Nutritive Evaluation of Nitrogenous Fractions in Leaves of *Gliricidia sepium* and *Caliandra calothyrsus* in Relation to Tannin Content and Protein Degradation by Rumen Microbes *in Vitro*

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A study of two fodder tree species, *Gliricidia sepium* and *Caliandra calothyrsus*, was undertaken to investigate the degradation of leaf protein (crude and soluble protein) by rumen microbes during *in vitro* fermentation and the effect of total tannin content on that degradation. Differences in DMD and protein degradation were observed between the two leaf species. *C. calothyrsus*, after extraction with 70% acetone, showed greater DMD and crude protein losses, 40% and 20% greater, respectively. The cumulative gas production of *C. calothyrsus* was increased after extraction with 70% acetone. It was concluded that the differences in protein degradation characteristics were mainly due to the inhibitory effects of tannins. The apparent increased resistance of glycoproteins to degradation appeared to be of relatively minor importance to the nutritive value of the fodder.

Keywords: *Protein degradation; tree leaf; rumen culture; in vitro DMD; tannins; glycoproteins*

INTRODUCTION

Tree leaves are an important source of feed for ruminants in many less developed countries. They can be an important protein supplement when used in conjunction with roughages, which are generally protein deficient. Many fodder trees contain tannins, which have long been associated with negative effects on nutritive value (Kumar and Singh, 1984; Mangan, 1988; Leiner, 1990). Conversely, tannins in some feeds are advantageous to ruminants, as they can protect protein from degradation by micro-organisms in the rumen and make it available in the lower gut (Barry et al., 1986). Tannins in protein-rich feeds can also reduce the occurrence of bloat, by reducing the stability of protein foam in the rumen and improving the efficiency of protein utilization (Rumbaugh, 1985).

Relatively little work has been carried out on monitoring the degradation of polypeptides in the rumen. Nugent et al. (1983) demonstrated that feed protein incubated in rumen fluid could be analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). Spencer et al. (1988) monitored the breakdown of individual polypeptides during their incubation in synthetic fiber bags immersed in rumen fluid, *in vivo* and *in vitro*. It was shown that the total protein loss from a system does not provide evidence of the resistance of individual protein components, as they are shown to have widely variable resistance to degradation. It has been shown (Cherney et al., 1992), using SDS−PAGE, that buffer type can selectively affect the extraction of soluble proteins, which has implications for *in vitro* work, due to the possible selective extraction of a nonrepresentative soluble protein sample.

Recently, some detailed work (Tanner et al., 1994) has described the effects of isolated proanthocyanidins (condensed tannins), purified from forage legumes, on the degradation of leaf protein incubated with strained rumen fluid *in vitro*. The metabolism of ribulose biphosphate carboxylase (EC 4.1.1.39, Rubisco) was monitored by SDS−PAGE, and it was shown that the presence or absence of proanthocyanidins markedly affected the metabolism of this protein. Using SDS−PAGE (Aufrere et al., 1994), characterization studies of the *in situ* degradation of lucerne proteins have been carried out according to forage type (green forage, hay, and silage). Rubisco was found to be rapidly degraded over a period of 48 h.

The objective of this work was to investigate the factors that govern the *in vitro* protein degradation of the fodder tree legumes *Gliricidia sepium* and *Caliandra calothyrsus* by rumen microbes. *G. sepium* is generally considered to be one of the most digestible of the tropical leguminous forages (Glover, 1989). *C. calothyrsus* has attracted interest as a fodder legume, but it is not widely used as a fodder in its native geographical range due to its inferior quality. *C. calothyrsus* is considered to be native to the New World, from the southwestern United States and Central America to Argentina and Chile in the warmer parts of South America (Paterson, 1994). *G. sepium* is considered native to the Pacific coast of Mexico and Central America (Bennison and Paterson, 1993). An understanding of the degradation of tree leaf protein and how it may be affected by tannins will assist in the screening of germplasm and the selection of fodder trees of improved nutritive value.

MATERIALS AND METHODS

Forages. The study was conducted on two different forages, *G. sepium* and *C. calothyrsus*, which were grown in greenhouses using natural light and day length and watered once a day. The trees were approximately 24 ± 1 months old and were maintained as small trees/shrubs with a height of 1.5−2 m. Mature leaves and pinnae were collected and frozen at −70 °C in a CamLab TT80 freezer for 4 h prior to freeze-drying to constant weight using an Edwards Modulyo freeze drier. The dried leaf material was ground to a fine particle size (<1 mm) in a coffee grinder and stored in a desiccator.

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Fresh leaves of *G. sepium* and *C. calothyrsus* were found to have 229 and 365 g of dry matter (DM) kg^{-1} of fresh material, respectively.

Crude protein (N × 6.25) was measured by Kjeldahl digestion (AOAC, 1980) using sulfuric acid, and a selenium catalyst, 0.1 ± 0.001 g dry wt, was digested with 4 mL of concentrated H_2SO_4 and a catalyst tablet containing 1.5 g of K_2SO_4 and 0.0075 g of selenium. The samples were heated for 2 h at 200 °C followed by 4 h at 350 °C. This process measures organic and ammonium nitrogen; nitrates and nitrites are removed during the heating stages. The resulting solution, after cooling, was diluted to 50 mL, and a 1 mL aliquot was analyzed on a Burkard SFA2 continuous flow analyzer. The indophenol-blue method was used (Rowland, 1983) whereby ammonium nitrogen is oxidized by sodium hypochlorite and coupled with a phenolic compound (sodium salicylate) to produce the indophenol-blue color.

Extraction of Tannins. The method of Wood and Plumb (1995) was used to extract tannins, although modifications were made to achieve a higher solvent/solid ratio. Approximately 10 g dry wt of each of leaf species (freeze-dried material) was extracted by agitation in a sealed flask on an orbital shaker, with 250 mL of 70% acetone for 15 min. The residue was filtered on a Hartly funnel and returned to the flask. The residue was extracted three more times to achieve a solvent/solid ratio of 100:1. The residue was filtered a final time and dried to a constant weight in a vacuum desiccator. The two leaf species, *G. sepium* and *C. calothyrsus*, were found to have losses, on extraction with 70% acetone, of 359.6 and 367.5 g/kg of DM, respectively.

***In Vitro* Fermentation.** The fermentation conditions described by Theodorou et al. (1994) as outlined by Prasad et al. (1994) were used. This consisted of the anaerobic fermentation of dried substrates in a buffered medium, at 39 °C, in stoppered 125 mL serum bottles. Sixteen replicate bottles of *G. sepium* and *C. calothyrsus*, each containing 1 ± 0.001 g dry wt of leaf material, were prepared for destructive sampling, in duplicate, at each of eight sampling times. In addition, readings of the gas produced during fermentation were monitored by means of a pressure transducer. To these bottles were added 90 mL of nitrogen-free Menke medium and 4 mL of reducing agent. The reducing agent was prepared by combining 95 mL of distilled water, 4 mL of 1 M NaOH, and 625 mg of sodium sulfide. To the serum bottles was added 5 mL of rumen inoculum. The innoculum was prepared by straining through four layers of muslin fresh rumen fluid, obtained from two fistulated sheep after an overnight fast. The two fistulated ewes were previously fed on a diet of hay and concentrates. The undigested material was macerated with a volume of nitrogen-free Menke medium equal to the volume of the rumen liqour, which after straining through four layers of muslin was added to the rumen fluid. During this procedure the rumen fluid and buffers used were maintained under CO_2 to preserve an anaerobic atmosphere. Two bottles containing no leaf material were analyzed as no-substrate controls (NSC). Leaf residue and the liquid phase were collected at 0, 1, 3, 6, 9, 24, 48, and 70 h after inoculation with rumen fluid and treated as follows: The residue was washed with deionized water and dried to a constant weight over phosphorous pentoxide in a vacuum desiccator. To aliquots of the liquid phase (5 and 25 mL for *G. sepium* and *C. calothyrsus*, respectively) were added polyvinylpolypyrrolidone (PVPP) and ascorbic acid [1 and 0.1% (w/v), respectively], and the method for soluble protein analysis, described below, was followed.

Soluble Protein Extraction. To 0.25 g of dry leaf material (or fresh equivalent) were added 0.2 g of PVPP, 7.5 mL of nitrogen-free Menke medium (Menke et al., 1979), and 0.15 g of ascorbic acid. The resulting slurry was mixed continuously for 15 min prior to filtration through two layers of Miracloth (CalBiochem). The filtrate was centrifuged at 14000*g* for 15 min. The pellet was discarded and the supernatant desalted against 100 mM carbonate/bicarbonate buffer (pH 9.65) on a DG-10 desalting column (Bio-Rad). The eluate from this column was added to a DEAE-Sepharose anion exchange column (Pharmacia Biotech) and washed with a further 20 mL of carbonate/bicarbonate buffer. The column was eluted with 8 mL of 2 M NaCl. The eluate was dialyzed overnight against deionized water and subsequently desalted against deionized water using a DG-10 desalting column.

Soluble protein concentrations were measured at this point according to the method of Bradford (1976), using Bio-Rad protein assay reagent.

Electrophoresis. The extracted soluble proteins were analyzed by SDS−PAGE according to the method of Laemmli (1970). Twenty microliter aliquots of the soluble protein extracts, from the method described previously, were diluted with 5 μL of electrophoresis sample buffer, which contained 10% glycerol, 2% SDS, 0.5% 2-β-mercaptoethanol, and 0.005% bromophenol blue.

The gels consisted of a stacking gel containing 4% acrylamide/bis(acrylamide) layered over a resolving gel containing 12% acrylamide/bis(acrylamide). Electrophoresis was carried out for approximately 45 min at 200 V, prior to staining with 0.2% Coomassie brilliant blue R in 10% acetic acid (AcOH) and 30% methanol (MeOH). Gels were destained with two solutions, 10% AcOH/30% MeOH for 30 min and 10% AcOH/5% MeOH overnight.

Gels were analyzed using a Sharp JX325 scanner and Pharmacia Biotech, ImageMaster one-dimensional analysis software. Molecular weights were determined with BDH Molecular weight markers 12 300−78 000 and commercial standard (Sigma Chemicals) Rubisco (MW = 55 000 and 15 400).

Tannin Analysis. Condensed tannins were determined after extraction of the dried leaf material with 70% acetone according to the acid/butanol method of Porter et al. (1986). As there is no widely accepted standard for condensed tannin analysis, the results are reported as the optical density (OD) at 550 nm given by 1 g of dry wt leaf material.

Total polyphenols were determined again after extraction with aqueous acetone (70%) according to the Prussian Blue method of Price and Butler (1977).

Protein precipitating activity (PPA) was estimated using the method of Hagerman (1987) as modified by Wood et al. (1994). PPA values were calculated as tannic acid equivalents.

Glycoprotein Analysis. Glycoproteins in the soluble protein extracts were identified using a Glycotrack carbohydrate detection kit, K-050 (Oxford Glycosystems), and by employing the protocol provided with the kit (Oxford Glycosystems, 1995). Proteins from SDS−PAGE gels were blotted onto nitrocellulose membranes and oxidized with periodate. Aldehydes generated by this oxidation were reacted with biotin hydrazide, which leads to the incorporation of biotin into the carbohydrate. The biotinylated compound was detected by reaction with a streptavidin−alkaline phosphatase conjugate. Glycoproteins are detected by using a substrate, which reacts with the alkaline phosphatase to form a colored precipitate.

RESULTS

Total and Soluble Protein. Analysis of the two leaf species used in this study (Table 1) showed *G. sepium* to contain 40% more crude protein (Kjeldahl N × 6.25) than *C. calothyrsus*.

Despite the large differences in soluble protein, seen in Table 1, the protein profiles of the two leaf species are similar (Figures 1 and 2). It can be seen that the major protein in both leaf species corresponds to the Rubisco standard. The relative intensities of the minor bands also show a high degree of similarity. Freeze-drying had little effect on the protein profiles of the two species, although the total extractable soluble protein of the freeze-dried material was higher than that of the fresh material, by 50 and 10%, for *G. sepium* and *C. calothyrsus*, respectively.

Tannin Analysis. The large differences in the tannin content of the two leaf species can be seen from Table 1. *C. calothyrsus* had a high concentration of phenols. The polyphenols present in *C. calothyrsus* were also shown to have PPA equivalent to 44.59 ± 1.22

Table 1. Composition of the Leaf Materials Used in This Study: Total Protein, Soluble Protein, Total Phenols, Condensed Tannins, and Protein Precipitating Activity (PPA)

	G. sepium	*C. calothyrsus*
total protein: freeze-dried material, g/kg of DM	290	203
total protein: acetone-extracted material, g/kg of DM	302	299
soluble protein: fresh material, g/kg of DM[a]	24.76 ± 0.55	0.599 ± 0.001
soluble protein: freeze-dried material, g/kg of DM[a]	38.24 ± 3.04	0.678 ± 0.025
soluble protein: acetone-extracted tracted material, g/kg of DM[a]	0.147 ± 0.004	0.111 ± 0.004
total phenols, g of gallic acid equiv kg^{-1} [a]	1.31 ± 0.01	10.63 ± 0.31
condensed tannins, OD[b]/g of DM[a]	N/D[c]	358.2 ± 22.2
PPA, g of tannic acid equiv/kg of DM[a]	N/D	44.59 ± 1.22

[a] Results are mean values ± one standard deviation. [b] OD/g = optical density at 550 nm given by 1 g dry weight of leaf material. [c] N/D, not detected.

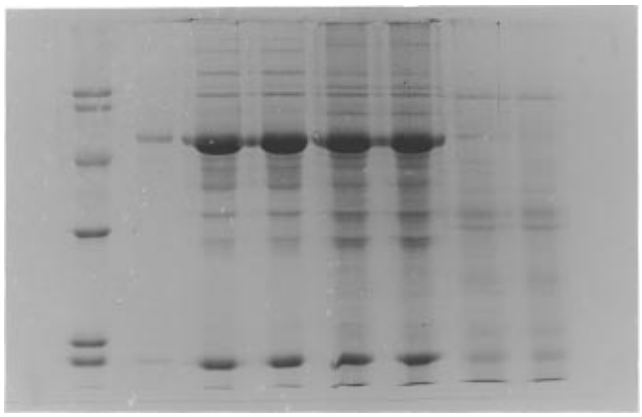

Figure 1. SDS−PAGE gel of *G. sepium*. Lanes, left to right: (1) BDH molecular weight markers; (2) Rubisco; (3, 4) fresh *G. sepium*; (5, 6) freeze-dried *G. sepium*; (7, 8) acetone-extracted *G. sepium*.

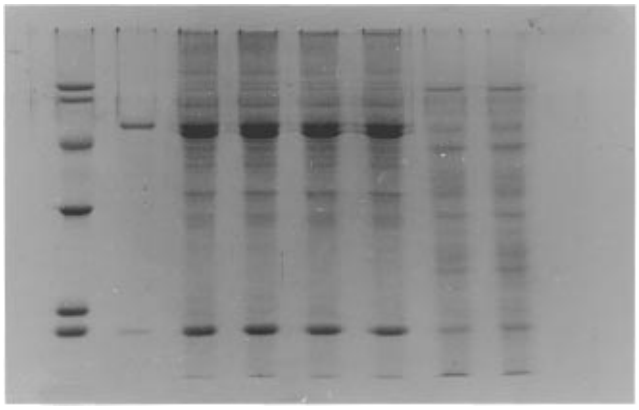

Figure 2. SDS−PAGE gel of *C. calothyrsus*. Lanes, left to right: (1) BDH molecular weight markers; (2) Rubisco; (3, 4) fresh *C. calothyrsus*; (5, 6) freeze-dried *C. calothyrsus*; (7, 8) acetone-extracted *C. calothyrsus*.

g of tannic acid/kg of dry wt. In contrast, *G. sepium* had a very low concentration of phenols, none of which could be detected as condensed tannins. There was no detectable PPA for the phenols found in *G. sepium*.

Extracted Material. After extraction of dried leaf samples with aqueous acetone (70%), the resulting material from both leaf species contained similar concentrations of crude protein and both species lost a similar proportion of dry matter. In the case of *C. calothyrsus* the extracted material had a protein content 96 g kg^{-1} higher than the freeze-dried material.

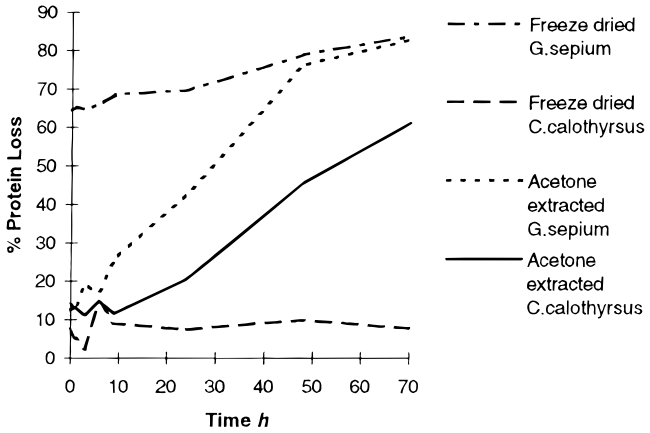

Figure 3. Graph showing the percent crude protein loss of the *G. sepium* and *C. calothyrsus* leaf material used in the fermentation experiment.

Extraction with 70% acetone greatly reduces the soluble protein concentration and has a dramatic effect on the protein profiles: bands that were predominant in the freeze-dried and fresh tissue were not observed or were greatly reduced in their relative intensities. Specifically, the larger band of Rubisco at 55 000, predominant in the fresh and freeze-dried material, was almost completely lost. The smaller Rubisco subunit (12 000) was still one of the most abundant proteins on the polyacrylamide gel of the acetone-extracted material. Bands that were of low intensity in the fresh and freeze-dried material were seen to become much more prominent in the acetone-extracted material, particularly those at approximately 22 000.

Extraction with 70% acetone reduced the crude protein content of the *G. sepium* and *C. calothyrsus* leaf residue by 33 and 29%, respectively.

***In Vitro* Fermentation.** *Cumulative Gas Production.* The cumulative gas production of both acetone-extracted and freeze-dried *G. sepium* was very similar over the 70 h duration of the fermentation experiment. Freeze-dried *G. sepium* reached a maximum cumulative gas production (CGP) of 176.82 mL, wheras acetone-extracted *G. sepium* reached a maximum CGP of 171.33 mL after 70 h.

Acetone-extracted *G. sepium* showed lower gas production in the initial stages of the experiment, but by 28 h CGP was equivalent to that of the freeze-dried material.

The CGP, on fermentation of *C. calothyrsus* residues, showed large differences between the freeze-dried and acetone-extracted residues. Over the first 9 h of fermentation CGP values were similar, 23.72 and 27.97 mL, respectively, for freeze-dried and acetone-extracted *C. calothyrsus*. After this time CGP for acetone-extracted *C. calothyrsus* increased rapidly in comparison to the freeze-dried material, until after 70 h freeze-dried and acetone-extracted *C. calothyrsus* gave CGP values of 89.87 and 140.64 mL, respectively.

Crude Protein Losses. The *G. sepium* residue showed a high initial protein loss of 60% (Figure 3). During the course of the fermentation experiment this figure rose steadily to *ca.* 85% by the final sample taken at 70 h. In contrast, the crude protein loss from the residue of *C. calothyrsus* was smaller, varying between 5 and 15%. There appeared to be no definite pattern of crude protein loss as the fermentation proceeded.

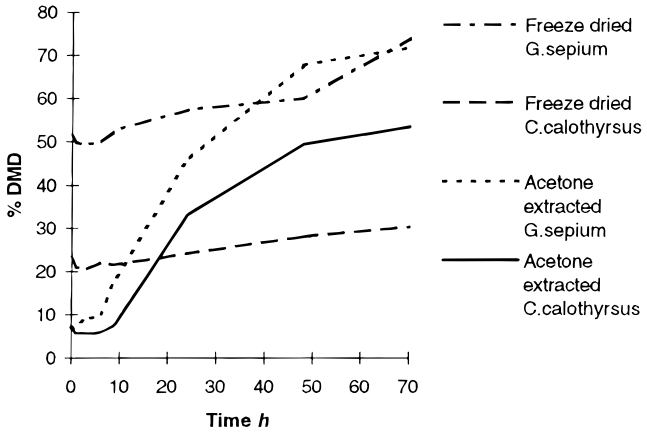

Figure 4. Graph showing the percent dry matter disappearance of the *G. sepium* and *C. calothyrsus* leaf material used in the fermentation experiment.

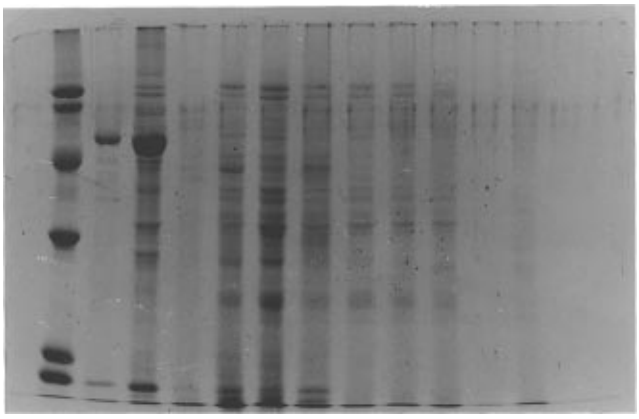

Figure 5. SDS−PAGE gel of *G. sepium* protein degradation during fermentation. Lanes, left to right: (1) BDH molecular weight markers; (2) Rubisco; (3) freeze-dried *G. sepium*; (4) no substrate control; (5) 0 h; (6) 1 h; (7) 3 h; (8) 6 h; (9) 9 h; (10) 24 h; (11) 48 h; (12) 70 h.

The residues of the *G. sepium* and *C. calothyrsus* material extracted with 70% acetone both showed similar patterns of protein loss over the course of the fermentation experiment. Both *G. sepium* and *C. calothyrsus* showed an initial loss of 10% followed by a steady increase in protein loss as the fermentation proceeded. The final protein losses were 80 and 60% for *G. sepium* and *C. calothyrsus*, respectively.

Dry Matter Losses. The dry matter disappearances of the freeze-dried leaf material were considerably different for the two leaf species (Figure 4). *G. sepium* showed a 50% initial loss, followed by a steady loss of dry matter up to 70−75% loss of dry matter after 70 h of incubation. *C. calothyrsus* showed a much smaller initial loss (23%) and a slow rate of further dry matter loss, rising to only 30% by 70 h of incubation.

The rates of dry matter disappearance of acetone-extracted leaf material from the two tree species are very similar. Both residues of acetone-extracted leaf species show little dry matter disappearance until 9 h, after which time the dry matter disappearance rose rapidly to 70 and 53% for *G. sepium* and *C. calothyrsus*, respectively.

Soluble Protein Degradation. The polyacrylamide gels of the total soluble protein profiles of freeze-dried *G. sepium* and *C. calothyrsus* monitored at intervals throughout the *in vitro* fermentation experiment (Figures 5 and 6) show major differences. The profile of *G. sepium* is complex and shows a number of bands which

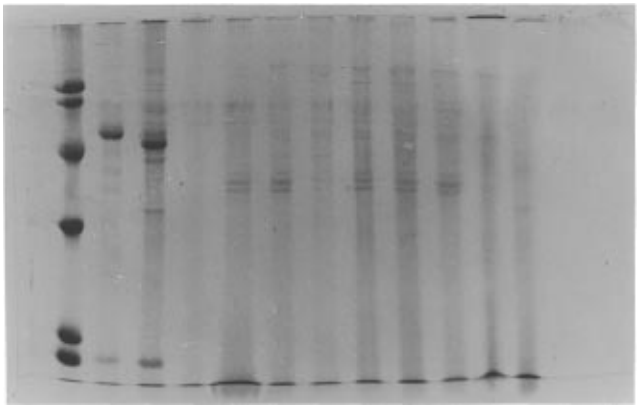

Figure 6. SDS−PAGE gel of *C. calothyrsus* protein degradation during fermentation. Lanes, left to right: (1) BDH molecular weight markers; (2) Rubisco; (3) freeze-dried *C. calothyrsus*; (4) no substrate control; (5) 0 h; (6) 1 h; (7) 3 h; (8) 6 h; (9) 9 h; (10) 24 h; (11) 48 h; (12) 70 h.

Table 2. Soluble Protein Remaining from the Substrates *G. sepium* and *C. calothyrsus* during *in Vitro* Fermentation Experiment[a]

time, h	*G. sepium*, μg/g of DM soluble protein	*C. calothyrsus*, μg/g of DM soluble protein
0	13100 ± 282	60.4 ± 30.2
1	10647 ± 293	115.3 ± 21.6
3	7573 ± 156	129.9 ± 7.8
6	5586 ± 586	249.4 ± 4.3
9	4317 ± 379	346.3 ± 17.3
24	3708 ± 207	192.1 ± 40.5
48	2708 ± 68	202.4 ± 25.9
70	146 ± 0	293.9 ± 3.4

[a] Results are mean values ± one standard deviation.

Table 3. Cumulative Gas Production Data (Corrected to 1 g of Substrate) for the *in Vitro* Fermentation of Acetone-Extracted and Freeze-Dried *G. sepium* and *C. calothyrsus*

	cumulative gas production, mL			
	G. sepium		*C. calothyrsus*	
time, h	freeze-dried	acetone-extracted	freeze-dried	acetone-extracted
0	0	0	0	0
3	14.24	7.74	7.99	7.49
6	27.72	15.48	15.48	16.48
9	39.21	25.97	23.72	27.98
12	48.95	37.21	31.95	38.97
16	59.69	50.19	40.44	50.96
20	70.93	63.93	48.43	63.2
24	80.41	80.66	55.42	73.94
28	90.91	99.14	62.42	88.43
33	105.89	118.87	67.91	100.67
39	118.88	137.61	72.4	112.41
45	131.36	150.1	76.14	122.4
52	146.6	159.33	80.14	129.4
60	162.58	165.58	85.13	135.14
70	176.82	171.32	89.87	140.64

are degraded at different rates varying from 1 to 24 h, indicating that different proteins have different resistances to degradation. The majority of the bands, and indeed the majority of the soluble protein (Table 2), is degraded by 6 h. After 24 h there is protein present in the liquid phase; however, no definite bands can be seen on the gel.

The profile of the degradation of the soluble protein from *C. calothyrsus* is less clear. The profile contains few bands (Figure 6), and indeed there is little soluble protein present (Table 2) due to the effects of complexation with polyphenols in the leaf material. In contrast

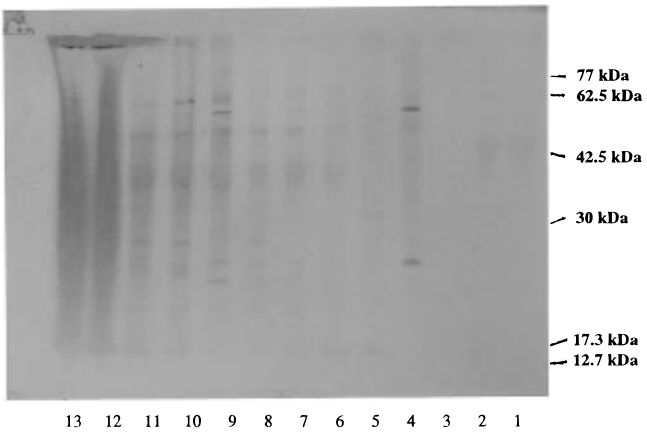

Figure 7. Carbohydrate stain of a protein blot on nitrocellulose membrane. Lanes, left to right: (1, 2) ovalbumin; (3) Rubisco; (4) no substrate control; (5) *C. calothyrsus* freeze-dried leaf matieral; (6) 0 h; (7) 1 h; (8) 3 h; (9) 6 h; (10) 9 h; (11) 24 h; (12) 48 h; (13) 70 h.

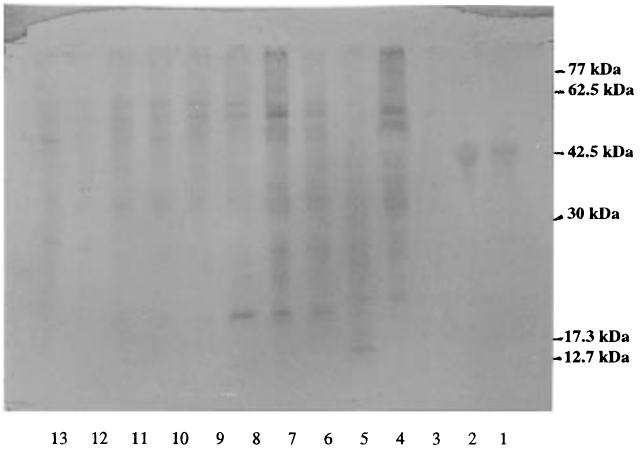

Figure 8. Carbohydrate stain of a protein blot on nitrocellulose membrane. Lanes, left to right: (1, 2) ovalbumin; (3) Rubisco; (4) no substrate control; (5) *G. sepium* freeze-dried leaf material; (6) 0 h; (7) 1 h; (8) 3 h; (9) 6 h; (10) 9 h; (11) 24 h; (12) 48 h; (13) 70 h.

to *G. sepium*, however, the soluble protein content of *C. calothyrsus* increases up to 9 h; this effect is seen on the gel by an increase in the number of bands present in the soluble protein profile and by an increase in the intensity of the bands already present. After 24 h, little protein was present in the liquid phase and no distinct bands could be seen on the gel.

The degree of glycosylation is one possible reason for the differences in the *in vitro* degradability of the proteins in the leaf material. The glycoprotein analysis was, however, inconclusive, although it showed clearly that neither band in the Rubisco standard is glycosylated. In the case of *G. sepium* (Figure 8) a number of the proteins with higher molecular weight are glycosylated, and on the glycoprotein stain of the *G. sepium* gel, the glycosylated proteins (approximate MW 60 000−70 000) remained undegraded after 24 h. The glycoprotein stain of the *C. calothyrsus* gel (Figure 7) showed an increase in the number and intensity of bands as the experiment progressed. After 24 h, individual bands in the sample lanes cannot be seen, as background staining is high. It is also clear that the crude protein extract of *C. calothyrsus* contains some glycosylated proteins, seen as distinct bands. These bands can be seen in the later stages of the experiment.

DISCUSSION

Previous work on *C. calothyrsus* and *G. sepium* forages, regarding the acceptability and digestibility of the leaf material, has concentrated on investigations to determine the usefulness of the two species for feed and feed supplement purposes (Paterson, 1994; Bennison and Paterson, 1993). Baggio and Heuveldop (1984) found *C. calothyrsus* to have a dry matter digestibility of 35.43%, compared to 30.24% for freeze-dried material. Extraction with acetone increased dry matter digestibility for *C. calothyrsus* to 51.77%.

In this study a modification of the *in vitro* fermentation of Theodorou et al. (1994) was used as an alternative to time-consuming, high-labor *in vivo* trials, which, to minimize experimental variation, require large numbers of animals of the same sex, age, and breed. This method has been used to assess *in vitro* digestibility and provide information on the kinetics of digestion of fodder tree species (France et al., 1993; Wood et al., 1993). Prasad et al. (1994) found that results obtained from this *in vitro* technique were comparable to those obtained by *in vivo* studies. *G. sepium* seemingly contains a high proportion of readily soluble material in comparison to *C. calothyrsus*. This material is probably carbohydrate and proteins that are readily degraded or solubilized under the conditions of the fermentation experiment. The effect of this high proportion of readily degradable material is to give a rapid rate of initial fermentation. In general, the leaf material of *G. sepium* is more readily degraded/solubilized or is more accessible to attack by rumen micro-organisms. The extraction with acetone appears to have had two distinct effects: to remove much of the readily degraded/solubilized material from the *G. sepium*, hence lowering the initial rate of dry matter loss and gas production, and to remove material from *C. calothyrsus* which is recalcitrant itself or prevents the breakdown of other material. This would indicate that the tannins in the *C. calothyrsus*, which formerly had a protectant effect, by complexation with degradable materials (carbohydrates, proteins, or both) or by blocking the attack of rumen micro-organisms, have been removed by the extraction with 70% acetone. This is supported by the reduction, either by solubilization or by degradation of proteinaceous material, in the crude protein of the residue. Freeze-dried *C. calothyrsus* leaf material showed a maximum crude protein reduction in the residue of approximately 15%. However, when the tannins were removed, a reduction of crude protein in the residue of over 60% was observed.

In this investigation the authors have developed a system to monitor the aspects of degradation of fodder leaf materials. This system is significant in that inhibitory effects on the degradation of soluble protein in the fermentation model can be monitored directly by the use SDS−PAGE. Previous work using SDS−PAGE has only attempted to show the resistance of different purified or semipurified proteins to degradation by rumen microbes. This variable resistance to degradation has been shown previously by Nugent et al. (1983), who showed that the order of rapidity of degradation for four proteins was as follows: casein, fraction I leaf protein, bovine serum albumin (BSA), bovine submaxillary mucoprotein. Casein was degraded approximately 15−20 times more rapidly than BSA. Individual soluble leaf proteins have been shown, in this paper, to have different degrees of resistance to degradation, although all soluble leaf proteins can be degraded after a certain

length of time, somewhere between 24 and 48 h. Aufrere et al. (1994) found that lucerne proteins, including Rubisco, were degraded in the rumen of fistulated sheep within a 48 h period. While some very faint bands may be seen even after 70 h, it is probable that it is proteins produced by the rumen microflora and the rumen microflora itself which account for the protein detected, since the bands were not observed in previous samples.

Proteins that showed some degree of glycosylation were among those which were most resistant to degradation in both leaf species. This gives some indication that in general glycosylated proteins may be more resistant to degradation than nonglycosylated proteins. The number and intensity of bands on the *C. calothyrsus* glycoprotein stain increase up to the 24 h sample. After 24 h, the level of background staining is very high, due to an increasing concentration of interfering material present in the protein extract. Although glycoproteins appear to be more resistant to degradation by rumen microbes, they represent only a small percentage of the total soluble protein, and as such may not be of great nutritional significance. Data from the glycoprotein stain are in direct agreement with the gel and soluble protein data and support the theory that proteins initially bound or insoluble are solubilized as the experiment progresses. This study clearly indicates that the differences in the protein degradation characteristics of *G. sepium* and *C. calothyrsus* are mainly due to the inhibitory effects of tannins.

There was a large difference between the two leaf species in terms of total (crude) protein, tannins, and soluble protein. *G. sepium* is higher in both crude and soluble protein, whereas *C. calothyrsus* is higher in tannins, which is consistent with reported data for proximate and fiber analyses of both species, summarized by Paterson (1994) and Bennison and Paterson (1993) for *C. calothyrsus* and *G. sepium*, respectively. The apparently low concentration of soluble protein in *C. calothyrsus* is probably due to the complexation of soluble protein by tannins, rendering them insoluble. Tannins also have the ability to complex carbohydrates, which may correlate with the ability to complex protein (Haslam, 1989). Constantinides and Fownes (1994) have studied the degradation of *C. calothyrsus* and *G. sepium* when applied to soil, with particular reference to the relationship between nitrogen mineralization and lignin and soluble polyphenol concentration. It was found that the leaves containing higher concentrations of polyphenols were broken at a much slower rate than those with lower polyphenol concentrations. The rate of nitrogen mineralization also showed a similar correlation with polyphenol concentration.

Incubations containing *G. sepium* showed a decrease in soluble protein as the fermentation experiment proceeded. In contrast, the incubations containing *C. calothyrsus* showed an increase in soluble protein up to the sample taken at 24 h, as shown in Table 2. This effect is presumably due to the solubilization of complexed or insoluble protein by the action of rumen microflora. This takes place either by the breakdown of the polyphenol−protein complex, formed initially, or by the release of protein bound to the structure of the leaf material.

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