: +86-10-8210-9572; Fax: +86-10-8210-5927;
E-mail: wanfh@mail.caas.net.cn

refolding or unfolding can be readily monitored by following the increase in light scattering in a solution [3]. Thus, when combined with *in vitro* assays, the function of sHSPs under stress conditions can be effectively demonstrated [17].

Accordingly, in this study, HSP17.6 and HSP17.7 from the tropical plant *Ageratina adenophora* were expressed in *Escherichia coli* and purified. Their effects on protein thermal aggregation and denaturation were then monitored. The recombinant HSP17.6 and HSP17.7 both inhibited CS aggregation during heat stress by binding with non-native proteins as a molecular chaperone, indicating their potential application as a protein protectant.

Materials and Methods

Enzymes and Reagents

The pig heart citrate synthase, lysozyme, Tris, and HEPES were obtained from the Sigma Chemical Company (St. Louis, U.S.A.). All the other chemicals were of analytical grade.

Plant Materials and Bacterial Strains

Seeds of *A. adenophorum* were obtained from Yunnan Province, China. The seeds were grown in a greenhouse until the seedlings were three months old. Fresh leaf tissue was then collected, frozen in liquid nitrogen, and stored at −70°C until use.

Escherichia coli (*E. coli*) strain Top10 was used to construct the recombinant plasmids, and *E. coli* strain BL21 (DE3) plysS (Novagen, Germany) was used as the expression host for the recombinant proteins.

Cloning of *hsp*17.6 and *hsp*17.7 Genes

The core region of *hsp*17.6 was amplified using the degenerate primers hspF1 and hspR1 (Table 1), designed according to the conserved regions in several plants. The 3' and 5' flanking sequences of the core region were amplified using a SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, U.S.A.) and in cooperation with the corresponding GSP primers (Table 1).

The full length of the *hsp*17.6 open reading frame (ORF) was amplified using the primers hspF2 and hspR2 (Table 1; EcoRI and NotI sites are underlined) according to the assembled sequences obtained above. Meanwhile, the full length of the *hsp*17.7 ORF was amplified using the primers hspF3 and hspR3 (Table 1; EcoRI and NotI sites are underlined) corresponding to the sequence published in the NCBI database (GenBank EF105483).

Expression Vector Constructs

The PCR products were both subsequently digested with EcoRI and NotI, and inserted into the same digested pET-30a (+) vector (Cat No. 69909-3; Novagen, Darmstadt, Germany) to generate pET-30a-*hsp*17.6 and pET-30a-*hsp*17.7. All the constructs were verified by DNA sequencing. The purified pET-30a-*hsp*17.6 and pET-30a-*hsp*17.7 plasmids were then transformed into *E. coli* BL21 (DE3) pLysS for expression.

Protein Expression and Purification of Recombinant HSP17.6 and HSP17.7

The two recombinant strains were both cultured in 1 l of an LB medium containing 50 μg/ml kanamycin and 34 μg/ml chloramphenicol with vigorous shaking at 37°C until the OD_{600} reached 0.6. A final concentration of 1.0 mM of isopropyl-β-D-1-thiogalactopyranoside (IPTG) was then added, followed by incubation at 220 rpm and 37°C for 4 h. The cells were harvested by centrifugation at 4,000 rpm for 15 min at 4°C and resuspended in 250 ml of a 100 mM potassium phosphate buffer (pH 7.5). Thereafter, the cells were disrupted by sonication and any insoluble cell debris was removed by centrifugation at 12,000 rpm for 20 min at 4°C. The supernatant fraction of the cell crude extract was loaded onto a Ni-NTA column according to the manufacturer's purification protocol manual (Novagen, Ni-NTA His•Bind Resins). The His-tag fusion proteins of HSP17.6 and HSP17.7 were then eluted using a wash buffer containing 200 mM imidazole, the purified protein was analyzed by 12.5% (w/w) SDS−PAGE, and the protein concentration was determined using the method of Bradford with bovine serum albumin (BSA) as the standard.

Thermal Aggregation Experiments

120 nM pig heart CS was inoculated with varying amounts of HSP17.6 or HSP17.7 in a buffer containing 50 mM HEPES-KOH (pH 7.5; total volume, 1 ml) in covered cuvettes in a water bath at 45°C for 60 min. Lysozyme was added at a concentration of 50 μg/ml in the absence of HSP17.6, HSP17.7, and the mixture. At the times indicated, the aggregation of CS upon thermal denaturation was determined by measuring the absorption due to increased turbidity from light scattering at 320 nm in a spectrophotometer. Each reaction was performed in three parallel replicate trials.

Table 1. Nucleotide sequence of primers used in this study.

Primers	Sequence	Size
hspF1	5'-CGAAAC(T/G)CCAGAAGCTCATGT-3'	21 bp
hspR1	5'-TGCAAC(T/G)CCAGAAGCTCATGT-3'	21 bp
3'GSP1	5'-AACACCAGAAGCTCATGTGT-3'	20 bp
3'GSP2	5'-CCGATCTTCCTGGAATCAAG-3'	20 bp
5'GSP1	5'-GGCCTTGAACACATGAGCTTCTG-3'	23 bp
5'GSP2	5'-CCTGTCGTCTTCAACCTCCACCT-3'	23 bp
hspF2	5'-GAATTCATGCCGATCGTCCCAAGCTT-3'	26 bp
hspR2	5'-GCGGCCGCCTAACCATTGATTTCAATGGATTTG-3'	33 bp
hspF3	5'-GAATTCATGTCGATCATTCCAAGCTTC-3'	27 bp
hspR3	5'-GCGGCCGCTTAGCCCGAAATGTCGAT-3'	26 bp

Thermal Inactivation Experiments

CS (120 nM) was inoculated, in the absence or presence of 600 nM HSP17.6, HSP17.7, or 50 μg/ml lysozyme, into a buffer containing 50 mM HEPES-KOH (pH 8; total volume, 1 ml) in covered cuvettes at 38°C in a water bath. The CS was then incubated at 38°C for 60 min and 25°C for 60 min. At various time points, 100-μl aliquot samples were removed and the CS activity was measured as described previously [17]. All the experiments were repeated at least three times.

Nucleotide Sequence Accession Number

The nucleotide sequence of *hsp*17.6 has been submitted to the GenBank under Accession No. EU269067.

RESULTS

Protein Sequence Comparisons and Identification of HSP17.6 and HSP17.7

The full length of the *hsp*17.6 gene was 468 bp coding 155 amino acids, and the full length of the *hsp*17.7 gene was 474 bp coding 157 amino acids. Their identity was 75%, indicating varying levels of amino acid conservation with the encoding sequence of sHSPs.

The deduced polypeptides were most identical to cytosolic class I sHSPs. Based on alignment with representative

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HSP17.7     MSIIPSFFTGRRSN-VFDPFSIEIWDPFQG--FSSAISNLPESSRETAAIANARIDWKET 57
HaHSP17.7   MSIIPSFFTGNGSN-IFDPFSSEIWDPFQG--LSSVINNLPESSRETTAIANTRIDWKET 57
NtHSP18     MAMIPSFFGGRRSN-IFDPFSLDIFDPFEGFPFSGTVANVPSSARETSAFANARIDWKET 59
HSP17.6     MPIVPSLFGGRRSN-IFDPLSFDVWDPFKDFPFPSSSI----VSNETSGFVNARVDWKET 55
HaHSP17.6   MSIIPSLFAGRRSS-VFDPFSLDVWDPFRDFPISSSSD----VSRETSALVNARVDWKET 55
GmHSP17.5   MSLIPSIFGGRRSN-VFDPFSLDVWDPFKDFHFPTS------LSAENSAFVNTRVDWKET 53
LeHSP17.6   MSLIPRIFGDRRSSSMFDPFSIDVFDPFRELGFPGTN------SGESSAFANTRIDWKET 54
AtHSP17.6   MSLIPSIFGGRRTN-VFDPFSLDVFDPFEGFLTPSGLAN--APAMDVAAFTNAKVDWRET 57
            *.::* :* .. :. :***:* :::***.    .         : : :.:.*:::**:**
Con-Type              S..V..PF...V
                      Class I region
HSP17.7     PEAHVFKADLPGLKKEEVKVEVEEGRVLQISGERSKENEEKNEKWHRVERSSGKFVRRFR 117
HaHSP17.7   PEAHVFKADLPGLKKEEVKVEVEEGRVLQISGERSRENVEKNDKWHRMERSSGKFLRRFR 117
NtHSP18     PDSHIFKMDVPGIKKEEVKVEVEEGRVLQISGERSREQEEKNDTWHRMERSSGKFMRRFR 119
HSP17.6     PEAHVFKADLPGIKKEEVKVEVEDDRVLQITGERNVEKEDKNDKWHRIERSSGKFTKRFR 115
HaHSP17.6   PEAHVFKADLPGIKKEEVKVEVEDGNILQITGERNVEKEDKNDKWHRVERSSGKFTRRFR 115
GmHSP17.5   PEAHVFEADIPGLKKEEVKVQIEDDRVLQISGERNLEKEDKNDTWHRVERSSGNFMRRFR 113
LeHSP17.6   PEAHVFKADLPGLKKEEVKVEVEEDRVLQISGERNVEKEDKNDKWHRVERSSGKFMRRFR 114
AtHSP17.6   PEAHVFKADLPGLRKEEVKVEVEDGNILQISGERSNENEEKNDKWHRVERSSGKFTRRFR 117
            *::*:*: *:**::******::*:..:***:***. *: :**:.***:*****:* :***
Con-Type            DLPGL.KEEV.V.V.....L.ISG.R                   F.RRF.
                    sHSP region II
HSP17.7     LPENAKLEGVKAAMENGVLTVTVPKAEEKKPEVKSIDISG 157
HaHSP17.7   LPENAKMDQVKAAMENGVLTVTVPKAEVKKPEVKAIDISG 157
NtHSP18     LPGNAKMEEIKAAMENGVLTVTVPKEEEKKSEVKAIDISG 159
HSP17.6     LPENAKLDQVKAAMENGVLTITVPKEEVKKTDVKSIEING 155
HaHSP17.6   LPENAKMDQVKAAMENGVLTITVPKEEVKKPDVKSIEISG 155
GmHSP17.5   LPENAKVEQVKASMENGVLTVTVPKEEVKKPDVKAIEISG 153
LeHSP17.6   LPENAKMDQVKASMENGVLTVTVPKEEVKKPEVKSIEISG 154
AtHSP17.6   LPENAKMEEIKASMENGVLSVTVPKVPEKKPEVKSIDISG 157
            ** ***:: :**:******::****   **.:**:*:*.*
Con-Type    .........V.A.M.NGVLTV.VPK
            sHSP region I
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Fig. 1. Multiple sequence alignment of sHSPs.

HSP17.7, *A. adenophora* HSP17.7(ABL10073); HaHSP17.7, *Helianthus annuus* HSP17.7 (AAB63311); NtHSP18, *Nicotiana tabacum* HSP18 (CAA50022); HaHSP17.6, *Helianthus annuus* HSP17.6 (CAC84406); HSP17.6, *A. adenophora* HSP17.6 (EU269067); GmHSP17.5, *Glycine max* HSP17.5 (P04793); LeHSp17.6, *Lycopersicon esculentum* HSP17.6 (AAD30454); AtHSp17.6, *Arabidopsis thaliana* HSP17.6 (AAF78436). The identified class I region, conserved regions, I and II, are in underline. (-) no amino acid residue; (*) identical residues; (:) conserved substitution; (.) semiconserved substitution.

sequences of many available sHSPs, HSP17.6 and HSP17.7 were both found to be composed of consensus regions I and II at the C-terminal, which are considered conserved domains for sHSPs (Fig. 1). Within consensus I, the residues were composed of Pro-X(14)-Gly-Val-Leu, which is a typical signature for almost all sHSPs. Interestingly, a similar motif, Pro-X(14)-X-Val/Leu/Ile-Val/Leu/Ile, also appeared in the consensus II region. The most conserved part, known as the α-crystalline domain, was close to the C-terminus, suggesting a role in the chaperone function (*i.e.*, interaction with partially unfolded proteins).

Expression and Purification of Recombinant HSP17.6 and HSP17.7

The amounts of 33.6 mg of HSP17.6 and 20.2 mg of HSP17.7 were purified from 1 l of each respective cell culture using a Ni-NTA column. The final preparation gave two distinct bands on SDS–PAGE with close molecular masses of approximately 23 kDa for HSP17.6 and HSP17.7 (Fig. 2). The recombinant HSPs were both 6.0 kDa heavier than predicted, which resulted from 52 extra amino acids at the N-terminus, including a 6×His-tag from the forward insert site EcoRI.

Prevention of CS Thermal Aggregation

Normally, CS is rapidly inactivated and aggregated at 45°C. When heated to 45°C, CS began to form insoluble aggregates that could be detected by light scattering (Fig. 3A–3B). HSP 17.6 and HSP 17.7 prevented this process at different ratios.

As determined by the absorption, the increasing trend slowed down at different HSP17.6/HSP17.7-to-CS ratios after 60 min at 45°C, indicating a reduction in the aggregation of CS. Thus, the aggregation was reduced in the presence

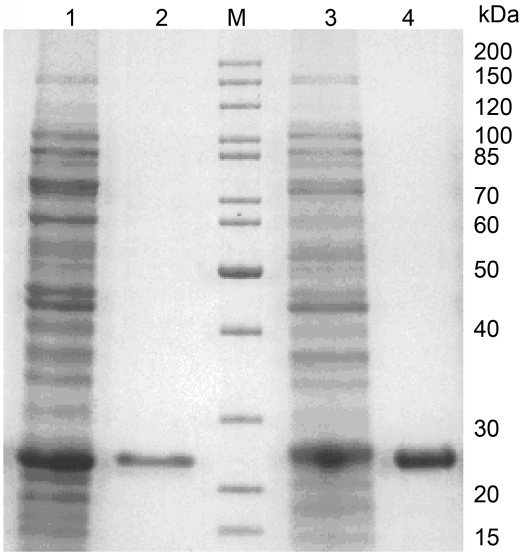

Fig. 2. SDS–PAGE analysis of expression and purification of HSP17.6 and HSP17.7 in *E.coli*.
Lane 1, crude extract of HSP17.6; lane 2, purified HSP17.6; lane M, protein standard; lane 4, crude extract of HSP17.7; lane 5, purified HSP17.7.

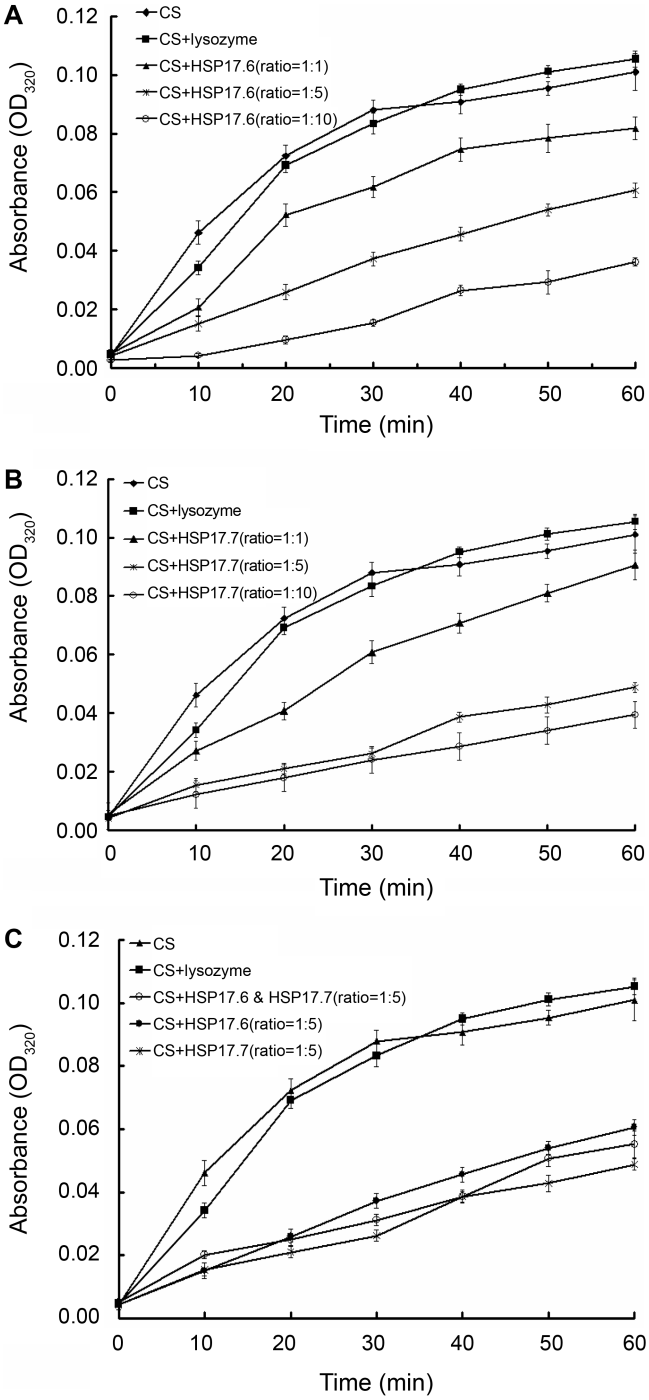

Fig. 3. Effects of HSP17.6 and HSP17.7 on thermal aggregation of CS.
CS monomers (120 nM) were incubated at 45°C in the absence or presence of different amounts of HSP17.6 (**A**) and HSP17.7 (**B**) at HSP17.6/HSP17.7-to-CS ratios of 1:1, 5:1, or 10:1, and mixture with HSP17.6 and HSP17.7 at sHSP-to-CS ratio of 5:1 (**C**). Data shown are mean values, with bars indicating the SD of the mean (n=3).

of the sHSPs and clearly inhibited at an HSP17.6-to-CS monomer ratio of 5:1 (Fig. 3A). The addition of 50 μg/ml lysozyme to the CS had no protective effect. Similarly, the suppression of CS aggregation by HSP17.6 at 45°C was observed at an HSP17.7-to-CS monomer ratio of 5:1, although HSP17.6 was far less efficient (Fig. 3B). Beyond this, increasing the amount of HSP17.7 had no further protective effect. A higher HSP17.7-to-CS monomer ratio of 10:1 only had a slight impact on preventing higher CS scattering when compared with the ratio of 5:1 (Fig. 3B). The maximal suppression by the HSP17.6/HSP17.7-to-CS monomer was also observed at a ratio of 5:1.

The addition of a mixture of HSP17.6 and HSP17.7 at an sHSP-to-CS monomer ratio of 5:1 enhanced the suppression of CS aggregation (Fig. 3C). However, when adding the mixture to the assay, although the CS protection was even better, the effect of the protection was not as strong as the same monomer ratio of HSP17.7.

Protection of CS from Thermal Inactivation

The effect of the recombinant HSP17.6 and HSP17.7 on the thermal inactivation of CS was investigated. When the CS was incubated at 38°C alone or in the presence of 50 μg/ml lysozyme, less than 12% of CS activity remained after 60 min (Fig. 4). To test the potential of the heat-inactivated CS to properly refold at a permissive temperature, the temperature was shifted to 25°C after 60 min of incubation at 38°C. After shifting the temperature of the samples to 25°C, the CS activity remained low after 60 min in the presence or absence of 50 μg/ml lysozyme.

In contrast, when the CS was similarly treated in the presence of HSP17.7 at an HSP17.7-to-CS monomer ratio of 5:1, the maximal CS activity at 38°C was 61%, and after 60 min at 25°C, approximately 87% of the original activity was regained. Under the same conditions, in the presence of HSP17.6, 42% of the CS activity was protected at 38°C and 67% of the original activity was regained (Fig. 4). No CS reactivation was detected after shifting the samples from 45°C to 25°C in the presence of either HSP17.6 or HSP17.7 (data not shown). As the CS experienced quantitative aggregation at 45°C, the reactivating effect of the sHSPs could be observed below 45°C [4, 17].

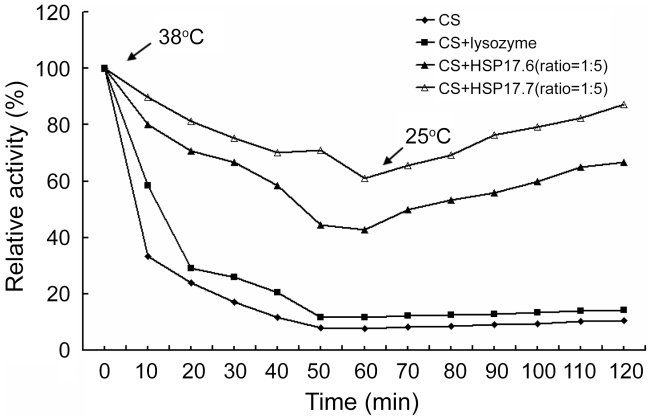

Fig. 4. Effects of HSP17.6 and HSP17.7 on thermal inactivation of CS.

CS monomers (120 nM) were incubated in the absence or presence of 50 μg/ml lysozyme, HSP17.6, and HSP17.7 at a HSP17.6/HSP17.7-to-CS ratio of 5:1. The activity of CS is expressed as the percentage of non-treated control. Data shown are mean values, with bars indicating the SD of the mean (n=3).

DISCUSSION

In this study, the C-terminal region was much more conserved than the N-terminal region in HSP17.6 and HSP17.7. However, a range of studies have indicated that the evolutionarily variable N-terminal arm is essential for substrate interaction [2, 11]. Moreover, the efficiency of the protein protection has been reported to be determined by the identity of the N-terminal arm in sHSPs for CS [3]. Thus, based on these findings, although HSP17.6 and HSP17.7 shared common features, such as a 75% amino acid similarity, it is likely that a similar divergence of the N-terminus determined their similar function, such as the thermal protective effect.

The ability of HSP17.6 and HSP17.7 to prevent thermal aggregation of the model substrate provides further support for their putative role in thermotolerance. The kinetics of the aggregation reactions, as monitored by the increase of absorbance, differed with various amounts of sHSPs, suggesting an efficient sHSP influence (Fig. 3A–3C). As previously reported, sHSPs are not only able to influence the folding and aggregation of CS, but also other substrates, such as firefly luciferase and malate dehydrogenase (MDH) [16, 20]. It has been suggested that sHSPs act universally *in vivo* to bind partially denatured proteins and thus prevent irreversible protein aggregation [32]. In the present study, despite their limited sequence homology, similar results were obtained when using a mixture of HSP17.6 and HSP17.7 to determine the thermal protection, thereby displaying the conservation of the functional properties among plant sHSPs.

The HSP17.6 and HSP17.7 activity remained stable over a relatively wide range of ratios. The aggregation was reduced in the presence of the sHSPs and completely suppressed at a molar ratio of 5:1 HSP17.6 or HSP17.7 to CS, similar to HSP18.1 at a molar ratio of 6:1 [3]. Owing to the interaction between the sHSPs and the substrates based on the binding sites, the molar ratio of the different sHSPs varied significantly. The efficiency of CS protection from thermal aggregation is determined by the identity of the N-terminal arm in sHSP–substrate proteins [2, 3, 7]. Thus, in this study, the identified divergence of the N-terminal in the sHSPs (Fig. 1) suggested differences in the

chaperone activity of the sHSPs for CS. Moreover, the fused extra residues may have partly affected the specificity of the sHSP–substrate interactions. A more precise molar ratio may be determined in future to build the complex structure of sHSPs and substrates during the interaction.

sHSPs have been reported to hold substrates (such as CS) in a folding-competent state until the temperature is lowered, at which point unfolded intermediates are formed [6, 17]. Through the *in vitro* assay, it was found that HSP17.6 and HSP17.7 displayed a similar behavior in protecting CS from irreversible heat inactivation. These findings are comparable to those described for HSP17.7 and HSP18.1 from peas [17]. The predominant effect of the sHSPs was to increase the potential of the heat-inactivated CS to refold properly at a permissive temperature of 25°C. The present data suggest that the presence of the sHSPs may have had important consequences during both the heat stress and the recovery. These results are also consistent with the observation that HSP17.6 and HSP17.7 were abundant in the *A. adenophora* tissue during and after heat stress (data not shown). Thus, HSP17.6 or HSP17.7 may have bound with CS to prevent the formation of large aggregates and the deterioration of transparency. In addition, the inactivated CS was more significantly reduced by HSP17.7 than by HSP17.6, suggesting that HSP17.7 may have been present prior to the high temperature. The greater ability of HSP17.7 to bind to CS may depend on the features of the sHSP–substrate interaction, which can be clarified by visualizing the complexes formed between the CS and the sHSPs [25].

Collectively, the current results indicated that HSP17.6 and HSP17.7 would appear to function as chaperones to other active proteins. Moreover, the present data provide evidence of cytosolic class I sHSP inhibition of active protein thermal aggregation, as well as irreversible inactivation in *in vitro* assays. Thus, the *in vitro* inhibition properties of the recombinant HSP17.6 and HSP17.7 indicate their potential as protein protectants for active protein applications, such as enzymes. However, elucidation of the protein protectant mechanism still requires further study.

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