Purification and Characterization of a Proanthocyanidin Polymer from Seed of Alfalfa (*Medicago sativa* Cv. Beaver)

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A soluble proanthocyanidin polymer (PAP; condensed tannin) has been histologically localized in the pallisade layer of the alfalfa seedcoat, purified, and characterized by spectroscopic, chemical, and biochemical procedures. The polymer consists principally (91–93%) of 4 → 8 linked (−)-epicatechin units with a mean degree of polymerization of 5. (+)-Catechin is the predominant terminal unit. Only traces of prodelphinidin units were detected. No significant quantities of low molecular weight flavan-3-ols could be detected in ethyl acetate fractions of seed extracts, but analysis of seed leachate indicated the presence of (+)-catechin, (−)-epicatechin, and higher molecular weight oligomeric flavanols. Time course studies indicated that PAPs were leached at rates similar to that of monomeric flavonoids. Comparison of binding of PAPs from alfalfa seed and sainfoin leaf with bovine serum albumin at pH 2.5 and 6 indicated no significant differences between the tannins; however, there was a significant pH effect at higher tannin concentrations with protein binding greater at pH 6.0 than at pH 2.5.

INTRODUCTION

The presence of proanthocyanidin polymers (PAPs; condensed tannins) in crop plants may profoundly influence their quality through effects on color, palatability, and digestibility. The presence of these compounds in the seedcoats of food and forage legumes has been associated with seed viability and color (Nozzolillo and De Bazada, 1984; Nozzolillo et al., 1989), antinutritional effects (Artz et al., 1986), and inhibition of rhizobial growth (Young and Paterson, 1980; Gottfred, 1981; Prevost et al., 1990). Legume seed tannins have been studied extensively; however, there are surprisingly few reports of their isolation and characterization (Martin-Tanguy et al., 1977; Paszkowski and Kremer, 1987; Hussein et al., 1990). Recent advances in the phytochemistry of PAPs indicates that these are polydisperse polymers exhibiting considerable variation in stereochemistry and hydroxylation pattern (Porter, 1988; Ferreira et al., 1992).

Surveys have indicated that while proanthocyanidins (PAs) are generally found in the seedcoats of forage legumes, their presence in leaves and other aerial parts is restricted to certain taxa (Goplen et al., 1980). Although there have been unsubstantiated reports of PAs in leaves of alfalfa (Milić, 1972), broad surveys have failed to detect their presence in leaf tissue (Goplen et al., 1980; Marshall et al., 1981). Young and Paterson (1980) have reported the presence of procyanidins in alfalfa seed on the basis of colorimetric methods. In the present study we have histologically identified the tannin-containing layer in the seedcoat and isolated and characterized the PAPs from alfalfa seed as part of a study examining regulation of biosynthesis of PAs in this species.

MATERIALS AND METHODS

Materials. Breeder seed of alfalfa (*Medicago sativa* cv. Beaver) was harvested in 1990 at the Agriculture Canada Saskatoon Research Station. Authentic standards of anthocyanidins and flavan-3-ols were obtained from Apin Chemicals (Abingdon, U.K.). Phloroglucinol was obtained from Aldrich Chemical Co. (Milwaukee, WI) and was recrystallized twice from hot water. Authentic samples of flavan-3-ol–4-phloroglucinol adducts were kindly provided by L. Y. Foo (DSIR, Petone, New Zealand). PA polymer was purified from sainfoin leaves as described earlier (Koupai-Abyazani et al., 1992). All solvents used were of analytical or HPLC grade. All embedding materials were obtained from J. B. EM Services Inc. (Pointe-Claire-Dorval, PQ). Safranin O, BSA, and MES were obtained from Sigma Chemical Co. (St. Louis, MO). Evaporation was carried out at <30 °C under reduced pressure.

Thin-Layer Chromatography. TLC was carried out on cellulose TLC sheets (E. Merck, Darmstadt, Germany) eluted with *tert*-butyl alcohol/acetic acid/water (3:1:1 v/v/v, TBA), 6% v/v aqueous acetic acid (6% HOAc), and acetic acid/water/concentrated HCl (30:10:3 v/v/v, Forestal). Flavan-3-ols were detected using 4% w/v vanillin in methanol/concentrated HCl (4:1 v/v).

HPLC Analysis. HPLC of flavan-3-ols and their phloroglucinol adducts was carried out as described previously (Koupai-Abyazani et al., 1992). Gel permeation chromatography (GPC) was performed on a Waters M600E system equipped with an M700 autosampler and a 991 photo diode array detector (PDA) [Millipore (Canada) Ltd., Mississauga, ON]. The PDA software was used to integrate the signal. Peracetate derivatives were chromatographed on an Ultrastyragel linear (7.8 × 300 mm) and an Ultrastyragel 10^3A column (7.8 × 300 mm) [Millipore (Canada) Ltd.] in series, eluted with tetrahydrofuran at 1.1 mL/min (25 °C). Molecular weights (MWs) were calculated after calibration with polystyrene MW standards (MW 687, 2000, 4136, 9000, and 32,660) (Aldrich) and phloroglucinol and catechin peracetates.

Tannin Analyses. Quantitative analysis of PAs was performed using the modified butanol–HCl assay (Porter et al., 1986) and a microadaptation of the modified vanillin–HCl assay (Broadhurst and Jones, 1978). The latter assays were performed in a 200-μL volume on Corning polystyrene 96-well microtiter plates with a 5-min incubation, and absorbance was read at 495 nm with a Titretek multiscan 3100 plate reader (Eflab Oy, Helsinki).

Condensed Tannin Polymer Isolation. Alfalfa seed (100 g) was ground to a fine powder in a coffee grinder and percolated in a glass column successively with 400-mL portions of chloroform and chloroform/methanol (3:1 v/v). The defatted flour was then extracted by percolation with 800 mL of 75% v/v aqueous acetone containing 0.1% w/v ascorbic acid. The aqueous acetone extract

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was reduced to the aqueous phase and extracted successively with 80% w/v aqueous phenol (2 × 80 mL), chloroform (2 × 100 mL), and ethyl acetate (3 × 100 mL). The combined ethyl acetate extracts were reduced to dryness and analyzed by 2D-TLC in TBA and 6% HOAc. The aqueous residue remaining after ethyl acetate extraction was evaporated until there was no smell of ethyl acetate and diluted to 50% with methanol and applied to a 3 × 20 cm column of Sephadex LH-20 equilibrated with methanol/water (1:1 v/v). The column was washed with a further 2 L of 50% aqueous methanol. Elution of colorless phenolic material from the column was monitored by observation of fluorescence bands under long-wavelength UV light, and flavanols were detected by a vanillin–HCl assay of fractions. Further elution was carried out with 80% v/v aqueous methanol, and proanthocyanidin polymer was then eluted with 75% aqueous acetone as a brown visible band. This was concentrated in vacuo and freeze-dried to yield 0.32 g of proanthocyanidin polymer.

Spectroscopic Analysis. Optical rotations of polymers dissolved in methanol/water (1:1 v/v) were measured in a 10 mm path length cell at 21 °C in a Jasco J710 spectropolarimeter. ^{13}C NMR spectra were recorded in water/acetone-d_6 (1:1 v/v) on a Varian XL (300 MHz) spectrometer. ^1H NMR spectra were recorded on a Bruker WH-400 (400 MHz) spectrometer. IR spectra were recorded in a KBr disk in a Perkin-Elmer 1600 FTIR spectrophotometer.

Chemical Analysis. Peracetate derivatives of polymers were prepared by reaction with acetic anhydride in pyridine (Williams et al., 1983). Complete acid hydrolysis of polymers was performed in 1-butanol/HCl (95:5 v/v) at 95 °C for 40 min, and anthocyanidin products were analyzed by cellulose TLC (Forestal). Estimates of procyanidin:prodelphinidin (PC:PD) ratios were obtained by visible scanning densitometry using an LKB Ultroscan XL scanner [Pharmacia (Canada) Inc., Baie D'Urfé, PQ] and quantitation with authentic cyanidin and delphinidin standards run in the same system. Mild acid hydrolysis of polymers was carried out under N_2 in 0.1 M HCl at 95 °C for 20 min. Flavan-3-ols were extracted into ethyl acetate and analyzed by 2D-TLC in TBA and 6% HOAc (Porter, 1989). Hydrolysis of polymers in the presence of phloroglucinol was carried out as described previously (Koupai-Abyazani et al., 1992). Hydrolysis of polymers with toluene-α-thiol and ^1H NMR analysis of products were performed as described by Cai et al. (1991).

Analysis of Leachate from Alfalfa Seed. For preliminary analysis of leachates, seed was surface sterilized by stirring in 70% v/v aqueous ethanol (3 min), blotted dry, and soaked in 150 mL of distilled water overnight. The aqueous ethanol used to sterilize the seeds showed no evidence of flavonoids when analyzed by TLC. The leachate was mixed with 3 volumes of acetone and filtered through Whatman No. 1 filter paper to remove precipitated proteins. Acetone was removed by rotary evaporation and glacial acetic acid added to give a final concentration of 5% v/v. The acidified aqueous residue was filtered through a glass fiber disk (Whatman 934-AH) and applied to a 400-mg C_{18} cartridge (E. Merck) previously equilibrated with 5% v/v aqueous acetic acid. The cartridge was washed with 5 mL of 5% v/v aqueous acetic acid, and bound phenolics were eluted with 1 mL of 70% v/v aqueous methanol. Aliquots of this extract were analyzed qualitatively for flavan-3-ols by 2D-TLC and quantitatively for proanthocyanidins by the modified butanol/HCl assay using purified alfalfa tannin as standard. For studies of the time course of leaching, 10-g samples of surface-sterilized seed were suspended in 50 mL of sterile distilled water and incubated at room temperature with occasional shaking. Aliquots of 1 mL were removed after 1, 2, 4, 6, 16, and 30 h of incubation and frozen at −20 °C until analysis. Samples were thawed, centrifuged (5 min at 10000g), acidified with 50 μL of acetic acid, and processed on C_{18} cartridges as described above. Aliquots of these samples were analyzed for PAs using the vanillin/HCl assay, and flavonoids in the samples were estimated spectrophotometrically by diluting aliquots in 1 M Tris (pH 10) on 96-well plates and measuring absorbance at 405 nm using rutin as standard.

Analysis of Tannin–Protein Binding. Microtiter plate assays of BSA binding were performed using purified sainfoin polymer (Koupai-Abyazani et al., 1992) and alfalfa seed polymer according to the method of Ittah (1991). Microtiter plate wells were coated with 0.2% BSA in 0.1 M acetic acid (pH 2.5) or 0.1 M MES buffer (pH 6), and the unbound BSA was removed with two washes. Aqueous solutions of condensed tannins and blanks were then applied to the wells and incubated for 1 h, and the wells were washed twice with buffer. Alkaline phosphatase (100 μL, 0.5 unit/mL) (Boehringer-Mannheim Canada Ltd., Lavel, PQ) was then adsorbed onto the bound tannin and any excess removed after 1 h of incubation. The activity of the alkaline phosphatase bound to the tannin was measured at 405 nm using p-nitrophenyl phosphate as the substrate (Ittah, 1991).

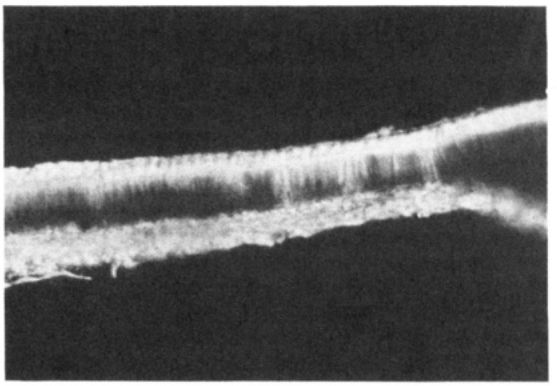

Figure 1. Testa removed from an alfalfa seed and stained with vanillin–HCl. The dark areas in the columnar cells are the positive cherry-red color reaction indicative of the presence of condensed tannins (×82). This figure is reproduced at 60% of the original.

Histochemistry. For gross localization of alfalfa seedcoat tannins, fresh cross sections of mature alfalfa seeds were infiltrated with 10% vanillin in EtOH/concentrated HCl (3:1). To determine the cellular location of alfalfa seedcoat tannins, seeds nearing maturity were dissected from hand-crossed purple-flowered alfalfa plants and fixed in 0.1 M sodium phosphate containing 6% glutaraldehyde for 3 days. The seeds were then washed in buffer until the smell of glutaraldehyde was not evident. The seeds were further fixed with 2% osmium tetraoxide in buffer (4 h) and rinsed with distilled water. The seeds were then dehydrated in aqueous acetone (10% steps, 30 min per step) followed by two 30-min periods in 100% acetone. The seeds were embedded in resin by transfer through three acetone/resin solutions [Araldite 502/Jembed 812/DDSA (dodecenyl succinic anhydride)/DMP-30 [tris(dimethylaminomethyl)phenol] (16:29:53:1 w/w/w/w)] with increasing resin content (2:1, 1:2, 0:3; 6, 16, 30 h). The seeds were then placed in the tips of resin-filled beem capsules, and the resin was polymerized for 48 h in a 60 °C oven. Thin sections cut from these seeds were stained for 5 min with Safranin O and viewed with a light microscope using bright-field conditions.

Statistics. Statistical comparisons were performed using SYSTAT routines (Wilkinson, 1990).

RESULTS AND DISCUSSION

In fresh-cut cross sections of alfalfa seeds stained with vanillin–HCl, only the seedcoat gave a positive reaction (Figure 1). The cherry-red color, indicative of condensed tannins, appeared to be in the columnar cells immediately below the cuticle layer in the testa; however, the exact location of tannins in the cell could not be resolved using this technique. Condensed tannins are known to be strongly osmiophilic and appear black in electron micrographs. In unstained thin sections prepared for electron microscopy, tannins can still be visualized as dark gray areas against a transparent background (Parham and Kaustinen, 1976). In our study, such thin sections were stained with safranin red to highlight cells and cell components. Light microscopy using bright-field techniques revealed condensed tannins as black material partially filling cell vacuoles in the pallisade cell layer of the seed testa (Figure 2).

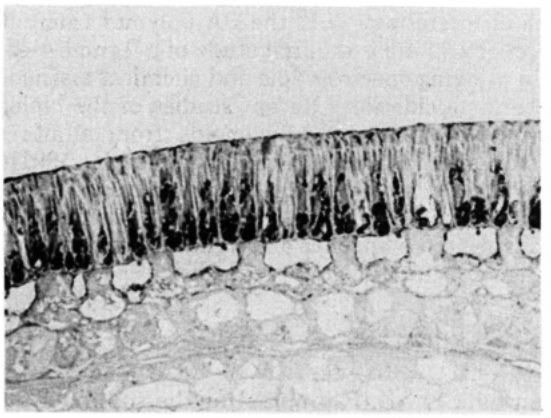

Figure 2. Thin section of a nearly mature alfalfa seed showing the testa with the typical cuticle, pallisade layer, and hourglass cells. Condensed tannins are shown as the dark, osmiophilic material located in the lower portion and partially filling the vacuole in each pallisade cell (×125). This figure is reproduced at 60% of the original.

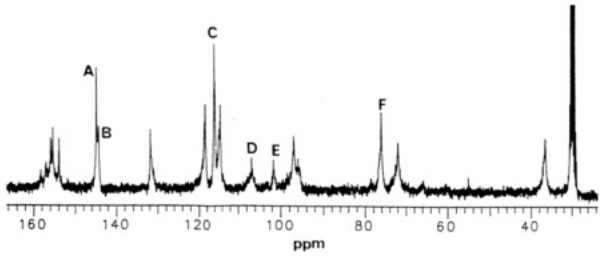

Figure 3. Determination of general structural features of alfalfa seedcoat polymer by 300-MHz ^{13}C NMR. A, C3′ and C5′ of PD units; B, C3′ and C4′ of PC units; C, C2′, C5′, and C6′ of PC units; D, C2′ and C6′ of PD units; E, C4a of 2,3-cis PC and PD units; F, C2 of 2,3-cis units.

Initial attempts to purify the proanthocyanidin polymers from alfalfa seed according to standard methods (Porter, 1989) yielded a product that was judged to be impure on the basis of TLC and UV–visible spectrophotometry. Introduction of an additional extraction of the aqueous extract with aqueous phenol permitted removal of additional protein and colored material. During the final chromatography step on LH-20, the presence of a strongly bound, blue fluorescent (UV) phenolic contaminant was detected. To separate the condensed tannin polymer from this contaminant, the LH-20 column was eluted with methanol/water (8:2 v/v). The ethyl acetate fraction, obtained during polymer purification, was found to contain a variety of flavonoids, but no flavan-3-ols could be detected by 2D-TLC.

The alfalfa polymer had a UV_{max} of 278 nm with a $E_{1\%}$ of 120, typical of a procyanidin polymer, and an optical rotation of $[\alpha]^{21}_{578}$ 134, which corresponds to a molar proportion of subunits with 2,3-cis stereochemistry of 91% (Porter, 1989). Complete acid hydrolysis yielded mostly cyanidin with a trace of delphinidin. When the hydrolysate was chromatographed on cellulose TLC, the PC:PD ratio was estimated by scanning densitometry to be 14:1.

These observations were further confirmed by IR and ^{13}C NMR spectroscopy (Figure 3). The IR spectrum exhibited bands at 1105 and 780–800 cm^{-1}, characteristic of a class A procyanidin polymer consisting predominantly of (−)-epicatechin units according to the classification of Foo (1981). The ^{13}C NMR spectrum also exhibited resonances typical of a polymer containing a predominance of subunits with 2,3-cis units (76 ppm) and 3′,4′-dihydroxylated B-rings (114–118 ppm), although the presence

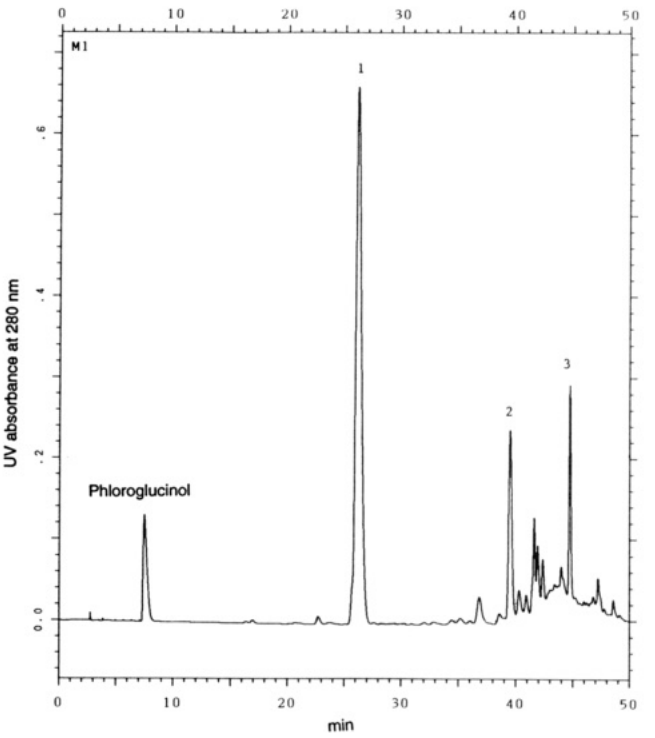

Figure 4. HPLC chromatogram of the hydrolysis products (in the presence of phloroglucinol) of alfalfa seedcoat polymer. Peaks: 1, (−)-epicatechin-4-phloroglucinol; 2, (+)-catechin; 3, (−)-epicatechin.

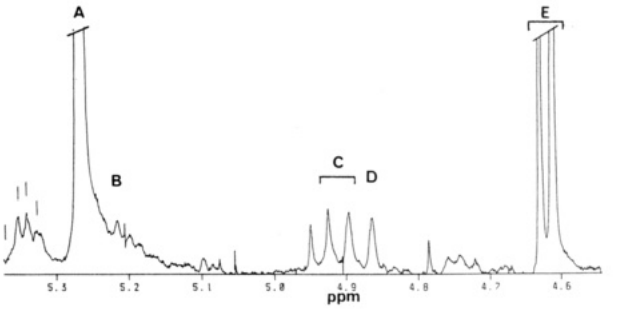

Figure 5. ^1H NMR spectrum of benzylthiol adducts of alfalfa seedcoat polymers. Peaks: A, (−)-epicatechin-4-benzylthiol; B, (−)-epigallocatechin-4-benzylthiol; C, (+)-catechin-4-benzylthiol; D, (−)-epicatechin; E, (+)-catechin.

of a small proportion of units with 3′,4′,5′-trihydroxylated B-rings could be clearly discerned (102 and 107 ppm).

Mild acid hydrolysis of the polymer yielded (−)-epicatechin (identified by cellulose 2D-TLC). Further quantitative information regarding the subunit composition of the polymer was obtained by degradation in the presence of the nucleophiles phloroglucinol and toluene-α-thiol. The adducts formed were analyzed by HPLC (Figure 4) and ^1H NMR (Figure 5), respectively. The major product in both cases was the 4-adduct of (−)-epicatechin. Traces of (−)-epigallocatechin and (+)-catechin benzylthiol adducts were detected by the NMR procedure. (+)-Catechin and (−)-epicatechin were detected in the hydrolysates by HPLC and ^1H NMR analysis. (+)-Catechin was present in larger amounts, indicating it was the predominant terminal unit. Integration of HPLC and NMR results gave estimates of the degree of polymerization of 4.4 and 5.1, respectively. GPC-HPLC analysis of the peracetate derivative of the alfalfa polymer indicated an average degree of polymerization of 6.5. These values are similar to the value of 6 recently reported by Hussein et al. (1990) for a tannin from Faba bean hulls.

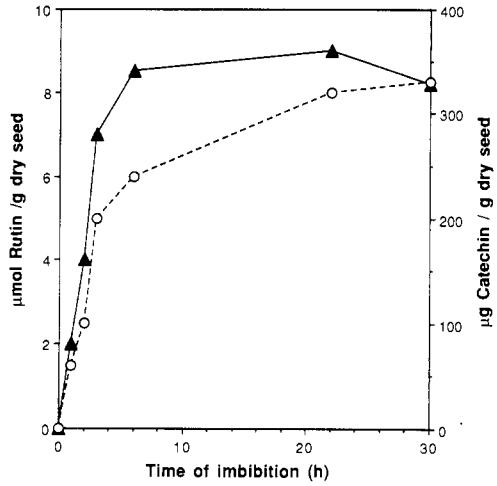

Figure 6. Time course of leaching of flavonoids (—) and flavan-3-ols (- - -) from imbibed alfalfa seeds as determined by vanillin–HCl analysis using catechin as the standard.

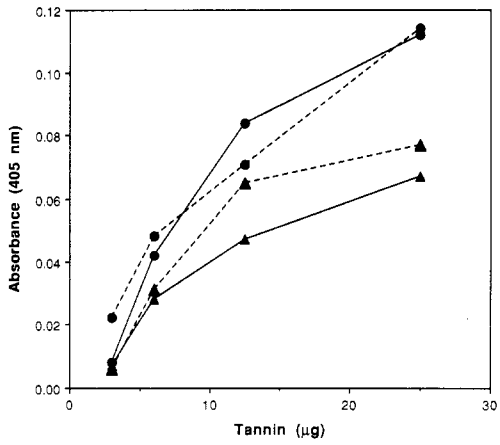

Figure 7. Activity of alkaline phosphatase as a measure of binding of sainfoin (—) and alfalfa (- - -) tannins with BSA at pH 2.5 (▲) and pH 6 (●).

Leaching of PAPs during Seed Imbibition. Preliminary analyses of seed leachates indicated that, in addition to a variety of flavonoids, these leachates contained vanillin–HCl reactive material with 2D-TLC mobility characteristic of PAs (immobile in TBA, basal streak in 6% HOAc), (–)-epicatechin, and an unidentified flavan (R_f 41, TBA; 50, 6% HOAc). Quantitative analysis of leachate showed a PA content of 50 µg of alfalfa tannin/g of dry seed as determined by the butanol/HCl assay. Analysis of the time course of release of PAs and other flavonoids indicated that these followed a similar time course (Figure 6) ($r = 0.96$, $p > 0.01$).

The binding of alfalfa and sainfoin tannins with BSA was examined at pH 6 and 2.5 using the method of Ittah (1991) to examine whether they differed in ability to bind protein under these conditions which approximate ruminal and abomasal pH values (Figure 7). There was no significant difference in binding between the two polymers, as measured by the activity of alkaline phosphatase immobilized on the bound tannin. Binding of the polymers was significantly greater at pH 6.0 [$p = 0.015$ ($n = 8$) by ANOVA] than at pH 2.5. These results are opposite to those obtained by Ittah (1991) with persimmon tannin, who observed significantly more binding of the tannin at acid pHs than at neutral pHs; however, our results are more in line with those observed for soluble protein–tannin interactions (Hagerman and Butler, 1981).

The characterization of the PA polymer from alfalfa seed reported here is the first study of a legume seedcoat tannin utilizing spectroscopic and chemical methods for structural elucidation. Recent studies of the biological activities of monomeric flavonoids from alfalfa seed (Hartwig and Phillips, 1991; Hartwig et al., 1991) and improved understanding of the structural diversity of proanthocyanidins (Porter, 1989) indicate the importance of utilizing more specific and validated procedures for analysis of legume seed polyphenols.

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LITERATURE CITED

Artz, W. E.; Swanson, B. G.; Sendzicki, B. J.; Rasyid, A.; Birch, R. E. W. Procyanidin interaction and nutritional quality of dry beans. In *Plant proteins: Applications, Biological effect, and Chemistry*; Ory, R. L., Ed.; ACS Symposium Series 312; American Chemical Society: Washington, DC, 1986; pp 126–137.

Broadhurst, R. B.; Jones, W. T. Analysis of condensed tannins using acidified vanillin. *J. Sci. Food Agric.* **1978**, *29*, 788–794.

Cai, Y.; Evans, F. J.; Roberts, M. F.; Phillipson, J. D.; Zenk, M. H.; Gleba, Y. Y. Polyphenolic compounds from *Croton lecheri*. *Phytochemistry* **1991**, *30*, 2033–2040.

Ferreira, D.; Steynberg, J. P.; Roux, D. G.; Brandt, E. V. Diversity of structure and function in oligomeric flavonoids. *Tetrahedron* **1992**, *48*, 1743–1803.

Foo, L. Y. Proanthocyanidins: Gross chemical structures by infrared spectra. *Phytochemistry* **1981**, *20*, 1397–1402.

Goplen, B. P.; Howarth, R. E.; Sakar, S. K.; Lesins, K. A search for condensed tannins in annual and perennial species of *Medicago*, *Trigonella*, and *Onobrychis*. *Crop Sci.* **1980**, *20*, 801–804.

Gottfred, N. P. The effect of seed pods, strains of *Rhizobium* and phenolic compounds on the growth, nodulation and nitrogen fixation of sainfoin (*Onobrychis viciafolia* Scop.). MSc. Thesis, University of Manitoba, 1981.

Hagerman, A. E.; Butler, L. G. The specificity of proanthocyanidin-protein interactions. *J. Biol. Chem.* **1981**, *256*, 4495–4497.

Hartwig, U. A.; Phillips, D. A. Release and modification of nodgene-inducing flavonoids from alfalfa seeds. *Plant Physiol.* **1991**, *95*, 804–807.

Hartwig, U. A.; Joseph, C. M.; Phillips, D. A. Flavonoids released naturally from alfalfa seeds enhance growth rate of *Rhizobium meliloti*. *Plant Physiol.* **1991**, *95*, 797–803.

Hussein, L.; Abdel Fattah, M.; Salem, E. Characterization of pure proanthocyanidins isolated from the hulls of faba beans. *J. Agric. Food Chem.* **1990**, *38*, 95–98.

Ittah, Y. Titration of tannin via alkaline phosphatase activity. *Anal. Biochem.* **1991**, *192*, 277–280.

Koupai-Abyazani, M. R.; McCallum, J.; Bohm, B. A. Identification of the constituent flavanoid units in sainfoin proanthocyanidins by reversed-phase high performance liquid chromatography. *J. Chromatogr.* **1992**, *594*, 117–123.

Marshall, D. R.; Broue, P.; Grace, J.; Munday, J. Tannins in Pasture Legumes 2. The annual and perennial *Medicago* species. *Aust. J. Exp. Agric. Anim. Husb.* **1981**, *21*, 55–58.

Martin-Tanguy, J.; Guillaume, J.; Kossa, A. Condensed tannins in horsebean seeds; Chemical structure and apparent effects on poultry. *J. Sci. Food Agric.* **1977**, *28*, 757–765.

Milić, B. L. Lucerne tannins. I. Content and composition during growth. *J. Sci. Food Agric.* **1972**, *23*, 1151–1156.

Nozzolillo, C.; De Bazada, M. Browning of lentil seeds, concomitant loss of viability, and the possible role of soluble tannins in both phenomena. *Can. J. Plant Sci.* **1984**, *64*, 815–824.

Nozzolillo, C.; Ricciardi, L.; Lattanzio, V. Flavonoid constituents of seed coats of *Vicia faba* (Fabaceae) in relation to genetic control of their color. *Can. J. Bot.* **1989**, *67*, 1600–1603.

Parham, R. A.; Kaustinen, H. M. Differential staining of tannin in sections of epoxy-embedded plant cells. *Stain Technol.* **1976**, *51*, 237–240.

Paszkowski, W. L.; Kremer, R. J. Identification and localization of tannins in velvetleaf (*Abutilon theophrasti* medik.) seeds. *Pamiet. Pulawski* **1987**, *90*, 49–55.

Porter, L. J. Flavans and proanthocyanidins. In *The Flavonoids, Advances in Research since 1980*; Harborne, J. B., Ed.; Chapman and Hall: New York, 1988; p 21.

Porter, L. J. Tannins. In *Methods in Plant Biochemistry. Plant Phenolics*; Harborne, J. B., Ed.; Academic Press: San Diego, 1989; Vol. 1, pp 389–420.

Porter, L. J.; Hritsch, L. N.; Chan, B. G. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry* **1986**, *25*, 223–230.

Prevost, D.; Jain, D. K.; Bordeleau, L. M. Growth inhibition of rhizobia isolated from arctic legumes (*Astragalus* and *Oxytropis* spp.) and sainfoin (*Onobrychis viciifolia*) by sainfoin seed diffusate. *Phytoprotection* **1990**, *71*, 113–119.

Wilkinson, L. *SYSTAT: The system for statistics*; Systat: Evanston, IL, 1990; 677 pp.

Williams, V. G.; Porter, L. J.; Hemingway, R. W. Molecular weight profiles of proanthocyanidin polymer. *Phytochemistry* **1983**, *22*, 569–572.

Young, H.; Paterson, V. J. Condensed tannins from white clover seed diffusate. *Phytochemistry* **1980**, *19*, 159–160.

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