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Characterization of the Condensed Tannin (Proanthocyanidin) from a Group II Sorghum

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Most of the tannin in seeds of high-tannin bird-resistant group III sorghums [*Sorghum bicolor* (L.) Moench] extracts readily into methanol or aqueous acetone. In contrast, the tannin of group II sorghums is not extracted by these solvents but is extracted by methanol containing 1% (v/v) HCl. Group II sorghums have been reported to be nutritionally less harmful than group III types, presumably due to differences in their tannins. We have purified tannin from a group II sorghum (IS 8768) and compared it to tannin purified from a group III sorghum (DeKalb BR 64). We do not find significant differences in the properties of the purified tannins. Tannin extractable only in acidic methanol is present along with methanol-extractable tannin in most group III sorghums, especially in immature grain. The differentially extractable tannins do not appear to have a precursor/product relationship.

One of the constraints on utilization of sorghum grain as food or feed is the occurrence, in some cultivars, of condensed tannins (proanthocyanidins, oligomers of flavan-3-ols). The presence of tannins is associated with decreased bird preference (Bullard and Elias, 1980) and lower digestibility (Jambunathan and Mertz, 1973; Hartigan, 1979) of the grain. Nutritionally superior low-tannin and tannin-free cultivars and hybrids are available but are more difficult to produce than high-tannin "bird-resistant" sorghums in areas where bird depredation is a severe problem (Tipton et al., 1970; Hoshino and Duncan, 1980). In addition to resistance to bird depredation, tannins may also confer resistance to preharvest germination (Harris and Burns, 1970) and molding (Harris and Burns, 1973).

Sorghum cultivars have been classified into three groups according to the tannin content of the grain and the genes which control it (Cummings, 1973; Price and Butler, 1977; Hartigan, 1979; Rooney and Miller, 1982). Group I sorghums do not have a pigmented testa layer and have no tannin, although other polyphenols may be present. Group II sorghums have a pigmented testa layer that contains condensed tannins. Most of these tannins are unusual in that they cannot be extracted from the seed with methanol or aqueous acetone. However, the tannins are readily extracted by methanol to which 1% concentrated HCl has been added (Maxson and Rooney, 1972). Group II sorghums, of which Hegari is an example, have dominant B_1 and B_2 genes with a recessive *s* gene resulting in the occurrence of polyphenols, including tannin, in the pigmented testa layer (Rooney and Miller, 1982). Group III sorghums have a pigmented testa layer (B_1–B_2–) and a dominant *S* gene resulting in polyphenols occurring in both the pigmented testa layer and the pericarp (Rooney and Miller, 1982). Group III sorghums, which include the well-known "bird-resistant" hybrids such as DeKalb BR 64, contain tannin readily extractable into methanol without HCl; many of them also contain tannin extractable only in acidic methanol (see Results).

Oswalt (1975), Hartigan (1979), and Bullard and Elias (1980) have suggested that tannins from group II sorghums may have less severe antinutritional effects than tannins from group III sorghums. The chemical characterization of tannins from group II sorghums is less complete than that of tannins from group III sorghums. Bullard et al. (1981) have analyzed polyphenols extracted by acetone, methanol, and aqueous methanol from three group II sorghums. We report here the purification and characterization from a group II sorghum (IS 8768) of those condensed tannins that do not extract into acetone, methanol, or their aqueous mixtures but that do extract

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into acidic methanol. We have compared this material, characteristic of group II sorghums, to the tannin purified from a group III sorghum. We will subsequently report on the nutritional quality and bird resistance of the group II sorghum from which the tannin was obtained.

MATERIALS AND METHODS

Sorghum grain was provided by Dr. John Axtell from the 1981 crop grown at the Purdue University Agronomy Farm, West Lafayette, IN. The cultivar IS 8768 was selected as the group II sorghum because its seed contains higher levels of tannins than other group II sorghums we analyzed. IS 8768 has a chalky white pericarp and purple testa. The group III sorghums used for comparison were the high-tannin, bird-resistance hybrids, DeKalb BR 54 and BR 64; we have not observed any significant differences in BR 54 and BR 64 with respect to the amount or composition of their tannin. Both have a red-brown pericarp and brown testa. Condensed tannin was purified from methanol extracts of BR 54 and BR 64 by the method of Hagerman and Butler (1980a).

Bovine serum albumin labeled with [^{14}C]formaldehyde (Amersham Chemical Corp.) by the method of Jentoft and Dearborn (1979) was prepared from fatty acid free, fraction V albumin (Sigma). All chemicals were reagent grade unless otherwise indicated and were used without further purification.

Chemical Assays. Total phenols were determined by the Prussian blue assay of Price and Butler (1977), flavan-3-ols and proanthocyanidins by the modified vanillin assay using glacial acetic acid as the solvent (Butler et al., 1982), proanthocyanidins (anthocyanidin formation) by the method of Watterson and Butler (1983), and protein precipitable phenols by the method of Hagerman and Butler (1978). Protein precipitating capacity was measured with radioisotope-labeled bovine serum albumin (BSA) as described by Hagerman and Butler (1980b) with the following modifications: BSA was labeled with ^{14}C (Jentoft and Dearborn, 1979), sample size was 1.0 mL, and radioactivity was determined on 0.4 mL of the unprecipitated protein by using [^{14}C]toluene as the internal standard. Assays for comparison of tannins from group II and group III sorghums were run on methanol solutions, 1 mg/mL, within 1 day of dissolving the purified tannin. All data presented are the average of duplicate assays.

Nitrogen analysis was performed by the microanalysis laboratory in the chemistry Department at Purdue using the Kjeldahl procedure.

Spectral Measurements. Absorbance spectra were obtained on solutions containing 1 mg/mL tannin in methanol by using a Uvikon 810/820 scanning spectrophotometer. IR spectra were run as KBr wafers on a Perkin-Elmer 137 sodium chloride spectrophotometer. ^{1}NMR spectra were obtained on a Perkin-Elmer XL-200 NMR spectrophotometer operating at 200 MHz and 35 °C with D_2O as the solvent (saturated solution) and Me_4Si as the external standard. ^{13}C NMR spectra were run under the same conditions but at room temperature.

Tannin Extraction and Purification. Sorghum grain was hand cleaned to remove glumes and damaged grains and was milled to pass a 0.6 mm mesh screen. Lipids were removed from 30 g of milled grain by stirring with diethyl ether, 3 mL/g, for 30 min. After the mixture settled, the ether was decanted and the residue dried under a stream of N_2. The residue was subjected to two extractions with methanol, 3 mL/g, for 30 min to remove non-tannin phenolics and alcohol-soluble proteins. The marc was then reextracted twice with 1% concentrated HCl in methanol (H^+/methanol), 3 mL/g, for 1 hr each, followed by centrifugation at 2000g for 10 min. The procedure was repeated and the supernatants containing the tannins were combined and the residues (pellets) were discarded.

A saturated aqueous solution of $NaHCO_3$, approximately 30% of the amount necessary to neutralize the acid as determined by titrating 1% HCl in water, was then slowly added to the pooled H^+/methanol extracts to produce a turbid purple-orange suspension. Not all preparations require the same amount of $NaHCO_3$. If a purplish sheen formed on top of the suspension, too much $NaHCO_3$ had been added; a few drops of H^+/methanol restorted the proper condition. After removal of insoluble material by centrifugation, the clear orange supernatant was evaporated under reduced pressure. The resulting red solid was dissolved in water equal in volume to the original supernatant. A small amount of red insoluble material, soluble in methanol but containing very little tannin, was removed by centrifugation and discarded.

For optimum results in the subsequent chromatography, the water was removed by rotary evaporation, the dried material was dissolved in a minimum volume of ethanol and allowed to stand at 4 °C overnight. The carbonate salts that crystallized were removed by centrifugation, leaving a deep red ethanol solution of polyphenols. The ethanol solution was applied to an 18 cm × 1.7 cm column of Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, NJ), previously equilibrated with ethanol, or was simply mixed with an LH-20 slurry (20 g in 100 mL of ethanol) for 30 min. The LH-20 was washed with approximately 100 mL of ethanol; the red-orange eluate contained little or no tannin. Tannin was then eluted with 1/1 (v/v) acetone/H_2O and recovered by rotary evaporation and lyophilization.

RESULTS

Extraction and Purification. Our attempts to purify the tannin from the H^+/methanol extract without neutralization of the acid invariably resulted in formation of intractable tarry deposits similar to the phlobaphenes described by Roux (1970). Neutralization of the acid (much less than a stoichiometric equivalent of base is required, suggesting that some seed component partially titrates the acid) increases the subsequent solubility of the tannin in water (Figure 1). Rapid and complete solubility in cold water is a characteristic of homogeneous proanthocyanidin preparations (L. J. Porter, personal communication). Dissolving the dried residue first in water and then in ethanol eliminates considerable non-tannin phenols. When the ethanol solution was chromatographed on Sephadex LH-20 under conditions described by Strumeyer and Malin (1975), the results were similar to those reported for tannin from a group III sorghum (Hagerman and Butler, 1980b) (Figure 2). Ethanol eluted phenolic materials but very little tannin; tannin was eluted by acetone/H_2O. A typical purification of tannin from IS 8768 is summarized in Table I. The overall recovery of tannin as measured by protein precipitable phenols is high because of the presence in the crude extract of materials that inhibit the assay.

When redissolved in methanol and reapplied to LH-20 columns, the tannin purified from IS 8768 (group II) and BR 54 (group III) gave the same results. Both preparations did not elute with ethanol, were only partially eluted with methanol, and were completely eluted with acetone/H_2O.

Thin-layer chromatography on dried, precoated silica gel plates (DC-Fertig platten Kieselgel 60 F-254 Merck) developed with 85% ethyl acetate, 8% H_2O, 6% formic acid, and 1% concentrated HCl (v/v) gave a long light smear. For the tannin from IS 8768 most of the material

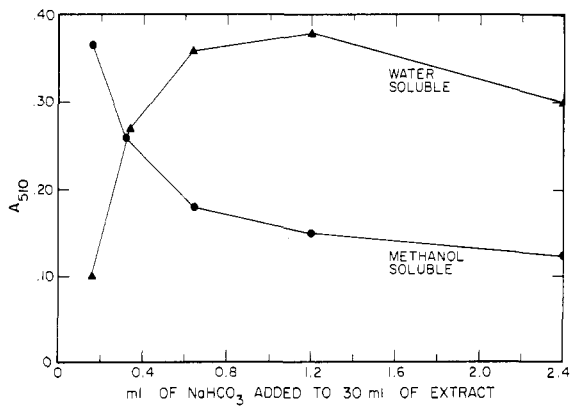

Figure 1. Effect of neutralization of the acid in extracts of a group II sorghum on subsequent solubility of the tannin. The amount of protein-precipitable phenols (Hagerman and Butler, 1978) dissolved by water is presented in the curve labeled "water soluble". The material not dissolved by water was dissolved in methanol and similarly assayed (curve labeled "methanol soluble"). The 1% HCl (v/v) in methanol extract (30 mL) of ground sorghum grain (IS 8768) was titrated with varying amounts of aqueous 0.83 M $NaHCO_3$ (saturated solution). (Complete neutralization of this amount of acid in water required 6.6 mL of the $NaHCO_3$ solution). The resulting precipitate was removed by centrifugation and discarded. The supernatant was taken to dryness on a rotary evaporator and then resuspended in 30 mL of water.

Table I. Purification of Tannin from a Group II Sorghum (IS 8768)

procedure	protein-precipitable phenols		total phenols	
	units	%	units	%
methanol extracts (discarded)	17.8		98	
H^+/methanol extracts	90.7	100	760	100
alkali precipitation	59.5	66	420	55
drying, water extraction	75.1	83	430	57
drying, ethanol extraction	94.4	104	207	27
LH-20 chromatography				
unbound and eluted with ethanol (non-tannins)	1.5	1.6	62	8.2
eluted with acetone/water (tannins)	92.5	102	126.5	16.6

applied remained at the origin, but a small spot formed at the solvent front. Tannin from BR 54 analyzed in the same way showed no spot at the solvent front but otherwise behaved similarly.

Chemical Assays. As can be seen in Table II, four independent assays gave similar results when applied to tannins purified from group II and group III sorghum. On a comparative per weight basis the tannins have nearly equivalent amounts of total phenolic hydroxyls (Prussian blue analysis), protein-precipitable phenols, total flavanol-3-ol units (proanthocyanidin assay), and terminal flavan-3-ol units (vanillin assay). The test for flavan-4-ols (Watterson and Butler, 1983) was negative for both.

Using the method of Butler et al. (1982) to estimate the average chain length of these oligomeric materials from the above results with the vanillin assay gave similar

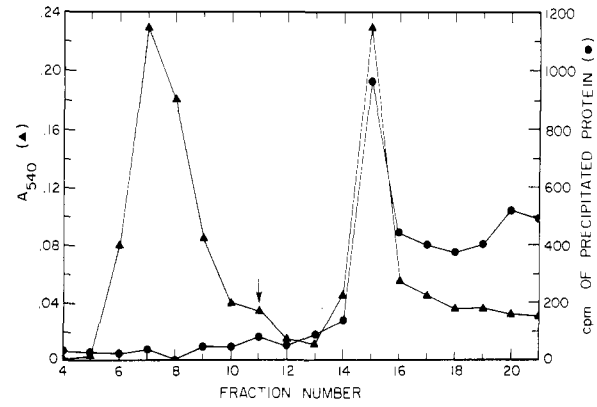

Figure 2. Chromatography on Sephadex LH-20. The 10-mL extract of sorghum IS 8768, prepared and dissolved in absolute ethanol as described under Materials and Methods, was applied to an 18 cm × 1.7 cm column Sephadex LH-20 equilibrated with ethanol. The column was washed with 33 mL of ethanol, and at the arrow the solvent was changed to 1/1 (v/v) acetone/H_2O to elute the tannin. The fraction size was 3.0 mL. The protein precipitation assay (●) was as described under Materials and Methods; absorbance at 540 nm (▲) was also monitored to detect non-tannin pigments and/or phenolics.

values, an average of 5.1 flavanol-3-ol units per molecule for the tannin from IS 8768 and 4.8 units per molecule for the tannin from BR 54. Gupta and Haslam (1978) have reported an average of 6–7 flavan-3-ol units per molecule for tannin purified from a different group III sorghum, NK 300, using an independent method.

Nitrogen content, a measure of the degree of contamination of the preparation by protein, averaged 0.4% on three separate preparations of tannin from IS 8768. Comparable values were reported for tannin from BR 54 as purified by Hagerman and Butler (1980b) by a more laborious procedure including extraction with aqueous phenol to remove proteins.

Spectral Analyses. Both tannin preparations showed major absoprtion peaks at 280 and 231 nm. In a boiling water bath in 2 N HCl both preparations produced cyanidin, which was identified by its absorption spectra with its characteristic shift with $AlCl_3$ (Riberau-Gayon, 1972). Thus, both oligomers are procyanidins, as previously reported for other group III (Gupta and Haslam, 1980) and group II (Bullard et al., 1981) sorghums.

IR spectra of tannin from both BR 54 and IS 8768 were essentially superimposable, showing the expected absorptions for –OH stretching as well as patterns consistent with the presence of substituted aromatics (Foo, 1981).

The ^{1}HMR spectra of BR 54 and IS 8768 tannins were also very similar, with broad signals characteristic of flavan-3-ol polymers (Thompson et al., 1972). A difference spectrum of the two polymers did not identify differences except for impurities and concentration.

Likewise, ^{13}CMR spectra of tannins isolated from group II and group III sorghums gave chemical shifts of all corresponding peaks within 2 ppm out of 200 ppm. Their positions match those observed by Karchesy and Hemingway (1980) for tannins obtained from loblolly pine bark. Like sorghum tannins, these tannins are believed to consist of chains of epicatechin units connected by C_4–C_8 (or C_4–C_6) linkages, terminating with a catechin unit (Kar-

Table II. Comparison by Chemical Assays of Tannins Purified from Group II and Group III Sorghums

tannin source	total phenols, A_{720}/mg	vanillin assay, A_{510}/mg	protein-precipitable phenols, A_{510}/mg	anthocyanidin formation, A_{550}/mg
IS 8768 (group II)	4.4, 4.1	3.55, 3.53	0.253, 0.26	2.78, 2.88
BR 54 (group III)	3.88, 3.8	3.53, 3.76	0.263, 0.265	2.2, 2.2

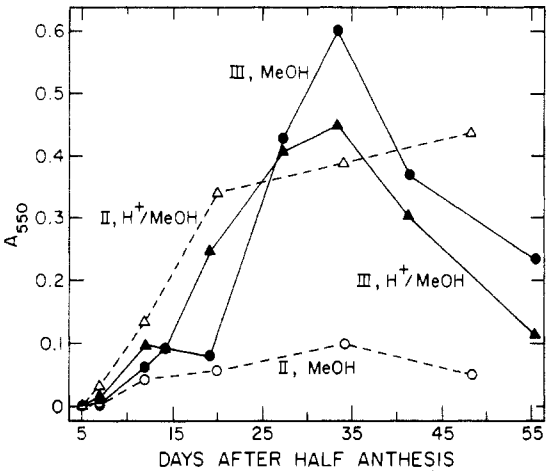

Figure 3. Extractable tannins throughout grain development. Sorghum BR 64 (group III) and IS 8768 (group II) were grown, collected, and extracted as previously described (Butler, 1982b). The fresh seeds were extracted without freezing or drying. Extraction with 1% HCl in methanol was done on the residue that remained after extraction twice with methanol; the total amount of tannin present is therefore the sum of the values for the methanol and acidic methanol extracts. Extracts were assayed by anthocyanidin formation (Watterson and Butler, 1983).

chesy and Hemingway, 1980). Although the tannins from group II and group III sorghums do not have completely superimposable ^{13}CMR spectra, they appear to consist of the same type of polymers.

Extractability of Tannin during Seed Development. Because we find that tannins purified from group II sorghums are chemically indistinguishable from those purified from group III sorghums, we have considered other explanations for the differences in extractability of their tannins. Figure 3 presents the amount of tannin obtained by extracting twice with methanol, and then by reextracting the residue twice with acidic methanol, during the period of seed development in these two sorghums. Tannins were assayed as proanthocyanidins by their conversion to anthocyanidins; similar data (not shown) were obtained with other tannin assays. The group III sorghum, BR 64, not only produced methanol-extractable tannin but also produced almost as much tannin which requires acidic methanol for extraction. Both populations of tannin occur in many but not all group III sorghums (Butler, 1982a). In contrast, most of the proanthocyanidins of IS 8768 are extractable only in acidic methanol, i.e., the characteristic that defines group II sorghums.

Figure 3 illustrates somewhat different development patterns for the two sorghums; the assayable tannin of the group III sorghum diminishes greatly as the grain reaches maturity, but the tannin of the group II sorghum does not. However, these patterns are not characteristic of all group II or group III sorghums. Wide variation in patterns of tannin in developing seeds are observed in both groups (Price et al., 1979; Bullard et al., 1980). In neither cultivar illustrated in Figure 3 nor in other cultivars (data not shown) does the time course suggest a precursor/product relationship between the tannins extractable in different solvents.

The relative degree of polymerization of the tannin molecules (Butler et al., 1982) throughout seed development (Butler, 1982b) was up to 2-fold higher in the acidic methanol extracts than in the methanol extracts of both lines (data not shown). The relative degree of polymerization of the corresponding extracts of the two sorghums was approximately the same. On drying of the seed the relative degree of polymerization of the tannin from the group II sorghum increased severalfold (data not shown) as was previously reported for the effect of drying on the group III sorghum (Butler, 1982b).

DISCUSSION

Maxson and Rooney (1972) showed that acidic methanol usually extracts more tannin from sorghum grain than does methanol. We showed (Butler, 1982a) that several sorghums contain tannins that extract only into acidic methanol as well as tannins that readily extract into methanol and the solvents employed by Bullard et al. (1981). In group II sorghums the acidic methanol extractable component predominates. Freezing the grain increases the amount of tannins extracted by acetone/H_2O or methanol, but the amount extracted into acidified methanol remains the same (Daly and Butler, 1982).

We conclude that after removal from the seed and purification, tannins from a group II sorghum and a group III sorghum are indistinguishable. A chemical explanation for the difference in extractability of the tannins has not been resolved; it may be due to an acid-labile bond such as a glycoside or an ester linking the tannin of group II sorghums to a methanol-insoluble component of the seeds. Another possibility for group II sorghums is the occurrence of an acid-labile structure that limits accessibility of the solvent to the tannin. The latter alternative is suggested in Table I where not only tannins but also other phenolics may require acidic methanol for extraction from seeds of group II sorghums; similar effects have been reported on extraction of other materials (Moore et al., 1982). The former alternative better explains our finding that calli of a group II sorghum produce and release condensed tannins that require acidic methanol to extract from the agar medium (Oberthur et al., 1983).

We are pursuing the biological significance of the group II sorghums. We are attempting to assess their nutritional value as compared to group III sorghums containing the same amount of tannin to evaluate reports that tannins from group II sorghums are less nutritionally harmful. We are also attempting to determine whether group II sorghums have desirable agronomic properties, such as bird resistance, often associated with the presence of tannins. Preliminary results suggest that they do not (Butler, 1982a). The structural characteristic that renders sorghum tannins extractable only in acidic methanol may represent a deactivation of these complex polyphenols that otherwise exhibit strong biological effects.

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Some Agronomic and Biochemical Characters of Brown Sorghums and Their Possible Role in Bird Resistance

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Chemical composition including tannin content and grain and agronomic characters including earhead length, head type, glume color, and threshability are reported for 18 sorghum genotypes with brown pericarp color, 15 of which had been reported to be bird resistant. Agronomic characters varied significantly among the genotypes. Variation in tannin content was much larger than variation in the other constituents. Detailed polyphenol analysis on selected genotypes indicated that some lines had insignificant levels of condensed tannins, that none of them was a group II sorghum, and that the levels of flavan-4-ols were relatively high. The possible role of polyphenolic components in relation to bird resistance is discussed.

Sorghum is a major staple food grain crop on the African and Asian continents. One of the major constraints on the production of grain sorghum is the severe bird depredation in many areas of Africa and many developing countries (Bullard and Elias, 1980). Sorghum produced in these areas is usually limited to bird-resistant cultivars, which are generally found to contain relatively high concentrations of polyphenols such as the condensed tannins (Tipton et al., 1970; Hoshino and Duncan, 1980). Brown-seeded hybrids have been reported to contain higher tannin levels than red- or yellow-seeded hybrids (Harris, 1969), and seed color of sorghum showed a highly significant positive correlation with tannin content (McMillan et al., 1972). However, Mabbayard and Tipton (1975) reported that pericarp color may not be a reliable indicator of tannin concentration.

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The astringency of the tannins is considered to be the principal means by which high-tannin sorghums effect resistance to bird depredation (Bullard and Elias, 1980). Resistance to bird depredation is a complex phenomenon that may be associated with non-tannin polyphenols, as well as tannins (Bullard et al., 1980). Flavan-4-ol monomers may contribute to bird repellency of high-tannin sorghums (Butler, 1982). Tannins in sorghum have also been associated with reduced preharvest germination and grain molding when wet weather prevails at the time of harvest (Harris and Burns, 1970). Besides the above advantages, high-tannin sorghums tend to be less digestible and nutritionally inferior to sorghums in which tannin is absent or is present at low levels (Maxson et al., 1973; Featherston and Rogler, 1975; Jambunathan and Mertz, 1973). However, tannin-containing varieties classified as group II may be nutritionally similar to non-tannin varieties (Oswalt, 1975).

The present report describes the variation in agronomic characters and chemical constituents of sorghum grains from 18 different genotypes. Fifteen genotypes selected in the study have been reported to be "bird-proof" sorghums (AICSIP, 1971) and their grains had brown peri-

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