Plant Physiology, May 1999, Vol. 120, pp. 237–244, www.plantphysiol.org © 1999 American Society of Plant Physiologists

Purification and Partial Characterization of a Dehydrin Involved in Chilling Tolerance during Seedling Emergence of Cowpea[1]

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Dehydrins are a family of proteins (LEA [late-embryogenesis abundant] D11) commonly induced by environmental stresses associated with low temperature or dehydration and during seed maturation drying. Our previous genetic studies suggested an association of an approximately 35-kD protein (by immunological evidence a dehydrin) with chilling tolerance during emergence of seedlings of cowpea (*Vigna unguiculata*) line 1393-2-11. In the present study we found that the accumulation of this protein in developing cowpea seeds is coordinated with the start of the dehydration phase of embryo development. We purified this protein from dry seeds of cowpea line 1393-2-11 by using the characteristic high-temperature solubility of dehydrins as an initial enrichment step, which was followed by three chromatography steps involving cation exchange, hydrophobic interaction, and anion exchange. Various characteristics of this protein confirmed that indeed it is a dehydrin, including total amino acid composition, partial amino acid sequencing, and the adoption of α-helical structure in the presence of sodium dodecyl sulfate. The propensity of dehydrins to adopt α-helical structure in the presence of sodium dodecyl sulfate, together with the apparent polypeptide adhesion property of this cowpea dehydrin, suggests a role in stabilizing other proteins or membranes. Taken together, the genetic, physiological, and physicochemical data are at this stage consistent with a cause-and-effect relationship between the presence in mature seeds of the approximately 35-kD dehydrin, which is the product of a single member of a multigene family, and an increment of chilling tolerance during emergence of cowpea seedlings.

A range of molecules have been found to accumulate during seed development and are thought to play a role in preventing damage to embryos during desiccation. These include soluble sugars (Koster and Leopold, 1988; Chen and Burris, 1990) and proteins, among which the LEA (late-embryogenesis abundant) proteins are typical (Blackman et al., 1991, 1995; Dure, 1993; Close, 1996; Ingram and Bartels, 1996). For example, studies with soybean indicated that accumulation of LEA proteins during embryogenesis might reduce the extent of desiccation-induced electrolyte leakage in immature seeds (Blackman et al., 1995).

The LEA D11 family (Dure, 1993), also known as dehydrins, includes some of the most commonly observed proteins induced by environmental stresses associated with dehydration or low temperature and that comprise an immunologically distinct family (Close, 1997). These proteins accumulate in dehydrating plant tissue, such as in seeds that are becoming mature or in leaves due to drought, salinity, or extracellular freeze-thaw cycles. Also, some specific genes in the dehydrin multigene family typically are induced by cold temperatures (Close, 1997). Distinct subclasses of dehydrins have been noted (Houde et al., 1995), and a "YSK" nomenclature scheme within the dehydrin family has been developed (Close, 1997). Several lines of evidence are consistent with a role of dehydrins in membrane interactions, including immunolocalization data that imply an endomembrane association of a basic YSK_2 maize dehydrin in the cytoplasm (Egerton-Warburton et al., 1997), a plasma membrane association of an acidic SK_3 wheat dehydrin (Danyluk et al., 1998), and adoption of an α-helical structure by several cereal dehydrins in the presence of SDS (T.J. Close, unpublished data). Dehydrins can also be present in nuclei (Asghar et al., 1994), which may require phosphorylation (Jensen et al., 1998). A role in protein stabilization has been proposed. It has been hypothesized that dehydrins function as surfactant molecules, acting synergistically with compatible solutes to prevent coagulation of colloids and a range of macromolecules (Close, 1997).

Cowpea (*Vigna unguiculata*) is a warm-season annual crop that is sensitive to chilling temperature during seedling emergence. Typically, soil temperature below 20°C can cause substantial reduction in seedling emergence under field conditions. Two closely related cowpea lines were found to vary in maximal emergence under chilling field conditions (Ismail et al., 1997). Seeds of the chilling-tolerant line 1393-2-11 were shown to contain a substantial quantity of an approximately 35-kD protein using immunoblot analysis (Ismail et al., 1997) with antibodies specific for the consensus K-segment of dehydrin proteins (Close et al., 1993), whereas this protein was not detected in seeds of the chilling-sensitive line. Based on studies with F_1 hybrids and their parents, Ismail et al. (1997) hypothesized that this protein confers an increment of chilling tolerance during emergence of cowpea that is not related to line differences

[1] This research was partially supported by the U.S. Department of Agriculture-National Research Initiative Competitive Grants Program (award no. 94-37100-0688 to A.E.H.) and by the National Science Foundation (IBN 92-05269) to T.J.C.

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Abbreviations: CD, circular dichroism; CNBr, cyanogen bromide.

in electrolyte leakage. Dehydrin purification methods have been described previously (Plana et al., 1991; Ceccardi et al., 1994; Kazuoka and Odeda, 1994; Houde et al., 1995; Jepson and Close, 1995; Lisse et al., 1996). In the present study we examined the developmental expression of the approximately 35-kD protein in developing cowpea seeds. We also purified it from dry seeds of cowpea line 1393-2-11 using the method of Ceccardi et al. (1994) with some modifications. We examined various characteristics of this protein, including total amino acid composition, partial amino acid sequence, and the effect of SDS on secondary structure. The results established that this protein is a dehydrin with properties that may be relevant to its physiological function.

MATERIALS AND METHODS

Developmental Expression of the Approximately 35-kD Dehydrin

Cowpea (*Vigna unguiculata* L. Walp.) line 1393-2-11 was sown in a greenhouse with day/night temperatures of 35°C/20°C. After anthesis pods were tagged daily until the oldest pod was fully mature and dry. Tagged pods were harvested and quickly moved to the laboratory for further analysis. From each pod, one to two seeds were removed and used for total protein extraction and dehydrin assay using SDS-PAGE and western blotting, as described by Ismail et al. (1997). The remaining seeds in each pod were weighed, dried at 105°C, and reweighed to determine their seed moisture content on a fresh weight basis.

Protein Purification

Dehydrin purification was carried out following the procedure of Ceccardi et al. (1994) with some modifications. Protein concentration throughout the purification was determined by a dye-binding assay (Harlow and Lane, 1988) using bovine γ-globulin (Bio-Rad) as a standard. Seeds of cowpea line 1393-2-11 were obtained from plants grown in field conditions during the summer of 1996 at Riverside, California.

About 250 g of dry seeds (1050 seeds) was ground to the consistency of flour using a coffee grinder (model IDS-50, Mr. Coffee, Bedford Heights, OH). The ground material was then mixed into 1.5 L of prechilled 25 mM Mes (2-[morpholino]-ethane sulfonic acid) buffer, pH 6.0, 20 mM NaCl, and 1 mM PMSF and stirred for 3 h at 4°C. The mixture was then blended for 1 min using a blender (model 31BL92, Waring) and stirred overnight at 4°C. The mixture was then centrifuged at 6000*g* for 20 min at 4°C and the supernatant was decanted and filtered through four layers of cheesecloth.

The supernatant was heated to 70°C in a boiling water bath with stirring, held for 10 min at 68°C to 72°C, cooled on ice, and filtered through a Whatman filter paper no. 1. The filtrate was concentrated to a final volume of about 200 mL using a Centriprep 10 concentrator with a 10,000 M_r cutoff (Amicon, Beverly, MA), and a clarified supernatant was produced by centrifugation at 30,000*g* for 1 h at 4°C.

To prepare for cation-exchange chromatography, the sample was dialyzed in a 6000 to 8000 M_r cutoff dialysis membrane (Spectra/Por, Spectrum, Laguna Hills, CA) against prechilled 25 mM Mes, pH 6.0, and 20 mM NaCl at 4°C. Two buffer changes were made with a minimum of 6 h for each dialysis. The sample was then filtered through a 0.2-μm filter (Nalge, Rochester, NY) and passed over a source 15S fast protein liquid chromatography column (Pharmacia LKB Biotechnology, Uppsala, Sweden) that had been equilibrated with 25 mM Mes, pH 6.0, and 20 mM NaCl. Under these conditions the approximately 35-kD protein became bound to the column. An NaCl concentration gradient was applied in the same buffer, and the approximately 35-kD protein eluted between 20 and 320 mM NaCl. Fractions of 10 mL were collected and stored at −20°C until completion of immunoblot analysis of the fractions. Fractions containing the approximately 35-kD protein were pooled.

For hydrophobic-interaction chromatography, pooled fractions were dialyzed against 50 mM KH_2PO_4/K_2HPO_4, pH 7.0, and 0.8 M $(NH_4)_2SO_4$, with two changes of buffer. Samples were filtered using a 0.2-μm filter (Nalge) and passed over a Phenyl Superose HR 10/10 fast protein liquid chromatography column (Pharmacia LKB Biotechnology) that had been equilibrated with 50 mM potassium phosphate, pH 7.0, and 0.8 M $(NH_4)_2SO_4$. The approximately 35-kD protein was retained on the column under these conditions and was eluted by a gradient of decreasing ammonium sulfate concentration from 0.8 to 0.0 M over 222 mL. Fractions of 1.5 mL each were collected and analyzed by immunoblotting, as before. Fractions containing the approximately 35-kD protein were pooled and stored at −80°C until the subsequent purification step.

For anion-exchange chromatography, pooled samples from hydrophobic-interaction chromatography were dialyzed in 6000 to 8000 M_r cutoff dialysis tubing against 20 mM Tris, pH 8.8, with two changes of the dialysis buffer for a minimum of 6 h each. Following dialysis, the samples were filtered using a 0.2-μm filter (Nalge) and then passed over a Source 15Q fast protein liquid chromatography column (Pharmacia LKB Biotechnology) previously equilibrated with 20 mM Tris, pH 8.8. Under these conditions the approximately 35-kD protein was retained on the column. The protein was then eluted by applying a gradient of NaCl from 0 to 300 mM. Fractions of 1.5 mL were collected and analyzed by immunoblotting with anti-dehydrin antibody as before and by 13% SDS-PAGE with colloidal Coomassie Brilliant Blue G250 staining (Harlow and Lane, 1988). Fractions containing the immunopositive approximately 35-kD protein were stored at −80°C.

Amino Acid Composition Analysis

To prepare for amino acid composition analysis, the protein in a sample of about 500 μL was concentrated three times using a Centricon-3 concentrator (Amicon) with a 3000 M_r cutoff to about 250 μL and then rediluted to 500 μL. Deionized water was used for dilution in the first cycle, and 10 mM Tris, pH 8.0, was used in the second and last cycles. These washes decreased the concentration of NaCl

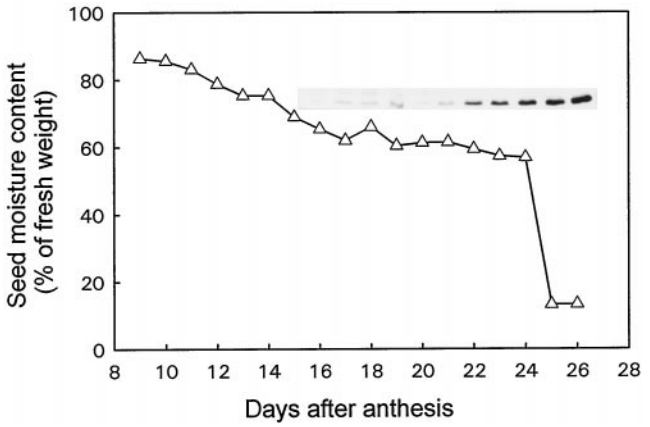

Figure 1. Changes in seed moisture content and dehydrin expression during seed development of cowpea line 1393-2-11.

in the sample from 200 to about 25 mM. The sample was then packed on dry ice and submitted to Beckman Research Institute of the City of Hope, Division of Immunology (City of Hope, CA) for total amino acid composition analysis.

Peptide Sequencing

CNBr digestion and peptide separation were performed following the procedures of Promega (Promega Technical Manual, 1993). A Centricon-3 concentrator (Amicon) was used to concentrate a sample of about 100 μg of protein in a volume of about 500 μL and to lower the concentration of Tris and NaCl by serial washes with 10 mM Tris (pH 8.0). The sample was then divided into five microcentrifuge tubes, each containing 100 μL, and lyophilized in a SpeedVac concentrator (model SVC 200, Savant, Farmingdate, NY). Two hundred microliters of CNBr solution (10 mg CNBr/mL in 70% formic acid) was added to each tube and incubated overnight at room temperature. Samples were then dried in a SpeedVac, dissolved in 45 μL of water, and redried. Thirty microliters of sample buffer was added to each tube, and the samples were combined and electrophoresed in a Tricine SDS-PAGE system (Schägger and von Jagow, 1987). Transfer of the fragmented peptides to a ProBlot PVDF sequencing membrane and staining were carried out following the protocol of Promega (Promega Technical Manual, 1993). One of the fragments was excised from the membrane and used for N-terminal amino acid-sequencing using a protein sequencer (Procise-492, Perkin-Elmer/Applied Biosystems, Foster City, CA) at the University of California, Riverside.

CD Analysis

A spectropolarimeter (model J715, Jasco, Easton, MD; laboratory of Carl Frieden, Washington University School of Medicine, St. Louis, MO) was utilized. Fraction 34, which was one of the three major chromatogram peaks following the final (anion-exchange) step of the purification, was analyzed. The sample was dialyzed against 20 mM KH_2PO_4/K_2HPO_4, pH 7.0, using Slide-A-Lyzer dialysis cassettes with a 3500 M_r cutoff membrane (Pierce). The buffer was changed four times with a minimum of 8 h during each dialysis. The sample was then concentrated using a Centricon concentrator to about 0.2 mg/mL and was subsequently dialyzed against 50 mM NaCl in preparation for CD analysis in the presence of SDS.

RESULTS

Accumulation of Approximately 35-kD Protein during Seed Development

The temporal accumulation of the approximately 35-kD protein was determined by western analysis of proteins extracted from developing seeds. The moisture content of developing cowpea seeds decreased progressively with time as shown in Figure 1. A sharp reduction in seed moisture content was observed 24 d after anthesis, when the seed moisture decreased to about 15% (on a fresh weight basis), approaching the typical moisture content of mature seed at harvest (Fig. 1). Just prior to this (21–22 d after anthesis) when the seed moisture content had decreased to about 60%, the dehydrin protein level rose considerably from a low initial level attained about 5 d earlier and continued to rise steadily as the seeds became mature (Fig. 1). The commencement of the later phase of accumulation coincided with the start of the color-break stage of pod development. Initially pods were green, but they began to develop a yellow color 21 d after anthesis, and by d 22 about 30% of the pod surface area was yellow. Coordination of maximal dehydrin accumulation with the dehydration phase of seed development is a unifying property of LEA proteins (Hughes and Galau, 1989; Dure, 1993). All cowpea genotypes that have been examined produce several dehydrin proteins during seed development (A.S. El-Kholy, A.E. Hall, and T.J. Close, unpublished data), but the approximately 35-kD protein is not detectable in many genotypes, and it is the major species detected by anti-dehydrin antibodies in genotype 1393-2-11 (Ismail et al., 1997).

Purification of the Approximately 35-kD Protein

Mature seeds were used as a source of the approximately 35-kD protein. The characteristic retention of solubility of dehydrins at high temperature was used in an initial purification step to obtain a dehydrin-enriched sample. Approximately 80% of the total soluble protein was precipitated by heating the sample to 70°C (Table I), whereas the approximately 35-kD protein remained in solution.

Table I. *Total protein and yield during purification of an approximately 35-kD cowpea dehydrin from dry seeds of line 1393-2-11*

Purification Step	Total Protein	Protein Yield
	mg	*%*
Crude extract	17,061.2	100.00
Heat treatment	3,482.7	20.41
Cation exchange	720.9	4.22
Hydrophobic interaction	17.9	0.11
Anion exchange	6.7	0.04

Three column chromatography steps were used to further purify the approximately 35-kD protein. The first step involved the use of a Source 15S cation-exchange chromatography column, where the approximately 35-kD protein eluted from the column over a wide range between 20 and 320 mM NaCl (Figs. 2A and 3). In the fractions containing the highest concentration of approximately 35-kD protein, the approximately 35-kD protein seemed to adhere to other proteins in a manner that was not fully disrupted by the Laemmli sample buffer and SDS-PAGE, as shown for fractions 21 to 35 in Figure 3. Fractions containing the approximately 35-kD protein were pooled and further separated on a Phenyl Superose HR 10/10 hydrophobic-interaction column. After this purification step, there was no further evidence of apparent adhesion to other proteins, and the approximately 35-kD protein eluted at approximately 270 to 540 mM ammonium sulfate (Figs. 2B and 4). Fractions 27 to 59 were then pooled.

The final purification step involved the use of a Source 15Q anion-exchange column. The approximately 35-kD protein eluted at approximately 150 to 280 mM NaCl (Figs. 2C and 5). Fractions 34 to 54, which covered this range, were stored at −80°C. Fractions collected were subjected to both Coomassie Blue staining and immunoblot analysis

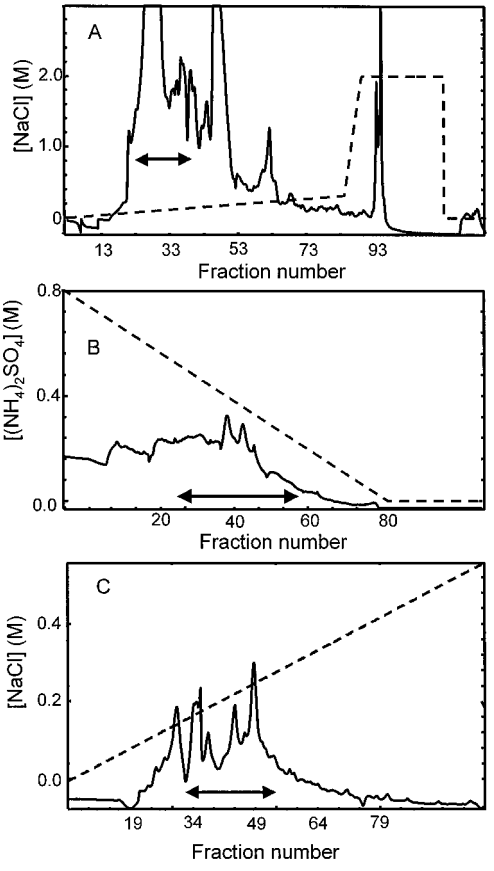

Figure 2. Chromatograms showing the different steps of dehydrin purification from cowpea seeds. Arrows indicate the fractions containing the dehydrin. A, Cation exchange; B, hydrophobic interaction; and C, anion exchange. Dashed lines indicate the salt gradient.

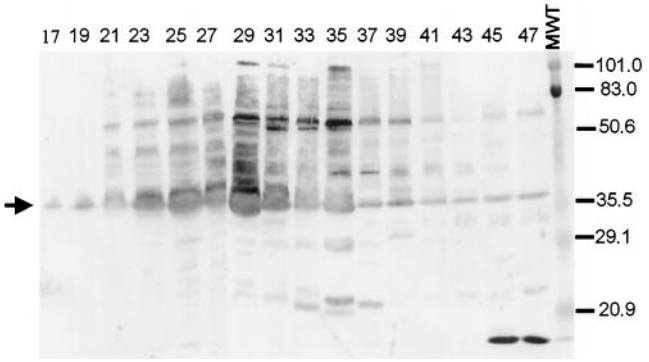

Figure 3. Immunobot of the fractions following cation-exchange chromatography, with 5 μL of each fraction loaded per lane. Fractions 22 to 35 were pooled and used for the next step. The arrow indicates the position of the approximately 35-kD dehydrin. Lane MWT, Low-range M_r markers (Bio-Rad).

(Fig. 5), which showed that the approximately 35-kD protein was free of detectable contaminants. Figure 2C shows the anion-exchange chromatogram, in which four peaks were observed. These peaks may reflect different levels of phosphorylation of the same polypeptide. An illustration of the relative purity at each major step is given in Figure 6. Total protein content and protein yield following each purification step are summarized in Table I. Approximately 0.04% of the extracted soluble protein was recovered as pure approximately 35-kD protein.

Peptide Sequencing and Amino Acid Composition

Efforts to perform N-terminal amino acid sequence analysis on the purified protein were not successful, presumably because of N-terminal blocking. Purified protein was then partially fragmented using CNBr, which cleaves the protein at the peptide bond involving the carboxyl group of Met (Gross, 1967). Cleavage products were separated using Tricine SDS-PAGE, as described by Schägger and von Jagow (1987), and transferred to a ProPlot PVDF sequencing membrane. After staining, three bands were observed as shown in Figure 7. The fragment with the lowest M_r was excised and used for N-terminal amino acid sequencing, and a sequence of 22 amino acids was obtained. Comparison of this sequence with other protein sequences in the National Center for Biotechnology Information database is shown in Figure 8. The greatest similarity was observed between this amino acid sequence and the de-

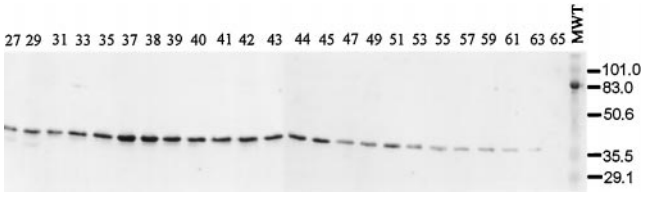

Figure 4. Immunobot of the fractions following hydrophobic-interaction chromatography, with 5 μL of each fraction loaded per lane. Fractions 27 to 59 were pooled and used for the next step. Lane MWT, Low-range M_r markers (Bio-Rad).

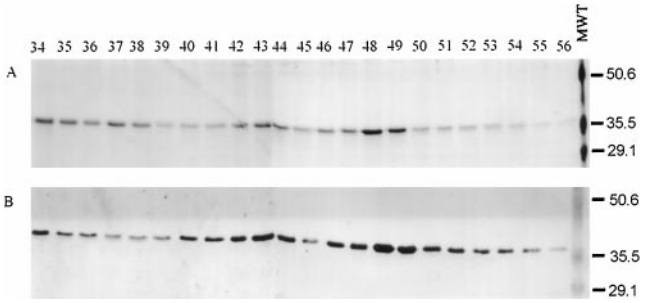

Figure 5. Fractions after anion-exchange chromatography with 5 μL of each fraction loaded per lane. A, 13% SDS-PAGE stained for total protein using colloidal Coomassie Brilliant Blue G250. B, Imunoblot analysis using anti-dehydrin antibodies as a probe. Lane MWT, Low-range M_r markers (Bio-Rad).

duced sequences of four previously identified LEA D11 proteins. One of these proteins is the CPRD22 dehydrin identified in leaves of cowpea plants subjected to drought (Iuchi et al., 1996). Two others, MAT1 (accession no. L00921; Y.J. Chyan, R.W. Rinne, L.O. Vodkin, and A.L. Kriz, unpublished data) and MAT9 (accession no. M94012; Chyan and Kriz, 1992), are maturation-associated proteins from soybean seeds and the fourth is a protein (accession no. U10111; N. Maitra and J.C. Cushman, unpublished results) from soybean leaves. The amino acid sequence of the latter protein is identical to that of MAT9, suggesting

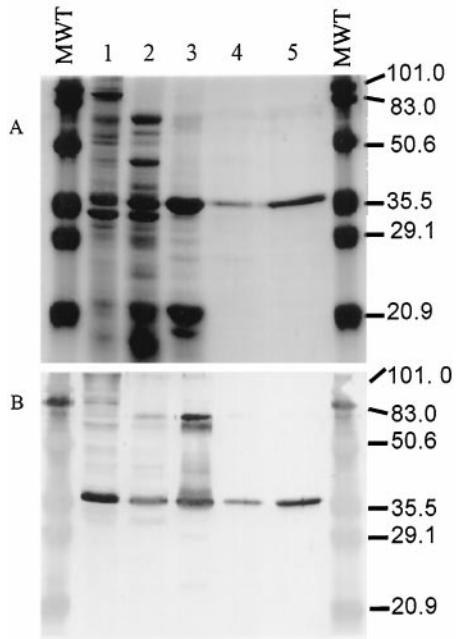

Figure 6. Steps in dehydrin purification. Lanes correspond to: 1, crude extract; 2, heat-treated; 3, cation-exchange; 4, hydrophobic-interaction; and 5, anion-exchange peaks. Lanes MWT, Low-range M_r markers (Bio-Rad). A, Colloidal Coomassie Brilliant Blue staining. Protein loaded was: lanes 1 and 2, 10 μg each; lane 3, 4.0 μg; lane 4, 2.8 μg; and lane 5, 1.9 μg. B, Immunoblot analysis using anti-dehydrin antibodies as a probe. Protein loaded was: lanes 1 and 2, 10 μg each; lane 3, 2.0 μg; lane 4, 1.0 μg; and lane 5, 0.6 μg.

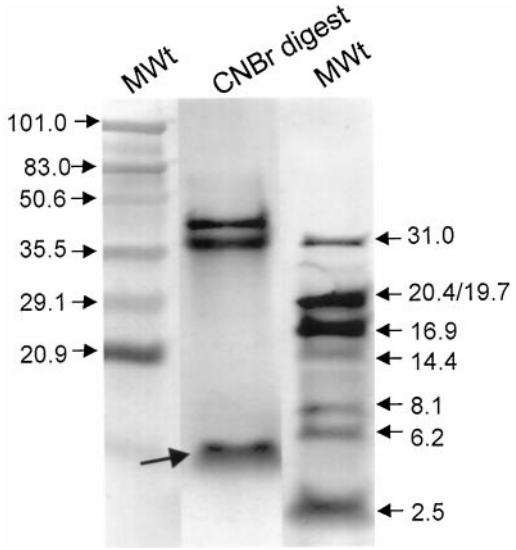

Figure 7. PVDF membrane stained with colloidal Coomasie Brilliant Blue G250 after the transfer of the protein from a Tricine gel using the western-blot technique. Lane MWt, left, Bio-Rad low-range M_r marker; lane CNBr digest, the fragments of the dehydrin after CNBr digestion; and lane MWt, right, the Promega low-range M_r markers. The large arrow shows the peptide used for the N-terminal amino acid sequence determination.

that these represent very similar or identical alleles of the same gene.

Total amino acid composition of the purified dehydrin showed that this protein is rich in Gly and polar and charged amino acids such as Thr, Asn, Gln, Ser, Asp, and Glu, suggesting that it is highly hydrophilic. It is also devoid of both Cys and Trp (Table II). This composition is in agreement with the common properties of dehydrin proteins (Close, 1997). A comparison was made between the amino acid composition of the purified cowpea approximately 35-kD protein and the deduced amino acid sequence of cowpea CPRD22 cDNA (Iuchi et al., 1996). A high level of similarity, but apparently not identity, was

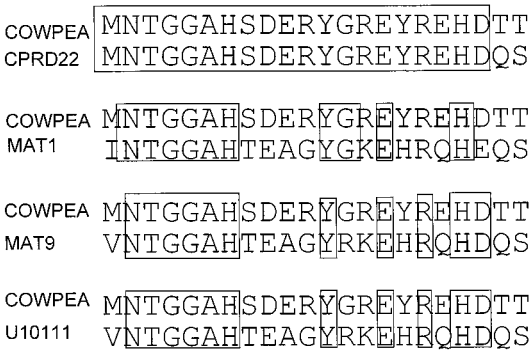

Figure 8. Comparison of the amino acid sequence of the cowpea dehydrin fragment with other dehydrin sequences in the National Center for Biotechnology Information database. CPRD22 is a drought-inducible protein produced in cowpea leaves. MAT1 and MAT9 are maturation-associated proteins from soybean seeds. U10111 is a drought-induced protein from soybean leaves. Boxes indicate conserved regions.

Table II. *Comparison of the total amino acid composition of the purified approximately 35-kD cowpea dehydrin with the deduced amino acid sequence of cowpea CPRD22 cDNA from Iuchi et al. (1996)*

Amino Acid	1393-2-11 Dehydrin	CPRD22
	mol %	
Gly	25.39	16.96
Thr	15.76	15.21
Asp + Asn	12.79	12.18
Glu + Gln	11.04	10.85
Ser	4.93	4.13
Ala	4.90	3.50
Lys	4.86	4.31
Tyr	4.71	7.71
Arg	4.27	7.98
His	2.47	6.10
Val	1.89	2.68
Leu	1.83	1.29
Phe	1.77	3.24
Pro	1.77	1.15
Ile	1.62	2.15
Met	0.25	0.98
Cys	0.00	0.00
Trp	0.00	0.00

observed between these two dehydrins (Table II). It is not possible from this information to decipher whether these two cowpea dehydrins represent alleles of a single gene or of two different cowpea dehydrin genes.

CD Analysis

The induction of α-helical structure by SDS, which has been observed previously for several cereal dehydrins (T.J. Close, unpublished data), was examined for the approximately 35-kD cowpea dehydrin. Far-UV CD was performed to estimate the structural conformation of the purified dehydrin in the presence and absence of 10 mM SDS. In the absence of SDS, the spectrum for fraction 34 contained a strong negative band near 197 nm and a weak band near 220 nm, which are characteristic of peptides lacking a well-defined secondary structure (Woody, 1992). The CD spectrum of fraction 34 was altered in the presence of SDS, such that the difference spectrum is typical of α-helical structure: positive bands in the range of 195 to 198 nm and a broad negative band in the range of 205 to 235 nm (Fig. 9).

DISCUSSION

Our previous studies with closely related cowpea lines and their F_1 hybrids (Ismail et al., 1997) indicated that an approximately 35-kD protein detected by anti-dehydrin antibodies confers an increment of chilling tolerance during seedling emergence. In this work we have established by partial amino acid sequencing that this protein is indeed a dehydrin. A high level of similarity between this protein and the deduced amino acid sequence of the cowpea dehydrin CPRD22 cDNA (Iuchi et al., 1996) was observed (Table II). Comparison of the 22-amino acid sequence of the fragment obtained after digestion with CNBr to other protein sequences in the National Center for Biotechnology Information database showed substantial similarity with four Fabaceae LEA D11 proteins (Fig. 8) and a lack of similarity to any other enzymes or proteins. The amino acid composition is also typical of dehydrins. We demonstrated that accumulation of this protein in seed of cowpea line 1393-2-11 is coordinated with the dehydration phase of embryo development. Detectable amounts were observed beginning when the moisture content of the developing embryo had decreased to about 65% on a fresh weight basis, about 5 d prior to the pod color-break stage (Fig. 1). At the pod color-break stage, when the moisture content had declined to about 60%, a further increase in abundance began, continuing to a maximal level of the approximately 35-kD protein at full-seed maturity. This coordinated expression between embryo maturation and desiccation is a typical property of LEA proteins (Hughes and Galau, 1989; Dure, 1993) and may be related to a function in protecting the embryo and cotyledons from damage during desiccation at maturity or during rehydration at germination. In this case, the consequences are apparent under chilling conditions during seedling emergence, particularly in seeds of low moisture content (Ismail et al., 1997).

Cowpea seeds contained several proteins detectable by anti-dehydrin antibodies, in addition to the approximately 35-kD dehydrin, which is typical of the multigene nature of this family of proteins. The approximately 35-kD protein appears to be the most abundant of these in genotype 1393-2-11 by our western-blot assay. Some of the other dehydrin cross-reactive proteins were collected from the chromatography steps and may be studied in the future. However, the approximately 35-kD dehydrin is of particular interest because of the genetic association that we demonstrated with seedling emergence under chilling conditions. We have previously mapped the genetic determi-

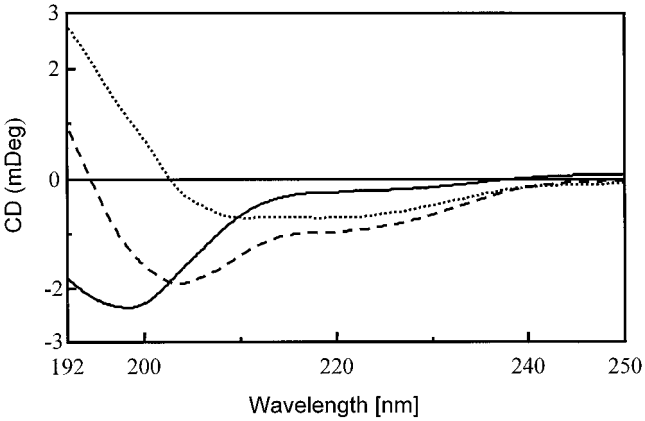

Figure 9. Effect of 10 mM SDS on secondary structure of the cowpea approximately 35-kD dehydrin. Path length, 0.1 cm; step, 0.2 nm; scan speed, 20 nm/min, six accumulations (noise-reduced and smoothed); bandwidth, 2 nm; sensitivity, 10 millidegrees; response, 2 s; and concentration = 0.19 mg/mL in 50 mM NaCl, pH 7.0, at 23°C. Solid line, 0 mM SDS; dashed line, 10 mM SDS; and stippled line, CD value in 0 mM subtracted from CD value in 10 mM SDS ("difference spectrum").

nant, which controls the presence of the approximately 35-kD dehydrin in mature cowpea seeds (data included in Menéndez et al., 1997). However, it is not yet known whether this mapped locus is a structural gene for the approximately 35-kD protein or a regulatory locus that controls expression of the approximately 35-kD dehydrin. To differentiate between these two possibilities, it is first necessary to obtain the cDNA that matches the approximately 35-kD protein and then determine its map position.

In the current study the approximately 35-kD protein was successfully purified using the characteristic high-temperature solubility of dehydrins as an initial enrichment step, followed by three sequential chromatography steps involving cation-exchange, hydrophobic-interaction, and anion-exchange chromatography. Compared with the G50 maize dehydrin, which has been purified by Ceccardi et al. (1994), the cowpea approximately 35-kD protein seems to be more hydrophobic. Its elution from the Phenyl Superose column required a gradient concentration of 0.54 to 0.27 M ammonium sulfate, which is much less than the concentration for elution of the G50 maize dehydrin, which eluted at about 1.0 M ammonium sulfate (Ceccardi et al., 1994). Hydrophobic interactions were also considered to be a possible explanation of the apparent adhesion of the approximately 35-kD dehydrin to other proteins in fractions from cation-exchange chromatography (Fig. 3). One possible explanation of this apparent protein-protein adhesion is that, at high dehydrin concentration and specific salt conditions, protein-protein complexes may form that are not disrupted by SDS, β-mercaptoethanol, and elevated temperature. Once the approximately 35-kD dehydrin has been bound to and eluted from a hydrophobic-interaction chromatography column, these putative protein-protein complexes are no longer evident.

The hydrophobic amino acid residues of the approximately 35-kD protein constitute only approximately 10% of the total amino acid composition (Table II). The capability of this protein for in vitro hydrophobic interactions may involve the formation of an amphipathic α-helix by the K-segment, analogous to the lipid-binding domain of exchangeable apolipoproteins, as suggested previously (Close, 1996). Evidence in favor of the formation of lipid-bound amphipathic α-helices was obtained by measuring the CD spectrum of the approximately 35-kD dehydrin in the presence of 10 mM SDS. As has been observed in other studies where it was shown that several cereal dehydrins form amphipathic α-helices in association with SDS (T.J. Close, unpublished data), the approximately 35-kD cowpea dehydrin also seems to share this propensity. In the absence of SDS, the CD spectrum of the approximately 35-kD cowpea dehydrin shows a strong negative band near 197 nm and a weak band at approximately 222 nm, which are characteristic of polypeptides that lack a well-defined secondary structure. These SDS-free CD data are equivalent to those obtained for a recombinant *Craterostigma plantagineum* dehydrin purified from an *Escherichia coli* expression strain studied in an SDS-free aqueous solution, from which the authors concluded that the native protein is generally unstructured (Lisse et al., 1996). However, the apparent structure-promoting effect of 10 mM SDS on the approximately 35-kD cowpea dehydrin (and others) suggests that dehydrins in vivo may contain α-helical structure(s) in a lipid-bound state.

Several proteins contain lipid-binding class A amphipathic α-helices (Segrest et al., 1990) resembling the dehydrin K-segment. In addition to exchangeable apolipoproteins, a more recently discovered analogy is α-synuclein. This protein has a role in both Alzheimer's and Parkinson's diseases, in the former case as the nonamyloid component of amyloid plaques and in the latter as a component of Lewy bodies. The α-synuclein protein binds to acidic phospholipids and vesicles with small diameters, which is accompanied by pronounced α-helicity (Davidson et al., 1998). There are numerous additional examples of proteins that appear to be "natively unfolded" in pure form but are structured in association with ligands of various types, including lipids, tubulin, and other proteins (for example, see table I of Weinreb et al., 1996). Perhaps by exploring hydrophobic interactions between dehydrins and their ligands, the physiological roles of what have often been referred to as "extremely hydrophilic" LEA and COR proteins (Thomashow, 1998) can also become better understood.

Further genetic and biochemical studies are currently underway to continue to test the apparent cause-and-effect relationship between the approximately 35-kD dehydrin and seedling emergence under chilling conditions and to define the interactions of the approximately 35-kD protein with other molecules, whether they be free fatty acids, membrane surfaces, proteins, or some combination.

ACKNOWLEDGMENTS

We thank Raymond D. Fenton for his excellent technical assistance, Dr. Carl Frieden for advice on CD and for use of the spectropolarimeter (Jasco) by T.J. Close while on sabbatical leave, and Dr. A. Clay Clark for first pointing out the parallels between LEA proteins and α-synuclein.

Received September 14, 1998; accepted January 15, 1999.

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