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In Vitro Protein Degradation of 38 Sainfoin Accessions and Its Relationship to Tannin Content by Different Assays

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ABSTRACT: This study compared 38 sainfoin and 2 *Lotus* accessions to their respective tannin contents, N buffer solubility, and in vitro protein degradation. Tannin contents were measured by a protein precipitation method using either bovine serum albumin or Rubisco and by the colorimetric HCl/butanol method. Precipitation of bovine serum albumin and Rubisco was highly correlated (R^2 = 0.939). Correlations between the protein precipitation variants and the HCl/butanol method were relatively low (R^2 < 0.6). Protein degradation was measured at 4 h of incubation in an inhibited in vitro system and could not be explained by any of the tannin assays (R^2 < 0.03) and only partially by N buffer solubility (R^2 ≤ 0.433). Decisive factors other than the quantity of tannins or their ability to precipitate proteins must be considered. Resistance of soluble protein toward degradation can possibly be caused by tannin protein binding.

KEYWORDS: *Onobrychis viciifolia, Lotus corniculatus, esparsette, Rubisco, bovine serum albumin, radial diffusion assay*

INTRODUCTION

Tannins comprise a large group of secondary metabolites in plants that are largely defined by their ability to bind to protein. Both positive and negative effects on animal nutrition have been reported as a result of protein−tannin complex formation.[1−3] Rumen-escape protein, which can increase protein utilization in high-producing dairy cows, has been attributed to protein complexing with condensed tannins in leguminous species such as *Lotus corniculatus*[4]) or sainfoin (*Onobrychis viciifolia* SCOP.,[5−8]). Methods to measure tannins quantitatively and qualitatively have been summarized by Hagerman.[9] They include colorimetric, protein precipitation, isotopic labeling, and electrophoretic methods. These methods have been used to relate tannin quality and/or quantity to nutritional characteristics such as in vitro protein degradation in ruminal fluid,[10,11] proteolysis during ensiling,[4,12] ruminal and postruminal degradation,[13] or carbohydrate digestion.[14,15] Yet, the decisive factor for efficient protein tannin binding other than the mere presence of tannins remains unknown. Factors such as the mean degree of polymerization, interflavanol linkages, or hydroxylation pattern of tannin subunits could be important.

The colorimetric HCl/butanol method[16] and the radial diffusion assay (RDA)[17] are commonly used to measure condensed tannin. It has been suggested that this method could be used within a single species for breeding purposes because a lower variation in tannin structure can be expected and could therefore be applicable in breeding programs.

Actual biological activity is measured as precipitation of bovine serum albumin (BSA) in an agar gel by the RDA. However, it is questionable if a unique blood serum protein can be representative of plant proteins of different sizes and structures. McAllister et al.[14] compared BSA to ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco), the predominant protein in plant leaves, in a study on the protein-precipitating capacity of tannins in nine different forage legumes, among them sainfoin. They found correlation between protein precipitation capacity regardless of whether Rubisco or BSA was used in the tannin assay. To our knowledge, there is no information available on how well values from RDA using BSA or Rubisco correlate to in vitro ruminal protein degradation of different sainfoin accessions.

The main objective of this study was to (a) compare the ability of BSA and Rubisco to precipitate tannins in different sainfoin accessions by RDA and (b) examine the relationship between rumen in vitro protein digestibility of 38 different sainfoin and two *Lotus* accessions and their buffer-soluble N (BSN) and tannin contents as measured by the HCl/butanol method and the RDA.

MATERIALS AND METHODS

Plant Accessions. Thirty-eight sainfoin and two *Lotus* accessions,[18] grown at the National Institute of Agricultural Botany, Cambridge, U.K. (NIAB), were harvested in August 2008, frozen, and transported to the Kungsängen Research Center (Uppsala, Sweden).

Extraction of Rubisco. Rubisco was extracted from fresh spinach leaves according to an abbreviated protocol from Anderson et al.[19] as follows: 100 g of stemless leaves was homogenized at highest speed four times for 30 s with 100 mL of 50 mM degassed Tris buffer at pH 7.5 (4 °C) in a precooled standard household blender (Moulinex, type Q50, 400 W, Alençon, France). The buffer contained 0.1 M NaCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, and 10 mg of phenylsulfonyl fluoride (protease inhibitor). The pH was adjusted to 7.5 with 0.1 M NaOH, and the homogenate was filtered through four layers of

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Table 1. Total N, Buffer-Soluble N (BSN), Dry Matter (DM), Protein Precipitation Area (PPA) by the Radial Diffusion Assay (RDA) Using Rubisco or Bovine Serum Albumin (BSA), Absorption Units ($AU_{(550)}$ = Absorbance/5 g DM/L/cm Path Length) by the HCl/Butanol Method, and Fraction of Undegraded Sainfoin Protein after 1 ($FUD_{(1)}$) or 4 ($FUD_{(4)}$) h of Fermentation in Vitro

	NIAB accession code	DM (g/kg)	total N (g/kg DM)	BSN (g/kg N)	HCl/butanol $AU_{(550)}$	RDA in PPA (mm^2)		$FUD_{(1)}$	$FUD_{(4)}$
						Rubisco	BSA		
sainfoin varieties									
Cotswold Common-1	1001	247	22.7	172	0.938	120.7	72.6	0.842	0.689
Perly-1	1005	249	20.9	259	0.528	50.6	29.3	0.798	0.616
unspecified-1	1007	na	15.8	274	0.369	38.7	21.8	0.813	0.658
Ambra	1012	275	18.9	247	0.399	26.7	14.5	0.832	0.645
Somborne	1013	268	20.4	188	0.495	40.0	20.2	0.851	0.709
Teruel	1017	263	19.5	269	0.426	39.7	23.5	0.800	0.624
Taja	1019	215	17.6	156	0.342	34.2	18.3	0.771	0.684
Buciansky	1026	268	20.6	238	0.411	53.9	36.3	0.727	0.663
Simpro	1028	266	23.2	190	0.838	115.9	73.6	0.771	0.666
Bivolari	1043	256	23.4	175	0.801	88.7	46.0	0.799	0.663
Hampshire Common	1071	254	25.1	196	0.944	129.5	76.5	0.809	0.637
Nova	1077	265	17.4	259	0.364	36.0	20.5	0.802	0.637
Korunga	1103	282	18.4	280	0.338	48.1	28.2	0.802	0.621
unspecified-2	1104	259	20.6	271	0.330	36.8	19.8	0.811	0.608
CPI 63750	1110	260	20.4	335	0.374	51.6	29.3	0.764	0.589
CPI 63753	1113	271	19.3	224	0.357	6.8	20.2	0.815	0.635
CPI 63758	1118	278	22.0	254	0.567	27.0	14.2	0.811	0.570
CPI 63767	1127	270	21.2	291	0.337	27.6	18.6	0.837	0.618
Dukorastushchii	1156	250	21.9	247	0.408	38.8	25.5	0.830	0.646
Miatiletka	1157	232	26.1	157	0.835	107.2	68.8	0.843	0.675
Giant	1163	275	21.1	249	0.453	56.6	30.2	0.801	0.605
Rees A	1165	271	21.6	270	0.947	141.0	93.4	0.796	0.609
CPI 63810	1169	282	26.6	226	0.872	119.0	70.7	0.814	0.612
CPI 63820	1179	234	28.9	239	0.837	59.5	31.2	0.813	0.617
CPI 63838	1197	227	21.6	263	0.421	60.1	37.0	0.791	0.618
CPI 63840	1199	259	21.1	300	0.347	29.5	24.8	0.780	0.574
CPI 63841	1200	275	22.4	216	0.855	85.5	53.5	0.809	0.550
Premier	1210	242	25.3	214	0.833	104.2	64.5	0.806	0.545
Perly-2	1211	245	22.3	243	0.471	60.5	43.1	0.799	0.515
CPI 63854	1213	235	24.5	231	0.626	60.9	39.5	0.795	0.624
247	1220	270	21.8	253	0.651	65.2	50.9	0.806	0.646
Visnovsky	1230	260	22.6	170	0.854	76.0	55.4	0.827	0.693
Tu86-43-03-1	1253	260	23.4	218	0.360	38.2	26.0	0.801	0.637
Wkt 10	1256	260	23.1	216	0.489	51.1	33.7	0.811	0.661
X93234	1260	na	19.7	102	0.602	70.0	56.6	0.869	0.773
line 107	1261	244	24.5	170	0.839	57.9	32.8	0.815	0.642
Cotswold Common-2	1262	257	24.8	175	1.038	102.0	64.1	0.821	0.700
Sisiani Local	1264	255	21.6	301	0.318	31.2	20.6	0.778	0.581
Lotus varieties									
Grassland Goldie	1293	221	25.8	194	1.172	89.9	52.9	0.808	0.646
Grassland Maku	1297	202	31.7	184	2.154	103.5	81.9	0.807	0.711

cheesecloth and centrifuged for 45 min at 18000*g* at 4 °C on a table-top centrifuge (Hermle, Z 36 HK, Gosheim, Germany). The supernatant was decanted and salted out slowly with ammonium sulfate to a saturation of 300 g/L. The pellet was discarded. The solution was centrifuged for 30 min at 18000*g* at 4 °C and the supernatant decanted. The pellet was discarded. Ammonium sulfate was added to the supernatant to reach 500 g/L saturation, and the solution was again centrifuged for 30 min at 18000*g* at 4 °C. The supernatant was discarded, and the white precipitate was redissolved in 10 mL of Tris buffer (without protease inhibitor). The protein solution was applied to a cooled Sephadex G-25 column (60 × 8 cm), which was equilibrated three times with Tris buffer (4 °C). Rubisco was detected at 280 nm and confirmed by gel electrophoresis (Agilent 2100 Bioanalyzer, Santa Clara, CA, USA). Protein content was measured according to the Kjeldahl procedure.

Chemical Analysis. Dry matter was determined in duplicate by drying at 105 °C to constant weight in a forced draft oven. Nitrogen analyses were done in duplicate on the freeze-dried material by a Kjeldahl procedure using a Kjeltec Analyzer 2400 and a 2020 Digestor (Foss, Hillrød, Denmark) with Cu as a catalyst. BSN was determined

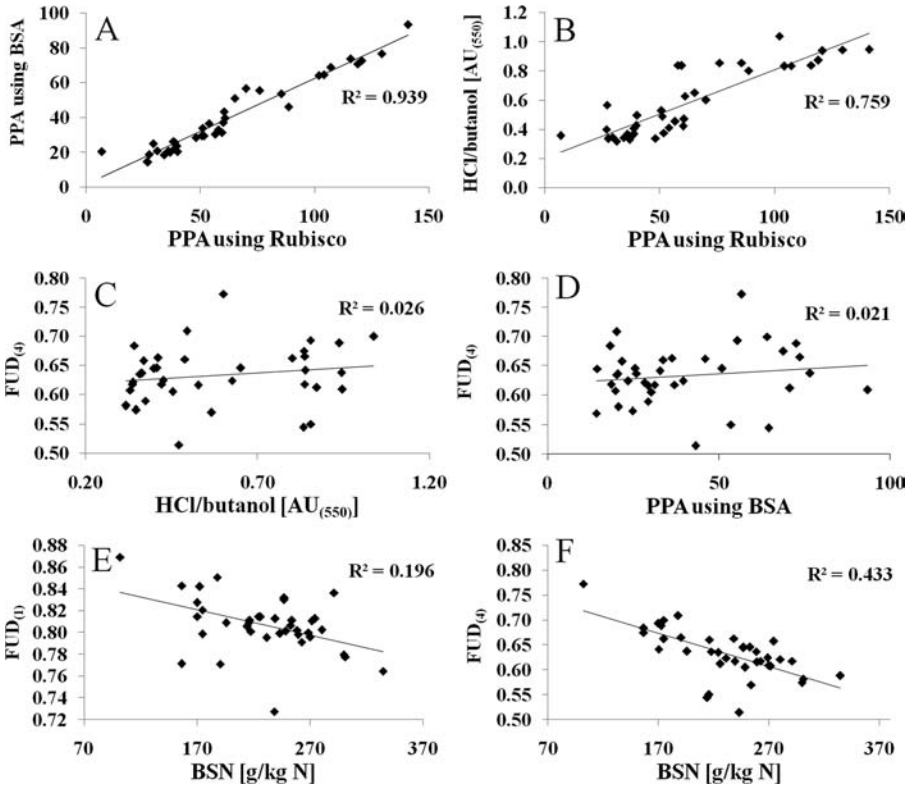

Figure 1. Regressions between protein precipitation area (PPA; mm^2) using Rubisco or bovine serum albumin (BSA), HCl/butanol response, in vitro undegraded protein fraction at 1 h ($FUD_{(1)}$) and at 4 h ($FUD_{(4)}$), and buffer-soluble N (BSN) content: (A) PPA with Rubisco vs BSA; (B) PPA with Rubisco vs HCl/butanol of sainfoin and *Lotus*; (C) HCl/butanol vs $FUD_{(4)}$; (D) PPA using BSA vs$FUD_{(4)}$; (E) BSN vs $FUD_{(1)}$; (F) BSN vs $FUD_{(4)}$.

by extracting freeze-dried samples with a borate phosphate buffer, pH 6.75, at 39 °C for 1 h according to a modified method by Licitra et al.[20] described in Lorenz et al.[5] Ammonia N was analyzed according to the method of Broderick and Kang[21] on an Auto Analyzer (Technicon, Dublin, Ireland) using ammonium sulfate (Merck, Darmstadt, Germany) as a standard. α-Amino acid N (AA-N) was analyzed according to a method by SEAL Analytical.[22]

Tannin Measurement Methods. Tannins were measured by an HCl/butanol method as follows. Freeze-dried samples (50 mg in triplicate) were weighed into 30 mL polypropylene test tubes. Five milliliters of HCl/butanol reagent (concentration of HCl/*n*-butanol = 1:20, v/v) were added to each sample and incubated in a water bath set at 100 °C for 60 min. Tubes were cooled to 20 °Cn and absorbance was read on a spectrophotometer (Pharmacia LKB, Uppsala, Sweden) at 550 nm in standard cuvettes (1 cm internal width). Tannin values were averaged and reported on the basis of absorbance units ($AU_{(550)}$ = absorbance/5 g DM/L/cm path length).

Protein precipitation capacity by RDA and using BSA was analyzed according to the method of Hagerman and Robbins[23] and for Rubisco from the adapted method by Giner-Chavez et al.[24] All tannin analyses were done in quadruplicates, and values were averaged. Values were expressed as protein precipitation area (PPA) in mm^2.

Inhibited in Vitro Protein Degradation Measurements. Protein degradation was measured in duplicate by the inhibited in vitro (IIV) method from Broderick.[25] An equivalent of 4 mg of N of freeze-dried plant material was weighed in a 30 mL polypropylene centrifuge tube. The material was wetted for 60 min at 20 °C in 10 mL of McDougal's buffer. Ruminal fluid was collected from two dry and fistulated cows after morning feeding, pooled, and strained through four layers of cheesecloth. Cows were fed a standard diet daily containing 3.4 kg DM grass hay with a crude protein content of 155 g/kg DM and 1.6 kg concentrates (CP = 200 g/kg DM). Strained ruminal fluid was preincubated with 8 g/L maltose, 4 g/L soluble starch, 4 g/L sucrose, 4 g/L xylose, 4 g/L pectin, and 5 g/L $NaHCO_3$ in a 39 °C water bath for 4 h during constant stirring. After preincubation, the pH was adjusted to 6.7 with 3 M NaOH. Bacterial growth was inhibited by adding 41.67 mL of hydrazine sulfate solution (72 mM), 41.67 mL of chloramphenicol solution (10 mM), and 0.234 g mercaptoethanol per liter of preincubated ruminal fluid. After an additional 30 min, 20 mL of ruminal fluid was added to the sample tubes and to four tubes (blanks) that contained only buffer. Final concentrations of hydrazine sulfate and chloramphenicol were 1.9 and 0.27 μM. Constant shaking and a temperature of 39 °C were maintained under anaerobic conditions by constant flushing with CO_2. Samples of 1 mL for NH_3-N and AA-N determination were collected every hour for 4 h and mixed with 0.1 mL of trichloroacetic acid solution (550 g/L) to stop fermentation. The degraded fraction after 1 h ($FD_{(1)}$) was equivalent to the content of NH_3 and AA-N from the plant sample plus the rapidly degraded N fractions after 1 h of incubation minus NH_3 and AA from blanks. The fraction of undegraded protein N at the respective time point *i* ($FUD_{(i)}$) was calculated as

$$FUD_{(i)} = 1 - FD_{(i)}$$

where $FD_{(i)} = (NH_3\text{-}N_{(i)} + AA\text{-}N_{(i)})/N_{(sample)}$. $N_{(sample)}$ = sample N; $NH_3\text{-}N_{(i)}$ and $AA\text{-}N_{(i)}$ are blank corrected with respective blanks.

Statistical Analysis. The IIV protein degradation data consisted of 40 (accessions) × 2 (incubation replicates) × 2 (analytical replicates) = 160 observations, which was averaged per accession (n = 40). Correlations of in vitro degradation values, tannin contents, and N fractions were performed with Minitab 16.1.0[26] and reported as the coefficient of determination (R^2).

RESULTS

DM, Total N, and BSN. DM, total N, and BSN are shown in Table 1. Total N of sainfoin was normally distributed with a mean of 21.9 and a standard deviation (SD) of 2.7 and ranged

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from 28.9 g/kg DM in CPI 63820 to 15.8 g/kg DM in the unspecified-1 sainfoin accession. BSN had a mean of 230 g/kg N (SD 48.8) and ranged from 335 g/kg N in sainfoin var. CPI 63750 to 102 g/kg N in sainfoin var. X93234.

Tannin Contents by HCl/Butanol and RDA. Tannin contents are shown in Table 1. Mean tannin content by HCl/butanol was 0.58 $AU_{(550)}$ (SD 0.23) and ranged from 0.32 in sainfoin var. Sisiani local to 1.04 in Cotswold Common-2. Mean tannin content by RDA, using Rubisco, was 62.8 (SD 33.5) and ranged from 6.8 for sainfoin var. CPI 63753 to 141.0 for sainfoin var. Rees A. Tannin precipitation with BSA had a mean PPA of 39.6 (SD 21.0) and ranged from 14.2 for sainfoin var. CPI 63758 to 93.4 for sainfoin var. Rees A. Precipitation of BSA was well correlated with that of Rubisco (R^2 = 0.939; Figure 1A). The regressions between HCl/butanol and RDA using Rubisco as the standard protein are shown in Figure 1B. R^2 was 0.759 and 0.713 for Rubisco and BSA (not shown), respectively. When *Lotus* was included in the calculation, R^2 decreased to 0.587 (Rubisco) and 0.521 (BSA).

IIV Protein Degradation. Proportions of undegraded protein after 4 h of degradation are shown in Table 1. Values for $FUD_{(1)}$ ranged from 0.727 for sainfoin Bukiansky to 0.869 for X93234 with a mean of 0.807 (SD 0.026). Values for $FUD_{(4)}$ ranged from 0.515 to 0.773, with the lowest value for sainfoin Perly-2 and the highest for X93234, respectively. Average $FUD_{(4)}$ for sainfoin was 0.633 (SD 0.049). There was only poor correlation between BSN and $FUD_{(1)}$ for sainfoin and *Lotus* (R^2 = 0.196; Figure 1E), but correlation increased to R^2 = 0.433 after 4 h of incubation ($FUD_{(4)}$; Figure 1F).

DISCUSSION

Condensed tannins are believed to have a potential for increasing animal productivity due to their anthelmintic properties and, in particular, an improved protein utilization by ruminants.[27,2,28] Predictions of nutritional effects of tannins require not only accurate and reproducible tannin assays but also biological relevance. The commonly applied colorimetric HCl/butanol method is widely used for measuring tannins in plants but suffers from drawbacks such as unsatisfactory reproducibility and varying color yield between different types of tannins. The protein-binding assays, on the other hand, are believed to yield more information on the affinity of tannins to proteins. In the traditional protein binding assay by Hagerman,[17] BSA is used as a standard protein. This has been questioned because the analogy of information about the reactivity of plant and animal proteins may not hold.[24] However, the comparison between these two proteins in our studies showed a high R^2 of 0.939. We therefore conclude that BSA and Rubisco have relatively similar abilities to precipitate sainfoin tannins. Extraction of plant protein may, therefore, not be needed to improve sainfoin tannin measurement by the RDA as suggested in earlier studies.[24] Both Rubisco and BSA are globular proteins with molecular weights of approximately 66 kDa for BSA and 14 and 51 kDa for the Rubisco subunits. It can be hypothesized that molecular weights of proteins do not affect protein−tannin binding, as has been suggested for the molecular weight of tannins.[29,30]

Variation in tannin values measured by both the HCl/butanol and the RDA were high. High variation of content and structure of sainfoin tannins was previously reported by Scharenberg et al.[31] and Gea et al.[32] A coefficient of determination of 0.759 between tannin quantification and protein precipitation is disconcerting. It is likely that the molecular structure of tannins is the main reason for the poor relationship between precipitation capacity and tannin content as suggested by Horigome[33] and Min et al.[34] The inclusion of the two *Lotus* samples decreased R^2 to 0.587. Earlier results by Stringano,[35] Meagher,[36] or Gea[32] shows that sainfoin and *Lotus pedunculatus* tannins are rich in prodelphinidins, whereas *L. corniculatus* tannins are rich in procyanidins, which could be the reason for lower correlation.

Furthermore, high PPA by *Lotus* tannin and their moderate absorption in the HCl/butanol methods support the hypothesis that structural differences between sainfoin and *Lotus*[37] have an impact on their binding behavior.

In vitro ruminal protein degradation is time-consuming and costly but has the potential to yield more realistic nutritional information than simple tannin protein precipitation by the RDA. Sainfoin in vitro protein degradation was poorly correlated to values from HCl/butanol and RDA (Figure 1C,D). The results suggest that even the extent of precipitation in the RDA is not a useful indicator of degradation in ruminal fluid.

BSN values were high compared to a previous study on different sainfoin varieties[5] and only poorly correlated to 1 h protein degradation by IIV (R^2 = 0.190; Figure 1E). In the Cornell Net Protein and Carbohydrate System (CNCPS[38]), the BSN fraction is considered to be rapidly degradable. However, BSN was not rapidly degradable in this study, possibly due to the formation of soluble tannin−protein complexes inaccessible by rumen bacteria. Even though correlation between BSN and FUD increased at 4 h of incubation (R^2 = 0.433; Figure 1F), the assumption of a rapid degradability of BSN in ruminal fluid may be erroneous at least for tanniniferous plants.

As a conclusion, it can be said that sainfoin tannin measurements by RDA using BSA or Rubisco were highly correlated, and despite differences in the molecular sizes of these globular proteins, binding to tannins did not seem to be affected. Correlation of in vitro protein degradation and tannin values was absent, which suggests that neither tannin assay is suitable for predicting tannin effects on protein degradation and that other factors besides the presence of condensed tannins should be investigated.

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ABBREVIATIONS USED

AA-N, α-amino-N; AU, absorbance units at 550 nm; BSA, bovine serum albumin; BSN, buffer-soluble nitrogen; DM, dry matter; $FUD_{(i)}$, fraction of undegraded protein at hour *i*; $FD_{(i)}$, fraction degraded at hour *i*; IIV, inhibited in vitro; NH_3, ammonia; PPA, protein precipitation area; RDA, radial

diffusion assay; Rubisco, ribulose-1,5-bisphosphate carboxylase oxygenase.

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