Protein Precipitating Capacity of Crude Canola Tannins: Effect of pH, Tannin, and Protein Concentrations

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The protein precipitating capacity of canola tannins was evaluated using the protein precipitation assay of Hagerman and Butler (*J. Agric. Food Chem.* **1978**, *26*, 809−812) and the dye-labeled bovine serum albumin (BSA) assay of Asquith and Butler (*J. Chem. Ecol.* **1985**, *11*, 1535−1543). Condensed tannins were isolated from hulls of Cyclone, Excel, and Westar canola cultivars. The tannin content in the hulls ranged from 98 to 1973 mg of catechin equivalents/100 g of hulls, as determined by the vanillin assay. The effect of pH on the affinities of dye-labeled and unlabeled BSA, fetuin, gelatin, lysozyme, and pepsin was monitored. The optimum pH for the precipitation of dye-labeled and unlabeled BSA was found to be 3.5 and 4.0, respectively. The optimum pH for the precipitation of proteins was found to be 0.3−3.1 pH units below the isoelectric points of the proteins. The crude tannin extracts contained about 20% proanthocyanidins, which were soluble in ethyl acetate as determined by the vanillin assay. Canola tannins showed definitive thresholds prior to the formation of insoluble tannin−protein complexes as determined by the protein precipitation assay. There was also a linear correlation ($r^2 = 0.975$) between the amount of tannin−protein complex formed and the amount of tannin added to the system. Ethyl acetate soluble proanthocyanidins contributed to the protein-precipitating capacity of crude canola tannins isolated from low-tannin Cyclone canola hulls.

Keywords: *Condensed tannins; canola; hulls; tannin−protein interactions*

INTRODUCTION

The presence of condensed tannins in rapeseed hulls was first reported by Bate-Smith and Ribereau-Gayon (1959). This finding was verified by Durkee (1971), who identified cyanidin, pelargonidin, and an artifact *n*-butyl derivative of cyanidin in the hydrolytic products of rapeseed hulls. Later Leung et al. (1979) reported that condensed tannins of rapeseed hulls contained leucocyanidin as their basic units.

Advances in dehulling of rapeseed (Sosulski and Zadernowski 1981; Greilsamer, 1983; Diosady et al., 1986) may soon bring about the introduction of dehulling to rapeseed/canola processing. The subsequent use of hulls as a component of feedstuffs may be one way for their utilization. Our studies (Naczk et al., 1994) indicated that canola hulls contained up to 1556 mg of tannins/100 g of hulls as determined by the vanillin assay, i.e., up to 8 times more condensed tannins than reported previously (Leung et al., 1979; Mitaru et al., 1982). Tannins may form soluble and insoluble complexes with proteins, which may be responsible for the antinutritional effects of tannin-containing ingredients in nonruminant (Martin-Tanguy et al., 1977) and ruminant (Kumar and Singh, 1984) feeds.

The available information on the biological activity of rapeseed tannins is still diverse and fragmentary. Mitaru et al. (1984) reported that condensed tannins isolated from rapeseed hulls were unable to inhibit the activity of α-amylase enzyme *in vitro*. On the other hand, Leung et al. (1979) found that tannins isolated from rapeseed hulls formed a white precipitate upon the addition of a 1% gelatin solution, but the authors did not attempt to quantify the protein-precipitating activity of tannins. Futhermore, Butler et al. (1982) and Fenwick et al. (1984) suggested that tannins may be responsible for the tainting of eggs laid by hens fed on rapeseed meal. They postulated that this tainting effect may originate via the formation of a tannin−trimethylamine (TMA) oxidase complex. The formation of this complex prevents the conversion of TMA to odorless and water-soluble trimethylamine oxide. Recently Naczk et al. (1994) reported that tannins isolated from canola hulls exhibit a protein precipitation capacity comparable to that of sorghum tannins. They also found that the protein precipitation capacity of tannins, expressed as a precipitation index (PI), correlated well with the content of tannins determined by the vanillin assay. The PI values for tannins isolated from the samples of high-tannin hulls did not exceed 5.0 mg of dye-labeled bovine serum albumin (BSA)/mg of tannins, but for tannins of low-tannin samples of canola hulls, the PI ranged from 17.7 to 40.7 mg of BSA/mg of tannins.

The objective of this study was to determine the effect of pH and tannin and protein concentrations on the protein-precipitating capacity of crude canola tannins extracted from low- and high-tannin canola hulls by utilizing protein precipitation methods commonly used for the quantification of tannins.

MATERIALS AND METHODS

Hulls of Westar, Excel, and Cyclone canola varieties, grown in western Canada in 1991−1994, were obtained according to

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the procedure described by Sosulski and Zadernowski (1981). Hulls were extracted with hexane for 12 h using a Soxhlet apparatus and then dried at room temperature.

The condensed tannins were isolated as follows. A (1 g) sample of hulls was extracted twice with 10 mL of 70% (v/v) aqueous acetone using a Polytron (Brinkman PT 3000) (60 s, 15 000 rpm) at room temperature. After each centrifugation for 10 min at maximum speed using an IEC Clinical Centrifuge (International Equipment Co., a Division of Damon, Needham Hts., MA), the supernatants were collected, combined, and evaporated almost to dryness at 40 °C under vacuum. The extracted phenolics were dissolved in 10 mL of methanol and centrifuged as described above. Any insoluble material was discarded.

The condensed tannins were assayed colorimetrically by the modified vanillin method of Price et al. (1978) as described by Naczk et al. (1994). To 1 mL of methanolic solution of condensed tannins, 5 mL of 0.5% vanillin in methanol containing 4% concentrated HCl (sample) or 5 mL of 4% concentrated HCl in methanol (blank) was added and mixed well. The absorbance of each sample against the blank was measured at 500 nm, after standing in the dark for 20 min at room temperature. The tannin content in the hulls was expressed as catechin [catechin (+) containing 3.5 mol of water/mol of catechin (Sigma Chemical Co., St. Louis, MO) was used as the standard] equivalents (mg per 100 g of hulls, on a dry basis) using the following equation: $C = k\,[1.684A_{500} - 0.039]$ (Naczk et al., 1994), with correlation coefficient $r^2 = 0.998$, where k is a dilution factor which ranged from 1000 to 2500 and C is the content of tannins in milligram of catechin equivalents per 100 g of oil-free canola hulls.

The protein precipitating capacity of condensed tannins of canola hulls was assayed as described by Hagerman and Butler (1978) with the following pH modification: To 1 mL of methanolic solution of crude tannin extract, 2 mL of standard BSA (Sigma, fraction V, initial fractionation by cold alcohol precipitation) solution was added (1 mg of protein/mL in 0.2 M acetate buffer, pH 4.0, and containing 0.17 M sodium chloride) and the solution was mixed well. After 15 min standing at room temperature, the solution was centrifuged for 15 min at maximum speed using an IEC clinical centrifuge. The supernatant was discarded, and the surface of the pellet and the tube walls were carefully washed with acetate buffer (pH 4.0) without disturbing the pellet. The pellet was then dissolved in 4 mL of sodium dodecyl sulfate (SDS)−triethanolamine solution [1% SDS and 5% (v/v) triethanolamine in distilled water], and 1 mL of ferric chloride reagent (0.01 M ferric chloride in 0.01 M HCl) was added to it and mixed. Fifteen minutes after the addition of the ferric chloride reagent, the absorbance (A_{510}) of the solution was read at 510 nm against a reagent blank (4 mL of SDS solution + 1 mL of ferric chloride reagent). The protein precipitating capacity of tannins was expressed as A_{510}/1 g of hulls.

The protein precipitation capacity of canola condensed tannins was also determined by the dye-labeled BSA assay of Asquith and Butler (1985), with the following pH modification: 4 mL of dye-labeled BSA solution containing 2 mg/mL of protein in 0.2 M phosphate buffer at pH 3.5 was added to either 1 mL of methanolic solution of the crude tannin extract (sample) or to 1 mL of methanol (reagent blank). The solution was then vigorously mixed at 1000 rpm for 5 min at room temperature using a shaker Type 50000 Maxi-Mix III (Barnstead/Thermolyne, Dubuque, IA). The protein−tannin complex thus formed was separated by centrifugation for 15 min at maximum speed using an IEC clinical centrifuge. The supernatant was carefully discarded; the surface of the pellet and the tube walls were washed with phosphate buffer (pH 3.5) without disturbing the pellet. (Note that we found that 0.2 M phosphate buffer of pH 3.5 maintains this pH during the formation of insoluble tannin−dye-labeled BSA complexes.) The pellet was then dissolved in 3.5 mL of 1% (w/v) SDS solution containing 5% (v/v) triethanolamine and 20% (v/v) isopropanol and the absorbance of the solution was read at 590 nm against the reagent blank. The protein precipitation capacity of tannins was expressed as A_{590}/g of hulls. The content of the dye-labeled BSA in the tannin−protein complex so produced was calculated using the following equation: $C = 4.902A_{590} - 0.024$, correlation coefficient $r^2 = 1.000$, where C is the content of dye-labeled BSA (mg) per assay (3.5 mL). The dye-labeled BSA was prepared according to the procedure described by Rinderknecht et al. (1968) as modified by Asquith and Butler (1985).

Table 1. Content of Condensed Tannins in Canola Hulls

canola variety	tannin content[a]
Westar	
sample 1	1556 ± 54[b]
sample 2	173 ± 12[b]
sample 3	142 ± 2[b]
sample 4	98 ± 5[b]
Cyclone	
sample 1	1307 ± 85[b]
sample 2	994 ± 45[b]
sample 3	1574 ± 58[b]
sample 4	695 ± 12
sample 5	1973 ± 82
Excel	144 ± 7[b]

[a] Tannin content in milligrams of catechin equivalents per 100 g of hulls. [b] Adapted from Naczk et al. (1994).

The effect of pH on the formation of canola tannin−protein complexes was monitored as described by Hagerman and Butler (1978). A series of solutions (1 mg/mL) of BSA (Sigma, fraction V, initial fractionation by cold alcohol precipitation), fetuin (Sigma, from fetal calf serum), lysozyme (Sigma, from chicken egg white), gelatin (Sigma, type B, from bovine skin), and pepsin (Sigma, 1:60 000, from porcine stomach mucosa) were prepared using a 0.01 M phosphate buffer at pH ranging from 2.0 to 11.0. The pH of the reaction mixture was checked after the addition of the tannins and proteins. Phosphate buffers were prepared by mixing 0.01 M phosphoric acid and 0.01 M trisodium phosphate in proportions to produce a buffer of a desired pH. The amount of precipitated, protein-bound polyphenols was determined according to the procedure described by Hagerman and Butler (1978). The effect of pH on the formation of canola tannin−protein complexes was also determined by the dye-labeled BSA assay of Asquith and Butler (1985). A series of 0.20 M phosphate buffers containing 1 mg/mL dye-labeled BSA were prepared at pH 2.0−8.0. Preparation of buffer solutions was carried out by mixing 0.20 M phosphoric acid and 0.20 M trisodium phosphate in the appropriate amounts required to attain a desired pH.

The effect of protein concentration on the formation of a tannin−protein complex was assayed by the protein precipitation method of Hagerman and Butler (1978) and the dye-labeled BSA method of Asquith and Butler (1985). A series of protein solution containing 0.5−10.0 mg/mL dye-labeled BSA in 0.20 M phosphate buffer, pH 3.5, and unlabeled BSA in 0.20 M acetate buffer, pH 4.0 and 4.9, were prepared.

All assays were conducted at room temperature (about 22 °C) using appropriate samples and blanks. Results presented in tables and figures are mean values of at least six determinations. The bars in the figures represent standard deviations.

RESULTS AND DISCUSSION

The content of tannins in samples of hulls of canola varieties used in this study is summarized in Table 1. The differences in tannin content within a canola variety may be due to the growing location, as well as the stage of seed development. The quantification of canola tannins by chemical methods has already been discussed by Naczk et al. (1994) and is represented by selected data in Table 1. This should facilitate the discussion of the results of the present study.

A number of methods are available for the determination of the protein precipitating capacity of tannins (Makkar, 1989). Of these methods, the dye-labeled BSA assay developed by Asquith and Butler (1985) and the protein precipitation assay developed by Hagerman and Butler (1978) were selected for the quantification of the protein-precipitating potential of tannins isolated from hulls of canola varieties. The dye-labeled BSA assay

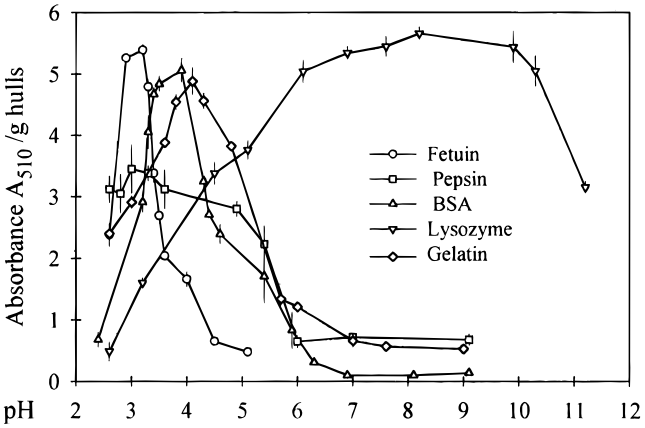

Figure 1. pH dependence of complex formation between canola tannins and several proteins as determined by the protein precipitation assay of Hagerman and Butler (1978). Tannins were extracted from hulls of Westar canola (sample 1).

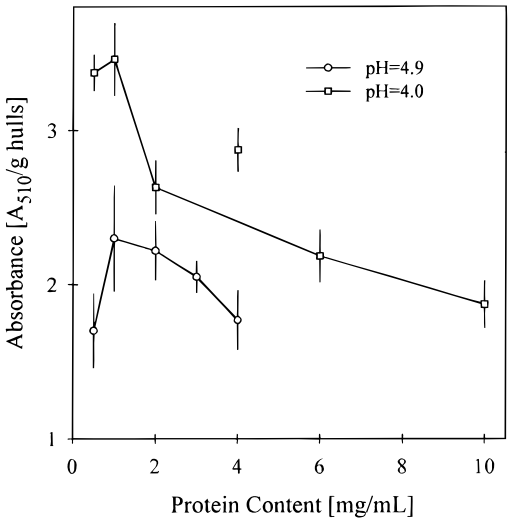

Figure 2. Formation of insoluble tannin−protein complexes as a function of BSA concentration using the protein precipitation assay of Hagerman and Butler (1978). Tannins were isolated from hulls of Cyclone canola (sample 4).

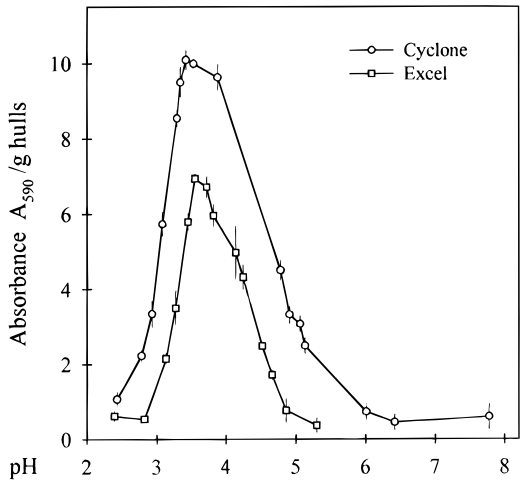

Figure 3. pH dependence of insoluble complex formation between canola tannins and dye-labeled BSA as determined using the dye-labeled BSA assay of Asquith and Butler (1985).

allows for the direct measurement of the amount of protein precipitated by tannins, while the protein precipitation assay allows for the estimation of the amount of precipitated, protein-bound polyphenols.

Van Buren and Robinson (1969) and Hagerman and Butler (1981) have reported that the pH optimum for the precipitation of a protein by polyphenols occurs near its isoelectric point. Accordingly, the effect of pH on the protein precipitating capacity of canola tannins for selected proteins was evaluated to determine the difference between the isoelectric point of a protein and the optimum pH for precipitation of that protein. The optimum pH is defined as the pH at which the maximum precipitation of a tannin−protein complex occurs. The proteins tested in this experiment were BSA, fetuin, gelatin, lysozyme, and pepsin. Figure 1 shows the effect of pH on the precipitation of these proteins by canola tannins as determined by the protein precipitation assay. The crude tannin extracts used in this experiment were isolated from the high-tannin sample of Westar hulls (Table 1; sample 1). BSA, fetuin, gelatin, and pepsin were effectively precipitated at pH values between 3.0 and 5.0. On the other hand, the optimum pH for precipitation of lysozyme was >8.0. These results indicate, therefore, that the precipitation of protein by canola tannins depends not only on the availability of unionized phenolic groups for hydrogen bonding but also on the kind of protein present in the reaction mixture. The pH optimum for the precipitation of BSA, pepsin, gelatin, fetuin, and lysozyme by canola tannins was found to be 0.3−3.1 pH units below the isoelectric points of the proteins. Hagerman and Butler (1978) reported a similar effect of pH on the formation of tannin−protein complexes by condensed tannins isolated from sorghum grains.

Figure 2 shows the formation of BSA−canola tannin complexes at pH 4.0 and 4.9 as a function of protein concentration. The crude tannin extracts used in this study contained 0.7 mg of tannin/mL (Table 1; Cyclone hulls, sample 4). The data indicate that at protein contents <1 mg/mL there may be an excess of tannin in the reaction mixtures. However, at concentrations >1 mg/mL less polyphenols were precipitated. Hagerman and Robbins (1987) reported a similar effect of protein concentration on the formation of tannin−protein complexes at pH 4.9 by tannins isolated from sorghum grains. According to Calderon et al. (1968), McManus et al. (1981), and Hagerman and Robbins (1987), the presence of excess protein leads to the formation of soluble tannin−protein complexes. Moreover, the results of the present experiments clearly illustrated that, in the case of canola tannins, pH 4.0 was optimum for carrying out the protein precipitation assay of Hagerman and Butler (1978) and not pH 4.9, as indicated by the authors for sorghum tannins. This pH value is in good agreement with that reported by McManus et al. (1981), who also found that pH 4.0 was optimum for the association of BSA with polyphenols.

The results of the protein precipitation assay carried out at pH 4.0 were subjected to a statistical analysis using the *t*-test. No statistically significant difference was found between the results obtained for 0.5 mg of BSA/mL and those for 1.0 mg of BSA/mL. However, the results obtained at 1 mg of BSA/mL were significantly different ($p \leq 0.03$) from those obtained at higher BSA concentrations.

Figure 3 shows the effect of pH on the formation of dye-labeled BSA−canola tannin complexes. The crude tannins used in this experiment were extracted from hulls of Excel (Table 1; low tannin) and Cyclone (Table 1; sample 1; high tannin) canola varieties. The data indicate that the maximum amount of dye-labeled BSA was precipitated at pH 3.5. Asquith and Butler (1985) proposed pH 4.8 for carrying out the dye-labeled BSA analysis using sorghum tannins. Table 2 shows the

Table 2. Effect of pH on the Precipitation of Dye-Labeled BSA by Canola Tannins[a]

canola variety	pH 4.8	pH 3.5
Westar		
sample 1	26.6 ± 1.1	73.5 ± 2.3
sample 2	4.0 ± 0.5	38.9 ± 1.6
sample 3	4.0 ± 0.5	29.8 ± 1.7
sample 4	5.1 ± 0.8	33.0 ± 2.2
Cyclone		
sample 1	27.2 ± 1.2	65.0 ± 1.2
sample 2	28.3 ± 1.5	55.7 ± 2.1
sample 3	33.4 ± 0.8	65.8 ± 1.5
sample 4	16.2 ± 1.2	42.6 ± 1.7
sample 5	42.3 ± 2.2	63.7 ± 0.9
Excel	6.1 ± 1.2	33.2 ± 0.5

[a] Percent of total BSA precipitated.

amounts of dye-labeled BSA precipitated at pH 3.5 and 4.8. In this experiment, the effect of these pHs on the precipitation of canola tannin−dye-labeled BSA complexes was illustrated using the crude tannin extracts obtained from selected samples of low- and high-tannin hulls of Cyclone, Westar, and Excel canola varieties. Note that the data presented in Table 2 indicate the following: (1) that there is a greater difference detected between the protein precipitating capacity for tannins isolated from high- and low-tannin canola hulls at pH 4.8 than at pH 3.5. Note however, that at pH 4.8, the protein precipitating capacity for samples of low-tannin canola hulls does not yield statistically significant differences (*t*-test) between the Westar and Excel canola varieties; (2) that from 3 times (for tannins isolated from high-tannin hulls) to up to 10 times (for tannins isolated from low-tannin hulls) more dye-labeled BSA was precipitated at pH 3.5 than at pH 4.8. Thus, the dye-labeled BSA assay, when carried out at pH 3.5, gives information about the maximum protein precipitating capacity of crude canola extracts. Accordingly, in the case of canola tannins, pH 3.5 was selected as the optimum for carrying out the dye-labeled assay of Asquith and Butler (1985), and not pH 4.8 as indicated by these authors for sorghum tannins. Moreover, the results of dye-labeled BSA (Figure 3) confirmed our previous findings (Naczk et al., 1994) that tannins isolated from low-tannin canola hulls (Excel; Table 1) showed greater affinity for dye-labeled BSA than those from high-tannin hulls (Cyclone, sample 1; Table 1). Porter and Woodruffe (1984) demonstrated that the ability of condensed tannins to precipitate proteins depends upon the molecular weights of the tannins. Accordingly, the differences in the tannin affinities for the dye-labeled BSA, found in this study, may originate in differences in molecular weight of tannins isolated from high- and low-tannin samples of canola hulls. Figure 4 shows the formation of dye-labeled BSA−canola tannin complexes at pH 3.5 as a function of protein concentration in the system. The crude tannin extracts used in this experiment contained 0.7 mg of tannin/mL (Table 1; Cyclone hulls, sample 4). The amount of dye-labeled BSA precipitated by tannins was expressed both in milligrams of dye-labeled BSA precipitated per gram of hulls and as percentage of the total proteins present in the system. At 0.5 mg of protein/mL, about 80% of the total protein in the system was precipitated by canola tannins. However, only about 20% of the total protein in the system was precipitated when the concentration of dye-labeled BSA was increased to 10 mg/mL. The data also indicate that a maximum of 9 mg of dye-labeled BSA was precipitated per assay at the concentration range of tannins used in this experiment.

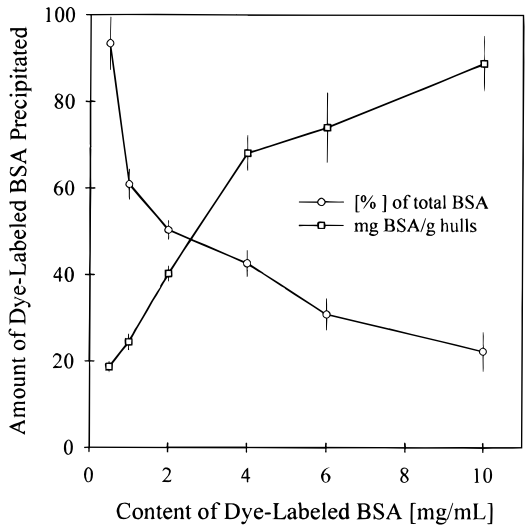

Figure 4. Formation of insoluble tannin−protein complexes as a function of the content of dye-labeled BSA as determined using the dye-labeled BSA assay of Asquith and Butler (1985). Tannins were isolated from hulls of Cyclone canola (sample 4).

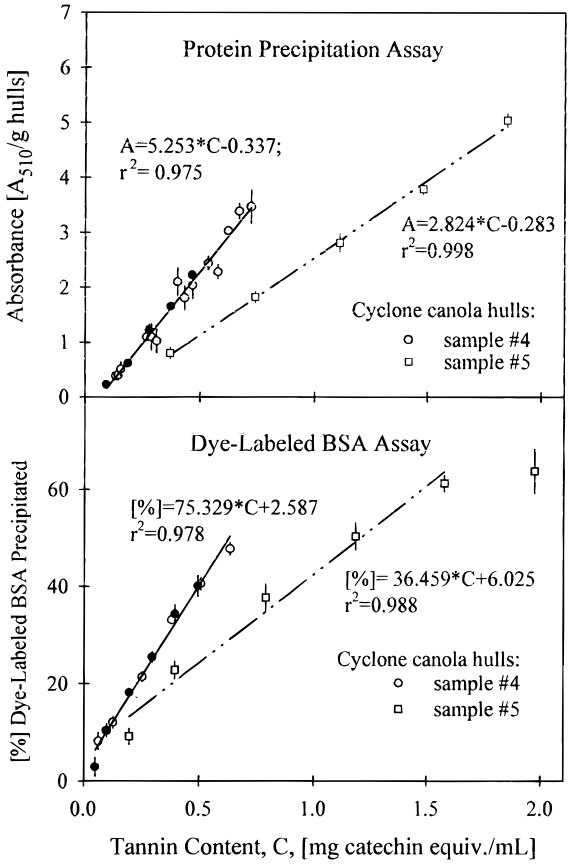

Figure 5. Titration curves of a known amount of protein with increasing amounts of canola tannins. Tannins were isolated from high- and low-tannin samples of Cyclone canola hulls. Dark symbols indicate partially purified tannins; open symbols indicate crude tannins.

Figure 5 shows the titration curves of a known amount of protein (1 mg/mL for the protein precipitation assay and 2 mg/mL for the dye-labeled BSA assay) with increasing quantities of canola tannins. The crude tannin extracts used in this experiment were isolated from high- (sample 5) and low-tannin (sample 4) hulls of Cyclone canola variety (Table 1). Selected crude tannin extracts (Cyclone, sample 4) were partially purified by extraction of their aqueous solutions with

ethyl acetate as described by Hagerman and Butler (1980) to remove low molecular weight phenolics. The aqueous tannin solutions, after extraction with ethyl acetate, were evaporated almost to dryness and dissolved in methanol. The crude tannin extracts (sample 4) contained approximately 20% of proanthocyanidins which were soluble in ethyl acetate, as determined by the modified vanillin assay of Price et al. (1978). According to Porter (1989), only monomeric and dimeric proanthocyanidins are highly soluble in ethyl acetate. No such attempts were made in this study to identify the phenolics soluble in ethyl acetate. The data presented in Figure 5 indicate that canola tannins did not show any threshold prior to binding labeled BSA but did show a definitive threshold prior to binding unlabeled BSA. For up to 0.8 mg of tannins/mL for tannin extracts from low-tannin hulls, and up to 1.5 mg of tannins/mL for tannin extracts from high-tannin hulls, a statistically significant ($p = 0.0001$) linear relationship was found between the amount of tannin−protein complex formed and the amount of tannin added to the reaction mixture. The titration curves for the dye-labeled BSA assay carried out for tannin extracts obtained from high-tannin Cyclone hulls, however, showed a saturation effect at higher concentrations of tannins. In this study, a maximum of 63.7% of dye-labeled BSA was precipitated in the presence of about 2.0 mg of canola tannins/mL. The observed differences between the titration curves obtained for crude tannins isolated from low- and high-tannin hulls may be due to differences in their affinities for proteins, as reported by Naczk et al. (1994). These authors found that the protein precipitating capacity of canola tannins isolated from high-tannin hulls did not exceed 5 mg of BSA/mg of tannins, while for tannins isolated from low-tannin hulls, this capacity ranged from 17.7 to 40.7 mg of BSA/mg of tannins. The data, presented in Figure 5, also indicate that ethyl acetate-soluble proanthocyanidins contribute to the protein precipitating capacity of crude canola tannins isolated from low-tannin hulls. It has been demonstrated (Bate-Smith, 1973; Artz et al., 1987) that tannins should have at least three flavonol subunits to be an effective protein precipitating agent: dimers were found to be less effective in precipitating tannins and monomers did not precipitate phenolics at all. No such attempts were made to determine the protein precipitating potential of canola hulls phenolics soluble in ethyl acetate.

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