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Purification and in Vitro Chaperone Activity of a Class I Small Heat-Shock Protein Abundant in Recalcitrant Chestnut Seeds[1]

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A 20-kD protein has been purified from cotyledons of recalcitrant (desiccation-sensitive) chestnut (*Castanea sativa*) seeds, where it accumulates at levels comparable to those of major seed storage proteins. This protein, termed Cs smHSP 1, forms homododecameric complexes under nondenaturing conditions and appears to be homologous to cytosolic class I small heat-shock proteins (smHSPs) from plant sources. In vitro evidence has been obtained that the isolated protein can function as a molecular chaperone: it increases, at stoichiometric levels, the renaturation yields of chemically denatured citrate synthase and also prevents the irreversible thermal inactivation of this enzyme. Although a role in desiccation tolerance has been hypothesized for seed smHSPs, this does not seem to be the case for Cs smHSP 1. We have investigated the presence of immunologically related proteins in orthodox and recalcitrant seeds of 13 woody species. Our results indicate that the presence of Cs smHSP 1-like proteins, even at high levels, is not enough to confer desiccation tolerance, and that the amount of these proteins does not furnish a reliable criterion to identify desiccation-sensitive seeds. Additional proteins or mechanisms appear necessary to keep the viability of orthodox seeds upon shedding.

Like other heat-shock proteins, smHSPs were originally identified as proteins, the expression of which is highly induced by elevated temperatures. Plant smHSPs are a structurally diverse protein family, and the members, with molecular masses between 15 and 30 kD, have been grouped into five major classes (for review, see Waters et al., 1996). In plant cells smHSPs have been localized to the cytoplasm (classes I and II), ER, mitochondria, and chloroplast (Vierling, 1991; Helm et al., 1993, 1995; Lenne and Douce, 1994; LaFayette et al., 1996). A recent phylogenetic analysis has revealed that gene duplication, sequence divergence, and gene conversion have been major events in the evolution of this protein group (Waters, 1995).

Little is known at present about the in vivo function of plant smHSPs. It has been shown that a smHSP-enriched protein fraction from heat-stressed soybean seedlings can protect soluble proteins from heat denaturation in vitro (Jinn et al., 1995). Molecular chaperone activity has also been reported for two recombinant pea smHSPs expressed in *Escherichia coli* (Lee et al., 1995). By crossing tolerant and nontolerant variants of *Agrostis palustris*, Park et al. (1996) have demonstrated that heat tolerance and the presence of certain 25-kD smHSPs are linked traits. On the other hand, a role in chilling tolerance has been proposed for heat-shock proteins of tomato fruit, including several low-molecular-weight components (Sabehat et al., 1996).

Besides heat stress, other environmental or developmental signals regulate the expression of smHSPs in plants (Waters et al., 1996). A well-characterized example of developmental induction is the accumulation of smHSPs during seed maturation (Vierling and Sun, 1989; Howarth, 1990; Almoguera and Jordano, 1992). Specific class I and II smHSPs have been reported to accumulate, under temporal and spatial control, during embryo development in the seeds of several plant species (Hernández and Vierling, 1993; Coca et al., 1994; DeRocher and Vierling, 1994; zur Nieden et al., 1995). More recently, heat-shock elements have been shown to be involved in heat-shock promoter activation during tobacco seed maturation (Prändl and Schöffl, 1996).

Several authors have suggested a role during the desiccation process for at least some seed smHSPs (Almoguera and Jordano, 1992; Coca et al., 1994; DeRocher and Vierling, 1994). In agreement with this proposal, Almoguera et al. (1993) have demonstrated that proteins homologous to class I and II seed smHSPs (HSPs 17.6 and 17.9, respectively), are specifically expressed in the stems and roots of water-stressed sunflower plants. Moreover, constitutive expression of proteins immunologically related to HSPs 17.6 and 17.9 has been reported in vegetative tissues of the resurrection plant *Craterostigma plantagineum* (Alamillo et al., 1995). Very recently, however, the analysis of seed smHSP expression in several ABA-insensitive mutants of *Arabidopsis thaliana* has led to the conclusion that wild-type levels of these proteins are not necessary for desiccation tolerance (Wehmeyer et al., 1996).

Although seed maturation typically involves a period of desiccation, certain species shed their seeds at high moisture contents. These seeds, which quickly lose their viability as they dry, cannot be stored under orthodox conditions

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Abbreviations: AS, ammonium sulfate; HSP, heat-shock protein; RP-HPLC, reversed-phase HPLC; smHSP, small (low-molecular-mass) HSP.

and have been termed "recalcitrant" (Roberts, 1973). A significant proportion of the species known to produce recalcitrant seeds is represented by woody perennials of economic importance. These include timber species of temperate latitudes, such as oak, chestnut (*Castanea* spp.), or horse chestnut, and tropical trees with commercial fruits, such as avocado, mango, or rambutan. The recalcitrant seeds of chestnut trees, which are highly sensitive to desiccation (Jaynes, 1974) and have moisture contents at shedding usually above 50% (Payne et al., 1983), are of particular interest in this context.

Here we report the isolation of an abundant protein in the cotyledons of European chestnut (*Castanea sativa*) that forms homododecameric complexes under nondenaturing conditions. This protein shows homology with class I smHSPs and can function as a molecular chaperone in vitro. The presence of immunologically related proteins in recalcitrant and orthodox seeds from several woody species has been investigated using monospecific antibodies.

MATERIALS AND METHODS

Mature seeds were harvested at shedding from chestnut (*Castanea sativa* Mill.), Norway maple (*Acer platanoides* L.), horsechestnut (*Aesculus hippocastanum* L.), eastern white cedar (*Biota orientalis* L.), beech (*Fagus sylvatica* L.), ash tree (*Fraxinus angustifolia* Vahl.), ginkgo (*Ginkgo biloba* L.), maritime pine (*Pinus pinaster* Ait.), holm oak (*Quercus ilex* L.), yew (*Taxus baccata* L.), and little leaf linden (*Tilia cordata* Mill). Seeds from *Annona cherimola* Mill., *Mangifera indica* L., and *Persea americana* Mill. were obtained from market fruits. All seeds were stored at −40°C.

Purification of Cs smHSP 1

Flour from *C. sativa* cotyledons was partially defatted with petroleum ether as previously described (Collada et al., 1991), and proteins were extracted at 4°C for 1 h with 0.0625 M Tris-HCl, pH 6.8, and 1 mM PMSF (10:1, v/w). After centrifugation at 30,000*g* for 30 min, the supernatant was subjected to differential AS precipitation. Cs smHSP 1 was the only polypeptide present in the 90 to 100% (percent of AS saturation) fraction. Prior to sequencing, Cs smHSP 1 was carboxymethylated and repurified by RP-HPLC on a Nucleosil C4 column (4.6 × 250 mm), using a two-step linear gradient of 0.1% trifluoroacetic acid in acetonitrile (20–50% acetonitrile in 50 min; 50–85% acetonitrile in 15 min; flow rate 0.5 mL/min). The peak containing Cs smHSP 1 was directly lyophilized.

Electrophoretic Procedures

SDS-PAGE was carried out according to Laemmli (1970) on Miniprotein II system minigels (Bio-Rad). Two-dimensional native pore-gradient PAGE × SDS-PAGE and IEF (dissociating and reducing conditions) × SDS-PAGE were performed as previously described (Collada et al., 1991).

Antiserum

Antibodies to Cs smHSP 1 were raised in rabbits. Every 2 weeks 200 μg of protein was injected in complete Freund's adjuvant (three injections in total), and blood samples were taken at 2-week intervals. The third bleeding was used to purify monospecific antibodies as previously described (Collada et al., 1992).

Immunodetection

Proteins from seed-storage tissues were extracted in all cases with 0.0625 M Tris-HCl, pH 6.8, supplemented with 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, and 8 M urea. Seed proteins were then fractionated by SDS-PAGE (reducing conditions), transferred to PVDF membranes, and probed with a 1:1000 dilution of anti-Cs smHSP 1 antibodies.

Protein Cleavage and Sequencing

Since the N terminus of Cs smHSP 1 was found to be blocked, this protein was digested with endoproteinase Asp-N (Boehringer Mannheim) for 5 h at 37°C in 50 mM sodium phosphate, pH 8.0. The resulting peptides were fractionated by RP-HPLC on a semipreparative Nucleosil C4 column (4.6 × 250 mm; particle size, 5 μm), using a linear gradient of 0 to 70% acetonitrile, 0.1% trifluoroacetic acid in water over a period of 70 min (flow rate, 0.5 mL/min). Two major peptides, Cs P1 and Cs P2, were sequenced by standard methods on a gas-phase sequenator (470A, Applied Biosystems).

Chaperone Activity Assays

Pig heart citrate synthase (Sigma) was denatured with guanidine hydrochloride as previously described (Lee et al., 1995), and then diluted 100-fold into refolding buffer in the presence of variable amounts of Cs smHSP 1 or lysozyme (negative control). At the times indicated in Figure 4, 25-μL aliquots were assayed for citrate synthase activity as described by Lee (1995). Thermal-inactivation experiments were carried out as described by Lee et al. (1995). Error bars in figures represent SE values from at least three replicate trials.

RESULTS

Isolation of a 240-kD Oligomer from Chestnut Cotyledons

A protein preparation from chestnut cotyledons, extracted with Tris-HCl buffer, was fractionated by differential AS precipitation and analyzed by SDS-PAGE (Fig. 1). The 90 to 100% saturation fraction showed a single band of approximately 20 kD (Fig. 1, lane 5). Further analysis of this fraction by two-dimensional electrophoresis (native pore-gradient PAGE × SDS-PAGE) revealed that the 20-kD band contained a single polypeptide that was able to form an oligomeric complex of approximately 240 kD (Fig. 2A). The absence of heterogeneity in this AS fraction, further corroborated by two-dimensional IEF (in the presence of 8 M

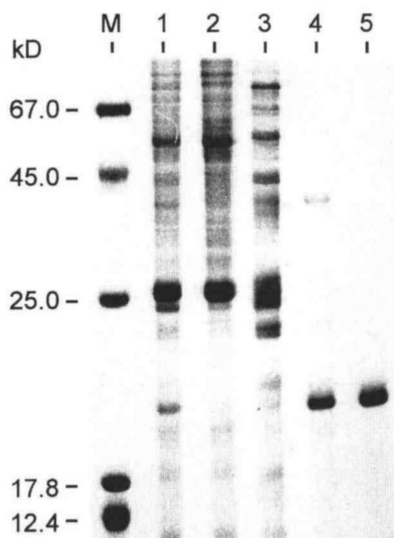

Figure 1. SDS-PAGE analysis of protein fractions obtained by differential AS precipitation. Lane 1, Total Tris-HCl extract from chestnut cotyledons; lanes 2 to 5, 0 to 65, 65 to 85, 85 to 90, and 90 to 100% (percent of AS saturation) fractions, respectively; and M, marker proteins (molecular masses are indicated on the left).

urea) × SDS-PAGE (results not shown), is consistent with the 240-kD complex in Figure 2A being formed by 12 identical subunits, although additional measurements would be required to confirm this point. The main seed storage proteins of chestnut, 11S globulins, also form oligomeric structures of approximately 240 kD (Collada et al., 1991). These complexes, which have subunits of apparent molecular masses of 33, 42, and 47 kD in SDS-PAGE (Collada et al., 1991), could be detected when total salt-soluble proteins of chestnut cotyledons (extracted with Tris-HCl buffer supplemented with 0.5 M NaCl) were analyzed by native pore-gradient PAGE × SDS-PAGE (Fig. 2B). The 20-kD subunit of the dodecameric complex was one of the most abundant components in the same two-dimensional map (Fig. 2B, arrowhead), and its identity was confirmed by co-electrophoresis of the total saline extract with the 90 to 100% AS fraction.

Homology of the 20-kD Subunit with Class I smHSPs

Prior to N-terminal sequencing, the dodecamer subunit was alkylated with iodoacetic acid and subjected to RP-HPLC. Elution of this subunit in a single peak (Fig. 2C) further confirmed the homogeneity of the AS fraction. Since its N terminus appeared to be blocked, the 20-kD subunit was cleaved with endoproteinase Asp-N, and two of the major resulting peptidic fragments, Cs P1 and Cs P2, were sequenced (Fig. 3). Peptide Cs P2 showed strong similarity with a highly conserved carboxyl-terminal region of class I smHSPs from plants (consensus II; Waters et al., 1996), whereas peptide Cs P1 could be aligned with a more divergent amino-terminal region of this protein family. According to these observations, the chestnut 20-kD polypeptide will be referred to as Cs smHSP 1.

In Vitro Chaperone Activity of Cs smHSP 1

The recent finding that recombinant pea HSP 18.1 and HSP 17.7 expressed in *E. coli* have in vitro chaperone activity (Lee et al., 1995) led us to investigate the effect of purified Cs smHSP 1 on the refolding of citrate synthase. This enzyme has been successfully used as a substrate to study chaperone-assisted protein folding (Horwitz, 1992; Jakob et al., 1993; Lee, 1995; Lee et al., 1995). In a set of experiments citrate synthase, a homodimer of 50-kD subunits, was chemically denatured in 6 M guanidine hydrochloride and then diluted 100-fold into a refolding buffer

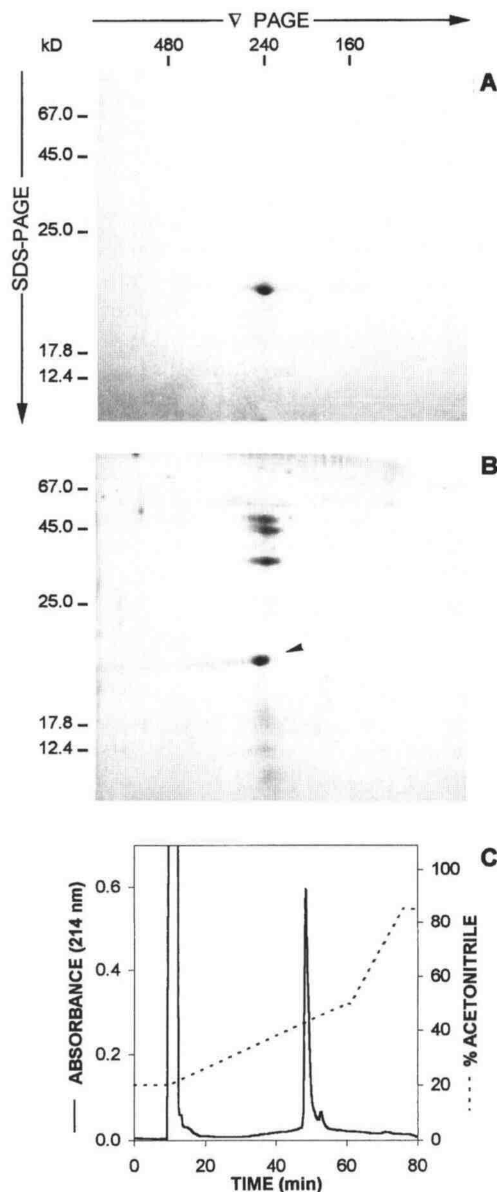

Figure 2. Two-dimensional (nondenaturing pore-gradient PAGE × SDS-PAGE) electrophoretic analysis of the 90 to 100% AS fraction shown in Figure 1 (A) and of total salt-soluble proteins from chestnut cotyledons (B). The single subunit of the dodecameric 240-kD complex (indicated by an arrowhead) was alkylated and repurified by RP-HPLC (C) prior to sequencing.

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Cs P1     DPFEGF....SAVANVPPS...ARET
At 18.2   DPFEGFFTPSSALANASTA....RDV   46
Dc 17.8   DPFKDFPLVTSSA..SEFG....KET   44
Gm 17.6   DPFKDFHVPTSSVSA.........EN   41
Os 17.4   DPFDGFPFG..SGSGSL.FPRANSDA   41
          ***  *

Cs P2     DGNVLQISGERSKEXE
At 18.2   DKNVLQISGERSKENE   98
Dc 17.8   EGKVLQISGERNKEKE   96
Gm 17.6   DDRVLQISGERNVEKE   93
Os 17.4   DGNVLQISGERIKEQE   93
            ********   *  *
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Figure 3. Alignment of amino-terminal amino acid sequences from peptides Cs P1 and Cs P2 (obtained by cleavage of Cs smHSP 1 with endo Asp-N) with class I smHSPs from *A. thaliana* (At 18.2), carrot (*Daucus carota*, Dc 17.8), soybean (*Glycine max* L., Gm 17.6), and rice (*Oryza sativa* L., Os 17.4). Database accession numbers are: X17295 (At), X53851 (Dc), M11317 (Gm), and D12635 (Os).

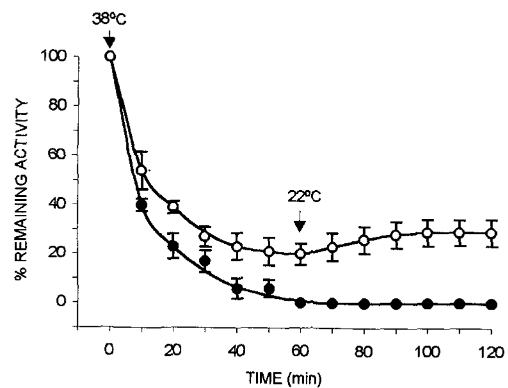

Figure 5. Influence of Cs smHSP 1 on the irreversible thermal inactivation of citrate synthase. This enzyme (150 nM) was incubated in 100 mM Hepes-KOH, pH 8.0, in the presence (○) or in the absence (●) of 150 nM Cs smHSP 1 (dodecamer). After 60 min at 38°C, samples were shifted to 22°C. At the times indicated, aliquots were taken and assayed for citrate synthase activity.

that was supplemented with or contained no Cs smHSP 1. As shown in Figure 4, when 150 nM Cs smHSP 1 (dodecamer) was added to denatured citrate synthase (at a monomer concentration of 150 nM) under renaturing conditions, the folding yields of this enzyme after 40 to 60 min of incubation were about four times higher than those of control reactions without Cs smHSP 1 (not shown) or with 150 nM egg white lysozyme (negative control; Lee, 1995). Up to a 60% reactivation (relative to an equivalent amount of nondenatured citrate synthase) was observed in the presence of Cs smHSP 1, whereas only 15% of activity was regained spontaneously (Fig. 4), even when the amount of lysozyme (in weight) equaled that of Cs smHSP 1 in the reaction mixture. Higher Cs smHSP 1 dodecamer-to-citrate synthase ratios did not result in increased enzyme reactivation, in agreement with the 1:1 stoichiometry previously proposed for pea HSPs 18.1 and 17.7 (Lee et al., 1995).

In another set of experiments we found that thermal inactivation of citrate synthase was partially prevented by the presence of Cs smHSP 1. Thus, after incubating this enzyme with a stoichiometric amount of Cs smHSP 1 for 60 min at 38°C, about 20% of the citrate synthase activity remained (Fig. 5), whereas the same treatment strongly inactivated (remaining activity < 5%) the enzyme alone or in the presence of an equimolar amount of lysozyme. Moreover, upon a temperature shift to 22°C, a small proportion of the original activity was regained in the presence of Cs smHSP 1, but not in the negative control (Fig. 5). The levels of citrate synthase protection shown in Figure 5 are lower than those reported by Lee et al. (1995) for pea HSPs 18.1 and 17.7 (up to 70% of the original value regained after 60 min at 22°C). Although this fact might reflect functional differences between Cs smHSP 1 and the pea smHSPs, the possibility of an irreversible complex formation between the former protein and citrate synthase cannot be ruled out.

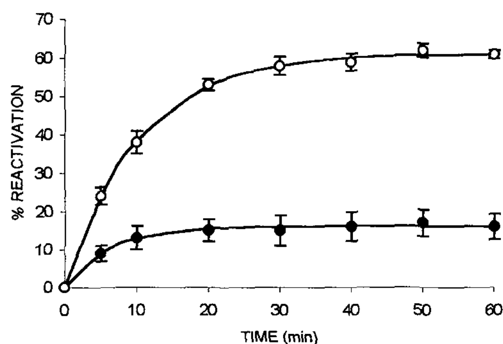

Figure 4. Effect of Cs smHSP 1 on the refolding of denatured citrate synthase. This enzyme (150 nM) was denatured in 6 M guanidine hydrochloride for 90 min and then placed under refolding conditions in the presence of 150 nM Cs smHSP 1 (dodecamer) (○) or 150 nM lysozyme (●). At the times indicated, aliquots were taken and assayed for citrate synthase activity.

Seed Proteins Immunologically Related to Cs smHSP 1

A single protein band was recognized when chestnut seed proteins (extracted with 0.0625 M Tris-HCl, pH 6.8, 2% SDS, 5% mercaptoethanol, and 8 M urea) were fractionated by SDS-PAGE and immunoblotted with the monospecific antiserum raised against Cs smHSP 1 (Fig. 6B, lane 1). Whereas homologous class I smHSPs accumulate at low to moderate levels in orthodox seeds (Coca et al., 1994; DeRocher and Vierling, 1994; zur Nieden et al., 1995), the amount of Cs smHSp 1 in recalcitrant chestnut seeds is comparable to that of 11S globulin subunits (Figs. 1 and 2). This observation led us to investigate the presence of Cs smHSP-like proteins in the seed storage tissue of other long-lived woody plants, including several species with recalcitrant seeds. The species chosen for this study shed their seeds at moisture contents ranging from 53 to 55% (*C. sativa* and *G. biloba*) to approximately 7% (*F. angustifolia*). According to these values, the seeds analyzed can be grouped in three categories: (a) orthodox, low-water content (<20% in weight) seeds from *A. platanoides*, *B. orientalis*, *F. sylvatica*, *F. angustifolia*, *P. pinaster*, *T. baccata*, and *T. cordata*; (b) orthodox, high-water content (>20%) seeds from *G. biloba* and *A. cherimola*; and (c) recalcitrant, high-

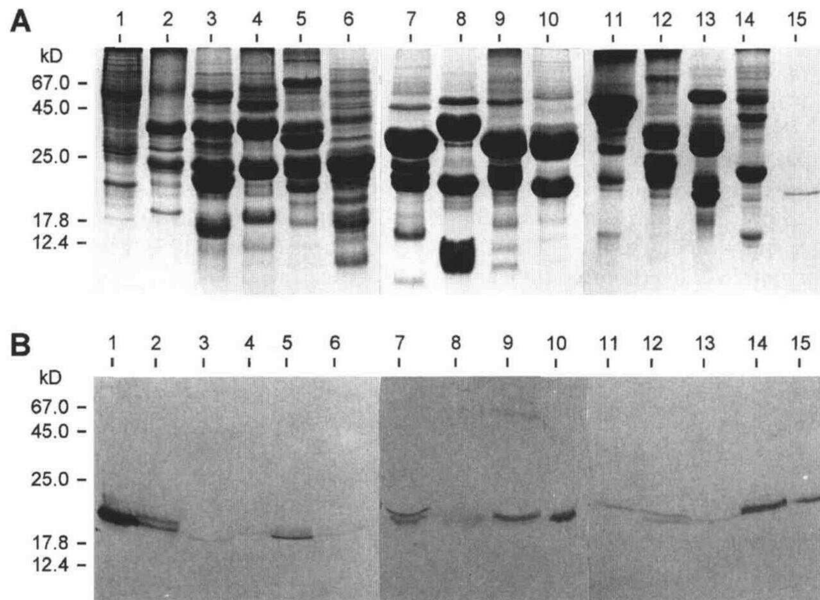

Figure 6. Western-blot analysis of cotyledon (angiosperms) or megagametophyte (gymnosperms) proteins extracted with 0.0625 M Tris-HCl, pH 6.8, 2% SDS, 5% mercaptoethanol, and 8 M urea. Proteins (approximately 100 μg) were fractionated by SDS-PAGE and either stained with Coomassie blue (A) or electrotransferred to PVDF and immunoblotted with monospecific antibodies against Cs smHSP 1 (B). Samples are as follows: lanes 1, *C. sativa* (recalcitrant); lanes 2, *Q. ilex* (recalcitrant); lanes 3, *F. sylvatica* (orthodox, low-moisture content); lanes 4, *A. platanoides* (orthodox, low-moisture content); lanes 5, *F. angustifolia* (orthodox, low-moisture content); lanes 6, *T. cordata* (orthodox, low-moisture content); lanes 7, *B. orientalis* (orthodox, low-moisture content); lanes 8, *P. pinaster* (orthodox, low-moisture content); lanes 9, *T. baccata* (orthodox, low-moisture content); lanes 10, *G. biloba* (orthodox, high-moisture content); lanes 11, *P. americana* (recalcitrant); lanes 12, *M. indica* (recalcitrant); lanes 13, *A. cherimola* (orthodox, high-moisture content); lanes 14, *A. hippocastanum* (recalcitrant); and lanes 15, purified Cs smHSP 1 (1 μg in A; 0.5 μg in B).

water content seeds from *A. hippocastanum*, *Q. ilex*, *M. indica*, and *P. americana*, in addition to chestnut. Proteins from these seeds, extracted as above, were fractionated by SDS-PAGE and probed with anti-Cs smHSP 1 antibodies. As shown in Figure 6B, the cross-reacting bands of 18 to 20 kD could be readily observed in the extracts of *A. hippocastanum*, *B. orientalis*, *F. angustifolia*, *G. biloba*, *Q. ilex*, and *T. baccata*. Minor responses to the antiserum, all in the same size range, were detected in the remaining samples. Although any quantitative conclusion from this experiment must be drawn with caution, it is noteworthy that the extract from recalcitrant *P. americana* seeds (51% moisture) reacted more weakly with the antiserum than the extract from orthodox *F. angustifolia* seeds (7% moisture) (Fig. 6B, compare lanes 11 and 5).

DISCUSSION

Cs smHSP 1, a protein of approximately 20 kD in SDS-PAGE, has been purified from recalcitrant chestnut seeds. This protein accumulates in mature cotyledonary tissue at levels comparable to those of major seed storage proteins of chestnut (Collada et al., 1991). Although its amino terminus appeared to be blocked, amino acid sequencing of two internal peptides revealed that Cs smHSP 1 is homologous to class I plant smHSPs previously described (see Waters et al., 1996).

The isolated protein, which showed no heterogeneity in two-dimensional electrophoresis, remains soluble at a high (>90% saturation) AS concentration. Additionally, its behavior in nondenaturing pore-gradient PAGE suggests that this protein can form 12-subunit aggregates. So far, the only demonstration of homododecameric complexes within the plant smHSP family had been obtained from bacterial expression of PsHSPs 18.1 and 17.7, two heat-inducible smHSPs from pea (*Pisum sativum* L.) leaves (Lee et al., 1995). Since plant smHSPs are often found as high-molecular-mass aggregates of 200 to 240 kD (Helm et al., 1993; Lenne and Douce, 1994; Osteryoung and Vierling, 1994; Jinn et al., 1995), our results further support the hypothesis, put forward by Lee et al. (1995), that such aggregates consist typically of 12 subunits. Indeed, this quaternary structure has been previously proposed for mammalian α-crystallins (Tardieu et al., 1986), a protein group related to animal and plant smHSPs.

Recent in vitro experiments have provided evidence that at least some plant smHSPs can function as molecular chaperones (Jinn et al., 1989, 1995; Lee et al., 1995). These observations led us to test whether purified Cs smHSP 1 could influence the in vitro folding properties of citrate synthase. As shown in Figure 4, refolding of this enzyme, previously denatured in guanidine hydrochloride, was enhanced in the presence of Cs smHSP 1, with this effect being maximal at dodecamer:enzyme ratios of 1:1 or

higher. Similar results have been obtained for recombinant pea HSPs 18.1 and 17.7 (Lee et al., 1995), although Cs smHSP 1 appears to be about 50% more efficient in terms of the highest refolding yield attained under equivalent experimental conditions. By contrast, Cs smHSP 1 prevented thermal inactivation of citrate synthase less effectively than HSPs 18.1 or 17.7. Whether such differences reflect some functional specialization among those members of the plant smHSP family having chaperone activity remains to be investigated. The experimental values reported here and elsewhere using citrate synthase as a model substrate are approximately of the same order for plant and animal smHSPs, which is supportive of shared functional properties in spite of their sequence divergence.

The accumulation pattern of class I smHSPs in orthodox seeds has led to the hypothesis that they are involved in the acquisition of desiccation tolerance (Coca et al., 1994; DeRocher and Vierling, 1994). This does not seem to be the case, however, for Cs smHSP 1, which accumulates abundantly in recalcitrant (that is, desiccation-sensitive) chestnut seeds. Rather, its levels might be somehow related to the high water content of chestnut seeds at shedding (>50%) and/or to their remarkable sensitivity to desiccation (Jaynes, 1974; Payne et al., 1983). To gain further insight into this latter hypothesis, the presence of immunologically related proteins was investigated in several forest seeds having different moisture contents (from 7 to 55% in weight), both orthodox and recalcitrant. Although monospecific antibodies against Cs smHSP 1 recognized proteins of 18 to 20 kD in all of the seed extracts that we tested, no correlation was found between moisture content and cross-reactivity with the antiserum. Likewise, the apparent levels of Cs smHSP 1-like proteins were unrelated to the desiccation-sensitivity of the seed. A potential bias of these conclusions may arise from the fact that proteins phylogenetically closer to Cs smHSP 1 would show stronger responses. Nonetheless, the cross-reactivities found for three out of four gymnosperms tested (*B. orientalis*, *T. baccata*, and *G. biloba*) exclude, at least in part, this possibility.

What becomes clear from our results is that the presence of Cs smHSP 1-like proteins in seeds, even at high levels, is not enough to confer desiccation tolerance. Although the expression of class I smHSPs in several *abi* (ABA-insensitive) mutants of *A. thaliana* has been recently found to be compatible with a role for smHSPs in the desiccation process, the desiccation-tolerant seeds of mutant *abi 3-1* contained 10-fold reduced levels of smHSPs (Wehmeyer et al., 1996). Our immunoblotting experiments suggest that the amount of Cs smHSP 1-like proteins at shedding does not furnish a reliable criterion to identify desiccation-sensitive (e.g. recalcitrant) seeds. Similar conclusions have been reached for dehydrin proteins, also thought to be implicated in desiccation tolerance (Finch-Savage et al., 1994). Thus, although a protective role during seed desiccation cannot be ruled out for Cs smHSP 1-like proteins, or some dehydrins, additional proteins or mechanisms are needed to keep the viability of orthodox seeds upon shedding. Relevant in this context is the recent identification of a developmentally regulated smHSP gene in sunflower; its activation during embryogenesis does not seem to be driven by water stress (Coca et al., 1996).

High-moisture seeds are in principle more sensitive to certain types of environmental damage than orthodox seeds and perhaps accumulate higher amounts of chaperones or, in general, proteins possessing a protective function. Among the species with recalcitrant seeds tested here, *C. sativa* and *Q. ilex* are particularly tolerant to low temperatures (Jaynes, 1974; Farrant et al., 1988). Unlike tropical species, the seeds of *Q. ilex* accumulate high levels of Cs smHSP 1-like proteins, and Cs smHSP 1 itself is highly abundant in chestnut. These observations are compatible with the recent finding that smHSP accumulation correlates with the acquisition of chilling tolerance in tomato fruits (Sabehat et al., 1996). It is interesting that certain seed chitinases, for which an antifreeze activity has been demonstrated in rye (Hon et al., 1995), are significantly abundant in chestnut cotyledons (Collada et al., 1992; Allona et al., 1996).

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