Stabilization of Soluble Proteins in Vitro by Heat Shock Proteins-Enriched Ammonium Sulfate Fraction from Soybean Seedlings

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The 70–100% ammonium sulfate fraction of postribosomal supernatant of heat shocked soybean seedlings contained a high percentage of all of the heat shock proteins. The proteins in this fraction were resistant to heat denaturation, as judged by their unpelletability after heat treatment. Moreover, this fraction, when added to the postribosomal supernatant from control (non-heat shocked) seedlings, showed a significant ability to protect the control proteins from heat denaturation. Heated at 55°C, some 50% of the control proteins, which were normally denatured after heat treatment, were protected for at least 1 h when heat shock proteins-enriched fraction was added. The degree of protection was proportional to the amount of heat shock proteins-enriched fraction added. However, when the ammonium sulfate fraction prepared from the seedlings with a heat treatment at 40°C for 3 h followed with a brief heat shock at 45°C which depleted most of the 15–18 kDa and partial 68–70 kDa, 24 kDa and 22 kDa heat shock proteins was added the effectiveness in preventing heat denaturation was lost. This suggests that the heat shock proteins of 15–18 kDa with those of 68–70 kDa and perhaps 24 kDa and 22 kDa are important for providing the protection from heat denaturation.

Key words: Heat shock protein — In vitro heat denaturation — Soybean seedling — Stabilization.

The induction of HSPs is a response to thermal stress in wide range of organisms (Schlesinger et al. 1982). When soybean seedlings are shifted from a normal growth temperature of 28°C to 40°C, there is a dramatic change in the pattern of protein synthesis: a new set of proteins, the HSPs, is rapidly synthesized (Barnett et al. 1980, Key et al. 1981).

While the physiological function of HSPs has not been documented, their induction is correlated well with the thermotolerance developed in a time and temperature dependent manner. Based on this correlation it has been hypothesized that accumulation of HSPs is an essential component of the protection process from heat damage (Gerner and Schneider 1975, Li and Werb 1982, Loomis and Wheeler 1980, McAlister and Finkelstein 1980, Mitchell et al. 1979, Petersen and Mitchell 1981). The kinetics of association or localization of the HSPs during HS and

of dissociation during recovery from HS in soybean seedlings (Lin et al. 1984) are also consistent with the notion that this dynamic localization process (Arrigo et al. 1980, Arrigo and Ahmad-Zadeh 1981, Cooper and Ho 1987, Levinger and Varshvsky 1981, Mitchell and Lipps 1975, Velazquez et al. 1980, Vincent and Tanquay 1979) is important for thermoprotection during HS and for resumption of cellular activities after HS.

During the purification of HSPs from PRS of heat shocked soybean seedlings by AS fractionation, we recovered the whole set of HSPs in a 70–100% AS fraction. Moreover, this fraction was very stable to heating (up to 65°C for 4 h).

Minton et al. (1982) proposed a model for the biological role of HSPs by stabilizing nonspecifically other proteins which are highly susceptible to inactivation or denaturation by heat.

In this study we used the AS fraction enriched in HSPs to test the hypothesis of Minton et al. (1982).

Materials and Methods

Plant material—Soybean seeds (*Glycine max* cv. Taita Kaoshiung #8) were germinated in rolls of moist paper

Abbreviations: AS, ammonium sulfate; DTT, dithiothreitol; HS, heat shock; HSPs, heat shock proteins; MW, molecular weight standard; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; PRS, postribosomal supernatant.

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towel at 28°C in a dark growth chamber.

Preparation of PRS—Two day-old seedlings (3–4 cm in length) without cotyledons were incubated in a medium containing 1% sucrose and 5 mM K-phosphate (pH 6.0) in a shaking water bath. For labeling, fifty seedlings per sample were incubated in 10 ml of the incubation medium at 28°C or 40°C for 3 h, labeled the last 2.5 h with 300 μ Ci of 3 H-leucine in the presence of 50 μ g/ml of chloramphenicol, and chased with 1 mM leucine at 28°C for 4 h. Labeled seedlings were rinsed thoroughly with 1 mM leucine before isolation of ribosomes. Seedlings were homogenized with a polytron equipped with PT-20 probe, in a medium containing 0.5 M sucrose, 0.2 M Tris-HCl (pH 8.8), 30 mM $MgCl_2$, 0.1 M KCl, 1 mM DTT, and 1 mM PMSF. The homogenate was filtered through a layer of Miracloth and centrifuged for 15 min at 23,000 $\times g$. Ribosomes were pelleted as described previously (Lin et al. 1984) but without the 1.7 M sucrose cushion.

AS fractionation—PRS was fractionated into precipitates at 30% saturation (AS I), 30–50% saturation (AS II), 50–70% saturation (AS III), and 70–100% saturation (AS IV). The pellet from each fraction was dissolved in a medium containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1% 2-mercaptoethanol, and the solution was dialyzed overnight against the same medium. All experimental procedures were carried out at 4°C.

Measurement of labeled proteins and quantitative estimation of proteins—For measurement of 3 H-proteins, a sample was blotted on 3 MM filter paper and processed as described by Mans and Novelli (1961). For quantitative analysis of proteins Lowry et al. (1951) method was used.

Gel electrophoresis and fluorography of in vivo synthesized proteins—Labeled proteins were extracted with 50 mM Tris-HCl (pH 8.5), 2% SDS, 2% 2-mercaptoethanol, and 1 mM PMSF at room temperature. The extract was precipitated with five volumes of acetone and stored at –20°C overnight. The precipitates were pelleted, dried, and dissolved in Laemmli's sample buffer

for SDS-PAGE according to Laemmli (1970). For gel electrophoresis equivalent amounts of protein samples were loaded unless otherwise specified. Fluorography of the gels was accomplished using ENHANCE (New England Nuclear) and Kodak film (XAR-5).

Assay for thermal denaturation of proteins—Protein samples (AS IV) 3 H-labeled at 28°C or 40°C were heated at the indicated temperature for the indicated period with shaking and at the end of the assay, the denatured proteins were pelleted at 23,000 $\times g$ for 15 min. The undenatured proteins remaining in the supernatant were measured for radioactivity. For the assay of stabilization of proteins by HSPs-enriched AS IV, 28°C 3 H-protein samples mixed with non-labeled AS IV were incubated at 55°C for 30 min or 1 h. After incubation the denatured 3 H-proteins were pelleted at 23,000 $\times g$ for 15 min. The radioactivity in the pellets were measured after suspension in Laemmli's sample buffer.

Results

AS fractionation of PRS—Four different concentrations of AS, 0–30% (AS I), 30–50% (AS II), 50–70% (AS III), and 70–100% (AS IV) were used to fractionate PRS. As shown in Table 1, the four AS fractionated PRS fractions differed little in protein distribution between samples from 28°C and from 40°C. However, in terms of radioactivity distribution, the AS IV showed marked differences. The specific activity (cpm/mg proteins) of the AS IV from 40°C was about twice that from 28°C (Table 1). HSPs were shown to be enriched in 40°C AS IV through SDS-PAGE analysis and fluorography (Fig. 1A). The molecular weight of soybean HSPs are 90 kDa, 83 kDa, 68–70 kDa, 27 kDa, 24 kDa, 22 kDa and 15–18 kDa shown in lane 2 of Fig. 1A as shown by Key et al. (1981). The presence of 15–18 kDa HSPs could be easily detected by Coomassie blue stain (Fig. 1B, lane 10). The AS IV from 40°C is therefore termed the HSPs-enriched fraction.

Table 1 AS fractionation of in vivo labeled 3 H-proteins from 28°C and 40°C heat treated soybean seedlings

AS	28°C		40°C	
	cpm	mg	cpm	mg
AS I (0– 30%)	181,000	2.68	257,000	2.53
AS II (30– 50%)	1,494,000	23.68	2,351,000	23.12
AS III (50– 70%)	4,664,000	19.72	6,051,000	19.04
AS IV (70–100%)	2,160,000	8.10	5,400,000	8.51

Fifty seedlings per sample were incubated in 10 ml of the incubation medium at 28°C or at 40°C for 3 h, labeled the last 2.5 h with 300 μ Ci of 3 H-leucine and chased with 1 mM leucine at 28°C for 4 h. The seedlings were harvested for isolation of PRS which were then subjected to AS fractionation.

Fig. 1 (A) Fluorographs and (B) Coomassie blue stains of in vivo labeled proteins. After AS fractionation, the fractions were separated into protein components by SDS-PAGE. 1 and 2, total proteins; 3 and 4, AS I; 5 and 6, AS II; 7 and 8, AS III; 9 and 10, AS IV. 1, 3, 5, 7 and 9 are proteins from 28°C and 2, 4, 6, 8 and 10 are proteins from 40°C. The MW are shown in the left lane of lane 1.

Table 2 Heat stability of AS IV fraction prepared from 28°C or 40°C heat treated seedlings

AS IV	cpm in supernatant (undenatured proteins)							
	65°C			77°C				85°C
	0 h	1 h	4 h	0.5 h	1 h	2 h	4 h	1 h
28°C	15,202 (100%)	12,173 (80%)	8,749 (58%)	5,493 (36%)	4,495 (30%)	3,642 (24%)	3,137 (21%)	2,485 (17%)
40°C	36,064 (100%)	35,786 (100%)	35,576 (100%)	24,382 (68%)	24,326 (67%)	21,119 (59%)	16,589 (50%)	9,195 (26%)

³H-labeled proteins of 28°C or 40°C AS IV fraction in 0.9 ml per sample (containing 1 mg of proteins) were heated at indicated temperature and time period, and 25 μ l duplicate aliquots of the supernatant containing undenatured proteins were measured for radioactivity.

Heat stability of HSPs-enriched AS IV—We found the 40°C AS IV, HSPs-enriched fraction, to be very resistant to heat denaturation as measured by unpelletability after heating (remained soluble in the supernatant) (Table 2). For example, when the 40°C AS IV was subjected to 65°C for 4 h 100% of the label remained soluble. When the fraction was heated at 77°C for 0.5 h and 4 h about 32% and 50% of the label was found in the pellet respectively. On the other hand when the 28°C AS IV was heated at 65°C for 4 h 42% of the proteins was pelleted (denatured); when heated at 77°C for 4 h 79% of the labeled proteins was pelleted. The undenatured proteins

remaining in the supernatant after the heating were analyzed by SDS-PAGE. We found that 15–18 kDa were more stable, followed by 27 kDa, whereas 68–70 kDa, 83 kDa show least stability (Fig. 2).

Protection ability of the 40°C AS IV (time and quantity)—Fig. 3A shows that the 40°C AS IV provided thermoprotection at 55°C during the 1 h incubation; the protection was about 50% higher than 28°C AS IV. When the temperature was at 65°C, the 40°C AS IV provided better protection only during the first 30 min. Fig. 3B provides evidence that the protection ability of the 40°C AS IV was proportional to the amount of proteins used.

Fig. 2 Fluorographs of 40°C AS IV undenatured proteins remained in the supernatant after heat treatment by SDS-PAGE analysis (based on equivalent amounts of tissue). Heat treatment at 55°C for 0 h (A) and 4 h (B); at 65°C for 1 h (C) and 4 h (D); at 77°C for 0.5 h (E), 1 h (F), 2 h (G) and 4 h (H); at 85°C for 1 h (I). The MW are shown in the left lane of lane 1.

Fig. 3 (A) Time course of 28°C ³H-protein denaturation by heat treatment. To 1 ml of 28°C ³H-proteins (containing 202,300 cpm and 2 mg proteins), 1 mg of AS IV fraction from 28°C or 40°C heat treated seedlings was added and the mixture was heated at 55°C or 65°C for indicated time period. After each heat treatment, samples were centrifuged at 23,000 × *g* for 15 min. The denatured protein pellets were dissolved in 500 μl of Laemmli's sample buffer, and 50 μl of aliquots in duplicates were measured for radioactivity. Heat treatment at 55°C by addition of unlabeled 28°C AS IV (Δ) and 40°C AS IV (▲); at 65°C by addition of unlabeled 28°C AS IV (○) and 40°C AS IV (●). (B) Dose-response of unlabeled 28°C and 40°C AS IV for thermoprotection of 28°C ³H-proteins from heat denaturation. Each sample with addition of 28°C AS IV (●) or 40°C AS IV (▲) were heated at 55°C for 30 min and processed as indicated in (A) for measurement of denatured ³H-proteins.

Protection ability of HSPs without 15–18 kDa and with reduced levels of 68–70 kDa, 24 kDa and 22 kDa—The 28°C AS IV had no HSPs, whereas the 40°C AS IV had a complete set of HSPs. In addition the 40°C → 45°C AS IV (seedlings that were first treated at 40°C for 3 h and then treated at 45°C for 30 min for induction of HSP association with organelles) was nearly depleted of the 15–18 kDa and only somewhat reduced in the 68–70 kDa, 24 kDa and 22 kDa HSPs (Fig. 4). Using these AS IV protein fractions, we asked “are the 15–18 kDa and partial 68–70 kDa, 24 kDa and 22 kDa HSPs involved in the protection against thermal denaturation?” The answer to this question is found in Table 3. The data suggest that the 15–18 kDa HSPs, which were present in the 40°C AS IV but

Fig. 4 Fluorograms of AS IV from three different treatments of soybean seedlings separated into protein components by SDS-PAGE. (A) 40°C → 45°C (seedlings were treated first at 40°C for 3 h and then at 45°C for 30 min for induction of HSP association with organelles), (B) 40°C and (C) 28°C.

Table 3 Stabilization of 28°C ³H-proteins against heat by addition of AS IV fraction

Addition AS IV	cpm	
	0.5 h	1.0 h
28°C	10,797 (26%)	12,253 (29%)
40°C	5,788 (13.7%)	7,743 (18.3%)
40°C → 45°C ^a	10,560 (25%)	12,730 (30.2%)

To each 0.9 ml of 28°C ³H-proteins (containing 422,100 cpm), 1 mg AS IV proteins from seedlings of three different heat treatment was added and heated at 55°C for indicated time period. After each heat treatment, the samples were centrifuged at 23,000 × g for 15 min. The pellets were suspended in 250 µl of Laemmli's sample buffer, and 25 µl in duplicates were measured for radioactivity.

^a Seedlings were treated first at 40°C for 3 h and then treated at 45°C for 30 min for induction of HSP association with organelles.

not in the 40°C → 45°C AS IV, were required for protection. We can not rule out the possibility that the 68–70 kDa, 24 kDa and 22 kDa are required in small amounts.

Discussion

In our earlier experiments to test the model proposed by Minton et al. (1982) that the biological role for HSPs may stabilize heat-sensitive proteins, PRS from 40°C treated seedlings were used as a source of HSPs. We found the PRS from 40°C treated seedlings were 50% more stable to heat denaturation than that from 28°C treated seedlings (unpublished data, presented as a poster at the 1st International Congress of Plant Molecular Biology, Savannah, Ga, U.S.A. 1985). This provided some of the first evidence for a possible role of HSPs in the acquisition of thermotolerance in intact plants.

In the present investigation, we used a HSPs-enriched fraction to attack the same problem. During purification of HSPs by AS fractionation the whole set of HSPs was found in AS IV fraction, although a small percentage was found in AS III.

The HSPs-enriched AS IV was itself resistant to heat denaturation as measured by pelletability after heating.

The 40°C AS IV also showed significant ability to protect non-HSPs from heat denaturation. Under our standard assay conditions, 26% of a 28°C soluble proteins was rendered pelletable by a 30 min incubation at 55°C. When 40°C AS IV was mixed with the 28°C soluble proteins, only 13% was pelletable after an identical heat treatment. In order to find more specifically which species of HSPs were contributing to the thermal stabilization, we used a protocol of seedling heat treatment (40°C → 45°C) which depleted 15–18 kDa and reduced 68–70 kDa, 24 kDa and

22 kDa HSPs in PRS (Lin et al. 1984). When this preparation of 40°C → 45°C AS IV was added in our standard assay the effectiveness in preventing normal non-HSPs from heat denaturation was lost entirely. The finding suggest that 15–18 kDa and perhaps 68–70 kDa, 24 kDa and 22 kDa HSPs contribute most to providing thermostability.

Recently we were able to demonstrate that isolated mitochondria are thermoprotected in vitro for O₂ uptake and tightly coupled to ATP formation at 42.5°C by association of 15–18 kDa and partial 68–70 kDa, 24 kDa and 22 kDa HSPs in soybean seedlings (Chou et al. 1989). While it appears that association of 15–18 kDa and perhaps 68–70 kDa, 24 kDa and 22 kDa HSPs with organelles during HS provides the basis of in vitro experiment for thermoprotection, the preparation of soluble proteins (PRS) we used in this study might contain some of the proteins from the organelles to which these HSPs are providing thermoprotection. We are now studying which component of the soluble proteins is preferentially thermoprotected by these HSPs.

The role of HSPs we observed here in stabilizing proteins by maintaining in a soluble, undenatured form of proteins may be analogous to the chaperones proposed by Hemmingsen et al. (1988) recently.

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