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Characterization and Physiological Function of Class I Low-Molecular-Mass, Heat-Shock Protein Complex in Soybean[1]

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Examination of an ammonium sulfate-enriched fraction (70–100% saturation) of heat-shock proteins (HSPs) by nondenaturing polyacrylamide gel electrophoresis revealed the presence of a high molecular mass complex (280 kD) in soybean (*Glycine max*) seedlings. This complex cross-reacted with antibodies raised against soybean class I low-molecular-mass (LMW) HSPs. Dissociation of the complex by denaturing polyacrylamide gel electrophoresis showed the complex to contain at least 15 polypeptides of the 15- to 18-kD class I LMW HSPs that could be detected by staining, radiolabeling, and western blotting. A similar LMW-HSP complex was observed in mung bean (*Vigna radiata* L.; 295 kD), in pea (*Pisum sativum* L.; 270 kD), and in rice (*Oryza sativa* L.; 310 kD). The complex was stable under high salt conditions (250 mM KCl), and the integrity was not affected by 1% Nonidet P-40 and 3 μg/mL RNase treatment. The size of the isolated HSP complex in vitro was conserved to 55°C; however, starting at 37.5°C, it changed to higher molecular forms in the presence of soluble proteins. The isolated HSP complex was able to protect up to 75% of the soluble proteins from heat denaturation in vitro.

The induction of HSP synthesis in response to thermal stress occurs in all organisms that have been examined, ranging from bacteria to humans (Schlesinger et al., 1982; Vierling, 1991). Whereas the physiological functions of HSPs have not been clearly established, these proteins are well correlated with the acquisition of thermotolerance in a time- and temperature-dependent manner. Under HS conditions the expression of the LMW HSPs in soybean (*Glycine max*) is increased, yielding final concentrations of up to 1% of the total cellular proteins (Hsieh et al., 1992). Based on this correlation, it has been hypothesized that accumulation of HSPs is an essential component of a process that prevents heat damage (Lin et al., 1984; Key et al., 1985; Kimple et al., 1990). Some HSPs become selectively localized in cellular organelles in a temperature-dependent fashion and relocate to the cytoplasm after a 28°C incubation period (Lin et al., 1984). Isolated mitochondria with associated HSPs are functional in oxidative phosphorylation under thermal stress, suggesting that association of HSPs with organelles provide protection from heat damage (Chou et al., 1989). Stabilization of soluble proteins can be demonstrated in vitro by an HSP-enriched fraction (Jinn et al., 1989); such HSP-enriched fractions are exchangeable among soybean, mung bean (*Vigna radiata* L.), and rice (*Oryza sativa* L.) in providing protein thermostabilization (Jinn et al., 1993). In soybean, depletion of the 15- to 18-kD HSPs in the HSP-enriched fraction resulted in the loss of the thermostabilizing ability, and this ability was again restored when these HSPs were added. The protection provided by these HSP-enriched fractions is effective mainly for membrane-associated proteins (Jinn et al., 1993).

The LMW HSPs of plants and all other eukaryotes have a conserved carboxyl-terminal region homologous to the α-crystallin of the eye lens at the amino acid sequence level (Ingolia and Craig, 1982; Augusteyn and Koretz, 1987; Lindquist and Craig, 1988). The LMW HSPs in vertebrates (Arrigo and Welch, 1987; Merck et al., 1993), *Drosophila* (Arrigo and Pauli, 1988), yeasts (Bentley et al., 1992), and recently in plants (Clark and Critchley, 1992; Helm et al., 1993; Chen et al., 1994) were shown to form higher order structures similar to the native molecular mass of α-crystallin.

In this study, we show by nondenaturing 5 to 20% PAGE that isolated class I LMW HSPs in soybean exist in an HMW complex of 280 kD. The subunit composition of this purified class I LMW-HSP complex was determined. We also show that this class of HSPs functions as molecular chaperones in thermostabilization by interacting with soluble proteins following heating in vitro.

MATERIALS AND METHODS

Plant Materials

Soybean (*Glycine max* cv Taita Kaoshiung No.8), mung bean (*Vigna radiata* L.), pea (*Pisum sativum* L.), and rice (*Oryza sativa* L. cv Tainong No. 67) were used in this study. All seeds were surface sterilized in 10% Clorox for 10 min, rinsed thoroughly in water, and then germinated in a roll of moist paper towel at 28°C in a dark growth chamber (Lin et al., 1984).

PAGE

One-dimensional SDS-PAGE was performed according to the method of Laemmli (1970) by using continuous 13.75% (w/v) acrylamide gels. For two-dimensional IEF/SDS-PAGE, the method of O'Farrell (1975) was used.

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Abbreviations: AS, ammonium sulfate; HMW, high molecular mass; HS, heat shock(ed); HSP, heat-shock protein; LMW, low molecular mass; PRS, postribosomal supernatant; PVDF, polyvinylidene difluoride.

One-dimensional, nondenaturing PAGE was carried out without SDS in nondenaturing conditions on 5 to 15% or 5 to 20% linear polyacrylamide gradients. To overcome any potential problem of slow migration, electrophoresis was performed at 150 V for at least 20 h at 4°C (Anderson et al., 1972; Lasky, 1978). Protein staining and fluorography were performed as described previously (Jinn et al., 1989).

Immunoblotting

For immunoblotting, proteins were transferred from polyacrylamide gels to Immobilon PVDF transfer membranes (Millipore) with Gly electrode buffer according to the method of Towbin et al. (1979). Protein bands cross-reacting with the LMW-HSP antibodies were identified by reaction with alkaline phosphatase conjugated to goat anti-rabbit IgG (Dakopatts, Denmark). Bound antibodies were visualized by reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium according to the manufacturer's specification (Bio-Rad).

Preparation of an HSP-Enriched Protein Fraction and Purification of the Class I LMW-HSP Complex

Two-day-old seedlings of soybean, mung bean, and pea without cotyledons and 5-d-old rice seedlings without endosperm were incubated in a medium containing 5 mM potassium phosphate buffer (pH 6.0) and 1% Suc at the indicated temperature with gentle shaking. Seedlings were harvested and homogenized with a Polytron in 0.2 M Tris-HCl buffer, pH 8.8, containing 0.5 M Suc, 0.1 M KCl, 30 mM $MgCl_2$, 1 mM DTT, and 1 mM PMSF. The homogenate was filtered through a layer of Miracloth (Calbiochem), and the filtrate was then centrifuged at 300,000*g* for 90 min, as described previously (Jinn et al., 1989) for the preparation of the PRS.

The PRS was fractionated by 70 to 100% saturation with AS. The precipitate was pelleted, dissolved in 50 mM Tris-HCl buffer, pH 8.8, containing 1 mM EDTA and 0.1% 2-mercaptoethanol (buffer D), and dialyzed overnight against the same buffer as described previously (Jinn et al., 1989).

The HSP-enriched protein fraction was further separated under nondenaturing conditions by using a linear gradient of 5 to 20% nondenaturing PAGE. The protein band equivalent to the class I LMW-HSP complex was cut out, eluted with Tris-Gly buffer using an Electro-Eluter (Bio-Rad, mode L422), and dialyzed against buffer D overnight. All experimental procedures were carried out at 4°C.

Assay for Thermal Denaturation of Soluble Proteins

The HSP-enriched protein fraction prepared from 70 to 100% AS saturation or the purified class I LMW-HSP complex was added to ^{3}H-labeled soluble proteins prepared from the PRS of non-HS seedlings, and the mixture was heated at 55°C for 30 min with shaking. The denatured proteins were pelleted at 16,000*g* for 15 min, and the radioactivity in the pellet was measured after suspension in Laemmli's sample buffer (Laemmli, 1970).

Measurement of ^{3}H- or ^{35}S-Labeled Proteins and Quantitative Estimation of Proteins

For measurement of ^{3}H- or ^{35}S-labeled proteins, a sample aliquot was blotted on a 3MM filter paper and processed as described by Mans and Novelli (1961). Proteins were measured according to the method of Lowry et al. (1951).

Antibody

Preparation of the antibody raised against a soybean LMW HSP has been described by Hsieh et al. (1992).

RESULTS

Identification and Determination of the Size of Class I LMW-HSP Complex

The class I LMW HSPs of soybean were enriched from a PRS by 70 to 100% AS fractionation and separated on a nondenaturing 5 to 20% gradient PAGE (Fig. 1). Coomassie blue staining (Fig. 1A) detected one major protein band that accumulated to a detectable level with a molecular mass of 280 ± 30 kD in the 70 to 100% AS fraction (lane 6 compared with lane 5). Under these conditions, one major [^{35}S]Met-labeled protein band with the same size was also observed (Fig. 1B, lane 6). This major band was characterized as a class I LMW-HSP complex because it immunoreacted with class I LMW-HSP antibodies from soybean (Fig. 1C, lane 2). The molecular size of this protein band, referred to as the class I LMW-HSP complex, was reproducible after elution from the nondenaturing gel and re-electrophoresis (Fig. 1D, lane 3).

Composition of the Class I LMW-HSP Complex

The subunit composition of the soybean class I LMW-HSP complex was examined using one- and two-dimensional PAGE (O'Farrell, 1975). The Coomassie blue-stained gels and the fluorogram in Figure 2, A, B and C, D, respectively, revealed the purity of the LMW-HSP complex. SDS-PAGE (Fig. 2, A, C, and E) and two-dimensional gels (Fig. 2, B, D, and F) were used to examine the subunit composition of the LMW-HSP complex. At least 15 polypeptides ranging from 15 to 18 kD were found in this complex. Soybean LMW-HSP antibodies immunoreacted with all of the 15- to 18-kD polypeptides in this LMW-HSP complex, confirming that the complex is composed of 15- to 18-kD, LMW-HSP subunits only (Fig. 2, E and F). Nondenaturing gradient PAGE as the first dimension followed by SDS-denaturing PAGE as the second dimension (Clark and Critchley, 1992) revealed that this 280-kD LMW-HSP complex has a 15- to 18-kD protein as a major band, and other HMW HSPs, such as 82, 72, and 68 kD, with their native mass (from 150–115 kD) are minor components (Fig. 3B).

The Native Mass of the Class I LMW-HSP Complex in Other Plant Species

Although a diverse array of LMW HSPs are synthesized in soybean, mung bean, pea, and rice (Mansfield and Key, 1987), the size of a native LMW-HSP complex ranges from

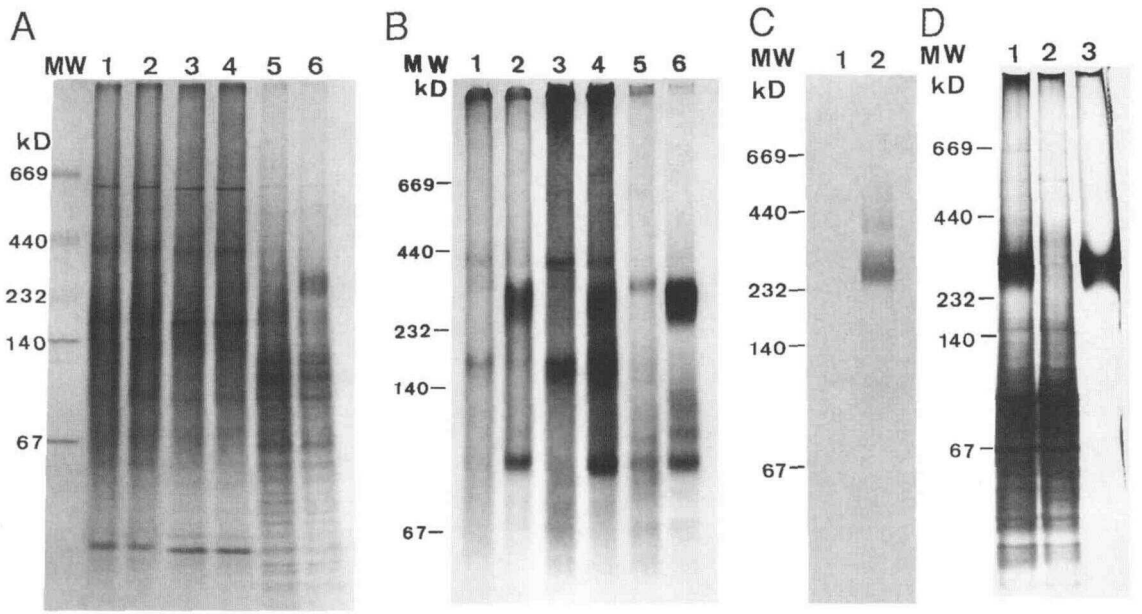

Figure 1. Nondenaturing PAGE (5–20% acrylamide gradient) separation of soybean proteins. A, Coomassie blue-stained gel; B, fluorogram; C, western blot; D, silver nitrate stain. A and B, The total soluble proteins from PRSs were fractionated into 0 to 70% and 70 to 100% saturation by AS. Lanes 1 and 2, Total proteins; lanes 3 and 4, 0 to 70% AS-saturated fraction; lanes 5 and 6, 70 to 100% AS-saturated fraction. Lanes 1, 3, and 5, Proteins from 28°C as a control; lanes 2, 4, and 6, proteins from 40°C for 2 h, followed by 28°C for 4 h as HS treatment. C, lane 1, 70 to 100% AS-saturated fraction from control; lane 2, 70 to 100% AS-saturated fraction from HS. D, lane 1, 70 to 100% AS-saturated fraction from HS; lane 2, 70 to 100% AS-saturated fraction from control; lane 3, the protein band from 280-kD complex (equivalent to the class I LMW-HSP complex) eluted and re-electrophoresed. Lanes MW, Molecular mass markers.

270 to 310 kD among these different plants (Fig. 4). In soybean there are at least 15 polypeptides of 15- to 18-kD LMW HSPs in the complex, and the molecular mass of the native form is 280 ± 30 kD (Fig. 4, lane 1); in rice, about 11 polypeptides of the 15- to 18-kD LMW HSPs are detected in this complex by two-dimensional PAGE, and the mass of the native form is 310 ± 10 kD (Fig. 4, lane 2); in mung bean there are at least 15 polypeptides of 15- to 18-kD LMW HSP subunits, and the molecular mass of the native form is 295 ± 20 kD (Fig. 4, lane 3); in pea there are at least 8 polypeptides of 15- to 18-kD LMW HSP subunits, and the molecular mass of the native form is 270 ± 30 kD (Fig. 4, lane 4). The class I LMW-HSP complexes from different plant species vary in the number of different subunits but share a similarity in size of the native form. The conserved size of the complex may therefore be important for its functions.

The Mass of the Class I LMW-HSP Complex during Recovery

To investigate the size and half-life of soybean HSP complexes during recovery, seedlings were heated (40°C for 3 h) and then transferred or planted at 28°C for varying time intervals. No change in the size of the native complex was detected after recovery at 28°C for 1 to 24 h (Fig. 5A). Planted seedlings were observed for up to 4 d at 28°C after heat treatment and still contained HSP complexes of 280 kD, suggesting that the stable form of the complex is 280 kD. An LKB laser densitometer was used to quantify this HSP complex (Hsieh et al., 1992); the half-life of the HSP complex in the cytoplasm was at least 3 d (Fig. 5B, lane 4). Since arsenite and the amino acid analogs azetidine or canavanine were shown to induce synthesis of a whole family of HSPs at 28°C in soybean (Lin et al., 1984; Edelman et al., 1988; Nover, 1990), we examined the formation of the HMW complex under these treatments. It is clear, as shown in Figure 6, lanes 2, 3, and 4, that the size of the higher-order structure formed during stress treatments with these chemicals at 28°C was the same as that formed during HS (shown in lane 1).

The Physical Properties of the Class I LMW-HSP Complex

The isolated soybean HSP complex itself was very heat stable for 15 min at 42.5, 45, and 50°C (Fig. 7A, lanes 2, 3, and 4, respectively) and was unaffected under high salt or detergent treatment such as 250 mM KCl, 1% Nonidet P-40, 250 mM KCl plus 1% Nonidet P-40 (Fig. 7B, lanes 2–4), or 3 μg/mL RNase treatments (Fig. 7A, lane 5). But the complex was sensitive to 0.1% SDS at room temperature for 2 h. Under these conditions the size decreased from 280 to 210 kD (Fig. 7B, lane 5) and could be restored to 280 kD again by dialysis against 50 mM Tris-HCl, pH 8.8, 1 mM EDTA, and 0.1% 2-mercaptoethanol at 4°C overnight (Fig. 7B, lane 6). In the presence of high salt concentrations and nonionic detergent, the complex keeps its integrity, suggesting that strong ionic interaction and hydrophobic forces stabilize these higher-order structures (Chen et al., 1994). But we

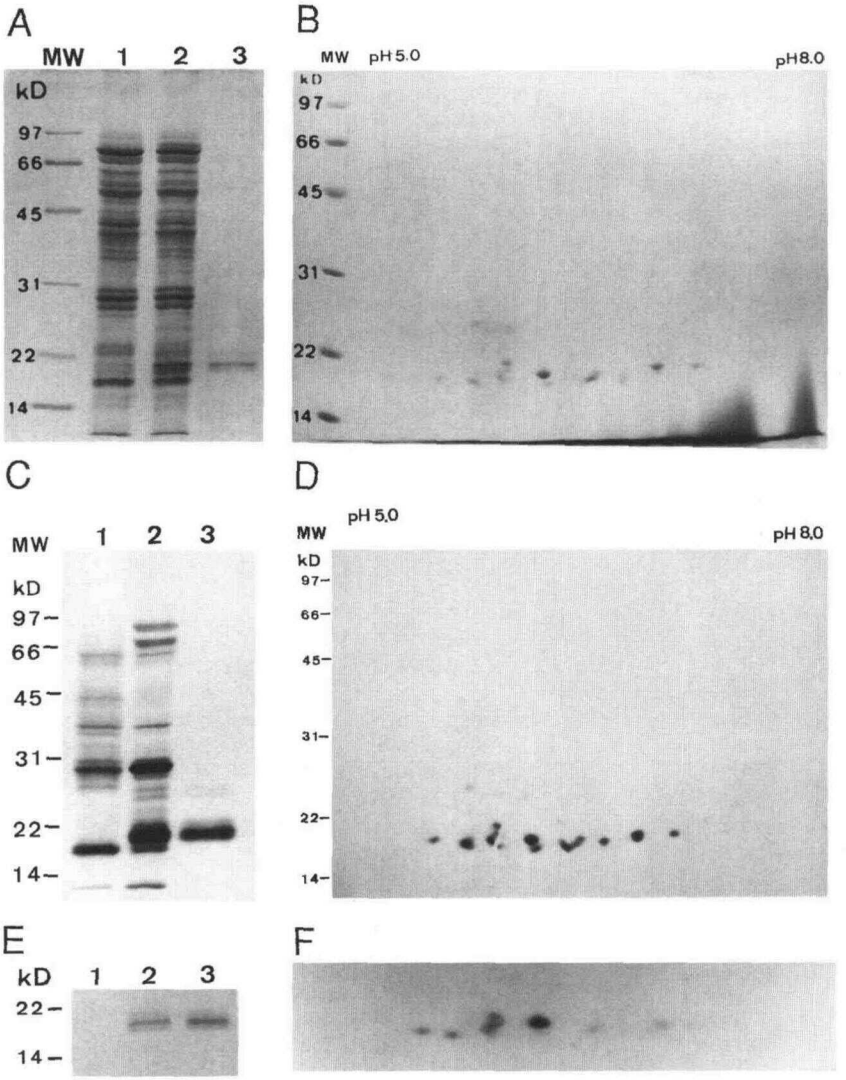

Figure 2. Composition of the soybean class I LMW-HSP complex by one-dimensional SDS and two-dimensional PAGE. A and B, Coomassie blue-stained gel; C and D, fluorogram; and E and F, western blot. Lane 1, 70 to 100% AS-saturated fraction from control; lane 2, 70 to 100% AS-saturated fraction from HS treatment; lane 3, the purified class I LMW-HSP complex. In the western blot (E and F), proteins were separated by one-dimensional SDS or two-dimensional PAGE, then blotted to Immobilon PVDF membrane, and reacted with the class I LMW-HSP antibodies, and the antibodies were detected by assaying alkaline phosphatase conjugated to the second antibody. Lanes MW, Molecular mass markers.

cannot exclude the possibility that the complex altered by KCl treatment may subsequently undergo structural rearrangements during electrophoresis dictated by the running conditions of the gel.

Merck et al. (1993) demonstrated the structural and functional similarities of bovine α-crystallin and mouse LMW HSP25; when mixed and dissociated in 6 M urea, the proteins had the ability to form mixed oligomers and combined with any αA-crystallin, αB-crystallin, and HSP25 subunits. In Figure 7C, the LMW-HSP complex from soybean, rice, and the mixture (lanes 1, 3, and 5, respectively) were dissociated in 6 M urea at 30°C for 2 h, followed by dialysis against 50 mM Tris-HCl, pH 8.8, 1 mM EDTA, and 0.1% 2-mercaptoethanol at 4°C overnight (lanes 2, 4, and 6). Dissociation of the LMW-HSP complexes was reversible (lanes 2, 4, and 6) and exchangeable (molecular mass of the mixture in lane 6 was between 280 and 310 kD). Using 6 M urea demonstrated that the LMW-HSP complex of soybean and rice kept the higher-order structure of 280 and 310 kD and may have played a similar functional and physiological role in vivo in heat-stressed plants.

Stabilization of Soluble Proteins in Vitro by the Class I LMW-HSP Complex

In our earlier reports (Jinn et al., 1989, 1993), we showed that a soybean HSP-enriched fraction (70–100% AS-saturated fraction) from seedlings treated at 40°C for 3 h and then allowed to recover at 28°C for 4 h protected approximately 50% of PRS proteins against denaturation at 55°C for 30 min in vitro. Another 70 to 100% AS-saturated fraction from a 40°C (3 h) → 45°C (30 min) treatment, which was depleted of the 15- to 18-kD HSPs (Lin et al., 1984), did not provide any thermoprotection, even in the presence of HMW HSPs in this fraction. These data suggest that only the 15- to 18-kD HSPs are required for thermoprotection of soluble proteins. In the present study, we used the LMW-HSP complex that contains no HMW HSPs to test thermoprotection of soluble proteins. The LMW HSPs provided a very significant thermostabilization of soluble proteins (Table I) against heat denaturation at 55°C for 30 min, and the degree of protection was proportional to the amount of this protein added (Table II). Up to 75% of the total soluble

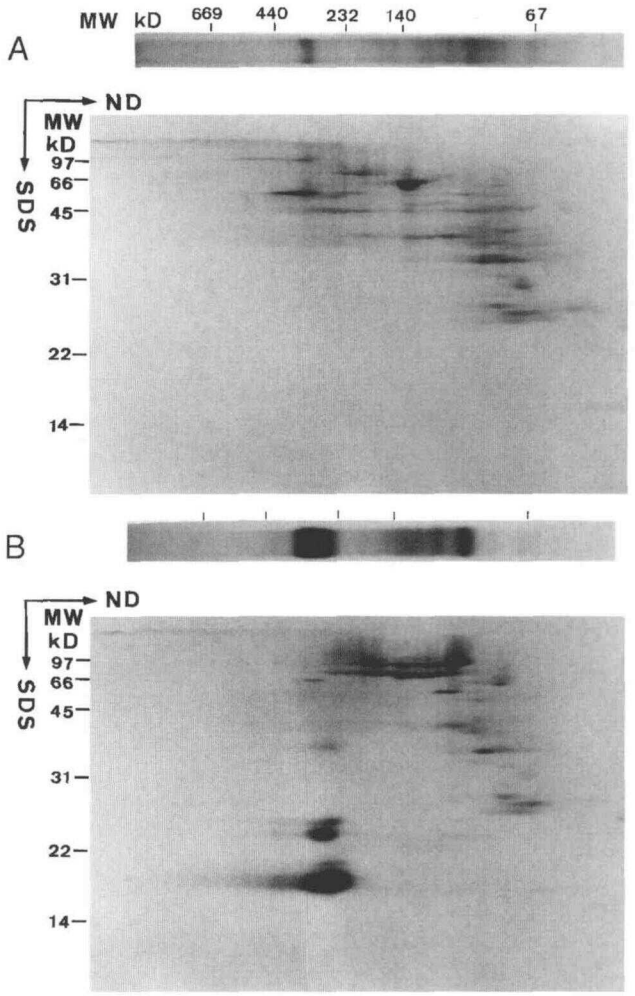

Figure 3. Fluorogram of nondenaturing (ND)/SDS-denaturing two-dimensional separation of the 70 to 100% AS saturation fraction from soybean. Samples from the control treatment fraction (A) or from the HS treatment fraction (B) were initially separated by nondenaturing PAGE (5–20% acrylamide gradient). Gel sections from the different temperature treatments were excised and equilibrated for 45 min in a denaturing buffer containing 3% SDS and 2% β-mercaptoethanol. The excised lanes were then layered horizontally across the top of an SDS-denaturing polyacrylamide gel. After SDS-PAGE, the gels were dried and the proteins were visualized by fluorography. Replicate samples of the first dimension (separated by nondenaturing PAGE) were also examined by fluorography. The native and denatured molecular mass standards (lanes MW) in kD are shown on top and the left of each figure, respectively.

proteins were protected from heat denaturation by the addition of the LMW-HSP complex.

Interaction between the Class I LMW-HSP Complex and Soluble Proteins

The LMW-HSP complex was added to the total soluble proteins from the PRS of 28°C seedlings, and this mixture was heated for 15 min at different temperatures, including 28, 30, 32.5, 35, 37.5, 40, 42.5, and 45°C. The size of the LMW-HSP complex started to increase beginning at 37.5°C (Fig. 8, lane 4) and became more evident as the temperature was increased (Fig. 8, lanes 5–7). This result correlated with the in vivo experiments by Lin et al. (1984). The size of the LMW-HSP complex by itself did not change up to 45°C with 15 min of heating (Fig. 7A, lane 3). However, the LMW-HSP complex apparently aggregated/interacted with soluble proteins after heating above 37.5°C for 15 min, thus causing the size of the LMW-HSP complex to change and increase in molecular mass. The size change of the LMW-HSP complex was irreversible during recovery at 28°C for 4 h, even when an ATP regeneration system was added (Fig. 9A, lane 5). This differs from the in vivo experiments that demonstrate that HSPs relocalized to the cytosol after heat stress was released (Lin et al., 1984). This protein interaction caused by heating also occurred with heterologous soluble protein systems, such as from mung bean (Fig. 9B, lane 1) or pea (Fig. 9B, lane 2). These results confirmed our previous report that the HSP-enriched fractions were exchangeable among soybean, mung bean, and rice for thermostabilization (Jinn et al., 1993). However, the LMW-HSP complex did not interact with BSA or chicken egg albumin (Fig. 9B, lanes 3 and 4). But, for now we do not know which kinds of proteins in the soluble protein fractions could interact with the HSP complex.

DISCUSSION

In the previous study of HSPs in soybean, the class of 15- to 18-kD HSPs that shuttle between the cytoplasm and

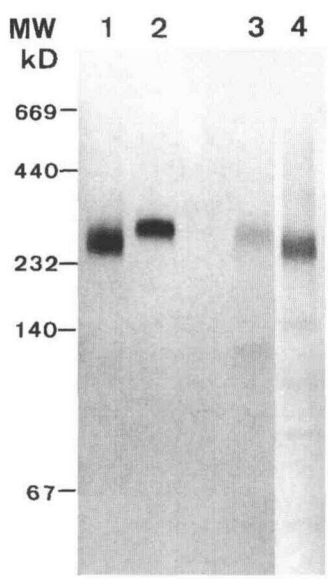

Figure 4. Identification of the class I LMW-HSP complex from different plant species. The purified class I LMW-HSP complexes from soybean (lane 1) and rice (lane 2) and the HSP-enriched fractions of 70 to 100% AS saturation from mung bean (lane 3) and pea (lane 4) were separated by nondenaturing PAGE, then blotted to an Immobilon PVDF membrane, reacted with the soybean class I LMW HSP antibodies (with the second antibody conjugated to alkaline phosphatase), and assayed for alkaline phosphatase activity by color development. One microgram of the purified complex and 50 μg of the HSP-enriched fractions were loaded. Lane MW, Molecular mass markers.

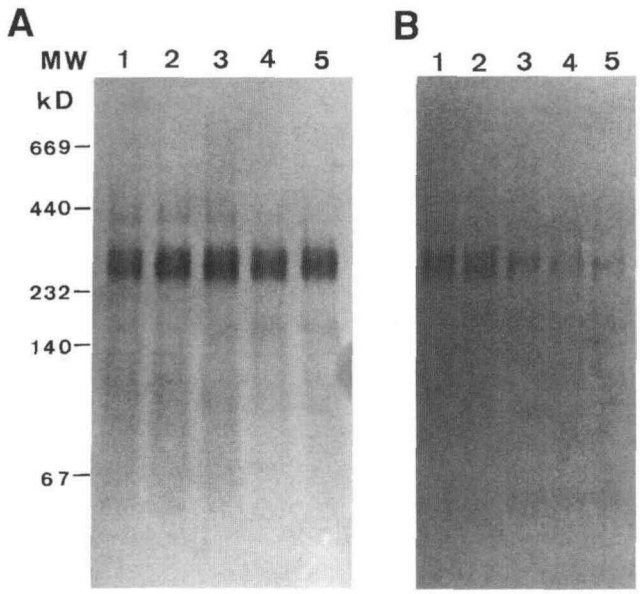

Figure 5. Persistence of the class I LMW-HSP complex during recovery from heat treatment. Soybean seedlings were treated at 40°C for 3 h and then transferred to 28°C for recovery from heat stress. Proteins were analyzed by western blot as follows: A, 28°C for 0, 4, 8, 16, and 24 h after the heat treatment in lanes 1 to 5, respectively; B, 28°C for 6 h and 1, 2, 3, and 4 d after the heat treatment in lanes 1 to 5, respectively. Total soluble proteins loaded were based on equal tissue amounts. Lane MW, Molecular mass markers.

organelles during HS and recovery from the HS (Lin et al., 1984) were enriched in the AS fraction of 70 to 100% saturation (Jinn et al., 1989). In this study, we used this preparation to demonstrate that these HSPs form a higher-order structural complex. The presence of the 15- to 18-kD class I LMW HSP in the 280-kD complex was confirmed by Coomassie blue staining, radioisotope labeling, and western blot analysis of two-dimensional PAGE gels. Other HSPs, ranging from 110 to 30 kD, formed lower molecular mass complexes, although occasionally the 22- to 24-kD HSPs were found in the higher complex form.

The composition of the class I LMW-HSP complexes in seedlings of rice, pea, and mung bean differed in the numbers, pI, and molecular mass of the individual LMW HSPs present, but the complexes formed had similar molecular structures in the native gels. Interestingly, recombinant rice 16.9-kD HSP expressed in *Escherichia coli* (C.H. Yeh, unpublished observations) alone forms a native complex of 310 kD. This suggests that the size of a native complex is not controlled by the number or size of the class I HSPs. This complex formation is not the consequence of HS as shown by treatment with arsenite and amino acid analogs at 28°C. The 25-kD HSP of mouse and the 28-kD of mammalian cells were found in HMW complexes of 400 to 800 and 200 to 800 kD (Arrigo et al., 1988), respectively. In the case of *Drosophila*, the four LMW HSPs of 22, 23, 26, and 27 kD also formed an HMW complex with RNA with properties similar to those of ribonucleoprotein particles (Arrigo and Ahmad-Zadeh, 1981). The relative LMW complex that we observed in this study appears to be different from the complex reported by Nover (1990) of large aggregates called HS granules. Although they proposed that the HS granules are formed under severe HS conditions by assembly of pre-HS granules, our native complex is different from the pre-HS granules in molecular mass and composition.

The soybean 280-kD HSP complex isolated from the nondenaturing PAGE is stable up to 250 mM KCl and 1% Nonidet P-40, but it is denatured by 0.1% SDS and 6 M urea. This complex is also heat stable, and the size of the native complex is unchanged by heating up to 50°C. However, in the presence of soluble proteins either from soybean, mung bean, or pea, as the incubation temperature of the mixture exceeds 37.5°C, the complex changes to the higher molecular form. In general, 37.5°C is the temperature at which the denaturation of proteins in vivo begins. These in vitro observations may offer an explanation of the earlier reports that the class I LMW HSP localized in the organellar fractions, depending on the severity of the HS treatments (Lin et al., 1984) and the thermostabilization (Jinn et al., 1989, 1993). Since the HSP complex alone does not change its molecular size upon heating, the formation of a complex in the organelles is more likely due to interaction of the HSP complex with the target proteins rather than aggregation of the HSPs themselves. The higher molecular mass complex

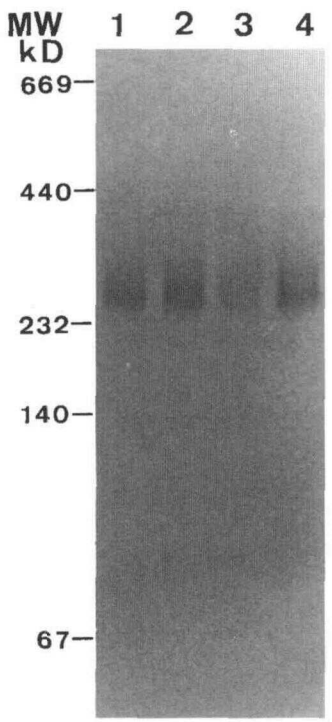

Figure 6. The class I LMW-HSP complex induced by amino acid analogs of azetidine, canavanine, and arsenite. Two-day-old soybean seedlings were treated at 28°C for 24 h with 5 mM azetidine (lane 2), with 5 mM canavanine (lane 3), and with 75 μM arsenite (lane 4). Seedlings treated at 40°C for 2 h, followed by 28°C for 4 h, were used as a reference (lane 1). The 70 to 100% AS-saturated fractions were separated by 5 to 20% nondenaturing PAGE and followed by western blot analysis with soybean class I LMW-HSP antibody. Lane MW, Molecular mass markers.

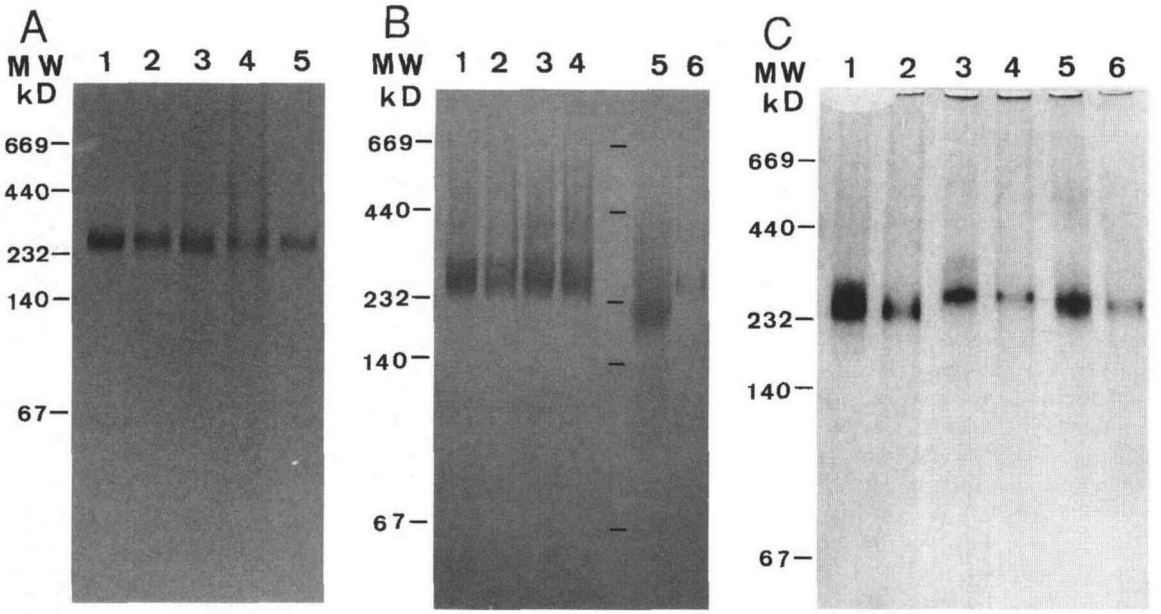

Figure 7. Physical properties of the class I LMW-HSP complex examined by western blot analysis. A, Heat stabilization of the class I LMW-HSP complex by heating at different temperatures for 15 min at 28°C (lane 1), 42.5°C (lane 2), 45°C (lane 3), and 50°C (lane 4) or with 3 μg/mL RNase at 28°C for 3 h (lane 5). B, Stabilization using high salt and nonionic detergent treatment at 28°C for 3 h; HSP complex alone as control (lane 1), with 250 mM KCl (lane 2), with 0.1% Nonidet P-40 (lane 3), with 250 mM KCl and 0.1% Nonidet P-40 (lane 4), with 0.1% SDS at 28°C for 3 h (lane 5); with same treatment as lane 5, followed by removal of SDS by dialysis (lane 6). C, Reformation of the LMW-HSP complex of soybean and rice after dissociation with 6 M urea was examined by silver nitrate stain. LMW-HSP complexes of soybean (lanes 1 and 2), rice (lanes 3 and 4), and the mixture of soybean with rice (lanes 5 and 6) were dissociated with 6 M urea at 30°C for 2 h, followed by dialysis against 50 mM Tris-HCl, pH 8.8, 1 mM EDTA, and 0.1% 2-mercaptoethanol at 4°C overnight. Lanes 1, 3, and 5 are before dissociation; lanes 2, 4, and 6 are after dissociation. Lanes MW, Molecular mass markers.

containing LMW HSPs and other proteins also remained during the recovery from HS in vitro even in the presence of ATP and its regeneration system. In contrast, the relocalization of this HSP complex in vivo to the cytoplasm is dependent on energy (NaF or anaerobiosis inhibited relocalization, C.Y. Lin, unpublished results). So it may need some factor(s) that is absent in these in vitro studies. We are now investigating the protein candidates that interact with the HSP complex.

We reported earlier that the HSP-enriched protein fraction prepared from the PRS of HS soybean seedlings provided thermostabilization of normal soluble proteins. These experiments were carried out based on the model proposed for the biological role of HSPs by Minton et al. (1982), which suggests that HSPs may act as heat-stable proteins to nonspecifically stabilize other proteins that are highly susceptible to inactivation or denaturation by heat.

Table I. *Thermostabilization of ^{3}H-proteins by addition of an HSP-enriched fraction and the purified class I LMW-HSP complex prepared from soybean seedlings*

To 1 mg of ^{3}H-proteins from the PRS (600,500 cpm), 1 mg of the 70 to 100% AS fraction from seedlings of two different treatments was added; the mixtures were heated at 55°C for 30 min. After the heat treatment, the samples were centrifuged at 16,000*g* for 15 min. The pellets were suspended in 250 μL of Laemmli's sample buffer, and 25 μL of duplicate samples were assayed for radioactivity. Each result is presented as the mean ± SE, $n = 2$.

Addition of 70–100% AS Fraction	Proteins Denatured
	cpm (%)
28°C (3 h)	181,500 ± 710 (100)
40°C (3 h)→28°C (3 h)	103,250 ± 1,290 (56.9)
Purified 280-kD LMW-HSP complex[a]	103,490 ± 2,090 (57.0)

[a] Under this treatment, 40 μg of this LMW-HSP complex was added.

Table II. *Thermostabilization of ^{3}H-proteins by addition of purified class I LMW-HSP complex from soybean seedlings*

To 500 μg of ^{3}H-proteins from the PRS (197,500 cpm), different amounts of purified class I 280-kD LMW-HSP complex were added; the mixtures were heated at 55°C for 30 min. After the heat treatment, the samples were centrifuged at 16,000*g* for 15 min. The pellets were suspended in 100 μL of Laemmli's sample buffer, and 10 μL of duplicate samples were assayed for radioactivity. Each result is presented as the mean ± SE, $n = 2$.

Addition of 280-kD LMW-HSP Complex	Proteins Denatured
μg	*cpm (%)*
No addition	39,620 ± 600 (100)
5	34,800 ± 400 (87.8)
10	30,560 ± 1020 (77.1)
25	24,720 ± 1240 (62.4)
50	20,300 ± 280 (51.2)
75	15,460 ± 320 (39.0)
100	8,900 ± 60 (22.5)

Under our standard assay condition, approximately 50% of the soluble proteins that are normally denatured by heating at 55°C for 30 min were protected when the HSP-enriched fraction was added. Subsequently, under closer to natural physiological temperature conditions, we obtained the same results by decreasing the heating temperature to 35°C for 16 h or 40°C for 9 h (Wu, 1992). Depletion of the 15- to 18-kD HSPs in the HSP-enriched fraction resulted in the loss of the thermostabilizing ability, and when the 15- to 18-kD HSPs were recovered in this fraction, the thermostabilizing ability was again restored (Jinn et al., 1993).

In this study the isolated soybean 280-kD HSP complex was used to test the biological role for the class I LMW HSPs in stabilizing heat-sensitive proteins as we have shown with the HSP-enriched fraction. We demonstrate here that the class I LMW HSPs in plants, which shuttle between the cytoplasm and cellular organelles during HS and recovery from HS, are responsible for functioning as molecular chaperones. The thermostabilization ability is dependent on the concentration of the HSP complex that is added. A molecular chaperone-like function was also shown for a mouse HSP of 25 kD (Jakob et al., 1993).

Although the physiological function of HSPs remains unclear, we have shown previously that synthesis and accumulation of LMW HSPs and their cellular localization are strongly correlated with the acquisition of thermotolerance in soybean seedlings (Lin et al., 1984). With the studies presented here using the isolated class I LMW-HSP complex, we may be able to establish a potentially important role for this class of HSPs in the protection of plants from thermal stress. It will be interesting to discover the in vivo significance of these observations on the ability of class I LMW-HSP complex to stabilize protein solubility in vitro.

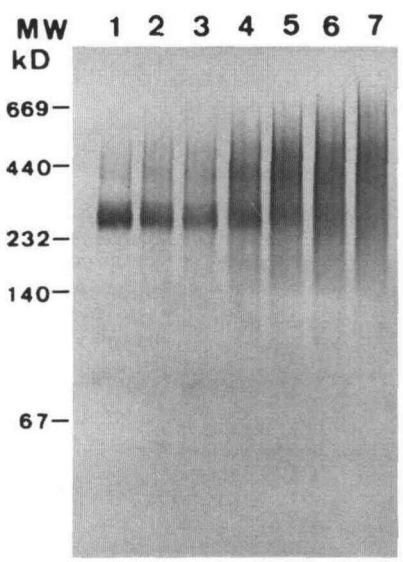

Figure 8. Western blot analysis of the soybean class I LMW-HSP complex interaction with soluble proteins. The class I LMW-HSP complex was added to the total soluble proteins of the PRS from control seedlings, and this mixture was heated for 15 min at different temperatures ranging from 30°C (lane 1), 32.5°C (lane 2), 35°C (lane 3), 37.5°C (lane 4), 40°C (lane 5), 42.5°C (lane 6), and 45°C (lane 7). Lane MW, Molecular mass markers.

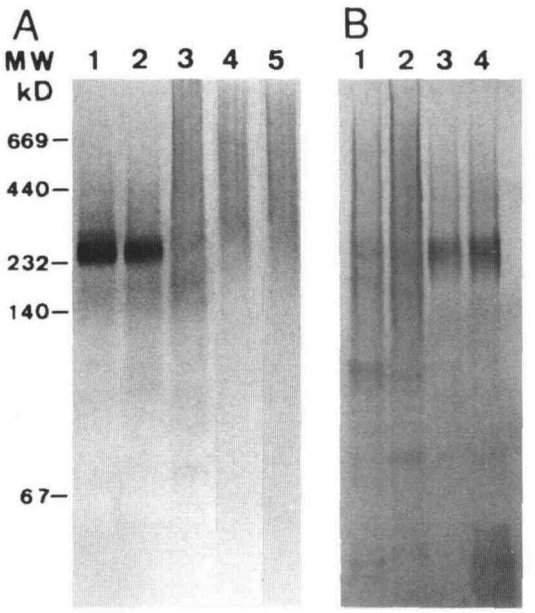

Figure 9. Western blot analysis of the soybean class I LMW-HSP complex interaction following heating after addition of different proteins. A, lane 1, The purified class I LMW-HSP complex alone; lane 2, the purified class I LMW-HSP complex heated at 42.5°C for 15 min; lane 3, the purified class I LMW-HSP complex with the total soluble proteins fraction from control treatment and heated at 42.5°C for 15 min; lane 4, same treatment as lane 3, followed by 28°C recovery for 3 h with phosphocreatine/creatine kinase ATP regeneration system; lane 5, same as lane 4 but without ATP regeneration system. B, The purified soybean class I LMW-HSP complex with soluble proteins from mung bean (lane 1), pea (lane 2), BSA (lane 3), and chicken egg albumin (lane 4) heated at 42.5°C for 15 min. The proteins were separated by 5 to 20% nondenaturing PAGE and analyzed by western blot. Lane MW, Molecular mass markers.

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