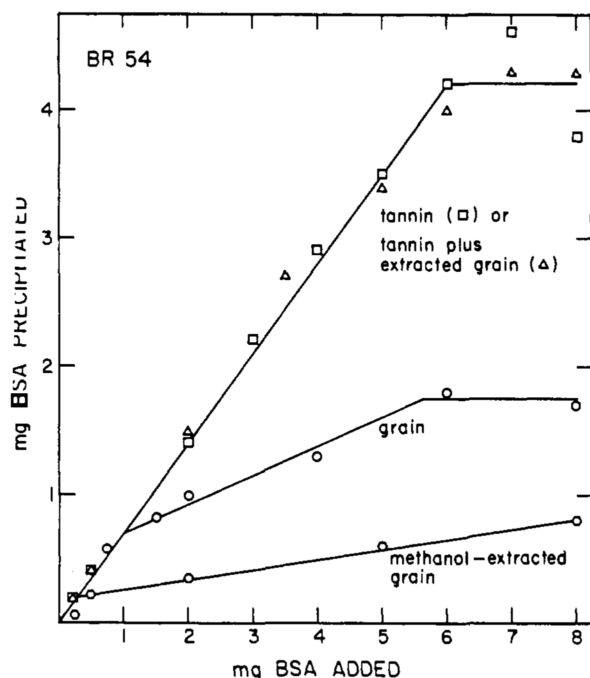


**Figure 3.** Titration of high tannin sorghum grain (variety BR 54) with iodine-125-labeled BSA. The amount of protein bound by 50 mg of ground high tannin grain (2.4% tannin) (○) and by 50 mg of methanol-extracted grain (○) was compared to the amount precipitated by 1.2 mg of purified tannin (□) or by 1.2 mg of purified tannin mixed with 50 mg of methanol-extracted grain (△).

more versatile than previously published methods and can be used to study tannin–protein interactions under a variety of conditions, providing insight into the mechanism of interaction. Investigations of protein binding by tannin in unextracted grain, which may lead to an understanding of the nutritional significance of dietary tannin, are readily accomplished with this new assay.

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# Condensed Tannin Purification and Characterization of Tannin-Associated Proteins

Ann E. Hagerman and Larry G. Butler*

The conventional isolation method has been modified in order to minimize protein contamination of tannin purified from high tannin sorghum. The two unique steps of the new procedure are preliminary extraction of the ground grain with ethanol and treatment of the partially purified tannin with phenol to remove traces of noncovalently bound protein. Tannin-associated protein removed by phenol treatment is not a random mixture of all the seed proteins, but consists of several discrete components which have been isolated and partially characterized. These proteins are quite hydrophobic, and one is rich in proline. With only minor changes, the purification method can be used to isolate tannin from seeds of other plants such as legumes.

Tannins, polyphenolic compounds found in many plants, are characterized by their affinity for proteins. Tannins interfere with isolation of organelles and proteins from plant tissue (Loomis and Battaile, 1966) and may be responsible for the decreased wieght gains of young animals fed tannin-containing grain (Featherston and Rogler, 1975). Although the general structures of hydrolyzable and condensed tannins (proanthocyanidins) have been established by the elegant work of Haslam (1979), the nature of tannin–protein interactions is still largely unknown. The lack of preparative scale methods for obtaining well characterized tannin has hindered investigation of the association, as has the absence of appropriate analytical techniques.

We have developed a scheme for preparing condensed tannin with minimum contamination by protein and have used this method to obtain tannin from sorghum grain [*Sorghum bicolor* (L.) Moench] and pinto beans [*Phaseolus vulgaris* (L.)]. Sufficient quantities of purified

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tannin have been obtained to permit preliminary investigations of tannin–protein interactions.

The proteins which are copurified with sorghum tannins have been partially characterized. They are extremely hydrophobic, suggesting that condensed tannin may associate with protein through hydrophobic bonds in addition to hydrogen bonds (Loomis, 1974; Hagerman and Butler, 1980).

## METHODS

All chemicals were reagent grade or the best grade available. Sephadex LH-20 was obtained from Pharmacia, Lipidex 5000 from Packard, and HP-K silica gel high-performance thin-layer chromatography plates from Whatman. Sorghum grain was grown in 1976 on the Purdue University agronomy farm. Tannin was purified from high tannin varieties NK 300 and BR 54. Pinto beans were obtained commercially.

A redox method, the Prussian blue assay (Price and Butler, 1977), was employed for determination of total phenolics at each stage of the purification. After addition of 50 mL of water to 0.1 mL of sample, the assay was performed as previously described (Price and Butler, 1977). The absorbance at 720 nm was recorded 19 min after addition of the second reagent, potassium ferricyanide. The amount of oxidizable material per milliliter of sample ($\Delta A_{PB}$/mL), 10 times the difference between the absorbance of the sample and the blank, was calculated for each sample.

Sample homogeneity was assessed with high-performance thin-layer chromatography using toluene–acetone–formic acid (60:60:10) as the mobile phase on silica gel (Lea, 1978). For the phenolic compounds examined with this system, the $R_f$ increased as molecular weight decreased (Lea, 1978). Tannin does not move from the origin.

The amount of protein precipitated by the tannin at each stage in the purification was measured with a modified version of a protein precipitation assay (Hagerman and Butler, 1978). Radioactively labeled bovine serum albumin (BSA) was mixed with the tannin-containing extract and the amount of protein precipitated determined by $\gamma$ counting (Hagerman and Butler, 1980).

**Purification Scheme.** The series of steps used in the purification of tannin from mature high tannin sorghum grain are shown in Figure 1. Glumes and dirt were removed from dry grain before coarsely grinding it. Extractions with ethanol and methanol containing 10 mM ascorbic acid were performed in the dark at 4 °C with stirring. Three milliliters of solvent per g of grain was used. The ethanol extract, which contained very little tannin, was discarded; 95% of the remaining tannin was extracted in the three subsequent methanol extractions, which were combined and mixed with an equal volume of 1 mM acetate buffer at pH 4. The methanol was removed by vacuum evaporation, and the remaining aqueous solution was extracted twice with an equal volume of ethyl acetate. The ethyl acetate fraction, which contained some amorphous solid material, was discarded. The aqueous phase was evaporated to dryness under reduced pressure, redissolved in a minimum volume of 80:20 ethanol–water (v/v), and mixed with 4 volumes of a Sephadex LH-20–ethanol slurry. The gel was washed repeatedly with ethanol until the 280-nm absorbance of the washes reached a constant minimum value. The gel was then washed with 50:50 acetone–water (v/v) until the absorbance at 540 nm reached a constant minimum value. The 50% acetone fraction, which contained the tannin, was evaporated under reduced pressure to remove the acetone, and the aqueous solution was extracted 3 times with an equal volume of liquefied phenol. The aqueous phase was washed with a small amount of diethyl ether to remove phenol, evaporated to dryness, and redissolved in a minimum volume of ethanol. This material was chromatographed on Sephadex LH-20 with an ethanol mobile phase, monitored at 280 nm. When a stable base line was reached, the mobile phase was changed to 50% aqueous acetone, monitored at 540 nm. The fractions containing tannin were pooled on the basis of retention time. The portions were lyophilized to yield light brown fluffy powders, which were soluble in methanol, 50% acetone, or cold water. No precipitation of purified tannin was observed in solutions up to 3 months old, although tannin prepared by conventional methods is reported to precipitate after 24 h (Davis and Hoseney, 1979).

The Prussian blue assay was used to determine yield at each stage of the purification of tannin from sorghum grain (Table I). Tannin specific activities (Hagerman and Butler, 1980) were calculated after extracts or lyophilized material dissolved in methanol was assayed with the protein precipitation method (Table I). The extent of protein contamination was determined by micro-Kjeldahl analysis.

Bean tannin was purified by essentially the same procedure as described above for sorghum tannin. Pinto beans were soaked for 18 h in 1 mL of methanol per g of beans, and the hulls were then removed manually. The methanol and hulls were mixed with methanol containing enough ascorbic acid to give a final concentration of 10 mM ascorbic acid. The mixture, containing 20 mL of methanol per g of hulls, was blended (Waring Blendor) for 5 min. It was then stirred for 12 h at 4 °C, filtered, and treated exactly like the crude methanol extract of sorghum grain.

The lyophilized product was light brown and fluffy. The yield and specific activity were determined after each step as described above (Table I).

**Protein Characterization.** Dialysis against water of the combined phenol phases from the phenol extraction step of BR 54 sorghum tannin purification yielded a pale brown solution containing brown particulate material. After evaporation under reduced pressure to a small volume (20 mL), the material was treated with sodium dodecyl sulfate ($NaDodSO_4$) and mercaptoethanol for several hours (Weber and Osborn, 1969). It was electrophoresed on $NaDodSO_4$–polyacrylamide gels, 6 mm in diameter (Weber and Osborne, 1969), which were stained with Coomassie blue after electrophoresis (Fairbanks et al., 1971). BSA, ovalbumin, and sperm whale myoglobin were used to prepare a molecular weight calibration curve.

By use of similar methods, preparative scale gels 10 mm in diameter were run to obtain protein for amino acid analysis. After the gels were frozen and sliced (Mickel gel slicer), the protein was eluted and dialyzed. Protein-containing slices were identified either by assay of an aliquot of the material eluted from the gels (Bradford, 1976) or by comparison with gels stained with Coomassie blue. After the protein was hydrolyzed (6 N HCl, 48 h, 110 °C), amino acid analysis was performed [Dionex (Durrum) D-500 automatic analyzer]; Trp was not determined.

Five protein fractions were obtained from a low tannin sorghum (variety 954063) by using the Landry-Moreaux extraction procedure as described by Jambunathan and Mertz (1973). The protein-containing extracts were dialyzed against water, lyophilized, and stored at 4 °C. $NaDodSO_4$–polyacrylamide gel electrophoresis (Weber and Osborn, 1969) showed that the major components of each fraction were similar to those previously reported (Jambunathan and Mertz, 1973). Several bands in the cross-linked prolamine fraction with mobilities similar to those

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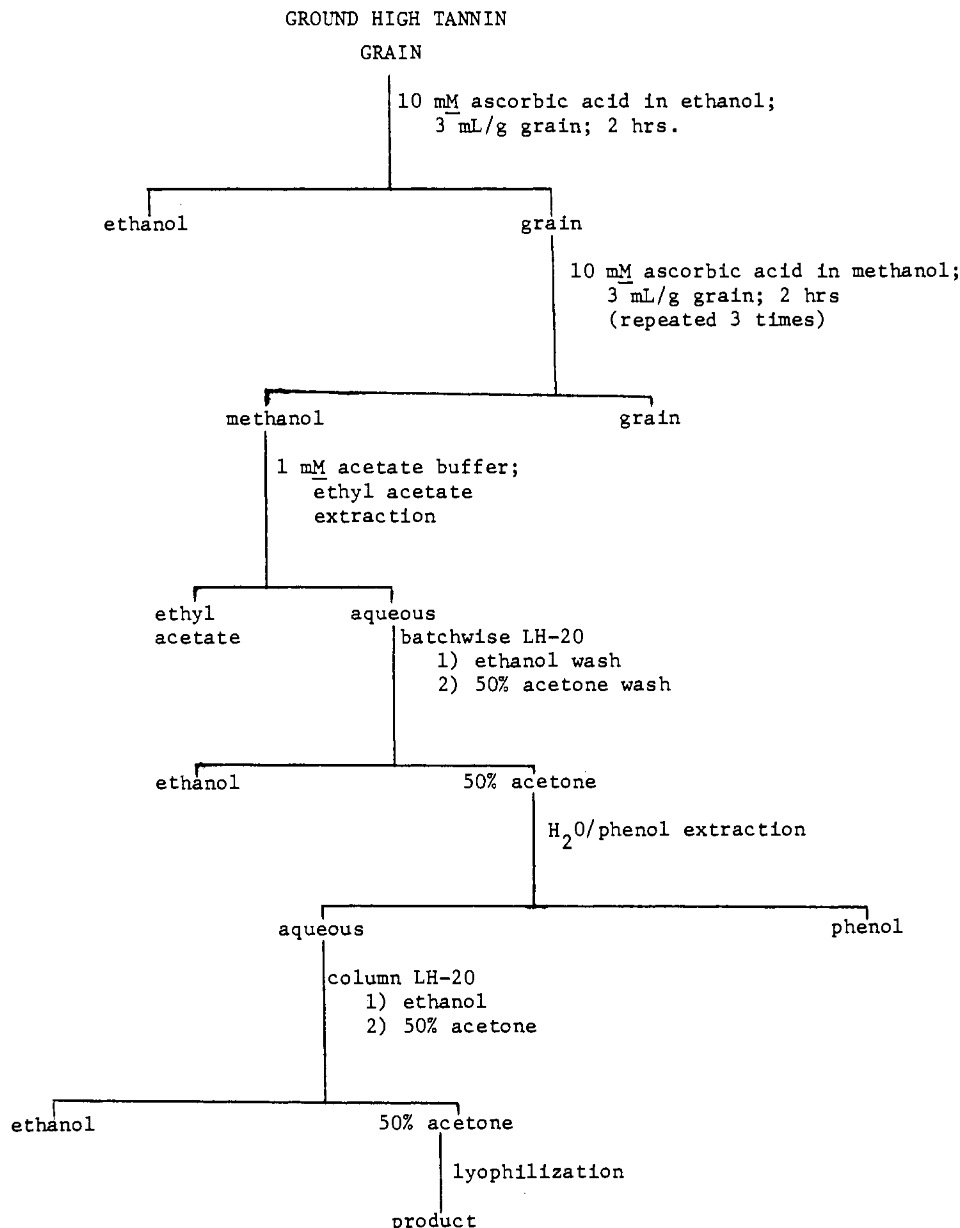


**Figure 1.** Scheme for the purification of tannin from sorghum grain. See text for explanation.

of the tannin-associated proteins were isolated by preparative scale $NaDodSO_4$–polyacrylamide gel electrophoresis, and the amino acid compositions were determined as described above.

## RESULTS AND DISCUSSION

**Purification.** Because of the characteristic affinity of tannin for protein, a major contaminant of tannin preparations is protein. For example, the tannins in the aqueous phase of an ethyl acetate fractionation may contain up to 30% protein while the nontannin phenolics found in the organic phase are protein free (Fletcher et al., 1977). Sorghum tannins prepared in our laboratory by conventional methods (Strumeyer and Malin, 1975; Davis and Hoseney, 1979) contain up to 20% protein (2–3% nitrogen).

Copurification of tannins and proteins is minimized with the scheme presented here (Figure 1). The first of the two unique steps is extraction of the ground grain with ethanol prior to extraction of the tannins with methanol. Absolute ethanol extracts only 10–15% of the methanol-extractable phenolic material from the grain, and the ethanol extract has very little BSA-precipitating activity. The ethanol apparently removes a fraction of the alcohol-soluble protein of the grain, preventing the simultaneous extraction and subsequent copurification of this protein with the tannin. Unlike cereals, legumes contain insignificant amounts of alcohol-soluble proteins (Long, 1961). The preliminary ethanol extraction is therefore omitted when preparing tannin from beans.

The second of the steps introduced to minimize contamination of tannin with protein is phenol fractionation. Phenol, a powerful protein solvent (Pusztai, 1966), is useful for the separation of protein from other water-soluble materials. Seed proteins, including the alcohol-soluble prolamines, are completely extracted from water by phenol, but flavanols are not. In addition, phenol dissociates tannin–protein complexes, removing 90–100% of the BSA from precipitated tannin–BSA complexes. None of the BSA is removed from the precipitate by similar treatment with aqueous buffer. Thus, phenol is an appropriate solvent to use for removing traces of protein from tannin preparations.

Nontannin contaminants which must be removed from the tannin-containing extract include low molecular weight phenolics and tannin precursors and the ascorbic acid

Table I. Purification of Tannin

| | ground sorghum (variety BR 54), 27 g | | | | | bean hulls (pinto bean), 30 g | | | |
|---|---|---|---|---|---|---|---|---|---|
| step | vol, mL | $\Delta A_{PB}$/mL | % yield | sp act., mg/$\Delta A_{PB}$ | % N | vol, mL | $\Delta A_{PB}$/mL | % yield | sp act., mg/$\Delta A_{PB}$ |
| ethanol extraction | 50 | 7.03 | (19) | 0.28 | ND[a] | | | | |
| methanol extraction | 192 | 9.46 | 100 | 1.5 | 1.2 | 340 | 6.05 | 100 | 3.3 |
| batchwise LH-20, 50% acetone wash | 68 | 6.03 | 23 | 2.7 | ND[a] | 380 | 1.18 | 22 | 3.7 |
| LH-20 column, 50% acetone elution | | | | | | | | | |
| pooled fraction A | 80 | 0.20 | 1 | ND[a] | 0.88 | 64 | 0.10 | <1 | 4.2 |
| pooled fraction B | 16 | 2.36 | 2 | 4.2 | 0.49 | 20 | 0.30 | <1 | 6.4 |
| pooled fraction C | 33 | 4.93 | 9 | 6.8 | 0.36 | 15 | 3.67 | 3 | 5.6 |
| pooled fraction D | 84 | 0.96 | 4 | 6.6 | 0.38 | 25 | 1.67 | 2 | 6.8 |
| pooled fraction E | | | | | | 81 | 0.54 | 2 | 6.1 |

[a] ND value not determined.

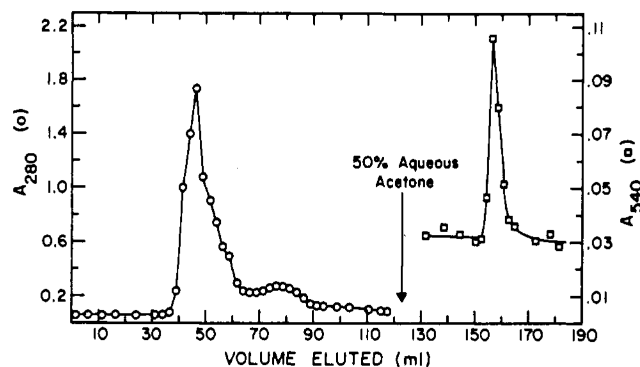


**Figure 2.** Chromatogram of tannin extracted from sorghum grain. 0.5 mL of extract (after ethyl acetate fractionation) was applied to Sephadex LH-20 equilibrated in ethanol. Column was 22.8 cm long, 1.7 cm in diameter.

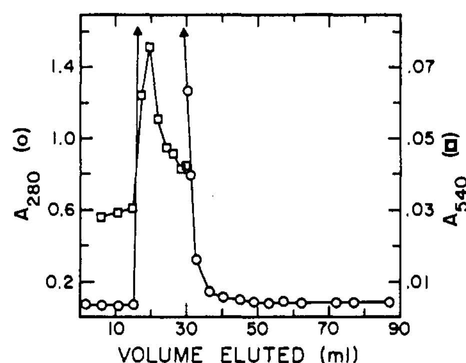


**Figure 3.** Chromatogram of tannin extracted from sorghum grain. 0.5 mL of extract (after ethyl acetate fractionation) was applied to Lipidex 5000 equilibrated in methanol. Column was 22.8 cm long, 1.7 cm in diameter.

added to prevent oxidation during the extractions. Some of these components are extracted into ethyl acetate, and others can be removed by adsorption chromatography with Sephadex LH-20, a hydroxypropyl derivative of Sephadex G-25 (Strumeyer and Malin, 1975). In absolute ethanol, the gel has a high affinity for aromatic materials such as tannin (Brooks and Keates, 1969). Nontannin phenolics are recovered from the gel during ethanol washes, and the tannins are recovered in the subsequent wash with aqueous acetone. Separation of the phenolic components of methanol extract of sorghum grain after treatment with ethyl acetate is shown in Figure 2. The separation, done either in batch or column mode, is clearly based on an adsorption mechanism (Brooks and Keates, 1969) rather than a gel filtration mechanism, and no conclusions can be drawn about the relative molecular weights of the separated components.

For sorghum tannin, the purification method described here results in a threefold reduction in nitrogen content from the starting material to give a product containing no more than 2–3% (by weight) residual protein. The overall yield of phenolic material is about 15%, and there is a three- to fivefold increase in tannin specific activity (Table I).

Similar yields are observed at each step in the purification of bean tannins, with an overall yield of about 7% and a twofold increase in tannin specific activity (Table I). The tannin specific activities of the purified sorghum and bean tannins are similar, although the specific activity of the crude bean extract is twice that of the crude methanol extract of sorghum. The crude bean extract must contain less nontannin oxidizable material than the crude sorghum extract.

A variation of the purification scheme described above employs Lipidex 5000, an alkoxy derivative of Sephadex LH-20 (Brooks and Keates, 1969). In contrast to Sephadex LH-20, Lipidex does not adsorb tannins, which can be eluted from the lipophilic gel with methanol. Tannins appear at the solvent front followed by other components. Separation by chromatography on Lipidex 5000 of the phenolics in an ethyl acetate treated methanol extract of high tannin sorghum is shown in Figure 3. Preliminary experiments indicate that the retention time is mainly a function of the polarity of the solute, with the least polar solutes retained for the longest time.

Lipidex 5000 is most useful as an alternate to the ethyl acetate extraction and the batchwise Sephadex LH 20 steps in the scheme in Figure 1. Several advantages accrue. The tannins are eluted at the solvent front and are thus recovered quickly, minimizing the possibility of oxidation and subsequent covalent condensation with proteins (Pierpoint, 1969). Furthermore, the inevitable losses encountered in adsorption chromatography are avoided with this technique. The modest resolution achieved in preliminary experiments is a disadvantage which might be overcome by the use of longer columns and slower flow rates.

The method described here is suitable for purifying tannin from a variety of sources. Its greatest advantage over existing methods is that protein contamination is minimized. The method permits preparation of large quantities of tannin, which may be quantitatively described by their tannin specific activities (Hagerman and Butler, 1980). Further investigation of tannin chemistry should be facilitated by these methods.

**Characterization of Tannin-Associated Proteins.** The affinity for tannin of the proteins copurified with tannin is quite strong, as is demonstrated by the fact that they are not removed by the extractions and chromatographic steps preceding phenol treatment. Removal of the

Table II. Hydrophobicity Parameters of Sorghum Proteins

| protein | apparent $M_r$ | NPS, % | $H\Phi_{av}$, cal/residue |
|---|---|---|---|
| TAP[a] A | 89 000 ± 5000 | 48 | 1410 |
| TAP B | 67 000 ± 6000 | 40 | 1200 |
| TAP C | 15 000 ± 1000 | 31 | 1000 |
| albumins and globulins[b] | | 16 | 515 |
| prolamines[b] | | 43 | 1210 |
| cross-linked prolamines[b] | | 39 | 1090 |
| glutelin-like[b] | | 24 | 593 |
| glutelin[b] | | 35 | 930 |
| cross-linked prolamine I | 90 000 ± 3000 | 53 | 1470 |
| cross-linked prolamine II | 74 000 ± 1000 | 40 | 1390 |
| cross-linked prolamine III | 18 000 ± 1000 | 47 | 1320 |

[a] TAP = tannin-associated protein. [b] Amino acid composition from Guiragossian et al. (1978).

proteins by phenol indicates that they are not covalently bound to tannin.

In an attempt to identify characteristics responsible for the high affinity of these proteins for tannin, the major tannin-associated proteins extracted by phenol from two BR 54 sorghum tannin preparations were examined. The components of the phenol layer migrated as distinct bands in $NaDodSO_4$–polyacrylamide gels and were comprised of discrete components rather than a mixture of all the proteins of the seed. The electrophoretic mobilities of the major components from the two tannin preparations were similar, although the minor components were not all the same. Apparent molecular weights were assigned to the three major tannin-associated proteins A, B, and C (Table II). Amino acid compositions of these proteins (Table III) are only approximate because of their poor recoveries from $NaDodSO_4$ gels and because of the difficulties encountered in staining proline-rich proteins in acrylamide gels (Meunzer et al., 1979).

Two hydrophobicity parameters were calculated from the amino acid compositions. The percent nonpolar side chains (NPS) is based on the mole percent of Trp, Ile, Tyr, Phe, Pro, Leu, and Val residues in the protein (Bigelow, 1967). Values for percent NPS range from 30 to 40% for most water-soluble proteins. The average hydrophobicity ($H\Phi_{av}$) of a protein is calculated from published values for the free energy of transfer of free amino acids from ethanol to water (Bigelow, 1967). $H\Phi_{av}$ has an average value of 1000 cal/residue for nonmembrane proteins and 1200 cal/residue for the more hydrophobic membrane-bound proteins (Vandlen et al., 1979). Hydrophobicity parameters for the three major tannin-associated proteins are presented in Table II.

The same parameters were calculated from the amino acid compositions reported for the five fractions of protein from sorghum grain (Guiragossian et al., 1978) (Table II). The alcohol-soluble prolamines and cross-linked prolamines characteristically contain only small amounts of basic amino acids but large amounts of Glu, Pro, Ala, and Leu (Wall and Paulis, 1978). The tannin-associated proteins have hydrophobicity parameters and amino acid compositions similar to those of the prolamines and cross-linked prolamines. Jambunathan and Mertz (1973) noted that in high tannin sorghum some of the albumins and globulins lose their characteristic water solubility and become glutelin-like, presumably as a result of interaction with tannin. Their amino acid compositions show that tannin-associated proteins A and B did not originate in the albumin or globulin class but are prolamines or cross-linked prolamines.

$NaDodSO_4$–polyacrylamide gel electrophoresis demonstrated that proteins with mobilities similar to those of tannin-associated proteins A and B were present only in the cross-linked prolamine fraction of low tannin sorghum grain. Proteins with low apparent molecular weight, like C, were present in all five protein classes of low tannin sorghum grain. From the amino acid compositions of the cross-linked prolamines with apparent molecular weights similar to A, B, and C (Table III), the hydrophobicity parameters were calculated and compared to those of the tannin-associated proteins (Table II). Cross-linked prolamines I and II have hydrophobicities similar to those of tannin-associated proteins A and B. However, they contain less of the basic amino acids and more Glu, Ala, and Leu than tannin-associated proteins A and B. Tannin-associated proteins A and B and cross-linked prolamines I and II are similar in their high proline and low tyrosine content. Cross-linked prolamine III is more hydrophobic than C, which does not have the high Glu, Pro, Ala, and Val content typical of the cross-linked prolamines. Tannin-associated proteins A and B are more similar to the cross-linked prolamines than to any of the other known types of seed proteins. However, C is dissimilar; it may be a globulin or albumin which has altered solubility properties because of association with tannin, as suggested by Jambunathan and Mertz (1973).

Since proteins similar to A and B are present in low tannin grain, the characteristics of A and B are probably

Table III. Amino Acid Compositions of Sorghum Proteins[a]

| amino acid | TAP[b] A | TAP B | TAP C | cross-linked prolamine I | cross-linked prolamine II | cross-linked prolamine III |
|---|---|---|---|---|---|---|
| lysine | 4.3 | 5.3 | 4.0 | 0 | 0 | 0 |
| histidine | 0.5 | 1.1 | 1.5 | 0 | 0 | 0 |
| arginine | 0.7 | 3.3 | 2.9 | 0 | 0.9 | 1.5 |
| aspartic acid | 7.4 | 6.2 | 9.0 | 3.5 | 5.0 | 5.7 |
| threonine | 4.0 | 2.2 | 7.1 | 4.7 | 1.8 | 2.1 |
| serine | 6.4 | 3.4 | 9.7 | 3.5 | 3.8 | 4.2 |
| glutamic acid | 9.6 | 9.6 | 8.8 | 12.6 | 17.1 | 18.4 |
| proline | 20.6 | 10.5 | 4.9 | 21.2 | 20.6 | 15.1 |
| cysteine | 6.7 | 1.3 | 1.7 | 0 | 0.8 | 1.2 |
| glycine | 10.4 | 13.4 | 12.0 | 4.6 | 3.8 | 5.1 |
| alanine | 6.4 | 14.2 | 10.4 | 18.0 | 16.7 | 14.6 |
| valine | 3.9 | 8.5 | 5.6 | 6.7 | 5.5 | 6.0 |
| methionine | 0 | 0.6 | 1.5 | 0 | 0 | 1.6 |
| isoleucine | 4.1 | 6.0 | 3.7 | 6.8 | 5.0 | 5.7 |
| leucine | 8.6 | 10.4 | 6.4 | 18.6 | 16.6 | 15.1 |
| tyrosine | 3.4 | 0 | 4.2 | 0 | 0 | 0 |
| phenylalanine | 7.0 | 4.4 | 6.4 | 0 | 2.4 | 5.0 |

[a] Expressed as residues/100 residues, assuming complete recovery of all amino acids. [b] TAP = tannin-associated protein.

not artifacts of their association with tannin. The major tannin-associated protein, A, had an unusually high proline content (20%) and a high apparent molecular weight. Protein A is very hydrophobic; it has larger $H\Phi_{av}$ values than have been reported for any common proteins and have been exceeded only by those for several cyclic peptides (Bigelow, 1967). Protein B has a lower apparent molecular weight, contains less proline, and is less hydrophobic than A. Protein C, with a low apparent molecular weight, has a low proline content and low hydrophobicity.

The prolamines and cross-linked prolamines are storage proteins and are found in protein bodies in the endosperm of the grain. Kafarin, the major prolamine of sorghum, contains about 40% helix when dissolved in *tert*-butyl alcohol (Wall and Paulis, 1978). Like zein, it may exist in a rodlike structure with a helical center and a shell of nonpolar amino acid side chains (Wall and Paulis, 1978). The high proline content of prolamines and cross-linked prolamines may be necessary for structural maintenance. Tannin-associated proteins, with their especially high proline content and extreme hydrophobicity, may exhibit a unique variation of this rodlike structure.

The factors which determine the relative affinities of proteins for tannin are unknown. A helical structure for condensed tannin has recently been proposed (Haslam, 1977). The possible existence of such a well-defined structure suggests that interactions with proteins might depend on the shape of the protein and its ability to interact properly with the tannin helix. The shape of the tannin-associated proteins may cause them to bind tightly to condensed tannin. Eggum and Christensen (1975) found that addition of 1.5% tannin to soybean meal specifically diminished the availability of proline, glycine, and glutamic acid to rats. Furthermore, the hydrolyzable tannin, tannic acid, interacts nonionically with polyproline but not with polymethionine (Meek and Weiss, 1979). These observations suggest that proline-rich proteins have a special affinity for the phenolic groups of tannin. Further studies on the relative affinity of tannin for various proteins are in progress, with hope that a better understanding of tannin–protein interactions will result.

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