(1957), which provides a qualitative estimate of darkening tendency based on the color change produced by the PPO–phenol interaction in a water-heated tissue slice. The second procedure is to quantify the phenol content of selections and establish a darkening scale based on these values. A third screening method is to measure the darkening of tissue homogenates of the selections of interest and establish a scale based directly on darkening values. The last two methods appear to be of potentially more use because they provide quantitative rather than qualitative comparisons among selections. Studies of factors such as harvest maturity, rate of fertilization, water application, etc. should provide information on the year-to-year and within-cultivar variability of PPO activity, phenol content, and darkening tendency, thus eliminating these concerns and allowing the unqualified use of either of the latter two screening methods.

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Determination of Protein in Tannin–Protein Precipitates

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The amount of protein precipitated by tannin under a variety of conditions is measured by using radioiodinated protein. The protein is mixed with purified tannin, finely ground plant tissue, or an unpurified plant extract; the mixture is centrifuged to remove the insoluble tannin–protein complex. An aliquot of the supernatant layer is counted to calculate the amount of protein precipitated. Complex formation is dependent on the pH and solvent composition. Tannin specific activity is useful for comparing tannin from various sources and for monitoring tannin purification. It is defined here as the ratio between the amount of protein precipitated and the amount of oxidizable material present.

Both the environmental role (Swain, 1979) and the nutritional effects (Price and Butler, 1980) of tannin might be better understood if adequate analyticaly techniques were available for quantitative study of tannin–protein interactions. With the protein precipitation assay we described previously (Hagerman and Butler, 1978) only precipitated tannin is measured. We have now modified our method so that the amount of protein precipitated is determined. By use of this new protein binding method in conjunction with our previous method (Hagerman and Butler, 1978), both components in the precipitated tannin–protein complex can be determined.

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METHODS

All chemicals were reagent grade. Condensed tannin was purified from sorghum [*Sorghum bicolor* (L.) Moench, variety BR 54] as described in the accompanying paper (Hagerman and Butler, 1980). Tannic acid was from Sigma Chemical Co. Methanol solutions of purified tannin or crude methanol extracts of grain were used within 24 h of preparation. For assays of unextracted grain, sorghum was ground in a electric hand-held coffee mill (Krups KM-75), Germany) and used within 3 days. Protein concentration was determined with the biuret assay (Layne, 1957), and radioactivity was measured with a Beckman Gamma 300 γ counter.

Bovine serum albumin (BSA) (Sigma Chemical Co.; fatty acid free, prepared from fraction V albumin) was labeled with iodine-125 by the Chloramine-T method

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(Greenwood et al., 1963) or the immobilized lactoperoxidase method (Bio-Rad Laboratories, 1978). The protein was separated from the unbound iodine by gel filtration on Sephadex G-25 in 0.2 M phosphate buffer, pH 7.2, and was immediately diluted with 1 mg/mL unlabeled BSA in buffer A (0.20 M acetic acid containing 0.17 M sodium chloride and adjusted to pH 4.9 with sodium hydroxide). The final specific radioactivity was about 10^5–10^6 counts per min (cpm) per mg of BSA. Trichloracetic acid (Cl_3-AcOH) precipitated 98% of the radioactivity, indicating that essentially all of the label was covalently attached to the protein. The radioactivity precipitable by Cl_3AcOH decayed slowly on storage but could be restored to 98% by dialysis against buffer A.

Labeled protein was added to unlabeled BSA in buffer A to obtain solutions of the desired protein concentration containing 2000–4000 cpm per mg of protein. Standard curves relating counts per minute to protein concentration were prepared at the beginning of each experiment. The precipitation reaction was carried out in 16 × 100 mm disposable culture tubes in a total volume of 2.25 mL, comprised of 2.0 mL of labeled protein in buffer A and 0.25 mL of tannin in methanol. After addition of the tannin to the protein solution, the samples were mixed immediately. They were maintained at room temperature for 15 min and then centrifuged for 15 min [IEC clinical centrifuge, maximum speed (5000g)]. A 1.0-mL aliquot of the supernatant was counted for calculation of the amount of protein precipitated. No protein was precipitated in control samples which contained methanol but no tannin.

For determination of the effects of various solvents on the precipitation, the assays were performed as above, using purified tannin dissolved in methanol. Aliquots of the solvents were added to the tannin and buffer before the protein was added. Controls showed that protein was not precipitated by the solvent in the absence of tannin.

The amount of protein bound by unextracted ground grain was measured by a similar method. In 1.5-mL plastic tubes with caps (Sarsedt, W. Germany) 50 mg of finely ground grain was mixed with 1.25 mL of labeled BSA in buffer A, gently rotated (Labquake mixer) for 30 min at room temperature, and then centrifuged as above. A 1.0-mL aliquot of the supernatant was counted for calculation of the amount of BSA bound by the grain. Low tannin grain bound less than 5% of the added protein.

The Prussian blue assay (Price and Butler, 1977) was used to determine oxidizable material. After addition of 50 mL of water to 0.1 mL of sample, the assay was performed as described (Price and Butler, 1977). The absorbance at 720 nm was recorded exactly 19 min after the addition of the second reagent, potassium ferricyanide. The amount of oxidizable material per milliliter of sample (ΔA_{PB}/mL) was calculated as 10 times the difference between the absorbance of the sample and the absorbance of the reagent blank. For a sample of purified tannin this value is directly related to the concentration. Nontannin oxidizable substances such as ascorbic acid and phenol give positive interferences in this assay.

RESULTS AND DISCUSSION

As a constant amount of tannin is added to increasing amounts of BSA, the amount of protein precipitated increases linearly until a point of maximum precipitation is reached (Figure 1). Beyond this point, addition of excess protein does not affect the amount of protein precipitated, although very large excesses of protein cause an apparent inhibition of precipitation. A similarly shaped curve is obtained with purified condensed tannin, hydrolyzable tannin, or crude tannin-containing extracts.

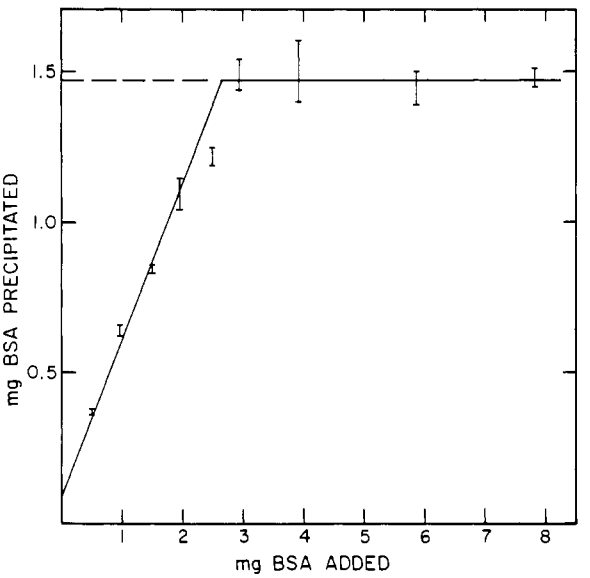

Figure 1. Titration of sorghum tannin with iodine-125-labeled BSA as described in the text. The concentration of the tannin solution was 1.17 ΔA_{PB}/mL. Bars indicate the range of three values. The line, drawn by the method of least squares, has the equation milligram of precipitate = 0.51 (mg added) + 0.09 and a correlation coefficient (r^2) of 0.98. The line with zero slope is drawn through the average of the values, and the dotted line indicates the value used for calculation of tannin specific activity (see text).

Table I. Amount of BSA Precipitated by Several Dilutions of a Tannin Solution

tannin concn, ΔA_{PB}/mL	BSA pptd, mg	ratio, mg/(ΔA_{PB}/mL)
1.40 ± 0.05[a]	0.9 ± 0.1[b]	0.7 ± 0.1[c]
4.53 ± 0.05	2.9 ± 0.3	0.64 ± 0.07
5.31 ± 0.05	3.6 ± 0.2	0.68 ± 0.05
8.59 ± 0.05	6.0 ± 0.4	0.69 ± 0.05

[a] Tannin concentration determined with the Prussian blue assay and expressed in absorbance units. Error is 1 SD. [b] Amount of BSA precipitated determined by averaging the values obtained in the region of zero slope. Error is 1 SD. [c] Error is 1 SD.

For each tannin preparation the ratio between the amount of protein precipitated in the region of zero slope and the amount of tannin present is approximately constant (Table I). This relationship may be expressed as the tannin specific activity, defined here as the ratio of the amount of protein precipitated to the amount of oxidizable material present:

$$\text{tannin sp act.} = \frac{\text{mg of BSA pptd}}{\Delta A_{PB}} \quad (1)$$

The tannin specific activity for a typical purified sorghum tannin can be calculated from eq 1 by using values obtained from Figure 1.

$$\text{tannin sp act.} = \frac{\text{mg of BSA pptd}}{(\Delta A_{PB}/\text{mL})(\text{mL of tannin})} = \frac{1.47 \text{ mg}}{(1.17\ \Delta A_{PB}/\text{mL})(0.25 \text{ mL})} = \frac{5.0 \text{ mg}}{\Delta A_{PB}}$$

This method can be used to determine the tannin specific activity of extracts containing either condensed or hydrolyzable tannin. If a sufficient quantity of purified tannin is available, a standard curve relating the Prussian

Table II. Effect of Methanol on Precipitation of BSA

methanol concn, % (v/v)	relative amount of BSA pptd[a]
0	65
4.4	75
11.0	100
20.0	104
30.0	117
40.0	130

[a] The amount of BSA precipitated under standard conditions (11% methanol) is set at 100.

blue absorbance units to weight of tannin can be prepared. The tannin specific activities of tannins purified from diverse sources can then be directly compared in terms of milligrams of protein precipitated per milligram of tannin. Purified condensed tannin from sorghum grain has a tannin specific activity of about 5.8 mg/ΔA_{PB} (Hagerman and Butler, 1980), which is equivalent to about 12 mg of BSA per mg of tannin. Tannic acid, a hydrolyzable tannin, has a tannin specific activity of 2.3 mg/ΔA_{PB}, or about 11 mg of BSA per mg of tannic acid.

Tannin purification can be monitored by determining the tannin specific activity of the preparation at various steps during the purification. As nontannin oxidizable materials are removed from the sample, the tannin specific activity increases. A three- to fivefold increase in tannin specific activity is obtained in the purification of sorghum tannin (Hagerman and Butler, 1980).

With our new method for determining the amount of protein precipitated by tannin, disadvantages of several previously described methods (Goldstein and Swain, 1965; Bate-Smith, 1973; Davis and Hoseney, 1979) are overcome. The protein is commercially available and need not be freshly prepared for each assay as is required for the haemanalysis method (Bate-Smith, 1973). Pigmented compounds do not interfere with the radiochemical determination as they do with colorimetric methods (Davis and Hoseney, 1979). The effects of altering pH and solvent composition cannot be rigorously studied with enzyme inhibition methods (Goldstein and Swain, 1965; Davis and Hoseney, 1979) but can be examined with our new method.

The interaction of BSA with condensed tannin is pH dependent. BSA precipitates only when the pH is between 4 and 7, and maximum precipitation occurs at about pH 5. This agrees with our previous work, in which maximum precipitation of tannin was observed at pH values close to the isoelectric point of the protein (Hagerman and Butler, 1978). For maximization of the precipitation of BSA in the protein binding assay described here, the system is normally maintained at pH 4.9.

Loomis and Battaile (1966) suggested that tannin–protein complexes might by disrupted by organic solvents. Addition of some solvents, including dioxane and *N*,*N*-dimethylformamide (DMF) to the assay system inhibits precipitation of BSA by tannin (Figure 2), but simple alcohols stimulate the precipitation (Figure 2, Table II). The strong hydrogen-bond acceptor DMF prevents formation of the tannin–protein complex (Figure 2), probably by inhibiting hydrogen bonding between tannin and the protein amide bond. Unlike the interactions of phenolic compounds with poly(vinylpyrrolidone), which are predominantly due to hydrogen bonding (Olsson and Samuelson, 1974), interactions of tannin with BSA are stimulated by the addition of simple alcohols (Figure 2, Table II). This implies that forces other than hydrogen bonding must be involved in the formation of tannin–protein complexes. Phenolic compounds interact hydrophobically with polystyrene resin (Gray, 1978; Loomis et al., 1979), suggesting that nonpolar forces may be important in tannin–protein interactions. This hypothesis is further supported by the observation that the nonpolar solvent dioxane inhibits BSA precipitation (Figure 2).

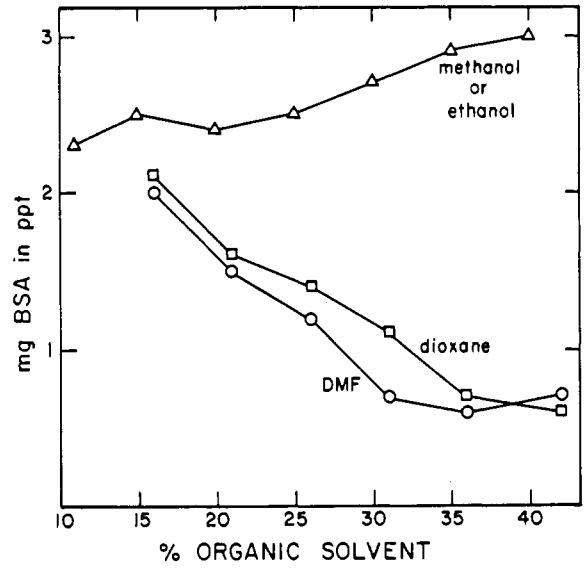

Figure 2. Precipitation of iodine-125-labeled BSA by purified sorghum tannin in the presence of organic solvents. To samples containing from 5 to 30% dioxane, DMF, or alcohol was added tannin in methanol to make the total organic solvent composition 16–41%. The amount of BSA precipitated was determined as described in the text.

Labeled BSA is not released from the tannin–protein precipitate when it is resuspended in buffer A containing unlabeled BSA, indicating that under these conditions the interaction is irreversible. However, covalent bonds between the protein and tannin do not form and the protein is completely released when the precipitate is treated with 1% sodium dodecyl sulfate ($NaDodSO_4$) in 5% triethanolamine, the solvent used to dissolve the precipitate prior to determination of precipitated tannin (Hagerman and Butler, 1978). Precipitation of BSA by sorghum tannin, a condensed tannin, is completely prevented by 0.1% $NaDodSO_4$. In contrast, Goldstein and Swain (1965) found that anionic detergents had no effect on the inhibition of enzymatic activity by hydrolyzable tannins.

Our new protein binding assay is easily adapted for direct measurement of the amount of BSA bound by plant tissue. As increasing amounts of labeled protein are added to finely ground high tannin sorghum grain, the amount of protein bound increases (Figure 3). Eventually a point of maximum binding is reached, and further addition of protein does not change the amount of protein bound. Relatively little BSA is bound by high tannin grain which has been extracted with methanol to remove the tannin (Figure 3). A mixture of methanol-extracted high tannin grain and purified tannin (final concentration, 2.4%) binds as much protein as do equivalent amounts of purified tannin in the absence of grain and significantly more protein than equivalent amounts of unextracted grain containing 2.4% tannin (Figure 3). Purified tannin alone or mixed with grain binds more protein than the equivalent of tannin which has not been extracted from the grain because the latter is not completely accessible to the added protein. Since this protein binding assay does not depend on extraction or purification of tannin, it may more accurately predict the nutritional quality of tannin-containing grain than do other assays.

We have developed a novel method for determining the amount of protein precipitated by tannin. The assay is

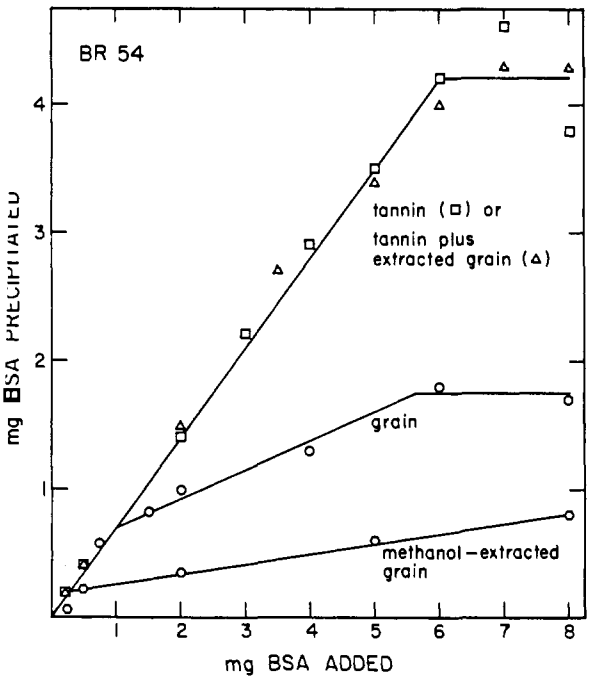

Figure 3. Titration of high tannin sorghum grain (variety BR 54) with iodine-125-labeled BSA. The amount of protein bound by 50 mg of ground high tannin grain (2.4% tannin) (○) and by 50 mg of methanol-extracted grain (○) was compared to the amount precipitated by 1.2 mg of purified tannin (□) or by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

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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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