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Protein precipitating capacity of condensed tannins of beach pea, canola hulls, evening primrose and faba bean

M. Naczk [a,*], R. Amarowicz [b], R. Zadernowski [c], F. Shahidi [d]

[a] *Department of Human Nutrition, St. Francis Xavier University, Antigonish, PO Box 5000, NS, Canada, B2G 2W5*
[b] *Division of Food Science, Institute of Animal Reproduction and Food Research of Polish Academy of Sciences, Olsztyn, Poland*
[c] *Faculty of Food Science, Warminsko-Mazurski University, Olsztyn, Poland*
[d] *Department of Biochemistry, Memorial University of Newfoundland, St. John's, NF, Canada, A1B 3X9*

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Abstract

Condensed tannins (CT) from beach pea, Cyclone canola hulls, evening primrose and faba bean were extracted into 70% (v/v) aqueous acetone. The lyophilized extracts were then purified on a Sephadex LH-20 column using first 95% ethanol as a mobile phase for elution of non-tannin phenolics and then 50% aqueous acetone to elute CT. Condensed tannins isolated from beach pea possessed shorter polymer chains than those isolated from canola hulls, evening primrose or faba bean. Bovine serum albumin (BSA) was effectively precipitated by beach pea CT at pH values between 3.5 and 5.0. CT of canola hulls, evening primrose and faba bean precipitated BSA at pH 4.0–5.0. A statistically significant ($P = 0.0001$) linear relationship existed between the amount of tannin-protein complex formed and the amount of CT added to the reaction mixture. The slopes of these lines indicated that evening primrose CT were the most effective protein precipitants, followed by canola hulls, faba bean and beach pea CT. Based on the amount of gelatin and BSA required to inhibit 50% of dye-labelled BSA-CT complex precipitation, gelatin was 10 times more effective as a precipitation inhibitor than unlabelled BSA.

Keywords: Condensed tannins; Tannin-protein interactions; Beach pea; Canola hulls; Evening primrose; Faba bean; Effect of pH; Protein precipitating capacity

1. Introduction

Tannins are complex polyphenolic compounds with molecular weights in the range of 500–20,000 Daltons. They are widely distributed in foods and feeds of plant origin (Shahidi & Naczk, 1995). Tannins may form soluble and insoluble complexes with proteins which may be responsible for the antinutritional effects of tannin-containing feed ingredients for non-ruminants (Martin-Tanguy, Guillaume & Kossa, 1977) and ruminants (Kumar & Singh, 1984).

The formation of tannin-protein complexes depends not only on the size, conformation and charge of the protein molecules, but is also affected by the size, length and flexibility of tannins involved (Shahidi & Naczk, 1995). Proteins with compact globular structures, such as ribonuclease, lysozyme or cytochrome C, exhibit low affinity for tannins, whereas conformationally open proteins such as gelatin or polyproline readily form complexes with tannins (Hagerman & Butler, 1981). It has also been demonstrated that tannins should possess at least three flavanol subunits to be effective protein-precipitating agents. Dimers are less effective precipitating agents, but simple flavanols do not precipitate proteins at all (Artz, Bishop, Dunker, Schanus & Swanson, 1987; Bate-Smith, 1973).

The precipitation of a tannin-protein complex is due to the formation of sufficient hydrophobic surface on the complex (McManus, Davis, Lilley & Haslam, 1981). At low concentration of proteins, the precipitation is due to the formation of a hydrophobic monolayer of polyphenols on the protein surface. However, at higher concentrations of proteins, a hydrophobic surface results from the combination of complexing of polyphenols on the protein surface and crosslinking of

* Corresponding author. Tel.: +1-902-867-2205; fax: +1-902-867-2389.
E-mail address: mnaczk@stfx.ca (M. Naczk).

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different protein molecules with polyphenols (Shahidi & Naczk, 1995).

Hagerman and Butler (1978) reported that the precipitation of tannin-protein complex was pH-sensitive. The lowest solubility of tannin-protein complex occurred at 0.3–3.1 pH units below the isoelectric point of the proteins. Bovine serum albumin (BSA), fetuin, collagen and pepsin were precipitated at pH values between 3.0 and 5.0, but the maximum precipitation of lysozyme occurred at pH > 8.0 (Naczk, Oickle, Pink & Shahidi, 1996).

The objective of this study was to determine the relative affinity of condensed tannins (CT) isolated from beach pea, Cyclone canola hulls, evening primrose and faba bean for selected proteins. Furthermore, the effect of pH and CT concentration on the protein-precipitating capacity of isolated CT was investigated using protein precipitation methods commonly employed for quantification of condensed tannins.

2. Materials and methods

Evening primrose (*Oenothera biennis* L.) and faba bean (*Vicia faba*) seeds were obtained from the Institute of Horticulture of the Warminsko-Mazurski University in Olsztyn, Poland. Mature pods of beach pea were collected from Bellevue Beach in Newfoundland in September and October of 1997. The seeds and pod shells were separated manually (Chavan, Amarowicz & Shahidi, 1999). Clean seeds were then stored in airtight containers for further analysis. Ground seeds of beach pea, evening primrose and faba bean were extracted with hexane for 12 h using a Soxhlet apparatus and then dried at room temperature. Cyclone canola hulls were prepared according to 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.

Condensed tannins from beach pea, Cyclone canola hulls, evening primrose, and faba bean were extracted twice at room temperature into 70% (v/v) aqueous acetone using a Waring Blender (Waring Products Division, Dynamics Corporation of America, New Hartford, CT) for 2 min at maximum speed. The extracts were combined, evaporated to near dryness under vacuum at 40°C and lyophilized.

The crude CT extracts of beach pea, Cyclone canola hulls, evening primrose and faba bean were purified according to the method described by Strumeyer and Malin (1975). A sample (550 mg) of crude CT extract was suspended in 5 ml of 95% (v/v) ethanol and applied onto a chromatographic column (2.3×40 cm) packed with Sephadex LH-20 and equilibrated with 95% (v/v) ethanol. The column was exhaustively washed with 95% (v/v) ethanol at a flow rate of 60 ml/h and then eluted with 50% (v/v) acetone at a flow rate of 60 ml/h. Acetone eluates were combined, the solvent was removed under vacuum at <40°C, then the purified CT were lyophilized and the residue was weighed.

The content of CT in extracts was determined using the modified vanillin assay (Price, van Scoyoc & Butler, 1978) and the proanthocyanidin assay (Naczk, Nichols, Pink & Sosulski, 1994); results were expressed as absorbance units per 1 g extract (A_{500}/g) and (A_{550}/g), respectively.

The effect of purified CT concentrations on the formation of CT-protein complex was assayed by the protein precipitation method of Hagerman and Butler (1978; at 1mg BSA per ml) and by the dye-labelled BSA assay of Asquith and Butler (1985; at 2 mg dye-labelled BSA per ml) with pH modifications as described by Naczk, Oickle, Pink, and Shahidi (1996). A series of methanolic solutions of purified CT extracts (0.1–2.0 mg/ml) were prepared. The effect of pH on the formation of tannin-protein complexes was monitored as described by Naczk, Oickle, Pink, and Shahidi (1996).

Relative affinities of beach pea, canola hulls, evening primrose and faba bean CT for BSA and gelatin were measured according to the procedure described by Asquith and Butler (1985) with the following modification. One millilitre of dye-labelled BSA solution, containing 2 mg of dye-labeled BSA instead of 1 mg recommended by the authors, was mixed with 0.6 ml of a solution containing 100–6000 μg of protein competitors (BSA or gelatin). To this mixture was added 0.4 ml of methanol containing 0.4 mg of CT extract. The relative affinity was calculated as the ratio between the weight of dye-labelled BSA present and the weight of the competitor which prevented 50% of dye-labelled BSA from precipitating.

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

3. Results and discussion

CT isolated from plant materials are mixtures of polymeric compounds that differ in their sensitivity towards the reagents used for their determination. The longer the polymer chain, the more anthocyanidin pigment is formed (Scalbert, 1992); on the other hand, less of the red condensation product of vanillin reaction is formed due to an increased steric hindrance towards the vanillin reagent (Mole & Waterman, 1987). It has been suggested by Goldstein and Swain (1963) that the ratio between the A_1^1 (the absorbance of a 1% solution of substance in a 1.0-cm cell) of proanthocyanidin (PrA) and A_1^1 of vanillin assay (V) is related to the length of CT chains. Table 1 shows the contents of CT expressed in absorbance (A)

equivalents per mg of extract (A/g) as determined by the vanillin and proanthocyanidin assays. These data suggest that CT isolated from beach pea possessed shorter polymer chains than those isolated from canola hulls, evening primrose or faba bean. Thus, more detailed analysis is required to determine the polymer chain lengths of CT from different sources used in this study.

A number of methods are available for the determination of protein-precipitating capacity of CT (Makkar, 1989). Of these methods, the dye-labelled BSA assay developed by Asquith and Butler (1985) and the protein precipitation assay developed by Hagerman and Butler (1978) were selected for quantification of protein-precipitating potential of CT isolated from beach pea, canola hulls, evening primrose and faba bean. The dye-labelled BSA assay allows for direct measurement of the amount of protein precipitated by CT, while the protein-precipitation assay allows for the estimation of the amount of precipitated protein-bound CT.

The effect of pH on the protein precipitating capacity of CT isolated from beach pea, Cyclone canola hulls, evening primrose or faba bean was evaluated in order to determine the optimum pH for precipitation of BSA by the CT examined. The optimum pH is defined as the pH at which the maximum precipitation of a tannin-protein complex occurs. Fig. 1 shows the effect of pH on the precipitation of BSA by CT, as determined by the protein precipitation assay. BSA was effectively precipitated by beach pea CT at pH values between 3.5 and 5.0, but the pH values were 4.0–5.0 for CT of canola hulls, evening primrose and faba bean (Fig. 1). A similar effect of pH on the formation of CT-BSA complexes was reported by Hagerman and Butler (1978) for CT isolated from sorghum grains and by Naczk, Oickle, Pink and Shahidi (1996) for crude extracts of canola hulls CT.

Figs. 2 and 3 show the curves (here referred to as titration curves) depicting the amounts of proteins or tannins precipitated as a CT-protein complex with increasing quantities of CT added to a solution containing a known amount of protein (1 /ml for the protein precipitation assay and 2 mg/ml for the dye-labelled BSA assay). A statistically significant ($P = 0.0001$) linear relationship existed between the amount of CT-protein complex formed and the amount of CT added to the reaction mixture for up to 0.5 mg CT/ml for tannin extract from evening primrose, up to 1.5 mg CT/ml for tannin extracts from canola hulls and faba bean, and up to 3.0 mg CT/ml for CT extract from beach pea. The intercept values of linear relationships presented in Figs. 2 and 3 indicate that beach pea, canola hulls, evening

Table 1
Content of condensed tannins in beach pea, canola hulls, evening primrose and faba bean extract fractionated on a Sephadex LH-20 column and their relative affinities for proteins

Condensed tannins extracts	Tannin content		Relative affinity	
	$[A_{500}/g]$[a]	$[A_{550}/g]$[b]	BSA[c]	Gelatin
Beach pea	1694±50	3324±273	0.58	4.24
Cyclone canola hulls	980±34	2260±145	0.48	4.33
Evening primrose	394±4	961±108	0.45	4.06
Faba bean	815±10	2139±100	0.43	4.24

[a] As determined by the vanillin assay (Price, van Scoyoc & Butler, 1978; Naczk, Nichols, Pink & Sosulski, 1994).

[b] As determined by the proanthocyanidin assay (Mole & Waterman, 1987; Naczk, Nichols, Pink & Sosulski, 1994).

[c] Bovine serum albumin.

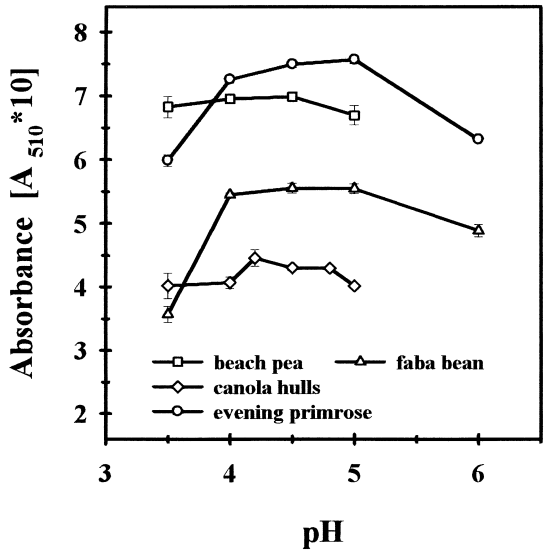

Fig. 1. The pH dependence of complex formation of condensed tannins of beach pea, canola hulls, Faba bean and evening primrose with bovine serum albumin determined by the protein precipitation assay (Hagerman & Butler, 1978).

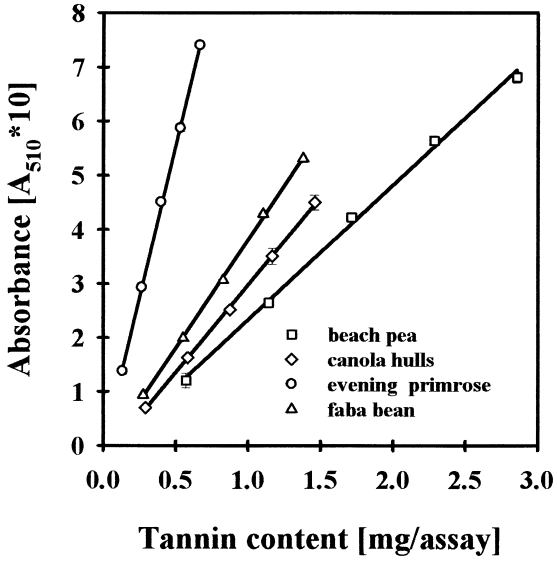

Fig. 2. Titration curves of a known amount of protein with increasing amounts of condensed tannins of beach pea, canola hulls, faba bean and evening primrose as determined by the protein precipitation assay (Hagerman & Butler, 1978).

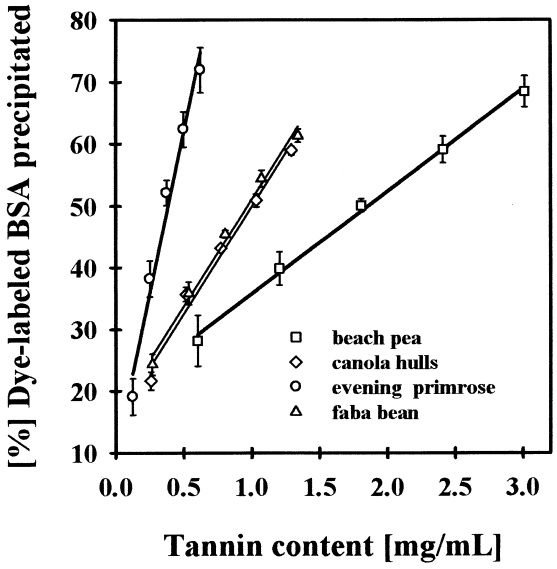

Fig. 3. Titration curves of a known amount of protein with increasing amounts of condensed tannins of beach pea, canola hulls, faba bean and evening primrose as determined by the dye-labelled bovine serum albumin assay (Asquith & Butler, 1987).

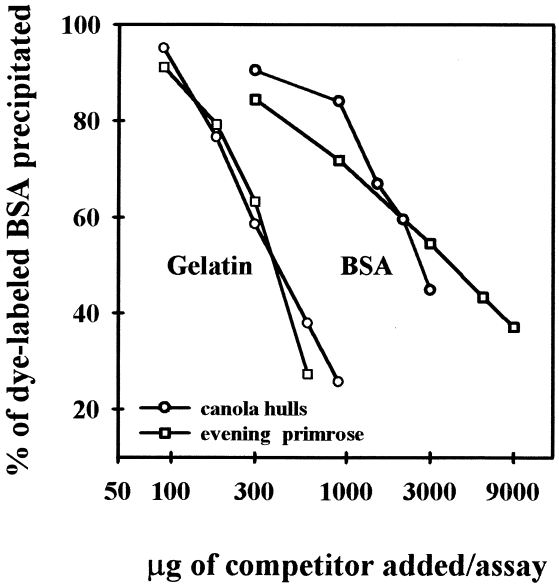

Fig. 4. Competition assays between dye-labeled bovine serum albumin (BSA) and standard proteins (BSA and gelatin) for condensed tannins of beach pea and evening primrose.

primrose and faba bean CT did not show any threshold prior to binding dye-labelled BSA (positive intercept values; Fig. 2), but did show a definitive threshold prior to binding unlabelled BSA (negative intercept values; Fig. 3). The numerical values of slopes of the titration curves obtained using the precipitation assay (Fig. 3) ranged from 2.49 to 11.3, and those of titration curves obtained using the dye-labelled BSA assay (Fig. 2) ranged from 19.2 to 104. The observed differences between the slopes of the titration curves obtained for CT isolated from beach pea, canola hulls, evening primrose or faba bean hulls may be due to differences in their affinities for proteins. Similar differences in the slopes were reported by Naczk, Nichols, Pink and Sosulski (1994) for the titration curves obtained for crude CT isolated from low- and high-tannin hulls of canola and rapeseed. According to Porter and Woodruffe (1984), the ability of CT to precipitate proteins depends on the molecular weights of the CT. Asquith and Butler (1985) have also suggested that precipitation of dye-labelled BSA was affected by the degree of CT polymerization. However, the chemical structures, polymer chain lengths, and molecular weights of beach pea, canola hulls, evening primrose and faba bean CT are still unknown and more detailed chemical analyses of these CT are still needed.

Fig. 4 shows the effect of the competitors (gelatin and BSA) on the precipitation of dye-labelled BSA by beach pea and evening primrose CT. Similar plots were reported by Hagerman and Butler (1981) and Asquith and Butler (1987) for sorghum tannins. The plots for gelatin and BSA are nonparallel. This, according to Creighton (1980), may be explained by co-operative binding between CT and protein or the heterogeneity of binding sites. Based on the amount of gelatin and BSA needed to inhibit 50% of dye-labelled BSA precipitation by CT, gelatin was 10 times more effective as an inhibitor than unlabelled BSA. The relative affinities of the tested CT for BSA and gelatin, calculated as the ratio between the weight of dye-labelled BSA present and the weight of competitor which prevented 50% of dye-labelled BSA from precipitating, are shown in Table 1. The relative affinities of the tested CT were similar to those reported by Asquith and Butler (1985) for sorghum and quebracho CT.

The results of our study indicate that CT isolated from evening primrose seeds were not only the most effective precipitants of dye-labelled BSA, but also showed the greatest affinity for unlabelled BSA. The optimum pH for precipitation of unlabelled BSA by beach pea, canola hulls, faba bean and evening primrose CT was in a pH range similar to that reported by Hagerman and Butler (1978) for sorghum CT and by Naczk, Oickle, Pink and Shahidi (1996) for crude canola hulls CT. The CT tested, exhibited a greater affinity for gelatin, with a conformational open structure, than that known for BSA with a compact globular structure.

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