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REGULAR PAPER

Molecular chaperone activity of tomato (*Lycopersicon esculentum*) endoplasmic reticulum-located small heat shock protein

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Abstract The gene encoding the small heat shock protein (sHSP), LeHSP21.5, has been previously cloned from tomato (GenBank accession no. *AB026983*). The deduced amino acid sequence of this tomato sHSP was most similar to that of other endoplasmic reticulum (ER)-localized sHSPs (ER-sHSP) and can be predicted to target the ER. We examined whether the gene product of LeHSP21.5 (probable ER-sHSP) can act as molecular chaperone. For functional analysis, LeHSP21.5 protein was expressed in *Escherichia coli* as His_6-tagged protein in the C-terminal and purified. We confirmed that ER-sHSP could provide thermal protection of soluble proteins in vitro. We compared the thermal stability of *E. coli* strain BL21 (DE3) transformed with pET-ER-sHSP with the control *E. coli* strain BL21(DE3) transformed with only the pET vector under heat shock and IPTG-induced conditions. Most of the protein extracts from *E. coli* cells expressing ER-sHSP were protected from heat-induced denaturation, whereas extracts from cells not expressing ER-sHSP were very heat-sensitive under these conditions. A similar protective effect was observed when purified ER-sHSP was added to an *E. coli* cell extract. ER-sHSP prevented the thermal aggregation and inactivation of citrate synthase. These collective findings indicate that ER-sHSP can function as a molecular chaperone in vitro.

Keywords Endoplasmic reticulum · *Lycopersicon esculentum* · Microsome · Molecular chaperone · Small heat shock protein

Abbreviations

CS	citrate synthase
ER-sHSP	endoplasmic reticulum-located small heat shock protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IPTG	isopropyl-1-thio-β-D-galacto-pyranoside
LDH	lactate dehydrogenase
ME	mercaptoethanol
PVPP	Polyvinylpolypyrrolidone
sHSP(s)	small heat shock protein(s)

Introduction

All organisms respond to high temperature by synthesizing a group of proteins called heat shock proteins (HSPs). Two species of small heat shock proteins (sHSPs) have been identified in humans, mice, and yeast; they have been localized in the cytosol (Arrigo and Landry 1994) and mitochondria (Morrow et al. 2000). sHSPs are abundant in plants and at least six classes (Scharf et al. 2001; Waters et al. 1996) have been identified in accordance to their amino-acid sequence, immunological cross-reactivity and

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intracellular localization (Waters et al. 1996). Three of the classes are found in the cytoplasm or nucleus (cytosolic I, II, and III sHSPs), while the other three classes are localized in the plastid, chloroplast, mitochondria, and endoplasmic reticulum (ER) (Sun et al. 2002). The abundance, intracellular distribution, and sequence conservation across divergent plant genera of the sHSPs suggest that they play an important part in thermotolerance (Sanmiya et al. 2004; Vierling et al. 1988; Yeh et al. 1997).

Several sHSPs have recently been detected in special plant organs, such as maturing pollen, grains, developing embryos (Wehmeyer et al. 1996), growing fruits (Sabehat et al. 1996), and germinating seeds (Arranco et al. 1997). In addition, cytosolic I (Magnard et al. 1996; Zarsky et al. 1995) and cytosolic II sHSPs (Atkinson et al. 1993; Kobayashi et al. 1994) have been detected in pollen during flower development.

Almost all the high molecular weight HSPs act as molecular chaperones in vitro and in vivo (Vierling 1991). Recently, several studies demonstrated that plant sHSPs also displayed molecular chaperone activity in vitro (Collada et al. 1997; Fujikawa et al. 2006; Giese et al. 2002; Joe et al. 2000; Lee et al. 1995; Liu and Shono 1999; Lopez-Matas et al. 2004; Smýkal et al. 2000; Sun et al. 2001, 2002; Vierling et al. 1991; Yeh et al. 1999). So far, the in vivo function of plant sHSPs has been shown only for cytosolic sHSPs (Forreiter et al. 1997; Giese et al. 2002; Kim et al. 2004; Smýkal et al. 2000; Sun et al. 2001) and mitochondrial sHSP (Sanmiya et al. 2004); however, the in vivo function of plant sHSPs from other classes has not been studied. The ER plays several vital roles in protein processing and secretion. Presently, a number of species have ER-localized sHSPs; however, only higher plants are known to have sHSPs localized in the ER (Atkinson et al. 1993; Boston et al. 1996; Cooper et al. 1983; Helm et al. 1993, 1995; LaFayette et al. 1996; Ukaji et al. 1999; van Berkel et al. 1994; Zhao et al. 2007).

The full-length cDNA encoding the small heat shock protein, LeHSP21.5, with GenBank accession no. *AB026983* has been previously isolated from tomato (*Lycopersicon esculentum* Mill., Zhao et al. 2007). The similarity between the deduced amino acid sequence of LeHSP21.5 and other reported ER-sHSP was well compared and documented by Scharf et al. (2001). The deduced amino acid sequence of this tomato sHSP was most similar to that of other ER-localized sHSPs (ER-sHSP) and can consequently be predicted to target the endoplasmic reticulum. Localization of LeHSP21.5 in the microsome fractions was confirmed in this study and recently by Zhao et al. (2007). The aim of the present study was to determine whether LeHSP21.5 protein from tomato (probable ER-sHSP) can function as a molecular chaperone to help elucidate the cellular function of ER-sHSP in plants.

Materials and methods

Subcellular fractionation

Tomato plants were grown in a greenhouse at 27°C and then were exposed to heat stress treatment using a growth chamber at 40°C for 2.0 h. One gram of young leaves was harvested and processed with mortar and pestle in 6 ml of extract buffer containing 50 mM Tris–HCl (pH 7.4), 250 mM sucrose, 5 mM dithiothreitol (DTT), 1 mM EDTA, a tablet of complete Mini EDTA-free protease-inhibitor-cocktail (Roche) and 0.5% PVPP. Crude extract was filtrated with four layers of Miracloth (Calbiochem), adjusted to 4 ml and then centrifuged at 3,000×*g* for 5 min. The pellet was removed, and the supernatant was centrifuged at 10,000×*g* for 20 min. The 10,000×*g* supernatant was further centrifuged at 200,000×*g* for 30 min to obtain enriched microsome fraction. The 200,000×*g* pellet was suspended in 4 ml of extract buffer without PVPP. Ten microliters of total proteins from each fraction were run on 12% SDS-PAGE and transferred to PVDF membrane for immunoblot analysis using anti-Le-HSP21.5 polyclonal antibody.

Construction of a recombinant protein expression plasmid

In order to express recombinant ER-sHSP in *Escherichia coli*, a pair of primers was designed on the basis of the Le-HSP21.5 sequence. The sequences of the forward and reverse primers were 5′-GTATCATATGAGGGTCAT-CAGC-3′ and 5′-TAAGAGCTCAGCTCTTCTCTAA CAG-3′, where the underlined nucleotide bases indicate restriction-enzyme digestion sites for *Nde* I and *Sac* I, respectively. Pyrobest polymerase (Takara), a high fidelity polymerase, was used for PCR amplification. After the amplified DNA fragment was purified with a PCR Geneclean kit (Qiagen), *Nde* I and *Sac* I were used to remove the cohesive ends. This fragment was directionally ligated with a pET-27b(+) vector (Novagen), which was digested with both *Nde* I and *Sac* I and processed with calf intestine alkaline phosphatase. The ligated plasmid was transformed into *E. coli* strain DH5α. After a positive clone was selected, the sequence of insert was confirmed, and the new constructed plasmid was named pET-ER-sHSP.

Induction and purification of recombinant His_6-tagged ER-sHSP and antibody preparation

E. coli strain BL21 (DE3), transformed with pET-ER-sHSP, was grown at 37°C in 100 ml of LB medium

containing 30 μg $ml^{-1}$of kanamycin to an OD_{600} of ≈0.6. Expression of recombinant protein was induced by the addition of 1 mM IPTG to the cultured cells for 1 h at 37°C. A cell pellet was harvested at 4°C by centrifugation at 2,000×*g* for 15 min and resuspended in 4 ml of ice-cold 1× binding buffer (0.5 M NaCl, 20 mM Tris–HCl, 5 mM imidazole, pH 7.9) containing a complete protease inhibitor tablet (Roche Applied Science) dissolved at the recommended concentration. The re-suspended cells were lysed at 4°C by sonicating three times for 30 s each, and then centrifuged at 39,000×*g* for 20 min. The resulting supernatant fraction was desalted by passage through a PD-10 column (Amersham Biosciences), and then applied to a 2-ml column of Ni-NTA His-Bind resin (Novagen). The column was pre-equilibrated with 1× binding buffer containing a complete protease inhibitor tablet dissolved as recommended. The Ni^{2+}-immobilized metal affinity-chromatography (IMAC) purification procedure was carried out at 4°C according to the manufacturer's instructions. LeHSP21.5 protein was eluted at 1 M imidazole, pH 7.9. The fractions possessing high protein concentration of recombinant protein were combined, desalted, and processed for antigen or stored at −20°C for future use. Rabbit anti-LeHSP21.5 antibody was produced by Sawady technology.

SDS-PAGE and immunoblotting

The protein samples were separated by 12.5% (w/v) SDS-PAGE according to the method of Laemmli (1970) and were stained with coomassie brilliant blue R-250 or transferred to PVDF membrane (Millipore) for immunoblot using anti-LeHSP21.5 primary antibody. Protein bands cross-reacting with the anti-LeHSP21.5 antibody were identified by alkaline phosphatase conjugated anti-rabbit IgG, followed by membrane incubation in Western Lightning CDP-*Star*.

Thermal stability of soluble extracts expressing ER-sHSP

Thermal stability of ER-sHSP was analyzed similarly to the method of Kim et al. (1998). *E. coli* strain BL21 (DE3) cells transformed with pET-ER-sHSP and pET vector only were grown at 37°C in LB medium containing 30 μg ml^{-1} of kanamycin to an OD_{600} of ≈0.6. Recombinant protein was induced by the addition of 1 mM IPTG for 1 h at 37°C. The cell pellet was harvested at 4°C by centrifugation at 2,000×*g* for 15 min, washed with buffer 25 mM Tris–HCl, pH 7.5, 2 mM DTT, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM antipain, and 5 μM leupeptin, then sonicated three times for 30 s, and centrifuged for 20 min at 39,000×*g*. The protein concentration of the cell extract was adjusted to 1 mg ml^{-1}. The cell extracts were heated at 50°C for 15, 30 and 60 min or at 70°C for 15 min. After being allowed to cool on ice, the soluble supernatants from each sample were centrifuged at 15,000×*g* to remove heat-denatured and aggregated proteins, and 15 μl soluble supernatants from each sample were analyzed on a 12% SDS-PAGE to evaluate the chaperone activity of ER-sHSP. The chaperone activity of ER-sHSP was estimated by SDS-PAGE, based on comparison of a retained, non-precipitated or non-aggregated protein (after heat treatment) in protein solution of the *E.coli* cell extract from control pET cells or cells expressing ER-sHSP.

Thermal protection of *E. coli* proteins by purified ER-sHSP

The thermal protection effect of ER-sHSP was analyzed similarly to the method of Kim et al. (1998). *E. coli* DH5α (10,000×*g* supernatant fraction, 2 mg ml^{-1}), used as a model strain for thermal protection assay, was prepared in 25 mM Tris–HCl, pH 7.5, 2 mM DTT, 1 mM EDTA, 1 mM PMSF, 10 μM antipain, and 5 μM leupeptin buffer. Forty micrograms of proteins in the cell extracts was mixed with 20 μg of purified ER-sHSP (final concentration of ER-sHSP in mixture was ≈19 μM) or 40 μg of Cyt C (final concentration of Cyt C in mixture was ≈71 μM) in a final volume of 40 μl. The samples then were incubated at 60°C for 15, 30, and 60 min or 70°C for 15 min. After cooling on ice, samples were centrifuged at 15,000×*g* to remove heat-denatured and aggregated proteins, and 10-μl soluble supernatants from each sample were analyzed on a 12% SDS-PAGE.

Thermal aggregation measurements

CS was used as a control target enzyme for the molecular chaperone activity of ER-sHSP according to the method of Lee (1995). Three hundred nanomolar porcine heart CS (Sigma) was incubated at 45°C in the absence or presence of 200, 400, 800, and 1,200 nM of ER-sHSP. BSA and lysozyme were added instead of ER-sHSP at the stated concentration. The aggregation of CS upon thermal denaturation was determined by measuring the absorption due to increased turbidity from light scattering at 360 nm in a Hitachi spectrophotometer at 45°C. Temperature was controlled using a circulating water bath and measured in the cuvette with a digital thermometer. All experiments were performed in 50 mM HEPES–KOH, pH 7.5, and in a total volume of 600 μl.

Thermal inactivation experiments

Thermal inactivation study was performed similar to the method of Lee (1995). For the protection assay of ER-sHSP from thermal inactivation, 150 nM CS was incubated in the absence or in the presence of 5 μM LeHSP21.5 protein or 5 μM lysozyme in 50 mM HEPES–KOH, pH 7.5 at 45°C for 40 min and then at 25°C. At various time points, 25-μl aliquots were removed and measured for CS activity. CS activity was measured as described previously (Lee et al. 1995).

Protein determination

The concentrations of protein were determined using the Bio-Rad protein assay with bovine serum albumin as the standard.

Results

Sequence analysis for the deduced amino acid of tomato LeHSP21.5 gene

A selected alignment of the deduced, full-length amino-acid sequences of LeHSP21.5 along with reported ER-sHSPs and the four classes of small HSPs (cytosolic-I, cytosolic-II, chloroplast and mitochondria) for tomato is depicted in Fig. 1. Of special note is that (1) the deduced amino acid sequence of tomato LeHSP21.5 has a predicted signal peptide at the N-terminus and (2) the last four amino acids at the C-terminus (i.e., REEL) compose a charged tetra-peptide suspected to be a retention sequence. The deduced amino acid sequence of LeHSP21.5 was similar to several reported ER-sHSPs, and the predicted primary structure of LeHSP21.5 is most similar to that of potato ER-sHSP at 87% identity (Fig. 1). Notably, two motifs, -PGL- and -GVL-, near the C-terminal of the peptides were highly conserved among all classes (Fig. 1) as well as in sHSPs from various organisms (Waters et al. 1996).

LeHSP21.5 protein is accumulated after heat stress treatment at 40°C

As mentioned above, the subcelluar localization of LeHSP21.5 in ER has been established recently (Zhao et al. 2007). To compare accumulation of LeHSP21.5 grown in tomato plant under normal and heat stress conditions, we fractionated protein extracts of tomato leaves by differential centrifugation. The presence of LeHSP21.5 protein in the fractions was determined by western-blot analysis using antibody raised against the recombinant LeHSP21.5 protein. Tomato plants were grown as described in "Materials and methods." A ≈21.5-kDa protein band corresponding to the deduced amino acid sequence of LeHSP21.5 gene was detected in the supernatant of 10,000×*g* (Supplementary Fig. 1, lane 4) and 200,000×*g* pellet (Supplementary Fig. 1, lane 5) of tomato plant, which was subjected to heat stress at 40°C for 2 h. This protein band was not detected in tomato plant grown in normal condition at 25°C. These results confirm that LeHSP21.5 protein is accumulated after sudden heat stress treatment at 40°C for 2 h, and the protein is localized in the microsomal fraction as shown previously (Zhao et al. 2007). The accumulation of LeHSP21.5 protein correlated well with LeHSP21.5 mRNA expression in tomato (Sanmiya et al. 2005). As has been shown in Supplementary Fig. 1, a 25-kDa protein band is also detected in lanes 1, 2, and 5; however, this band may not be ER-sHSP, because the deduced amino-acid sequence of LEHSP21.5 gene is 21.5 kDa.

Purification of recombinant ER-sHSP

In order to express recombinant LeHSP21.5 protein (probable ER-sHSP), the corresponding ORFs were sub-cloned into the *E. coli* expression vector pET-27b(+) and transformed into *E. coli* strain BL21(DE3) as described in "Materials and methods." The expressed recombinant protein was found to be highly soluble, and His-tagged recombinant ER-sHSP was effectively purified from clarified cell extracts by Ni^{2+}-IMAC and appeared as a 26-kDa polypeptide on SDS-PAGE (Fig. 2, lane 11). The recombinant protein was expressed with a signal sequence and His tag with 29 extra amino acids between LeHSP21.5 protein sequence and six histidine residues at the C-terminal of protein sequence. Therefore, the molecular mass of the recombinant LeHSP21.5 is larger than the original protein.

ER-sHSP protected *E. coli* extract soluble proteins from heat denaturation

To study the thermal stability and thermal protection effect (see below) of ER-sHSP, the bacterial cell extract samples were prepared, heat-treated and analyzed on SDS-PAGE as described in "Materials and methods." Figure 2 shows that although many soluble proteins were precipitated or were rapidly degraded in the control cells (transformed with pET vector only) during the heat treatment (Fig. 2, lanes 3, 5, 7, and 9), this effect was delayed and quantitatively less pronounced in pET-ER-sHSP cells (Fig. 2, lanes 4, 6, 8, and 10). Most protein bands from transformed cells seemed

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tomato          -------------------------MRVISKLTLLIISIACIFQVSSLS---ADGSSLV  31
potato          -------------------------MRVISKLTLLIISIGCIFQVSSLG---ADASSLL  31
pea             ----------------------------MSLKPLNMLLVPFLLLILAADFPLKAKASLL  31
soybean         ----------------------------MRLQQLNLF---FLLLCVA-----KANGSLL  23
arabidopsis     ----------------------------MMKHLLSIFFIGALLLGNIK----TSEGSLS  27
cytIsHSP        ------------------------------------------------------MSLI   4
cytIIsHSP       ------------------------------------------------------MDLRLL   6
mitsHSP         ---------------------MATLALRRATASSLFNRLVNPVRSASAFRSFNTNTQMT  38
chlsHSP         MAYTSLTSSPLVSNVSVGGTSKINNNKVSAPCSVFVPSMRRPTTRLVARATGDNKDTSVD  60

tomato          PLIID---------------------QMISSNPANTFL---------DPFKVLEQIPFGL  61
potato          PLILD---------------------QMIGSNPANTFL---------DPFKVLEQIPFGL  61
pea             PFIDS--------------------PNTLLSDLWSDRFP---------DPFRVLEQIPYGV  63
soybean         PFMDP-------------------PITLLADLWSDRFP---------DPFRVLEHIPFGV  55
arabidopsis     SALETT-----------------PGSLLSDLWLDRFP---------DPFKILERIPLGL  60
cytIsHSP        PRIFG--------------------DRRSSSMFDPFSI---------DVFDPFRELGFPS  35
cytIIsHSP       GIDNTP-----------------LFHTLHHMMEAAGE---------DSDKSVNAPSRNY  39
mitsHSP         AYDQDDRGVDVDRRSDRSVSRRDAFPSLFSDVFDPFSPPIRSVSQLLNMMDQMMDSPFVA  98
chlsHSP         VHHSSAQGGNNQGTAVERRPTRMALDVSPFGVLDPMSPMRTMRQMIDTMDRLFEDTMTFP 120

tomato          EN----TL---LARVDWKETAKGHVISVEVPGLNKDDIKIEIEENRVLRVSGERKKEEEK 114
potato          ENREETTLPLSIARVDWKETAEGHVISIDVPGLKKDDIKIEIEENRVLRVSGERKKEEEK 121
pea             EKH-EPSITLSHARVDWKETPEGHVIMVDVPGLKKDDIKIEVEENRVLRVSGERKKEEDK 122
soybean         DKD-EASMAMSPARVDWKETPEGHVIMLDVPGLKREEIKVEVEENRVLRVSGERKKEEEK 114
arabidopsis     ER--DTSVALSPARVDWKETAEGHEIMLDIPGLKKDEVKIEVEENGVLRVSGERKREEEK 118
cytIsHSP        TNS-GESSAFANTRIDWKETPEPHVFKVDLPGLKKEEVKVEVEEDRVLQISGERNVEKED  94
cytIIsHSP       VRD---AKAMAATPADVKEYPNSYVFVVDMPGLKSGDIKVQVEEDNVLLISGERKREEEK  96
mitsHSP         APRAMGAGVGARRGWDVKEDDNALYIKMDMPGLDKENVKVAVEEN-TLIIKGEGEKESE- 156
chlsHSP         GRNRASGTGEIRTPWDIHDDENEIKMRFDMPGLSKEDVKVSVEND-MLVIKGEHKKEEDG 179

tomato          NDEENHWHCVERSHGKFWRQFRLPENA-DIDTMKAKLENGVLTISFAKLSADRIKGPKVV 173
potato          NDEQNHWHCVERSYGKFWRQFRLPENA-DIDTMKAKLENGVLTISFAKLSADRIKGPKVV 180
pea             KGD--HWHRVERSYGKFWRQFKLPQNV-DLDSVKAKMENGVLTLTLHKLSHDKIKGPRMV 179
soybean         KGD--HWHRVERSYGKFWRQFRLPQNV-DLDSVKAKLENGVLTLTLDKLSPGKIKGPRVV 171
arabidopsis     KGD--QWHRVERSYGKFWRQFKLPDNV-DMESVKAKLENGVLTINLTKLSPEKVKGPRVV 175
cytIsHSP        KND--KWHRMERSSGKFMRRFRLPENA-KMDQVKASMENGVLTVTVPK---EEVKKPEVK 148
cytIIsHSP       EGA--KFIRMERRVGKFMRKFSLPENA-NTDAISAVCQDGVLTVTVQKLPPPEPKKPKTI 153
mitsHSP         ---------NEEYRRRYSTRLEIPQNIYKLDGIKAEMKNGVLKVAVPKVKQEERKDVFDV 207
chlsHSP         RDK----HSWGRNYSSYDTRLSLPDNV-VKDKIKAELKNGVLFI---------------- 218

tomato          SIESKQQGKESSVREEL---- 190
potato          SIESKQEGKESSVREEL---- 197
pea             SIVEEDDKPSKIVNDELK--- 197
soybean         SIAGEDHQQGNLNNDGAKQEL 192
arabidopsis     NIAAEEDQTAKISSSESKEL- 195
cytIsHSP        SIEISG--------------- 154
cytIIsHSP       EVKVA---------------- 158
mitsHSP         KIE------------------ 210
chlsHSP         ----------------------
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Fig. 1 Selected amino-acid sequence alignment of ER-sHSP from tomato and representative plant sHSPs. Deduced amino-acid-sequence alignments of tomato ER-sHSP, together with representative plant sHSPs, were performed using ClustalW program. The abbreviated name of each aligned sequence is as follows: tomato, ER-sHSP from tomato (GenBank accession no. *AB026983*); potato, sHSP from potato (GenBank accession no. *AAB30525*); pea, sHSP from pea (GenBank accession no. *M333898*); soybean, sHSP from Glycine max (GenBank accession no. *X63198*); Arabidopsis, sHSP from Arabidopsis (GenBank accession no. *U11501*); cytIsHSP, cytosolic-I sHSP from tomato (GenBank accession no. *X56138*); cytIIsHSP, cytosolic-II sHSP from tomato (GenBank accession no. *U72396*); mitsHSP, mitochondria sHSP from tomato (GenBank accession no. *AB017134*); chlsHSP, chloroplast sHSP from tomato (GenBank accession no. *U66300*). The deduced, carboxyl-terminal tetrapeptide REEL in tomato and potato sHSPs, which is similar to known ER retention signals, is *underlined*. The deduced amino acid sequence of tomato LeHSP21.4 has a predicted transmembrane sequence at the N-terminus, R^2-S^{24}, which is *underlined*. Two conserved motifs, -PGL- and -GVL-, near the C-terminal of the peptides were highly conserved in sHSPs from various organisms and are enclosed by *squares*

to remain soluble after heating at 50°C for 60 min (Fig. 2, lane 8), but these levels were gradually decreased after heating at 50°C for 30 min (Fig. 2, lane 6). On the other hand, proteins from control cells started to precipitate at 50°C for 15 min (Fig. 2, lane 3). Protein assay showed that treatment of the wild-type strain at 50°C for 15 min (Fig. 2, lane 3) led to a significant loss (≈50%) of soluble proteins, whereas similarly treated extracts from cells expressing ER-sHSP experienced minimal loss (less than 1%) of soluble protein (Fig. 2, lane 4). This finding indicates that ER-sHSP expressed in *E. coli* is capable of protecting *E. coli* proteins from heat-induced denaturation.

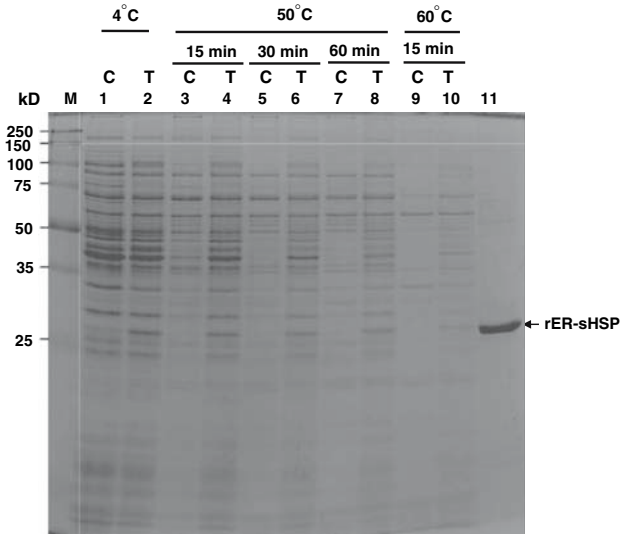

Fig. 2 Thermal stability of an *E. coli* crude extract expressing ER-sHSP. Extract of soluble *E. coli* strain BL21(DE3) proteins from transformed with pET vector only (control) or pET-ER-sHSP cells expressing ER-sHSP were prepared as described in "Materials and methods." Aliquots of cell extract (1 mg ml^{-1}) were heated at 50°C for 15, 30, and 60 min or at 60°C for 15 min as indicated. Soluble proteins recovered by centrifugation were analyzed by SDS-PAGE. *Lanes*: *1* and *2*, unheated cell extracts, kept at 4°C; *1*, *3*, *5*, *7*, *9*: soluble cell extract proteins from *E. coli* strain BL21 (DE3) transformed with pET-27b(+), controls, indicated as *C*; *2*, *4*, *6*, *8*, *10*: soluble cell extract proteins from *E. coli* strain BL21 (DE3) transformed with pET-ER-sHSP, indicated as *T*; *11*: 5 μg of Ni^{2+}-IMAC column purified recombinant ER-sHSP. *M*: molecular mass markers

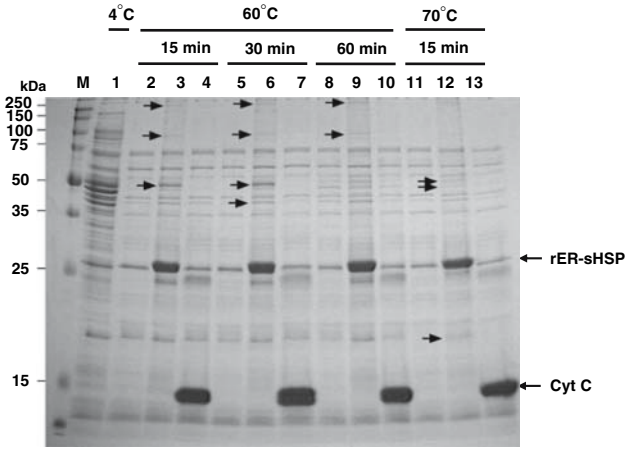

Fig. 3 Thermal protection of *E. coli* extract proteins by purified ER-sHSP. Cell extract was prepared from *E. coli* strain DH5α and incubated with a purified ER-sHSP at a 2:1 (w/w) ratio or with Cyt C protein at a 1:1 (w/w) ratio as described in "Materials and methods" and then heated at 60°C or 70°C at various times as indicated. Soluble proteins recovered by centrifugation were analyzed by SDS-PAGE. *Lanes*: *1*: *E. coli* extract (*E*), 4°C; *2*: E, 60°C, 15 min; *3*: E + ER-sHSP, 60°C, 15 min; *4*: E + cytochrome C, 60°C, 15 min; *5*: E, 60°C, 30 min; *6*: E + ER-sHSP, 60°C, 30 min; *7*: E + Cyt C, 60°C, 30 min; *8*: E, 60°C, 60 min; *9*: E + ER-sHSP, 60°C, 60 min; *10*: E + Cyt C, 60°C, 60 min; *11*: E, 70°C, 15 min; *12*: E + ER-sHSP, 70°C, 15 min; *13*: E + Cyt C, 70°C, 15 min; *M*: protein standards. The *arrows* indicate protein bands that were significantly protected by ER-sHSP

In order to confirm that recombinant ER-sHSP was responsible for these observations, we tested the effect of purified recombinant ER-sHSP on the thermal protection of *E. coli* extract proteins. Kim et al. (1998) reported that Cytochrom C provided no protection effect when *E. coli* cell extract was incubated with Cyt C at 1:1 (w/w) ratio; therefore, for a control, the cell extract was incubated with Cyt C at a 1:1 ratio (w/w). As shown in Fig. 3 (lanes 4, 7, 10, and 13), in the condition of our experiments, Cyt C was not able to protect cell extract from heat denaturation; however, purified ER-sHSP added to *E. coli* cell extract at a 1:2 ratio protected proteins from thermal precipitation. Figure 3 shows that some proteins were significantly protected from denaturation (those bands are shown by arrows). These collective findings show that thermal protection of soluble proteins is conferred by ER-sHSP.

Inhibition of thermal aggregation of CS by ER-sHSP

We tested the effect of ER-sHSP on thermal aggregation of CS. CS, a dimmer of identical 43.5-kDa subunits, was chosen as the substrate for this study because it is a commonly used model for folding studies, its thermal aggregation behavior is well characterized, and it has a simple activity assay method (Lee 1995). When heated to 45°C, CS began to form insoluble aggregates that could be detected by light scattering at 360 nm; however, addition of purified ER-sHSP prior to heat treatment effectively inhibited the thermal aggregation of CS at 45°C in a concentration-dependent manner (Fig. 4a). Nearly complete protection from heat-induced denaturation was observed with ≈600 nM CS monomer and 1,200 nM ER-sHSP, with a 1:2 molar ratio of CS to ER-sHSP. Little to no suppression of CS aggregation was observed when comparable concentrations of lysozyme or BSA were substituted for ER-sHSP prior to heat treatment, implying that our observations were not due to change in protein concentration.

ER-sHSP prevents thermal inactivation of CS at 45°C

Since the thermal aggregation of CS is effectively suppressed by ER-sHSP, we further tested whether the enzymatic activity of CS was protected. Liu and Shono (1999) reported that tomato mitochondria-located sHSP was able to prevent the thermal inactivation of CS when 150 nM CS was incubated in the presence of 1.8 μM

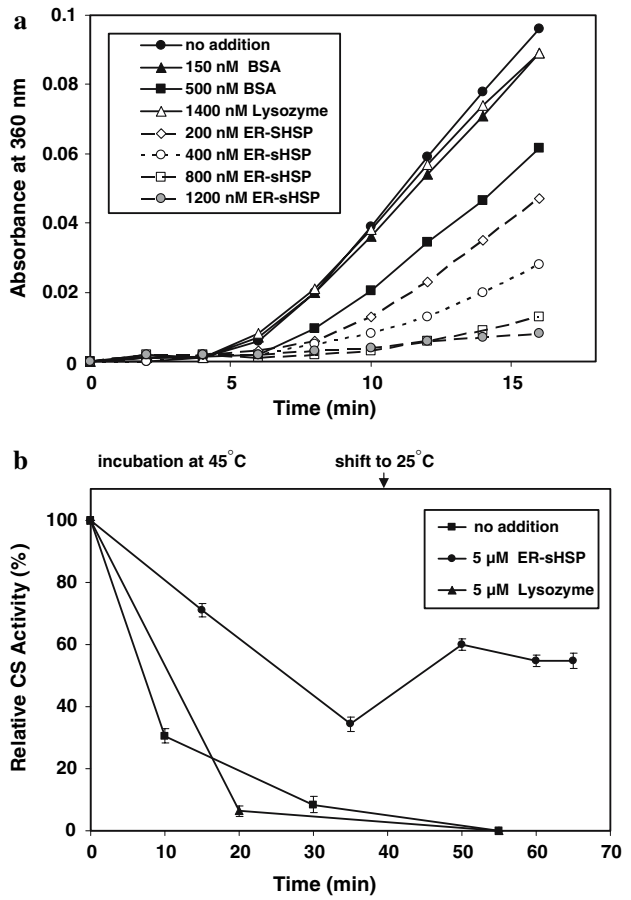

Fig. 4 Prevention of aggregation (**a**) and thermal inactivation (**b**) of CS at 45°C by ER-sHSP. **a** Three hundred nanomolar Porcine Heart CS dimers were incubated at 45°C in the absence or presence of increasing amounts (200, 400, 800, and 1,200 nM) of ER-sHSP as indicated. Where indicated, BSA (150 and 500 nM) and lysozyme (1,400 nM) were added in the absence of ER-sHSP. Relative light scattering indicative of CS aggregation was measured as the apparent absorbance at 360 nm. Each point is representative of the average value of three separate experiments. **b** Three hundred nanomolar of CS was incubated at 45°C in 50 mM HEPES–KOH buffer, pH 7.5 in the absence and presence of 5 μM ER-sHSP and 5 μM lysozyme, as indicated above. Samples were shifted to 25°C after 40 min. Each data point represents the mean ± standard error of the mean of at least three separate experiments. The graph with *error bars* was accomplished using Origin 6 software (RockWare Inc.)

recombinant protein. To compare this effect with the other tomato sHSP (MT-sHSP), we used comparable concentrations of CS and ER-sHSP in this study. When 300 nM CS was incubated at 45°C alone or in the presence of 5 μM lysozyme, less than 5% of the original activity remained after 30 min (Fig. 4b). In contrast, when CS was heat-treated in the presence of 5 μM recombinant ER-sHSP, CS activity was protected at 45°C, and approximately 40% of activity was retained. Shifting the temperature to 25°C resulted in a detectable renaturation of CS when treated with ER-sHSP.

Discussion

Analysis of the amino acid sequence showed that LeHSP21.5 protein belongs to the sHSP family. In addition, signal peptide at the N-terminal and the C-terminal tetrapeptide REEL, which is similar to known ER retention signals, suggests that LeHSP21.5 is targeted to the ER in heat-stressed tomato. The primary sequence of LeHSP21.5 proteins had high homology to other ER-localized sHSPs, including PsHSP22.7 in pea, GmHSP22.0 in soybean, and AtHSP22.0 in *Arabidopsis*. Similar to LeHSP21.5, the primary structure of WAP20 had two consensus sequences of sHSP, a transit peptide in the N-terminal region and a putative ER-retention signal, KQEL, in the C-terminal region (Fujikawa et al. 2006). Western blotting analysis confirmed the localization of LeHSP21.5 in microsome fraction. Previous reports showed co-sedimentation of sHSP with endomembrane fractions from heat-stressed maize and barley (Helm et al. 1993; Merck et al. 1993; Sticher et al. 1990). Helm et al. (1993) reported the endomembrane localization of the sHSPs, PsHSP22.7 and GmHSP22.0, in heat-stressed pea and heat-stressed soybean, respectively. In potato tubers, a cold-induced gene, *C119*, has been shown to have homology to genes of heat-shock-induced sHSPs localized in the ER of pea (Helm et al. 1993), suggesting that the *C119* gene product exists in the ER (van Berkel et al. 1994). The localization of sHSPs in the endomembrane also has been suggested (Helm et al. 1995) in *Arabidopsis* and in cortical parenchyma cells of mulberry trees (Ukaji et al. 1999; van Berkel et al. 1994). All these findings suggest that ER-localized sHSPs accumulate in all higher plants, and that these sHSPs might have important functions in protecting the ER proteins from heat-induced damage.

The predicted molecular mass of tomato LeHSP21.5 protein was approximately 21.4 kDa. Antibody raised against tomato LeHSP21.5 cross-reacted with a ≈21.5-kDa protein in tomato plant that was subjected to heat stress at 40°C for 2 h. Proteins of a similar molecular mass, 20 and 21 kDa, are accumulating in heat-stressed pea (Helm et al. 1993). Antibody raised against WAP20, a cold-inducible ER-localized sHSP found in the cortical parenchema of mulberry tree, reacted with two proteins of 20 and 21 kDa (Ukaji et al. 1999).

Recently, many studies demonstrated that plant cytosolic and mitochondrial sHSPs displayed the molecular chaperone's properties in vitro. Although localization of sHSPs in ER has been reported for a number of species (Atkinson et al. 1993; Boston et al. 1995; Cooper and Ho 1987; Helm et al. 1993, 1995; LaFayette et al. 1996; Ukaji et al. 1999; van Berkel et al. 1994), so far only one recent study (Fujikawa et al. 2006) has revealed the function of this protein in the ER. In this study the functional role of

WAP20 as molecular chaperone was shown in vitro using a recombinant WAP20 (rWAP20) expressed in *E. coli*. It was shown that rWAP20 recovered from thermal inactivation and suppressed thermal aggregation of CS by refolding (Fujikawa et al. 2006).

In this paper we have shown that tomato ER-sHSP can act as a molecular chaperone. To our knowledge, this paper is the first to describe the molecular chaperone function of heat-inducible ER localized sHSP in vitro. The molecular chaperone activity of ER-sHSP in vitro was examined from different aspects. Our results strongly suggest that ER-sHSP can confer thermal protection of *E. coli* extract proteins in vitro as found in other sHSPs. In addition, purified recombinant ER-sHSP had a similar protective effect on heat-treated *E. coli* proteins, indicating that the effect is specifically due to the presence of recombinant ER-sHSP. These results also suggest that ER-sHSP exhibits broad substrate specificity, as shown by the protection of many proteins in *E. coli* cell lysate, as observed with murine HSP25 (Ehrnsperger et al. 1997) and Mj HSP16.5 (Kim et al. 1998). Thermal aggregation experiments showed that ER-sHSP protein was a strong chaperone for CS, requiring about 2 mol of ER-sHSP subunit per 1 mol of CS monomer to almost completely prevent its thermal aggregation at 45°C. A significant (>25%) suppression of CS aggregation by rWAP20 was observed at 37°C in monomer molar ratio of 1:1 of CS:WAP20 protein (Fujikawa et al. 2006). Lee et al. (1997) demonstrated that pea HSP18.1 has a large binding capacity for heat-denatured malate dehydrogenase monomer at 40°C; however, the molar ratio required for protection from heat treatment can depend on the sHSP and substrates. For example, at 40°C, a monomer molar ratio of 1:40 of CS:Mj HSP16.5 was necessary to prevent the thermal aggregation of CS (Kim et al. 1998), whereas 14–15 *C. elengans* HSP16-2 molecules are needed to protect the CS monomer (Leroux et al. 1997). Tomato ER-sHSP also has the ability to prevent thermal inactivation of CS at 45°C. In conclusion, tomato ER-sHSP has several activities that are typically found in chaperones:

- Inhibition of temperature-dependent aggregation of proteins.
- Protection of enzymes against activity lost during thermal inactivation.

These collective findings demonstrated that ER-sHSP can function as a molecular chaperone, and further studies may help elucidate the role of ER-sHSP in plant heat tolerance.

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