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Fractionation, Characterization, and Protein-Precipitating Capacity of the Condensed Tannins from *Robinia pseudo acacia* L. Leaves

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Condensed tannins (proanthocyanidins) from black locust (*Robinia pseudo acacia*) leaves were fractionated into five different molecular sizes on a column of Sephadex LH-20 using 70% aqueous acetone as eluent. All five fractions yielded four anthocyanidin pigments upon acid hydrolysis, and their degree of polymerization measured by the vanillin assay in acetic acid ranged from 1.53 to 4.12. Both the percentage of protein-precipitable phenolics and the protein-precipitating capacity of the tannin fractions increased with the increase in the degree of polymerization.

INTRODUCTION

The leaves of *Robinia pseudo acacia* (black locust) are being utilized as fodder in some countries (Negi et al., 1979; Bonciarelli, 1980; Papageorgiou et al., 1981; Sheikh and Khan, 1983). The trees are heavily lopped for fodder as the black locust leaves are rich in crude protein (17–25%), calcium (1.93–3.49%), and phosphorus (0.14–0.22%) (Singh, 1982). The supplementation of black locust leaves with rice straw (Lee and Kang, 1980), barley (Kang and Yoo, 1978), and maize and defatted soybean meal diets (Takada et al., 1980) have been found useful for the sheep, goat, and poultry, respectively. However, when offered as a sole feed the black locust leaf meal was found to have the low digestibility for crude protein, organic matter, and phosphorus (Horton and Christensen, 1981; Negi et al., 1979). The leaves have also been found toxic when eaten in excess (Everist, 1969). Horigome et al. (1984) and Negi et al. (1979) reported that the low nutritional value of the black locust leaves was due to the presence of condensed tannins. Since the most relevant antinutritional property of condensed tannins is their ability to interact and precipitate feed and enzyme proteins, this largely depends upon the chemical nature of the tannins (Kumar, 1983; Porter and Woodruffe, 1984). This paper describes the fractionation, characterization, and protein-precipitating capacity of the condensed tannins from black locust leaves.

EXPERIMENTAL SECTION

The tannin from four separate batches of leaves collected in June 1984 was purified, with identical results each time. The tannin was extracted from 400 g of leaves with 1.4 L of acetone–water (70:30, v/v) containing 0.5% ascorbic acid (Jones et al., 1976). The acetone and water were separated into immiscible phases by saturating the extract with NaCl. Acetone was removed in vacuo at less than 40 °C, and the extract was diluted with an equal amount of water. The aqueous phase was extracted three times with diethyl ether followed by ethyl acetate to remove the pigments, lipids, and low molecular weight polyphenolic compounds, and the solution of crude tannins was dialyzed against water containing 0.5% ascorbic acid. To the dialyzed solution was added an equal amount of methanol. The 50% methonolic solution of crude tannins was applied to a column of Sephadex LH-20 (2.6 × 7.9 cm) preswollen in 50% methanol. The adsorbed tannins were washed with 800 mL of the same solvent and were eluted with 50% aqueous acetone as a discrete visible band. The acetone was removed in vacuo and water removed by freeze-drying to yield light, tan, fluffy solids that gave a single spot at the origin when two-dimensional paper chromatography was carried out in butanol–acetic acid–water (6:1:2) and 2% acetic acid.

For fractionation, a sample of tannin (0.55 g) in 3.0 mL of 70% acetone was loaded onto a column of Sephadex LH-20 (2.6 × 61 cm) that had been equilibrated with 70% acetone. The column was eluted at a flow rate of 1.4 mL/min with 70% acetone and 3.2-mL fractions were collected. The absorbance at 350 nm was monitored for each fraction. The fractions 29–35 (A), 38–47 (B), 53–60 (C), 64–71 (D), and 74–81 (E) were pooled. After removal of the acetone, pooled fractions were lyophilized to yield light, white fluffy solids that were used in the following tests.

The degree of polymerization of fractions A–E was determined by the vanillin assay in acetic acid (Butler et al., 1982). One milliliter of tannin solution (1 mg/mL in MeOH) was diluted at 25 mL with glacial acetic acid. The 1-mL diluted solution was mixed with 4.0 mL of freshly prepared vanillin reagent (4% concentrated HCl and 0.5% vanillin in acetic acid), and the absorbance was determined at 510 nm. Catechin (Sigma) dissolved in acetic acid was used as standard for the calculation of degree of polymerization.

For anthocyanidin analysis, 10-mg tannin fractions were mixed with 1.0 mL of 5% HCl in butanol in sealed tubes and the resultant mixtures heated in a boiling water bath for 2 h. The digest was applied as a streak to a sheet of

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Table I. Degree of Polymerization, Average Absorbance Values (nm) of Prussian Blue and Protein-Precipitation Assays, Protein-Precipitable Phenolics, and the Protein-Precipitating Capacity of Fractionated Tannins from the Black Locust Leaves

	A	B	C	D	E
deg of polymn	4.12	3.00	2.13	1.91	1.53
Prussian Blue assay, A_{720}	0.53	0.62	0.68	0.72	0.79
BSA-precip assay, A_{510}	0.99	0.96	0.94	0.80	0.74
% protein-precip phenols	74.71	61.93	55.29	44.44	37.56
% inhibn of β-glucosidase	51.9 ± 2.3	27.4 ± 2.6	22.7 ± 0.9	17.4 ± 1.3	11.0 ± 0.9

3-mm paper and developed in Forestal solvent. The anthocyanidin band was eluted in ethanol, rechromatogarphed in Forestal solvent, and eluted with ethanol containing 0.01% HCl. Anthocyanidins were identified by R_f values in paper chromatography and absorption spectra (Bate-Smith, 1954).

Protein-precipitable phenolics were determined as described by Hagerman and Butler (1978). A 0.5-mL portion of tannin solution (1 mg/mL in MeOH) was mixed with 3.0 mL of bovine serum albumin (BSA) solution (1 mg/mL in 0.2 M acetate buffer, pH 4.8). After 15 min the precipitate was centrifuged and washed twice with methanol. The washed precipitate was dissolved in 4.0 mL of sodium dodecyl sulfate–triethanolamine solution. One milliliter of the $FeCl_3$ reagent (0.01 M, in 0.01 N HCl) was added and vortex mixed; 20 min later the absorbance at 510 nm (A_{510}) was recorded. For quantitative incorporation of tannins into precipitate, at least twice as much protein as tannin (by weight) should be used (Hagerman and Butler, 1978). In the present study excess protein (6 times) was used to ensure that all the protein-precipitable phenolics were detected. For Prussian Blue assay, 0.2 mL of tannin solution (1 mg/mL in MeOH) was diluted with 100 mL of water; the assay was conducted as suggested by Price and Butler (1977). The absorbance was recorced at 720 mm (A_{720}).

The protein precipitation assay, Prussian Blue assay, and the Vanillin assay in acetic acid were performed in triplicate using the same stock solutions of the various tannin fractions. All the assays were conducted at the same time and within 6 h after the preparation of the stock solutions. The absorbance values obtained for all the assays were reproducible with a maximum variation of 0.005 absorbance unit. Since the assay conditions for all the tannin fractions were identical, the percent of protein-precipitable phenolics present in the various tannin fractions was calculated as $100[\text{av } A_{510}/(2.5 \times \text{av } A_{720})]$.

β-Glucosidase precipitation assay in triplicate was effected by essentially following the method of Goldstein and Swain (1965). One milliliter of tannin solution (1 mg/mL) in 0.2 M acetate buffer (pH 5.0) was added to 4.0 mL of β-glucosidase (1.0 mg of almond emulsin/mL; Sigma G-8625, activity 6 units/mg) solution in acetate buffer, and the resultant mixture was mixed and left at room temperature for 15 min. Standard was prepared by adding 1.0 mL of acetate buffer to 4.0 mL of enzyme solution. After the tannin–enzyme preparations was centrifuged, the activities of the enzyme in supernatants and standards were determined by combining 40-μL aliquots with 3.0 mL of acetate buffer containing esculin hydrate (0.5 mM) and $AlCl_3$ (3.75 mM) at room temperature and measuring the changes in absorbance at 385 nm over 3 min by using the Hitachi Model L 120-39 spectrophotometer. The changes in absorbance were linear over this time interval, and the protein-precipitating capacity of tannins was calculated by percent inhibition of enzyme activity as follows: $\{\Delta A_{385}(\text{st}) - \Delta A_{385}(\text{supernat})\} \times 100/\Delta A_{385}(\text{st})$.

Absorption spectra of tannin fractions in water and of anthocyanidins in ethanolic HCl were recorded on a Hitachi Model L 120-39 spectrophotometer fitted with a scanning attachment.

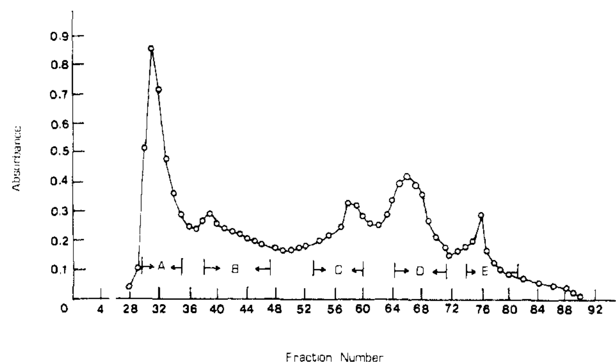

Figure 1. Fractionation of purified black locust tannins by gel filtration on Sephadex LH-20 using 70% acetone as eluent.

RESULTS AND DISCUSSION

Gel Filtration and Degree of Polymerization. Figure 1 shows the elution profile of the purified tannins in Sephadex LH-20 using 70% acetone as eluent. This profile was reproducible for the tannins purified from all the batches of leaves, and in this system there was no evidence of tannins binding to the gel. Some condensed tannin polymers have been observed to be fractionated according to the molecular size when eluted from Sephadex LH-20 with 70% acetone (Czochanska et al., 1980). Further, fractionation of the condensed tannins in Sephadex LH-20 using ethanol (Thompson et al., 1972), ethanol followed by methanol (Kolodziej, 1984), and 50% acetone (Strumeyer and Malin, 1975) as eluents has also been reported. However, in the present study, attempts to fractionate black locust tannins either with 50% acetone or with methanol always resulted in binding of tannins to the gel. The degree of polymerization of fractionated tannins indicates their polymeric nature (Table I), and it seems their separation in Sephadex LH-20 with 70% acetone as eluent is primarily dependent upon the molecular size. Moreover, UV spectra of tannins fractions A–E in water consisted of maxima at 205 and 272 nm with a shoulder around 240 nm and were overlapping, which indicated the homogeneous composition of all five fractions. A similar absorption spectrum has been reported for the tannins in *Ribes sanguineum* leaf by Czochanska et al. (1980).

Anthocyanidin Formation. Oligomeric proanthocyanidins, when treated with acid, yield anthocyanidins due to the lability of interflavan C–C bonds (Haslam, 1975). Paper chromatography and spectra of the anthocyanidins formed when tannin fractions A–E were treated separately with 5% HCl in butanol revealed the presence of delphinidin (R_f 0.38; A_{max} 558 nm), cyanidin (R_f 0.54; A_{max} 547 nm), and two unidentified pigments (R_f 0.74 and 0.83; A_{max} 549–551 and 538–542 nm, respectively). Further, the size and intensity of bands on the paper chromatogram indicated that all the fractions had the same proportions of the four pigments, which also suggests the homogeneous composition of the fractionated tannins. Reed et al. (1982) also reported the similar production of delphinidin,

cyanidin, and two unidentified pigments by condensed tannins of cassava leaves.

Protein-Precipitable Phenolics. The percent of protein-precipitable phenolics of the various tannin fractions was estimated, as described in the Experimental Section, by using the absorbance values obtained in the Prussian Blue assay (Price and Butler, 1977) and in the protein-precipitation assay (Hagerman and Butler, 1978). The Prussian Blue assay is based on the reduction by phenolics of ferric ion to ferrous ion followed by the formation of the ferricyanide–ferrous ion complex commonly known as Prussian Blue. Total phenolics in a solution can be determined with Prussian Blue assay, in which the amount of colored complex formed is proportional to the concentration of phenolics in the solution. The BSA-precipitation assay selectively measures the protein-precipitable phenolics, or tannins. Therefore, the ratio of absorbance of the above assays as described in the Experimental Section should give a relative value indicating the percentage of total phenol present in a particular tannin fraction that are precipitated by BSA. The results presented in Table I clearly demonstrate that the percentage of BSA-precipitable phenolics is maximum (74.7) in the highly polymerized tannin, fraction A, whereas, in fraction E, which has the lowest degree of polymerization, the minimum percentage (37.6) of the total phenolics precipitates. This suggests that the incorporation of phenolics in the tannin–protein precipitate, along with several factors such as pH, ionic strength, nature of protein, etc. (Kumar and Singh, 1984), may also depend upon the molecular size of the tannins and increases with the increase in molecular size.

Protein-Precipitating Capacity. The unique property of tannins is their ability to precipitate proteins, which is generally attributed to hydrogen-bonding and hydrophobic interactions (Haslam, 1974; Hagerman and Butler, 1980). The protein-precipitating capacity of various tannin fractions measured as β-glucosidase inhibition is dependent upon the degree of polymerization and increases regularly with the increase in molecular size (Table I). Although the relationship between the enzyme β-glucosidase inhibition and the formation of enzyme–tannin insoluble complexes is not fully understood, Haslam (1974) noted that the inhibition of β-glucosidase is caused by the precipitation, not by the residual soluble phenols. Data obtained in the present study clearly demonstrate that the enzyme β-glucosidase is maximally inhibited by the highly polymerized fraction A tannin followed by tannin fractions B–E (Table I). These results corroborate the findings of Haslam (1974) and Porter and Woodruffe (1984) who, by using the purified tannins from different plant sources, also showed that the capacity of tannins to precipitate proteins is primarily dependent upon the molecular weight of tannins.

The results obtained for the percent BSA-precipitable phenolics are qualitatively similar to β-glucosidase inhibition, but quantitatively quite distinct. Approximately 5 times as much β-glucosidase is inhibited by fraction A tannin as by fraction E, but only 0.3 times as much protein-precipitable phenolic is found in fraction A as is in fraction E. The most reasonable rationalization of these observations is that the increase in degree of polymerization of tannins affects the protein precipitation more markedly than the corresponding of precipitation of tannin phenolics.

On the basis of these observations, it is presumed that the proanthocyanidins in the leaf of black locust could exist in five molecular sizes and that their protein-precipitating capacity and the percentage of protein-precipitable phenolics increase with the increase in the degree of polymerization.

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