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Red Clover Extracts Inhibit Legume Proteolysis*

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Abstract: Unwilted red clover and alfalfa herbage were hand-chopped, inoculated, ensiled into laboratory silos and opened after 0, 1, 3 and 7 days. Proteolysis was 40% lower with the red clover silage than alfalfa silage after 7 days of ensiling. The lower extent of proteolysis with the red clover silages could not be explained by differences in dry matter content, pH decline nor final pH of the silage. It was determined that red clover has a buffer soluble protein that could inhibit proteolysis in red clover and alfalfa. The soluble protein is possibly a polyphenol oxidase.

Key words: ensiling, alfalfa, lucerne, Medicago, Trifolium, silage, proteases, oxidases, fermentation, lucerne.

INTRODUCTION

Proteolysis of ensiled alfalfa results in the degradation of 44–87% of the forage's protein (Papadopoulos and McKersie 1983; Muck 1987). This translates into an economic loss for ruminant producers due to the additional cost of protein supplementation or due to the increased cost of adding rapidly fermented carbohydrates in the ration to maximize the use of the non-protein nitrogen. Interestingly, red clover, a legume of similar protein content, will have only 7–40% of its original protein degraded during ensiling (Papadopoulos and McKersie 1983). This reduction in proteolysis results in the red clover silages having higher true protein content and consequently an economic benefit to producers.

Prior research by Papadopoulos and McKersie (1983) demonstrated that red clover and alfalfa had similar proteolytic rates of azocasein hydrolysis. They postulated that the reason for the lower extent of proteolysis in red clover silage was related to the red clover proteases functioning below their optimum pH and that the proteases were not stable at low pH. Jones *et al* (1994) demonstrated that alfalfa and red clover extracts had similar proteolytic rates and that these activities had similar pH and temperature optima and stabilities. The following research was undertaken to establish that red clover does have lower extents of proteolysis during ensiling than alfalfa and to investigate whether the lower extent of proteolysis is related to a soluble factor.

MATERIALS AND METHODS

Two ensiling studies were performed comparing red clover and alfalfa, one using plants grown in a greenhouse and the other forage from field plots. Forage types grown in the greenhouse and field plots, respectively, were cultivars C136 (Dr R R Smith, US Dairy Forage Research Center, Madison, WI) and Marathon for red clover (*Trifolium pratense*) and clones of TB F1 (Dr E T Bingham, Dept of Agronomy, UW Madison, WI) and cultivar Arlington for alfalfa (*Medicago sativa*). Greenhouse conditions were 22 : 18°C for the respective day : night temperatures and 14 h of artificial light. Legumes were grown in a silt loam soil mixture (1 : 1 : 1 v/v/v loam/perlite/peat) and fertilized biweekly with 10 : 10 : 10 (% total N, available phosphoric acid, water-

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soluble potash) soluble fertilizer. Field grown legumes (1-year-old stand) were grown in silt loam soil and fertilized to maintain maximum yields. Forage was harvested on 6 October which was the fourth cutting for alfalfa and the third cutting for red clover. All legumes were harvested at early bud, chopped by hand (1·2 cm length), inoculated with lactic acid bacteria (10^3 live bacteria per gram herbage, Chris Hansen's Laboratory, Milwaukee, WI), and ensiled into laboratory silos according to the method of Muck (1987). A sample of the chopped, inoculated herbage was taken and frozen until analyzed. Silos from both studies were incubated at 30°C for 0, 1, 3 and 7 days. Duplicate silos were removed from the incubator and frozen until analyzed. Thawed fresh herbage and silages were analyzed for dry matter (DM) content by drying at 60°C for 48 h. A 10 g sample of the silage or herbage was diluted 10-fold in a blending jar and macerated (30 s). The macerated sample was filtered and a pH taken immediately. To a 20 ml aliquot of the filtered sample, 5 ml of 25% (w/v) trichloroacetic acid were added to precipitate protein. After centrifugation, the supernatant was analyzed for ammonia (NH_3), free amino acids (FAA) and soluble nonprotein nitrogen (NPN) (Muck, 1987). Fresh herbages were also analyzed for total nitrogen (Brotz and Schaeffer 1984). Means were calculated across silos.

Two separate experiments from the ensiling studies were performed using legume extracts. Leaves of greenhouse-grown red clover and alfalfa were harvested, ground in liquid nitrogen and suspended in 50 mM $KHPO_4$ buffer (pH 7·0; 333 g leaves liter^{-1} buffer). The slurry was filtered through cheesecloth (four layers), and the filtrate was centrifuged to remove particulates (20 000 × *g*, 25 min). Supernatants were collected, kept on ice and used as the legume extracts. Prepared legume extracts were used in two experiments. Treatments in the first experiment were as follows: Trt 1, untreated alfalfa extract; Trt 2, untreated red clover extract; Tr 3, alfalfa extract with 20 mM ascorbate (Ascor) and 2 mM dithrothreitol (DTT); Trt 4, red clover extract treated with 20 mM Ascor and 2 mM DTT; Trt 5, untreated alfalfa and red clover extracts mixed 1 : 1, v/v; and Trt 6, untreated alfalfa and boiled red clover extracts mixed 1 : 1, v/v. The release of free amino acids over time (0, 0.25, 0.5, 1, 2 and 4 h; pH 7·0; 30°C) in treated legume extracts was determined by a ninhydrin assay (Moore and Stein 1954; ninhydrin reagent Sigma #N 1672) after precipitation of proteins by the addition of 0·92 M trichloroacetic acid (TCA) to a final concentration of 0·31 M. Proteins were removed by centrifugation (1200 × *g*, 10 min). Treatment means were averaged across replicates. Treatment differences were determined by LSD ($P = 0·05$) using ANOVA.

Treatments in experiment 2 were as follows: Trt 1, untreated alfalfa extract; Trt 2 untreated alfalfa and red clover extracts mixed 1 : 1; Trt 3, untreated alfalfa mixed 1 : 1, v/v with boiled (under laboratory air) red clover extracts; and Trt 4, untreated alfalfa extract mixed 1 : 1, v/v with boiled (under nitrogen) red clover extract. The experiment was completed in the same way as experiment 1.

RESULTS AND DISCUSSION

Red clover and alfalfa, harvested from plants grown in the greenhouse, were ensiled at DM contents of 206 and 169 g kg^{-1} herbage and total nitrogen content of 37·3 and 40·5 g kg^{-1} DM, respectively. Legumes harvested from the field were of lower dry matter content (134 and 133 g kg^{-1}) due to rainfall on days prior to harvest, while the total N contents were similar for alfalfa and red clover, respectively (40·5 and 34·9 g N kg^{-1} DM). Table 1 displays the pH and nonprotein nitrogen composition of the fresh herbages and silages. The pH (of silages from the greenhouse material) did not decline substantially with the red clover and increased with alfalfa during ensiling due to poor growth of the lactic bacteria. Although the number of live bacteria in the inoculum was determined at ensiling (serial dilutions of the inoculum were plated on Rogosa SL agar), the lack of growth during ensiling appeared due to residual insecticidal soap from routine spraying of the forages. Lactic acid bacteria (LAB) were plated with insecticidal soap (at the application concentration, diluted 10- and 100-fold) and without insecticidal soap. Insecticidal soap inhibited LAB growth by 95%, yet this inhibition was reduced when the insecticidal soap was diluted (13 and 0% inhibition for 10- and 100-fold dilution, respectively). Even though the forages were harvested several days after application, the residual insecticide appeared sufficient to inhibit the LAB.

Legumes harvested from the field plots ensiled better than the legumes harvested from the greenhouse as noted by the lower pH (Table 1), yet neither legume silage attained low pH. Failure of the silage pH to reach 4 allowed substantial proteolysis to occur in all silages (Table 1). Red clover silage had consistently and significantly ($P < 0·05$) lower FAA-N, NH_3-N and NPN than alfalfa silage.

Peptide N was similar between the legumes in the greenhouse study, but was lower for red clover than alfalfa silage in the field study. Research has reported that not only the amount of NPN but also the proportion of NPN as peptide, FAA-N and NH_3-N could be important in N utilization by ruminants (Cotta and Hespell 1986; Wallace 1994). In the field study, both legume silages had a similar proportion of the NPN as NH_3-N, FAA-N and peptitde (13–14%, 71–73% and 15%, respectively). However, red clover silage in the greenhouse study had higher peptice (25 vs 16%) and lower NH_3-N (8 vs 15%) than alfalfa silages. Both silages had similar proportions of the NPN as FAA-N (15%). Apparently, the mechanism inhibiting proteolysis

TABLE 1
pH and nitrogen fractions (g kg^{-1} total N) of red clover and alfalfa herbage and silage (mean ± SD, $n = 2$)

	Red clover					*Alfalfa*				
	pH	*FAA-N*[a]	*NH_3-N*	*NPN*	*Peptide*[b]	*pH*	*FAA-N*	*NH_3-N*	*NPN*	*Peptide*
Greenhouse study										
Initial herbage	6·48 ± 0·011	39·5 ± 1·21	3·2 ± 0·01	135·4 ± 11·85	92·7	6·17 ± 0·026	59·5 ± 1·89	3·8 ± 0·17	160·8 ± 15·88	97.1
Ensiled 1 day	5·99 ± 0·216	158·3 ± 10·9	8·9 ± 0·94	337·7 ± 12·66	170·5	6·40 ± 0·019	234·9 ± 5·96	11·1 ± 2·42	520·8 ± 8·48	274·8
Ensiled 3 day	5·02 ± 0·129	251·3 ± 8·36	35·1 ± 5·03	408·9 ± 17·20	122·5	6·29 ± 0·203	470·5 ± 20·1	123·5 ± 10·41	730·1 ± 50·02	136·1
Ensiled 7 day	5·56 ± 0·114	319·8 ± 6·26	36·4 ± 4·97	475·0 ± 9·06	118·8	6·63 ± 0·078	493·6 ± 80·73	110·7 ± 7·3	717·4 ± NA[c]	113·1
Field study										
Initial herbage	6·12	35·1	3·5	113·5	74·9	6·30	47·2	6·7	144·5	90·6
Ensiled 1 day	5·02 ± 0·144	144·3 ± 9·28	35·3 ± 0·63	326·9 ± 4·40	147·3	5·50 ± 0·127	261·2 ± 2·74	33·7 ± 4·60	483·1 ± 7·53	188·2
Ensiled 3 day	4·46 ± 0·143	254·3 ± 8·41	49·3 ± 7·08	407·6 ± 8·94	104·0	4·85 ± 0·108	408·8 ± 6·06	60·3 ± 5·35	588·3 ± 10·90	119·2
Ensiled 7 day	4·36 ± 0·051	324·9 ± 15·22	62·5 ± 3·93	458·3 ± 23·67	70·9	4·86 ± 0·051	528·1 ± 2·98	91·3 ± 7·17	728·8 ± 23·67	109·4

[a] FAA-N, free amino acid nitrogen; NH_3-N, ammonia nitrogen; NPN, nonprotein nitrogen.
[b] Peptide nitrogen is calculated as NPN-(FAA-N + NH_3-N).
[c] Value represents only one silo, no standard deviation calculated.

in red clover silages may also alter the composition of the NPN in the silage. This observation will need further validation.

When essentially all proteolysis has occurred, after 7 days of ensiling (Muck 1987), red clover silage was consistently 40% lower in NPN increase than alfalfa silage. Both dry matter and pH may explain a portion of this difference in proteolysis between the two legume silages. Increasing DM of forages will decrease the initial rate of proteolysis at ensiling (Muck 1987; Jones *et al* 1992). A simulation model of ensiled alfalfa (Muck 1987; Muck and Dickerson 1988) was used to determine if the differences in extent of proteolysis between the red clover and alfalfa silages could be explained by DM or pH. Legume DM differences were only noted with the greenhouse study. Yet using the simulation, if the red clover was ensiled as the same DM as the alfalfa, it was predicted that there would only be an increase of 31 g N kg^{-1} total N in NPN (Table 2). This amount only accounts for 14% of the difference in NPN increases due to ensiling between the two legumes.

The second factor that will influence proteolysis during ensiling is pH. The silage pH for the greenhouse grown legumes were greatly different (Table 1) and resulted in red clover staying near its otpimum pH for proteolysis whereas alfalfa stayed near a pH that resulted in only 60–75% of its optimum activity (Jones *et al* 1995). Stability of the legume proteases is also influenced by pH. Alfalfa at pH 6 and 7 will have 20 and 44% residual proteolytic activity after 24 h, respectively, whereas red clover has 20% residual activity at both pH 5 and 5·5 (Jones *et al* 1995). Based on these two factors, optimum pH activity and stability, no substantial pH effect would be expected on the extent of proteolysis.

A slightly different observation is noted with the ensiled legumes that were harvested from the field. Here the pH declined with ensiling of both legumes but red clover was significantly ($P < 0·05$) lower (Table 1). This

TABLE 2
Comparison of proteolysis between a computer simulation and the actual ensiling values of alfalfa and red clover

	Dry matter (*g kg^{-1}*)	*Increase in NPN*[a] (*g N kg^{-1} total N*)	
		Actual	*Predicted*
Greenhouse study			
Alfalfa	169	556	456
Red clover	206	339	465
Red clover[b]	169	—	496
Field study			
Alfalfa	133	584	472
Red clover	134	345	482

[a] Values calculated as NPN after 7 days of ensiling minus initial value.
[b] Simulation was run using the red clover data except changing the dry matter content to that of alfalfa.

lower pH should result in less proteolysis occurring because of being further from both the optimal pH for activity and stability (McKersie 1985; Jones *et al* 1995). Using the computer simulation (Table 2), there was minimal predicted difference in the NPN during ensiling due to pH when NPN is expressed as g N kg^{-1} total N (comparing alfalfa and red clover treatment at the same DM). In fact, it would appear that there was greater proteolysis in red clover. However, when the numbers are expressed as g N kg^{-1} DM, increases in NPN of alfalfa silages were predicted to be 18·46 and 19·81 while red clover silages were 17·34 and 16·80 for the greenhouse and field studies respectively. Thus since red clover has a lower crude protein content, the effect of a lower pH on proteolysis is masked by expressing NPN on a total N basis. Regardless of the unit of expression, it is clear that pH had a small effect ($\leq 15\%$) on the difference in extent of proteolysis measured between red clover and alfalfa silages. Combining the maximal effects for both DM and pH, this could explain up to 29% of the difference in extent of proteolysis between red clover and alfalfa silages. An additional factor(s) must be present in red clover to explain the remaining 71% of the difference in NPN content of red clover and alfalfa silages.

Figure 1 shows proteolysis that occurred in various treatments of alfalfa and red clover extracts. There was no significant difference ($P > 0{\cdot}05$) among the treatments with the 0·25 h incubation. All of the following comments refer to 0·5–4 h incubation period. Untreated alfalfa extract (Trt 1) and when treated with Ascor and DTT (Trt 3) or boiled red clover extract (Trt 6) had maximum proteolytic activity and were not significantly different except at 0·5 h (Trt 1 and 6 were significantly different than Trt 3). In comparison, untreated red clover (Trt 2) had the lowest activity and was significantly different from Trt 1, Trt 3, Trt 4 and Trt 6 at 1–4 h incubations. Mixing untreated alfalfa and red clover extracts significantly reduced alfalfa proetolysis by 70% (comparing Trt 1 with Trt 5) and was significantly lower than red clover treated with Ascor and DTT (Trt 4). Yet when boiled red clover extract was mixed with untreated alfalfa extract (Trt 6), there was no effect on alfalfa proteolysis at any time point of the incubation (comparing Trts 1 and 6). Inactivation of the red clover factor by boiling suggests that the factor is a protein. In addition this protein in red clover is inhibited by Ascor and DTT (Trt 4) but after 0·5 h the inhibitors (Ascor, DTT) were oxidized resulting in a browning of the extract (data not shown) and prompt inhibition of red clover proteolysis. Inhibition of the red clover protein by Ascor and DTT along with the browning of the red clover extract suggests that the inhibitory protein is a polyphenol oxidase. Research is being conducted to validate the presence of a soluble polyphenol oxidase in red clover extracts.

Red clover contains several phenols that could undergo oxidation (Wong 1962; Horigome and Kandatsu 1968) and bind to proteins (Horigome and Kandatsu 1968). Boiling the red clover extract in air may oxidize these phenols, bind the oxidized phenols to red clover proteins and lose the inhibitory response in the

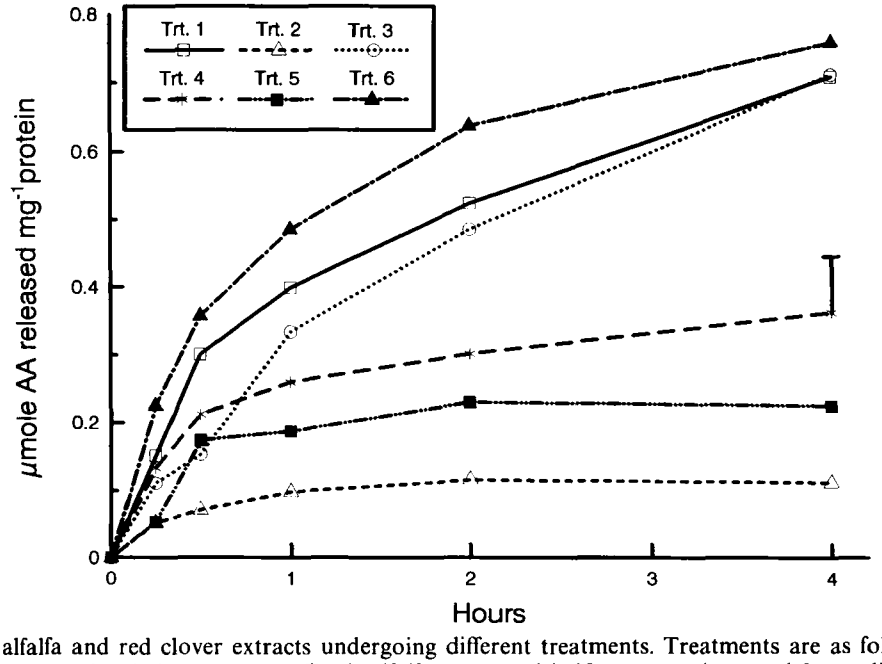

Fig 1. Proteolysis of alfalfa and red clover extracts undergoing different treatments. Treatments are as follows: Trt 1, untreated alfalfa extract; Trt 2, untreated red clover extract; Trt 3, alfalfa extract with 20 mM ascorbate and 2 mM dithiothreitol; Trt 4, red clover with 20 mM ascorbate and 2 mM dithiothreitol; Trt 5, untreated alfalfa and red clover extracts mixed 1 : 1; Trt 6, untreated alfalfa and boiled red clover extracts mixed 1 : 1. Proteolysis was monitored by the release of free amino acids. The bar represents the greatest standard deviation.

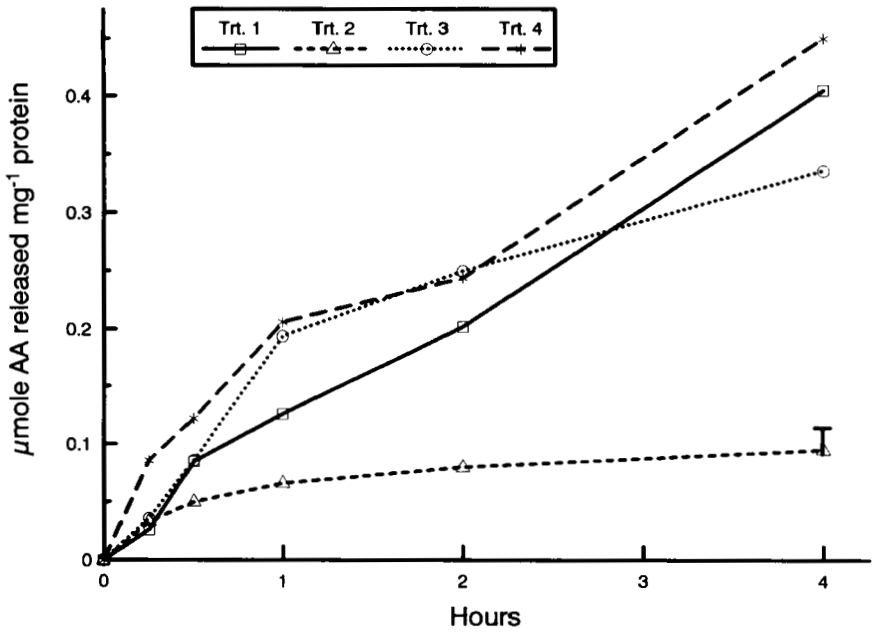

Fig 2. Proteolysis of alfalfa extract mixed with red clover and boiled red clover extracts. Treatments are as follows: Trt 1, untreated alfalfa extract; Trt 2, untreated alfalfa and red clover extracts mixed 1 : 1; Trt 3, untreated alfalfa and boiled (under air) red clover extracts mixed 1 : 1; Trt 4, untreated alfalfa and boiled (under nitrogen gas) red clover extracts mixed 1 : 1. Proteolysis was monitored by the release of free amino acids. The bar represents the greatest standard deviation.

red clover extract. To determine if phenols were oxidized, two red clover extracts were boiled (15 min); one under air and the other under nitrogen. The boiled extracts were mixed with untreated alfalfa extract, and proteolysis was monitored by the release of free amino acids. Untreated red clover and alfalfa were mixed 1 : 1, v/v, as a positive control. Data are shown in Fig 2. Boiling of the red clover extract destroyed the inhibitory factor in red clover extract with no significant difference ($P > 0{\cdot}5$) between the boiling treatments of the red clover extract (comparing Trt 3 and Trt 4). Since both boiling treatments were ineffective in inhibiting alfalfa proteolysis (comparing Trt 1, Trt 3 and Trt 4), this suggests that the factor is a protein and not auto-oxidized phenols.

In conclusion, red clover silage has lower extents of proteolysis than alfalfa, and this difference can not be explained by differences in dry matter content, pH decline or protease activity and stability. It is proposed that the inhibitory factor in red clover is a protein and possibly a polyphenol oxidase.

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