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Characterization of a Soybean Leaf Protein That Is Related to the Seed Lectin and Is Increased with Pod Removal[1]

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ABSTRACT

Levels of several polypeptides in addition to the vegetative storage protein (VSP) increase in soybean leaves following depodding. Two of these polypeptides interact specifically with antibodies raised against the seed lectins of *Phaseolus vulgaris* and soybean. The two polypeptides, which had apparent molecular masses of 29,000 daltons and 33,000 daltons, were present in the sink-deprived plants but not in control podded plants and were the subunit polypeptides of a glycoprotein designated lectin-related protein (LRP). Soybean LRP was purified to near homogeneity by a combination of ammonium sulfate precipitation and gel filtration. Dialysis of the resuspended ammonium sulfate precipitate caused LRP to reprecipitate, and LRP was soluble only in the presence of molar NaCl. The native relative molecular mass of LRP was 119,000 daltons, a size consistent with a tetrameric organization of the two polypeptides. LRP precipitated during dialysis in association with a 28,000 dalton polypeptide. The protein coprecipitating with LRP was identified as the dimer of the 28,000 dalton subunit of VSP, one of three native isomeric forms of VSP occurring in leaves of depodded plants. Although the specific association between LRP and VSP was intriguing, an *in vivo* interaction between LRP and VSP was doubtful. LRP was shown to be immunologically similar to soybean agglutinin but did not have detectable hemagglutinating activity. LRP also was shown to be made up of polypeptides distinct from soybean agglutinin.

It is now well established that vegetative tissues contain glycoproteins that have similarities to seed storage proteins (6, 27). Some of these glycoproteins are immunologically similar to the seed lectins isolated from the same species (6, 27), but others appear to be immunologically distinct from any seed protein (for reviews, see refs. 6, 12, 14). Glycoproteins that are similar to seed lectins have been identified in various leguminous plants, including *Dolichos biflorus* (20, 24), *Griffonia simplicifolia* (11), and *Sophora japonica* (9, 10), and the level of some of these proteins may be related to stress responses in the plants (20).

Attempts have been made to identify lectins or lectin-like proteins in vegetative soybean tissues, but the results have been inconclusive. Pueppke *et al.* (17), using radioimmunoassay techniques, were unable to detect a protein that was cross-reactive to antibodies raised against soybean agglutinin, the major lectin of soybean seeds (14), in plants older than 16 d postgermination. However, in more recent experiments, peptides recognized by antibody to soybean agglutinin were demonstrated in Western blots of soybean leaf and root extracts (25). Also, agglutinating activity that may have been caused by lectin activity has been reported in membrane fractions of roots, stems, and leaves of soybean (3, 18).

The levels of lectins isolated from the barks of *Sambucus nigra* and *Robinia pseucoacacia* fluctuate seasonally and have been implicated as vegetative storage proteins (16). In soybean leaves (*Glycine max* [L.] Merr.), a glycoprotein has also been identified that accumulates markedly when reproductive sink demand is lowered by depodding (26). However, soybean VSP[3] appears to be distinct from the major seed lectin of soybean (23, 27). Peptide sequences derived from nucleic acid sequences of the VSP subunits indicate only limited homology with soybean agglutinin (23), and no hemagglutinating activity has been reported for the purified protein.

Depodding of soybean plants also increases the levels of several polypeptides that have not been characterized and, therefore, may be distinct from VSP (8, 27). In this report, we characterize a LRP that accumulates in leaves of soybean with lowered sink demand. LRP appears to be immunologically related to the seed lectins isolated from both soybean and *Phaseolus* (14) but distinct from VSP.

MATERIALS AND METHODS

Supplies

DEAE-Bio-Gel-A and electrophoresis supplies were obtained from Bio-Rad.[4] Sephacryl S-200 gel permeation resin was obtained from Pharmacia. All other biochemicals were purchased from Sigma.

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[3] Abbreviations: VSP, soybean vegetative storage protein; LRP, soybean lectin-related protein; Kav, gel permeation elution coefficient.

[4] Mention of a trademark of proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or the North Carolina Agricultural Research Service and does not imply its approval to the exclusion of other products that may also be suitable.

Plant Material

Soybean (*Glycine max* [L.] Merr.) seed was generously provided by Dr. T. E. Carter, Jr., USDA-ARS, Raleigh, NC. The variety used was near to isogenic with the cultivar Miles and segregated for the male-sterile mutation MS1. Plants were grown under greenhouse conditions with supplemental lighting provided by 1000 W metal halide lamps to obtain a 14 h photoperiod (1). Male-sterile plants were identified at flowering and did not set seed. Fertile plants were either allowed to set seed normally (control) or depodded daily. Leaves were harvested from all plants 25 d after flowering, immediately frozen in liquid nitrogen, and stored at −80°C until used.

Protein Isolation

Proteins were purified by a modification of the procedure of Wittenbach (27) with all steps being carried out at 4°C. All centrifugation steps were at 27,000*g* for 15 min, and samples were concentrated in an Amicon ultrafiltration cell with a PM-30 membrane. Frozen leaves (100 g) were homogenized in 500 mL of homogenization buffer (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 30 mM ascorbic acid, 28 mM β-mercaptoethanol, 15 g insoluble polyvinyl-polypyrolidone). The homogenate was passed through cheesecloth and centrifuged. Ammonium sulfate was added sequentially to the supernatant to final concentrations of 1.7, 2.0, and 2.8 M, with each precipitate being removed by centrifugation. The 2.8 M precipitate was redissolved in Tris-buffer (20 mM Tris-HCl [pH 7.5], containing 14 mM β-mercaptoethanol) and dialyzed overnight. The precipitate that formed during dialysis consisted of crude LRP and was removed by centrifugation from the crude VSP in the supernatant.

The crude LRP precipitate was washed with Tris-buffer, dissolved in 10 mL of Tris-buffer containing 1 M NaCl, and applied to a Sephacryl S-200 column (2.5 × 100 cm) connected to a Pharmacia FPLC. The column was eluted in Tris-buffer containing 1 M NaCl at a flow rate of 1.0 mL min^{-1}, and three fractions were collected. The fraction containing purified LRP was either left in Tris-buffer containing NaCl or allowed to reprecipitate with dialysis against Tris-buffer, and stored at −80°C.

The crude VSP supernatant fraction was applied to a DEAE-Bio-Gel-A column (2.8 × 38 cm) preequilibrated in Tris-buffer. The column was then washed with Tris-buffer until the A_{280} approached zero. Bound protein was eluted with a 0 to 300 mM NaCl linear gradient, at 1.5 mM mL^{-1} and a flow rate of 0.5 mL min^{-1}. Bound VSP eluted as three peaks which were pooled separately, concentrated, and dialyzed against Tris-buffer containing 50 mM NaCl.

VSP proteins were purified further with Sephacryl S-200 as described above for LRP, except that the column was preequilibrated and eluted in Tris-buffer containing 50 mM NaCl. Protein from the center of the single eluent A_{280} peak was concentrated and dialyzed against Mes-buffer (25 mM Mes [pH 6.5], 1 mM $CaCl_2$, 0.5 M NaCl). The VSP isomers were affinity purified on concanavalin A Sepharose 4B (40 mL bed volume), preequilibrated in Mes-buffer and eluted in Mes-buffer containing 50 mM 1-*O*-methylglucopyranoside. The purified proteins were concentrated and dialyzed against Tris-buffer.

The VSP used for production of antibodies was purified through a Pharmacia Mono-Q column prior to the Sephacryl S-200 step. The column was preequilibrated in Tris-buffer and eluted as described above for the DEAE column.

Native Mol Wt Determination

Relative native molecular masses of proteins were determined by filtration through a Sephacryl S-200 (1.8 × 120 cm) column under the conditions described above for LRP and VSP purification. M_r was estimated from a plot of K_{av} (elution coefficient) *versus* log M_r for the protein standards: Cyt *c* (12,400), chymotrypsinogen-A (25,000), ovalbumin (43,000), bovine serum albumin (67,000), and alcohol dehydrogenase (150,000).

Antibodies

Rabbit antibody to purified VSP was made commercially (Environmental Diagnostics, Inc., Burlington, NC). Protein (0.1 mg) in Freund complete adjuvant was injected into three New Zealand white rabbits at 0, 4, and 6 weeks, and antisera were collected 1 month after the final injection. Preimmune serum collected prior to injection did not interact with VSP on Western blots. Deglycosylated VSP was prepared with trifluoromethane sulfonic acid (7), and antibodies specific to deglycosylated VSP were isolated from crude anti-VSP serum by the affinity purification technique of Smith and Fisher (19). Rabbit antisoybean agglutinin antibody (25) was a generous gift of Dr. L. O. Vodkin, University of Illinois, Chicago. Mouse antideglycosylated-*Phaseolus* agglutinin antibody was a generous gift of Dr. T. C. Osborn, University of Wisconsin, Madison.

Gel Electrophoresis and Western Blot Hybridization

Samples were subjected to SDS-PAGE (13% acrylamide resolving gel), electroblotted to a nitrocellulose membrane, and probed for immunoreactive peptides as previously described (21). Subunit apparent molecular mass was determined from a plot of relative mobility *versus* log molecular mass for the protein standards: phosphorylase b (97.4 kD), bovine serum albumin (66.2 kD), ovalbumin (42.7 kD), carbonic anhydrase (31 kD), soybean trypsin inhibitor (21.5 kD), and lysozyme (14.4 kD). Peptide maps of the 28 kD VSP polypeptide and each of the subunit polypeptides of soybean agglutinin and LRP were made by the procedures of Cleveland *et al.* (4) with protein bands cut from SDS-PAGE gels and digested with *Staphylococcus* V8 protease.

Other Procedures

Agglutination of trypsin-treated or untreated blood from New Zealand White rabbits was assayed as previously described (15). Carbohydrate content of proteins was determined according to Dubois *et al.* (5) with fetuin as a standard. Protein was determined according to Lowry *et al.* (13) with BSA as a standard.

RESULTS

Enhancement of Proteins in Sink-Deprived Leaves

As previously reported (26), depodding of soybean plants increased the level of several polypeptides in the leaves (Fig. 1, lanes 2 and 3). The increase was due to sink deprivation and not the wounding that occurred with depodding, because the same polypeptides increased in leaves of isogenic male-sterile plants that failed to set pods (Fig. 1, lane 4). The major changes observed were increases in the level of two polypeptides with molecular masses of 28,000 and 30,000 (Fig. 1, lanes 2–4) which corresponded to the two subunits of VSP (27). However, the level of an unidentified polypeptide at 33 kD also was observed to increase with depodding. When electroblotted soybean leaf extracts were probed with antibodies raised against deglycosylated *Phaseolus* agglutinin, the polypeptide at 33 kD and another at 29 kD were observed in leaves of both depodded and male-sterile plants but not in control plants (Fig. 1, lanes 8–10). These polypeptides were also recognized by antibodies raised against soybean agglutinin (Fig. 1, lanes 12 and 13) and, therefore, appear to be subunits of a soybean leaf protein which will be referred to as the lectin-related protein (LRP).

Sera raised in rabbits against purified VSP reacted broadly with many peptides in crude extracts including LRP (our unpublished results). However, when the antibody preparation was affinity purified with deglycosylated-VSP, only a few minor antibody interactions were observed with polypeptides other than VSP (Fig. 1, lanes 5–7). Neither of the antibody preparations to the *Phaseolus* or soybean agglutinin showed any interaction with VSP peptides (Fig. 1, lanes 8–13).

Characterization of LRP

When the 2.0 to 2.8 M ammonium sulfate-precipitated fraction of a depodded leaf homogenate was dialyzed, a substantial white precipitate formed. When this precipitate was dissolved in 10 mM NaOH and subjected to SDS-PAGE, three peptides with molecular masses of 28, 29, and 33 kD were observed (Fig. 2, lane 3). Western blots showed that the two larger peptides cross-reacted with the anti-*Phaseolus* agglutinin antibody (Fig. 2, lane 10), whereas the lower band cross-reacted with the anti-VSP antibody (Fig. 2, lane 7). Only the 28 kD VSP-polypeptide precipitated with LRP, as only trace amounts of the 30 kD VSP subunit were detected in the precipitates that formed during dialysis of protein preparations between 0.35 to 11 mg mL^{-1}. Composition of the precipitate was the same after dialysis against buffers at pH 7.5 or pH 9.5 (50 mM bis-Tris Propane) or buffers containing 100 mM mannose, galactose, or 20% ethylene glycol. Also, the LRP precipitate did not dissolve in the above solutions, 70% ETOH, or 50% saturated $(NH_4)_2SO_4$ but did dissolve in Tris-buffer containing 1 M NaCl.

The NaCl-solubilized sample eluted from Sephacryl S-200 as three protein peaks (S1, S2, and S3 of Fig. 3). The peptide composition of each of these fractions was determined by SDS-PAGE (Fig. 2, lanes 5 and 6), and the polypeptides were identified on Western blots. Fraction S2 contained the 29 and 33 kD LRP-peptides which cross-reacted with anti-*Phaseolus* and antisoybean agglutinin antibodies (Fig. 2, lanes 11 and 13), and S3 contained the 28 kD VSP-peptide which cross-reacted with the anti-VSP antibody (Fig. 2, lane 9). The void volume (S1) contained all three peptides (Fig. 2, lane 4) and some contaminating proteins. The LRP fraction had an elu-

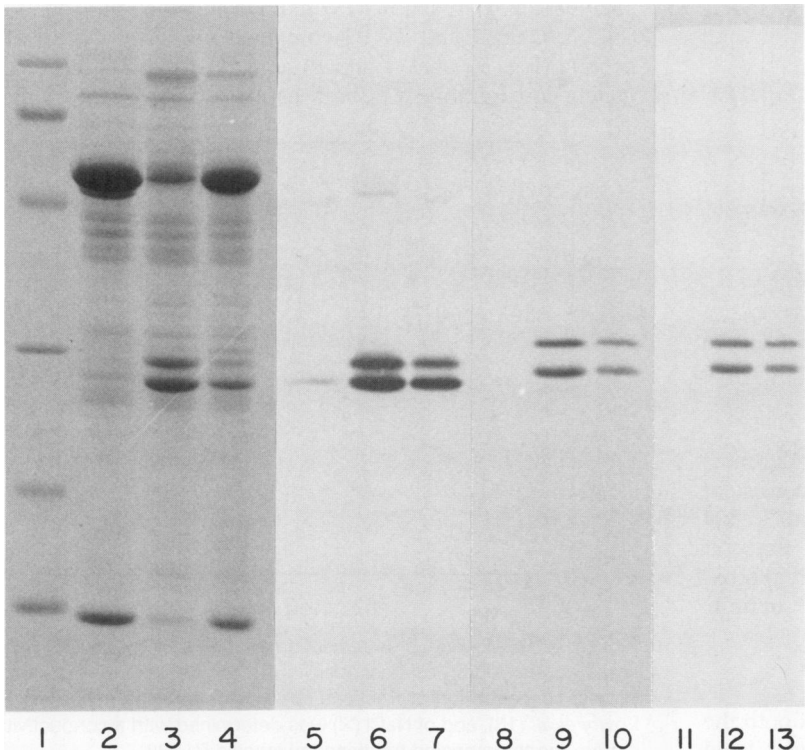

Figure 1. Resolution of polypeptides in crude leaf homogenates by SDS-PAGE and Western blot hybridization. Protein samples were subjected to SDS-PAGE and part of the gel was stained with Coomassie blue (lanes 2–4; 50 μg protein/lane). The other part of the gel (20 μg protein/lane) was electroblotted to nitrocellulose. The blot-membrane was cut into three sections that were probed separately with antibody raised against VSP (lanes 5–7), deglycosylated-*Phaseolus* agglutinin (lanes 8–10), or soybean agglutinin (lanes 11–13). Samples from control plant are in lanes 2, 5, 8, and 11, from depodded plant in lanes 3, 6, 9, and 12, and from male-sterile plants in lanes 4, 7, 10, and 13. Mol wt standards identified in the "Materials and Methods" are shown in lane 1.

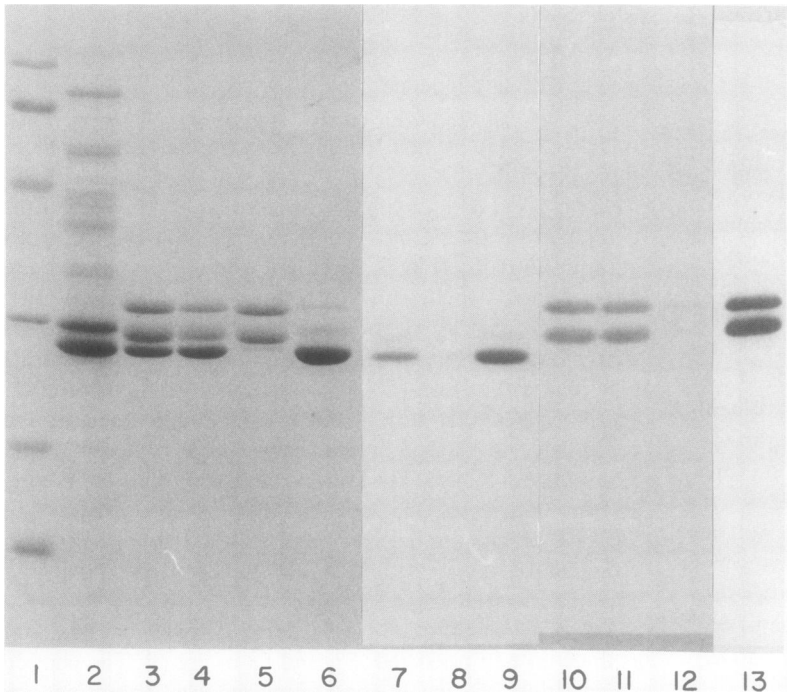

Figure 2. Resolution of polypeptides in proteins that precipitated during dialysis of the 52 to 73% $(NH_4)_2SO_4$ fraction of leaf homogenate from depodded plants. Polypeptide bands were resolved by SDS-PAGE and part of the gel was stained with Coomassie blue (lanes 1–6; 20 μg protein/lane). The other part of the gel (5 μg protein/lane) was electroblotted to nitrocellulose. The blot-membrane was cut into three sections that were probed separately with antibody raised against VSP (lanes 7–9), deglycosylated-*Phaseolus* agglutinin (lanes 10–12), or soybean agglutinin (lane 13). Lane 2 contains the soluble supernatant fraction and lanes 3, 7, and 10 contain the dialysis precipitate redissolved in 10 mM NaOH. Sephacryl S-200 gel permeation column fractions shown in Figure 3 are as follows: S1, lane 4; S2, lanes 5, 8, 11, and 13; and S3, lanes 6, 9, and 12. Lane 1 shows mol wt standards identified in the "Materials and Methods."

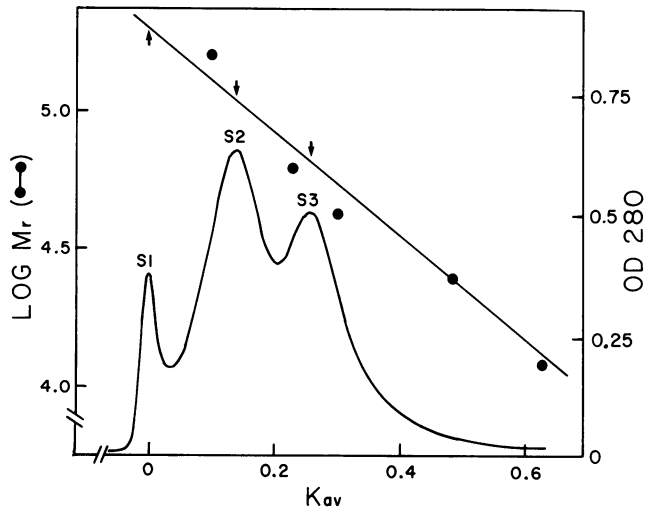

Figure 3. S-200 Sephacryl gel permeation chromatography of the dialysis precipitate dissolved in Tris-buffer containing NaCl. Arrows indicate log M_r for proteins of the eluted peaks estimated as described in the "Materials and Methods." The three pooled fractions are labeled S1–S3.

tion coefficient (Kav) which corresponds to a molecular mass of 119 ± 3 kD (Fig. 3), a size that is approximately consistent with a protein consisting of a tetramer of 29 and 33 kD polypeptides. In contrast to LRP, the VSP fraction eluted at an apparent molecular mass of 62.7 ± 1.1 kD which approximately corresponds to a homodimer of 28 kD VSP subunit.

Characterization of VSP

VSP was previously described as a heterodimer of both the 28 and 30 kD subunits (27), but the protein that precipitated with LRP during dialysis appeared to be a homodimer of the 28 kD subunit. This discrepancy coupled with the observation that the 28 kD subunit specifically precipitated with LRP during dialysis prompted us to examine in greater detail the native structure of VSP. DEAE anion-exchange chromatography resolved three VSP-containing fractions (P1, P2, and P3 of Fig. 4). The VSP in each of the three fractions was affinity purified followed by SDS-PAGE and Western blot analysis (Fig. 5). Fraction P1 contained only the 28 kD peptide (Fig. 5, lanes 3 and 7), P3 contained primarily the 30 kD peptide (Fig. 5, lanes 4 and 8), and P2 contained approximately equal amounts of both peptides (Fig. 5, lanes 5 and

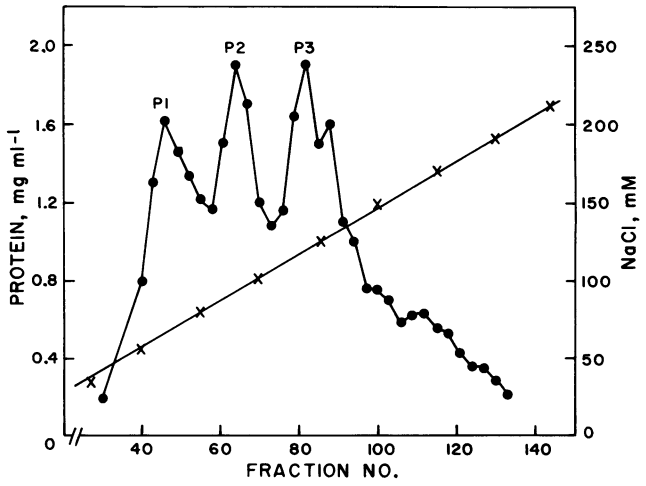

Figure 4. DEAE-Bio-Gel-A resolution of VSPs. The column was prepared and eluted as described in the "Materials and Methods." Protein concentration of fractions (●) was determined according to Lowry *et al.* (13), and of NaCl (×) was determined with a conductivity meter. The three pooled fractions are labeled P1–P3.

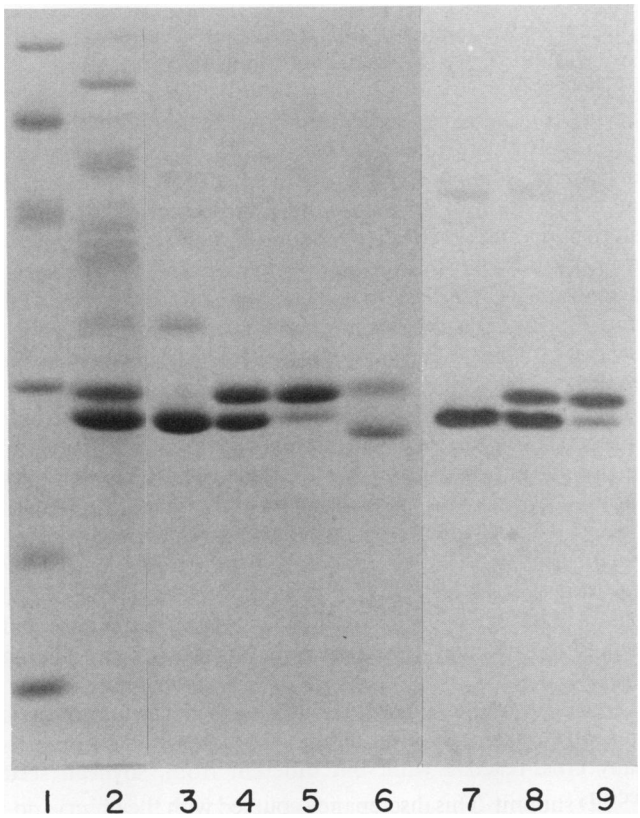

Figure 5. Identification of VSP protein fractions from DEAE chromatography. Polypeptides were resolved by SDS-PAGE (lanes 2–5), and the bands were identified by Western blot hybridization (lanes 7–9). Protein samples (40 μg in lane 2, 20 μg in lanes 3–5, and 5 μg in lanes 7–9) were subjected to SDS-PAGE, and part of the resultant gel was stained with Coomassie blue (lanes 1–6). The other part of the gel was electroblotted to nitrocellulose, and the resultant membrane was probed with anti-VSP antibody (lanes 7–9). Lane 2, supernatant from the dialyzed 52 to 73% saturated $(NH_4)_2SO_4$ fraction that was applied to the DEAE column; lanes 3 and 7, VSP fraction P1 from Figure 4; lanes 4 and 8, VSP fraction P2; lanes 5 and 9, VSP fraction P3. Lane 6 contains 10 μg of deglycosylated VSP. Lane 1 shows mol wt standards identified in the "Materials and Methods."

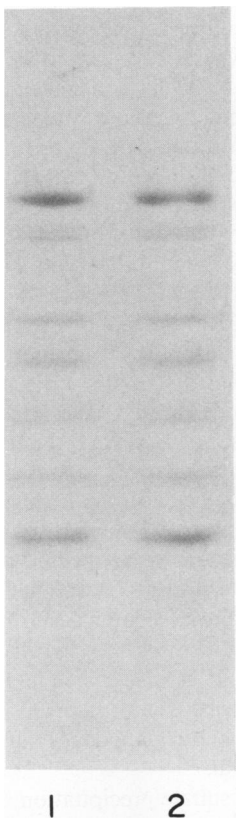

Figure 6. Protease digest peptide maps of soluble VSP 28 kD homodimer (lane 1) and the 28 kD homodimer that coprecipitated with LRP (lane 2). Each protein (30 μg) was initially subjected to SDS-PAGE in a 1.5 mm thick, 12% acrylamide resolving gel. The 28 kD polypeptides were excised in gel segments and electroeluted into a second, 3 mm thick gel. Peptide fragments produced from the polypeptides during a 30 min incubation with V8 protease in the stacking gel were resolved in a 15% acrylamide resolving gel.

9). In the Western blots, each purified VSP was immunologically recognized by anti-VSP antibody (Fig. 5, lanes 7–9) but not by anti-*Phaseolus* agglutinin or antisoybean agglutinin (results not shown).

The native molecular mass of each of the three VSP isoforms separated by DEAE chromatography was determined by gel filtration. All three iso-forms had similar molecular masses of 59.1 ± 3.3, a size that is approximately consistent with either the two homodimers of the 28 and 30 kD polypeptides or the heterodimer of both polypeptides.

Other Characterizations

Homology between the 28 kD peptides obtained from the precipitate that formed during the dialysis step of LRP isolation and from DEAE-resolved VSP (fraction P1 of Fig. 5) was examined by comparing peptide maps of these proteins after protease digestion (Fig. 6). The peptide maps were indistinguishable and suggested that the polypeptides were the same. Homology between either of the two polypeptides of LRP and the two peptides of soybean agglutinin was examined by comparing peptide maps of the four polypeptides. Only two of the peptides produced by protease digestion of one of the LRP subunits corresponded to any of the peptides from either of the subunits of soybean agglutinin (data not shown), indicating that neither of the polypeptides in LRP was directly homologous to one of the soybean agglutinin polypeptides.

Most lectins, including soybean agglutinin, have been shown to be glycoproteins (6), and VSP also has been shown to be a glycoprotein (27). Therefore, the probable carbohydrate content of LRP was determined and compared to VSP. The total carbohydrate contents of both VSP subunits and LRP were not discernably different, averaging 3.3 ± 0.4%. Chemical deglycosylation of VSP heterodimer reduced the carbohydrate content to less than 0.2% and yielded polypeptides with molecular masses of 31 and 27 kD (Fig. 5, lane 9). No agglutination of trypsin-treated or untreated rabbit erythrocytes was detected with crude homogenates or either purified LRP or VSP.

DISCUSSION

Several reports have shown that the level of VSP increases dramatically in soybean leaves during depodding (22, 23, 26).

VSP accumulates in the vacuole of paraveinal mesophyll cells of soybean leaves where it appears to serve the function of a storage protein (8). The level of a few other proteins also increases with depodding, and a major one of the increased proteins, LRP, is immunologically related to soybean agglutinin. In contrast to VSP, which can reach 45% of the total leaf protein in depodded plants (27), LRP accumulates to only about 0.5% of the total soluble leaf protein. Therefore, the levels of LRP that accumulate would appear to be too low for it to function as a storage protein.

The increase in LRP was not a reaction to the wounding that occurred with depodding, because both LRP and VSP accumulated in leaves of a male-sterile soybean variety that was unable to set pods. In soybean plants which are allowed to set seed normally, levels of VSP fluctuated in response to normal developmental changes in sink demand (22). However, LRP was not detected in the leaves of vegetative plants with antibodies specific for *Phaseolus* or soybean agglutinin. Therefore, it is uncertain what specific physiological conditions stimulate LRP synthesis or whether LRP also responds directly to sink demand. Recently, a lectin-related protein was shown to increase in *Dolichos biflorus* cell cultures in response to heat stress (20). It is also possible that LRP levels in vegetative soybean plants are changed by stress, and the accumulation observed in depodded plants is only an aberration of sink removal.

After ammonium sulfate precipitation, LRP reprecipitated during dialysis and subsequently required NaCl at 1 M to remain in solution. Another protein coprecipitated with LRP, and immunological cross-reactivity tests demonstrated that this protein was exclusively VSP. However, all the forms of VSP isolated from soybean leaves were soluble without added salt. The VSP that coprecipitated with LRP contained only a 28 kD polypeptide that was indistinguishable from the 28 kD polypeptide of soluble VSP in peptide maps. However, the data of Wittenbach (27) indicated that the native VSP protein existed as a dimer of both the 28 and 30 kD polypeptides. This apparent difference in VSP forms was resolved by DEAE chromatography of VSP. DEAE separated three dimeric forms of VSP consisting of the homo- and heterodimers of the 28 and 30 kD subunit polypeptides, and this occurrence of multiple isomeric VSP forms was analogous to other plant lectins and storage proteins, including conglycinin of soybean seeds (12) and *Phaseolus* agglutinin (14).

While the specificity of the association between LRP and the 28 kD VSP is intriguing, we are hesitant to attribute physiological significance to this observation. Previous work has suggested that lectins, including soybean agglutinin, tend to bind glycoproteins within the same species and may serve *in vivo* to organize protein complexes (2). LRP could conceivably act as a lectin and bind specifically to the 28 kD VSP. However, inability of galactose or mannose to prevent coprecipitation of LRP and the 28 kD dimer suggests that this association is not dependent upon a LRP lectin activity of the type common to most legume lectins (14). Also, LRP that was isolated free of VSP remained insoluble. Alternatively, LRP and VSP interaction may result from differences in protein charge, since the pI of the 28 kD peptide is significantly more basic than that of the 30 kD peptide (Dr. H. Mason, Department of Biochemistry and Biophysics, Texas A & M University, personal communication).

LRP shares a number of similarities with the soybean agglutinin, the major seed lectin of soybeans. Both are tetramers with native molecular masses of 120 kD and contain subunits with molecular masses of approximately 30 kD. LRP and soybean agglutinin are both glycoproteins and contain similar amounts of covalently bound carbohydrate (14). However, peptide maps of the polypeptides in LRP and soybean agglutinin showed few similar peptides. The fact that the 29 and 33 kD LRP subunits are recognized by antibodies raised against either soybean or *Phaseolus* agglutinin must be due to only partial sequence homology between all three proteins (14). LRP also may be related to a soluble 33 kD polypeptide in soybean leaves that was cross-reactive with antisoybean agglutinin antibody (25) or to lectin activities that were previously reported in soybean vegetative organs (3, 18). Although we were unable to detect agglutination activity, interaction between LRP and VSP could potentially mask LRP agglutination activity in crude homogenates of depodded plants. Alternatively, the purification procedure used could denature LRP and thus destroy its carbohydrate binding properties.

In conclusion, we provide compelling evidence for synthesis in soybean leaves of a protein (LRP) which is immunologically cross-reactive with, but different from, soybean seed lectin.

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