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Genetic variation in red clover for rumen protein degradability☆

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Abstract

Protein in red clover (*Trifolium pratense* L.), a forage that does not contain condensed tannins, has been found to be degraded less extensively in the rumen than the protein in other non-tannin legumes. The objective of this study was to determine if there are genetic differences in rumen degradability of the protein in red clover forage. Field grown forage was harvested from 133 red clover entries (117 plant introductions and 16 cultivars or experimental lines) plus one cultivar of lucerne (*Medicago sativa* L.). Forages were analyzed for total N and for crude protein (CP; N × 6.25) that was undegraded at 0 h, as well as for rate of protein degradation and rumen protein escape estimated using a rumen in vitro system. There were small but significant ($P = 0.007$) differences in total N among the 133 red clover entries but no differences in proportion of CP that was undegraded at 0 h. Protein degradation rate was more rapid for the lucerne cultivar than for any of the red clover entries; this difference was significant ($P < 0.05$) for 132 of the red clovers. Protein degradation rate ranged from 0.088 to 0.146/h and rumen protein escape ranged from 287 to 409 g CP/kg CP among red clover entries; effect of accession was highly significant ($P < 0.001$) for both traits. The frequency distributions for the red clover entries for protein degradation rate and rumen protein escape were normal, showing relatively little skewness. The consistency and

Abbreviations: B_0, intact protein present at 0 h; CP, crude protein; DM, dry matter; IIV, inhibitor in vitro; k_d, degradation rate; k_p, passage rate; N, nitrogen; PI, plant introduction; PPO, polyphenol oxidase; TCA, trichloroacetic acid; TN, total nitrogen; TAA, total amino acids

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distribution of these results suggested that conventional plant breeding techniques could be used to develop lines of red clover forage with improved protein utilization in ruminants.

Keywords: Red clover; *Trifolium pratense* L.; Protein degradation (in vitro); Genetic variation

1. Introduction

Although N from degraded protein may be reincorporated by rumen microbes, excessive degradation of forage proteins usually is wasteful due to excessive ammonia formation in the rumen. There is considerable variation in protein degradability among forage legumes (Broderick and Albrecht, 1997). Part of this variation can be explained by the presence of condensed tannins in some species, which depresses protein degradation either by altering the forage proteins and/or by inhibiting microbial proteases (Barry et al., 1986). However, protein in legumes not containing tannins also vary in susceptibility to proteolytic attack. Lucerne (*Medicago sativa* L.) is the most commonly fed non-tannin legume forage; the protein in lucerne is extensively degraded in the rumen (NRC, 2001). Feeding studies have confirmed that lucerne protein often is poorly utilized by lactating dairy cows (Broderick, 1985; Broderick, 1995). Silage made from red clover (*Trifolium pratense* L.), which also has no detectable tannins, contained less non-protein N than that made from lucerne and from birdsfoot trefoil (*Lotus corniculatus*), a forage containing detectable amounts of tannin (Albrecht and Muck, 1991). Rumen in vitro assays on a number of commercial cultivars of forage legumes indicated that red clover protein was less degradable than that in lucerne, white clover (*Trifolium repens* L.), cicer milkvetch (*Astragalus cicer* L.), Canada milkvetch (*Astragalus canadensis* L.), crownvetch (*Coronilla varia* L.), and birdsfoot trefoil over two harvest years (Broderick and Albrecht, 1997).

The objective of this study was to determine if there is genetic variation for protein degradability in red clover forage. A rumen in vitro system was used to screen a large number of germplasm entries and several cultivars of red clover for protein degradability by rumen microbes.

2. Materials and methods

2.1. Forage materials

One hundred forty-two plant introductions (PI) and 17 commercial cultivars (commercially available in the USA or experimental lines) of red clover, plus one common commercial cultivar of lucerne (‘Dart’), were planted in single row plots two meters long and spaced 0.76 m apart. Plots were sown on 30 April 1993 at the University of Wisconsin Arlington Agricultural Research Station into Plano silt loam (fine-silty, mixed mesic typic Agriudoll) soil in two replications. Soil pH was 6.7 and fertility was adjusted by P and K application to meet soil test recommendations for lucerne on that soil type. Irrigation was applied as needed to insure successful establishment and vigor for sampling the following

year. On 1 June 1994, when most red clover entries had a few stems with flowers, individual stems ranging in maturity from buds showing some purple color to stems having a few open florets were harvested 3 cm above the soil surface. Approximately, 10 stems per plot were harvested with an attempt to sample as many plants within the plot as possible. Samples were immediately put into plastic bags and on ice and then stored at −20 °C until they were freeze-dried. Samples then were ground to pass a 1 mm screen of a cyclone mill. Air-equilibrated ground samples of herbage were analyzed for dry matter (DM) at 105 °C (AOAC, 1980) and for total N (TN) using a combustion assay (Leco FP-2000; Leco Instruments, Inc., St. Joseph, MI, USA).

2.2. Rumen protein degradability

Rates of rumen protein degradation (k_d), and fractions degraded at 0 h (fraction A_0) and potentially degradable (fraction B_0) were determined on herbage samples in the inhibitor in vitro (IIV) method. Rumen fluid was obtained 2 h after feeding from two lactating dairy cows that were fitted with permanent, 10 cm rumen cannulae and fed a standard diet (Luchini et al., 1996). The using limited-substrate IIV system described earlier (Broderick, 1987) was used with the following modifications: (1) before adding inhibitors and mercaptoethanol, equal volumes of strained rumen fluid from each donor cow were blended and pre-incubated with mixed soluble carbohydrates [8, 4, 4, and 4 g/l of, respectively, maltose, soluble starch, xylose and pectin (Sigma Chemicals, St. Louis, MO)] at 39 °C for 3 h to reduce background levels of NH_3 and total amino acids (TAA) (Broderick et al., 1998); (2) incubations were conducted in 50 ml centrifuge tubes containing 1/20 of the substrate and volumes used earlier in spinner flasks [per tube, 1.875 ± 0.025 mg of substrate N, 5 ml McDougall's buffer (McDougall, 1948), and 10 ml inoculum (containing 5 ml rumen fluid)]; and 3) incubations were conducted separately for 0 and 2 h and stopped by adding only trichloroacetic acid (TCA), to a final concentration of 5% (w/v), and holding tubes on ice for 30 min. Four standard proteins were included in every incubation: casein (Sigma Chemicals, St. Louis, MO), solvent and expeller soybean meals (Broderick and Clayton, 1992), and lucerne hay (Hristov and Broderick, 1996). The 0 h "incubations" were made by soaking herbage and standard proteins (1.875 ± 0.025 mg N) in 5 ml McDougall's buffer for 60 min at 39 °C. Then 10 ml of warm (39 °C) McDougall's buffer was added and samples treated with TCA as in the incubations with rumen fluid. Both 0 and 2 h samples were centrifuged (15,300 × *g*, 15 min, 4 °C) and supernatants analyzed for NH_3 and TAA using a semi-automated method (Broderick and Kang, 1980) modified to include dialysis in both manifolds and automated data collection. Four blank tubes (no added protein) were incubated for 2 h for each 50 protein-containing tubes; blanks for 0 h samples were TCA-treated McDougall's buffer. Duplicate tubes were incubated at both 0 and 2 h for standard proteins and all herbage samples. Net release of NH_3 and TAA was defined as the concentration differences between protein-containing tubes and blanks. Fraction degraded at 0 h (A_0) was defined as the proportion of crude protein (CP; total N × 6.25) present as NH_3 plus TAA in 0 h incubations, computed as described earlier (Broderick, 1987) except that a ratio of TAA: total N of 50 μmol mg^{-1} N was used for all forages. The undegraded fraction present at 0 h (B_0) was defined as $1 - A_0$. The fraction remaining undegraded at 2 h (B_2) was defined as $1 - A_2$, where A_2 was computed as described for A_0. Undegraded CP fractions were not

corrected for an undegradable fraction. Degradation rate (/h) was computed:

$$\text{Degradation rate } (k_d) = \frac{\ln B_2 - \ln B_0}{2} \quad (1)$$

Estimated rumen protein escape was computed:

$$\text{Estimated protein escape } \left(\frac{\text{g CP}}{\text{kg CP}}\right) = B_0 \times \left[\frac{k_p}{k_d + k_p}\right] \quad (2)$$

where B_0 was expressed as g CP/kg CP, k_p, the rumen passage rate, was assumed equal to 0.06/h. Passage rates of 0.05–0.07/h have been reported for rumen DM in early lactation dairy cows (Hartnell and Satter, 1979; Shaver et al., 1986).

Accessions from replicate plot harvests were incubated at 0 and 2 h in two separate sets of three IIV incubation runs; thus, effect of plot replicate was confounded with effect of run. For 18 accessions (17 PI and one cultivar), only one of the two plots survived the winter; neither plot survived for eight PI. Data were analyzed only for the 134 accessions [117 PI (29 from the red clover core collection; Koume and Quesenberry, 1993) and 16 cultivars or experimental lines of red clover plus one cultivar of lucerne] for which there were results from both plot replicates.

The General Linear Model of SAS (1989) was used in separate statistical analyses of data for: (1) all red clover accessions, (2) the 16 named cultivars or experimental lines plus the lucerne cultivar, and (3) the 29 PI from the core collection plus the lucerne cultivar. The models used for N concentration and proportion of CP in fraction B_0 included (df for accessions and cultivars only) accession (132, 29 and 16) and plot replicate (1 and 1). The models used for degradation rate and rumen escape included (df for accessions and cultivars or experimental lines only) accession (132, 29 and 16), field replicate (1 and 1), run-within-replicate (4 and 4) and accession-by-replicate interaction (132, 29 and 16). Where significant effects ($P < 0.01$ for accessions and the core collection, and $P < 0.10$ for cultivars and experimental lines only) were detected due to accession, mean separation was by LSD at $\alpha = 0.05$. Only mean results from the 133 accessions are presented; however, individual results from all accessions are available from the authors on request.

3. Results and discussion

Mean TN concentration ranged from 24.8 (PI 255184) to 32.8 g/kg DM (PI 245141); the effect of accession on TN content was significant ($P = 0.007$; Table 1). Fractions B_0 (proportions of CP present as intact protein) were in a very narrow range, from 959 to 972 g CP/kg CP, and were not significantly different among accessions ($P = 0.091$; Table 1). The effect of field plot replicate on the magnitude of fraction B_0 was significant ($P < 0.001$). However, numerical differences between plot replicates were very small: fraction B_0 averaged 965 and 969 g CP/kg CP for replicates 1 and 2, respectively; differences between the plot replicates for the different accessions ranged from 0 to 15 g CP/kg CP. Differences of this magnitude have little effect on the proportion of total protein escaping the rumen undegraded. Total N concentration among the PI from the core collection of red clovers ranged from 26.7 to 32.8 g/kg DM and was 31.9 g/kg DM for 'Dart' lucerne; effect of

Table 1
Mean composition and rumen in vitro crude protein (CP) degradation data for all samples of red clover germplasm (with data for 'Dart' lucerne presented separately)

Trait	Total *N* (g/kg DM)	Fraction B_0 (g CP/kg CP)	Degradation rate (/h)	Rumen escape (g CP/kg CP)
'Dart' lucerne	31.9	964	0.153	278
Red clover mean	28.1	967	0.108	355
S.E.M.	1.1	2	0.003	8
Minimum	24.8	959	0.088	287
Maximum	32.8	972	0.146	409
LSD (0.05)	3.0	6	0.009	22
Probabilities				
Accession	0.016	0.090	<0.001	<0.001
Replicate	0.372	<0.001	<0.001	<0.001
Run (replicate)			<0.001	<0.001
Accession × replicate			<0.001	<0.001

cultivar on TN content was significant ($P < 0.001$; Table 2). Fractions B_0 among core collection red clovers ranged only from 963 to 972 g CP/kg CP and were not significant; however, the small differences due to field plot replicate again were significant ($P < 0.001$; Table 2). Among the 16 cultivars and experimental lines, TN concentration ranged from 24.8 to 29.2 g/kg DM ($P = 0.089$) and fractions B_0 from 962 to 970 g CP/kg CP ($P = 0.732$; Table 3).

Significant effects ($P < 0.001$) of accession and plot replicate were found for both rumen degradation rate and estimated escape using the IIV system (Table 1). Values for these traits varied widely among the red clover germplasm: degradation rate ranged from 0.088 to 0.146/h and estimated rumen protein escape ranged from 287 to 409 g/kg CP (data not shown). A striking finding was that, numerically, the degradation rate observed for 'Dart' lucerne was more rapid (0.153/h), and estimated rumen escape was lower (278 g/kg CP), than for any red clover entry. Including the lucerne entry with the red clover accessions indicated that the protein degradation rate for that lucerne cultivar was significantly more rapid than 132 of the 133 red clover germplasm and rumen protein escape was significantly lower than 129 of the 133 red clovers (data not shown). Because plot replicate was confounded by IIV run, it was not possible to assess independently the statistical significance of plot effects on degradation traits. Previously, differences in microbial degradative activity among individual runs with different batches of rumen inoculum resulted in substantial differences in estimates of both rate and extent of protein degradation (Broderick et al., 1992). However, statistical analysis removed the effect of incubation run and small differences in degradation were detected (Broderick et al., 1992). Effects of run-within-replicate and accession-by-replicate also were significant ($P < 0.001$) for degradation rate and rumen escape. Significant effects ($P \leq 0.001$) of accession and plot replicate also were found for protein degradation traits for 'Dart' lucerne and the 29 red clovers from the core collection (Table 2) and the 16 red clover cultivars and experimental lines (Table 3). Only one of 29 core red clover PI was not degraded more slowly and did not have greater estimated rumen escape than 'Dart' lucerne (Table 2); degradation rates were slower and rumen escapes greater

Table 2
Summary of composition and rumen in vitro crude protein (CP) degradation data for 29 plant introductions (PI) from the red clover core collection plus 'Dart' lucerne

PI	Country of origin	Total N (g/kg DM)	Fraction B_0 (g CP/kg CP)	Degradation rate (/h)	Rumen escape (g CP/kg CP)
207520	Afghanistan	27.3gh	967	0.088p	396a
234836	Germany	29.5bcdefgh	970	0.092nop	391ab
217507	Denmark	27.8efgh	965	0.091op	389ab
245141	Switzerland	32.8a	969	0.093mnop	384abc
187284	United Kingdom	27.6fgh	972	0.094lmnop	383ab
307948	Spain	32.2ab	966	0.097jklmnop	381abcd
189174	The Netherlands	28.1defgh	967	0.096klmnop	380abcde
418889	Italy	29.0defgh	971	0.097jklmnop	377abcdef
228160	Former USSR	27.5gh	966	0.096klmnop	376abcdef
449326	Chile	28.4defgh	966	0.101ijklmn	373bcdef
220856	Portugal	29.1cdefgh	965	0.100ijklmno	370bcdefg
286116	Canada	28.0defgh	969	0.102ijklmn	365cdefgh
294481	Austria	26.7h	970	0.107fghij	364cdefgh
225119	Germany	27.7efgh	964	0.101ijklmn	363cdefgh
293591	Poland	30.1abcdefg	969	0.104hijkl	362cdefghi
250899	Iran	30.4abcdef	963	0.103ijklm	361defghi
384058	Poland	29.4bcdefgh	968	0.106ghijk	361defghi
371959	Bulgaria	27.3gh	963	0.106ghijk	358efghi
376880	New Zealand	28.3defgh	967	0.107fghij	356fghij
196424	Denmark	27.9efgh	966	0.108efghi	350ghijk
253583	Spain	27.4gh	965	0.108efghi	350ghijk
440737	Russian Federation	29.4bcdefgh	972	0.113defgh	346hijkl
306677	Ecuador	30.8abcd	968	0.116cdef	342hijklm
401469	Romania	29.0defgh	968	0.114cdefg	340ijklm
315538	Denmark	30.5abcdde	968	0.120cd	335jklm
286222	Canada	30.1abcdefg	968	0.117cde	333klm
419294	Greece	27.6fgh	964	0.122cd	326lm
266047	Poland	29.8bcdefg	967	0.123c	321m
315533	Bulgaria	27.8efgh	968	0.141b	293n
Dart (lucerne)	USA	31.9abc	964	0.153a	278n
Mean		29.0	967	0.107	357
S.E.M.		1.0	3	0.004	8
LSD (0.05)		2.9	8	0.010	23
Probabilities					
PI		0.008	0.641	<0.001	<0.001
Replicate		0.202	<0.001	<0.001	<0.001
Run (replicate)				<0.001	<0.001
PI × replicate				<0.001	<0.001

Means within columns for forage varieties with different letters differ ($P < 0.05$).

than the lucerne for all 16 named cultivars and experimental lines (Table 3). Others have found greater rumen degradability of protein in lucerne than red clover herbage (Broderick and Albrecht, 1997; Cassida et al., 2000). As in the analysis of all accessions, plot replicate and run-within-replicate were confounded by IIV run. The cultivar-by-replicate interaction

Table 3
Summary of composition and rumen in vitro crude protein (CP) degradation data for 16 samples of red clover cultivars or experimental lines plus 'Dart' lucerne

Cultivar or experimental line	Total N (g/kg DM)	Fraction B_0 (g CP/kg CP)	Degradation rate (/h)	Rumen escape (g CP/kg CP)
Arlington	25.6cd	967	0.098f	388a
Isi-84-Hk[a]	26.7bcd	962	0.100ef	370ab
Marathon	26.6bcd	967	0.101ef	373ab
Red Star	27.2bcd	969	0.102ef	365abc
Cherokee	29.2ab	966	0.103ef	364abc
Renegade	27.2bcd	966	0.103ef	362abcd
Acclaim	26.5bcd	968	0.104def	359bcd
FUS[a]	27.1bcd	969	0.105def	359bcd
Kenstar	26.2bcd	968	0.105def	357bcd
Atlas	26.9bcd	966	0.106def	356bcd
Persist	28.0bcd	968	0.108cde	356bcd
Concorde	28.2bc	967	0.109cde	349bcd
Redland III	27.7bcd	970	0.110cde	346bcde
Scarlett	27.3bcd	967	0.114bcd	339cde
VS-895[b]	28.2bc	970	0.117bc	336de
Reddy	24.8d	966	0.124b	321e
Dart (lucerne)	31.9a	964	0.153a	278f
Mean	27.4	967	0.110	352
S.E.M.	1.1	2	0.004	10
LSD(0.05)	3.3	7	0.011	28
Probabilities				
Cultivar	0.089	0.732	<0.001	0.001
Replicate	0.152	0.021	<0.001	<0.001
Run (replicate)			<0.001	<0.001
Cultivar × replicate			0.199	0.176

Means within columns for forage varieties with different letters differ ($P < 0.05$).
[a] Germplasm from Cebeco International Seeds, Halsey, OR.
[b] Germplasm from Cal West Seeds, Woodland, CA.

was not significant for either degradation trait ($P \geq 0.18$; Table 3) in the analysis of only the cultivars and experimental lines. However, PI-by-replicate interaction was significant ($P < 0.001$; Table 2) for both degradation rate and estimated rumen escape for the core collection of red clovers. Detection of significance for this interaction was surprising and may have been related to the power of the statistical analyses. The mean squares for PI were 2.4 (rate) and 2.0 (escape) times those for the same traits for the PI-by-replicate interaction. A substantial amount of variation was accounted for by the run-within-replicate term in the model. Omitting this term yielded a simpler analysis in which both PI and replicate were still highly significant ($P < 0.001$) for rate and escape, but in which the terms for PI-by-replicate interaction were not significant for either rate ($P = 0.371$) or escape ($P = 0.402$).

There was a wide range of degradation rates and rumen protein escapes estimated for the red clover germplasm in this sample set. The most rapidly degraded red clover accession (PI 306192) had a degradation rate 65% faster than the most slowly degraded red clover accession (PI 210370). Among the core collection of red clovers (Table 2), a similar difference in rate was observed between the most slowly degraded (PI 207520; Afghanistan) and most rapidly degraded red clover (PI 315533; Bulgaria). Rumen protein degradation is an important factor contributing to inefficient protein utilization in dairy cows. An increase from 287 to 409 g CP/kg CP for rumen protein escape would be of major nutritional importance. The NRC (2001) recommended feeding diets with undegraded CP contents of about 35% for lactating dairy cows. Diets based on forage with undegraded CP of 29% would require supplementation with either costly proteins that are resistant to rumen degradation or with greater amounts of typical proteins with higher rumen degradabilities, such as solvent soybean meal (undegraded CP = 31%; NRC, 2001). A diet based on a forage with undegraded CP of 41% could be fed without costly protein supplements.

Frequency distributions of degradation rates and estimated rumen protein escapes for the 133 red clover entries are shown in Fig. 1. These distributions were normal for both degradation traits, with only slight skewness to more rapid degradation rates (Fig. 1A) and lower estimated protein escapes (Fig. 1B). Previously, screening of accessions of *M. sativa* showed a pattern skewed toward slower rates of rumen protein degradation (Broderick and Buxton, 1991). Results from that trial (Broderick and Buxton, 1991), and another involving the screening of a large number of lucerne cultivars using a similar IIV technique (Tremblay et al., 2000), indicated that it would be feasible to select for reduced protein degradation in lucerne. The wide range of rumen degradability among this large set of red clover entries shown in Fig. 1 suggests that selection among and/or within accessions of red clover could effect genetic shifts for this characteristic, allowing development of lines of red clover germplasm with improved protein value for ruminants.

It is not known what accounted for the observed differences in protein degradability among the red clover entries. However, these effects may be related partly to the lower protein breakdown that occurs when red clover is ensiled (Albrecht and Muck, 1991; Owens et al., 1999). Preliminary evidence (Jones et al., 1995a) suggests that *o*-quinones are generated from reaction of caffeic acid and similar compounds with a soluble enzyme system, polyphenol oxidase (PPO), naturally present in red clover. Interaction of the *o*-quinones with both proteases and substrate proteins is believed to account for the consistently lower non-protein N content of red clover silage (R.D. Hatfield, personal communication). A range of PPO activities of from 6 to 9 units/g leaf tissue was reported in a set of 24 red clover accessions (Jones et al., 1995b). Presence of the PPO in red clover tissue may have generated *o*-quinones that reacted with fraction 1 and other proteins that are substrates for microbial proteases in the rumen. Fraction 1 protein, which is principally the photosynthetic enzyme ribulose bisphosphate carboxylase, is the most abundant protein in legume tissue (Mangan, 1982). Fraction 1 protein was degraded very rapidly by mixed rumen organisms (Nugent et al., 1983). Zhu et al. (1999) have suggested a role in rumen protein degradation for the proteases released from plant cell rupture; if this were correct, *o*-quinones from PPO action could have inhibited red clover proteases in a manner analogous to what happens in the silo and, thus, may have reduced protein breakdown in the rumen.

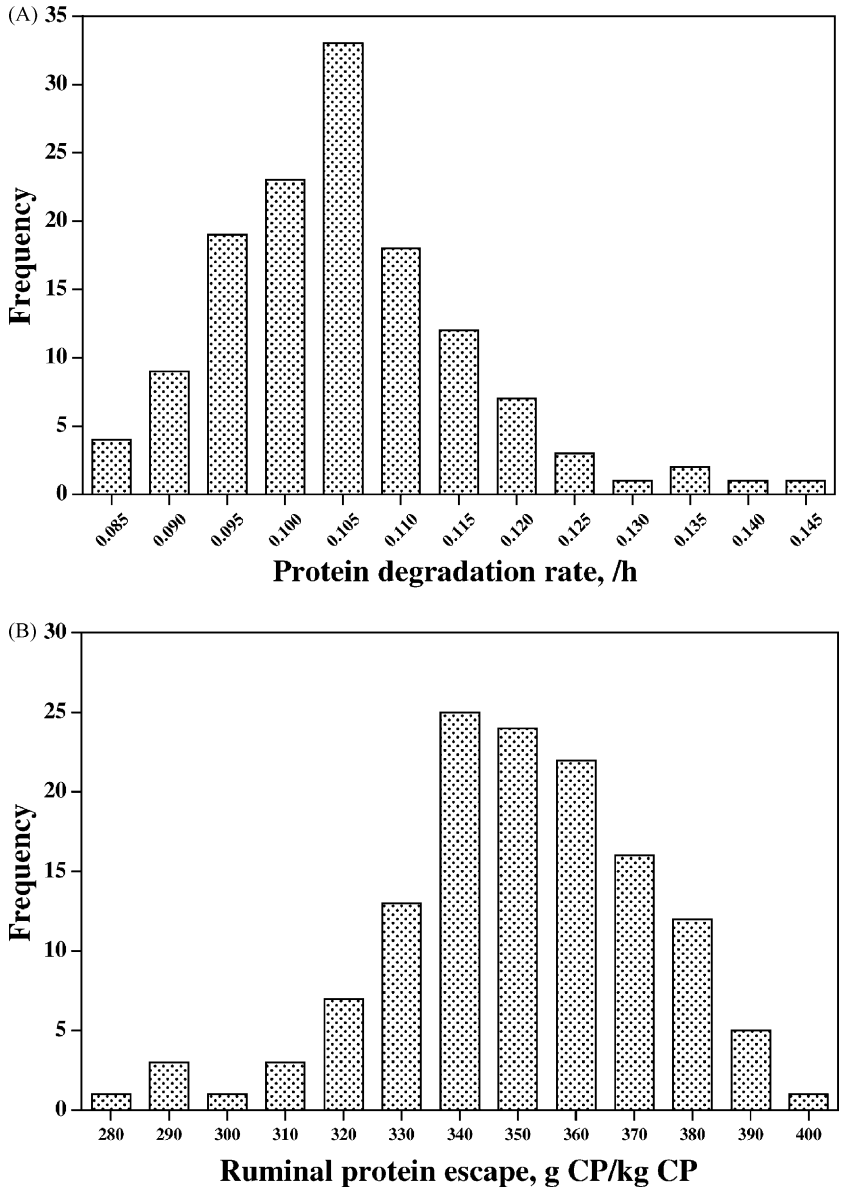

Fig. 1. Frequency distributions of: (A) in vitro rates of rumen protein degradation, and (B) estimated rumen protein escape for 133 entries of red clover germplasm (*T. pratense* L.). Frequencies for protein degradation rates: (A) are given for ranges of 0.005/h, from 0.085–0.090 to 0.145–0.150/h, and for rumen protein escapes; (B) are given in crude protein (CP) ranges of 10 g CP/kg CP, from 280–290 to 400–410 g CP/kg CP.

4. Conclusions

In vitro and chemical analyses were conducted to assess the potential protein value of forage variation in rumen protein degradability among 133 entries of red clover relative to a standard cultivar of lucerne. There were only small differences in total N content and no differences in degraded protein present at 0 h (fraction A) among the red clover entries. Protein degradation rate, determined in rumen in vitro incubations, was more rapid for the lucerne cultivar than for any red clover entry. Protein degradation rate ranged from 0.088 to 0.146/h and estimated rumen escape ranged from 287 to 409 g CP/kg CP among the red clover entries. The frequency distributions for the red clover entries for protein degradation rate and rumen protein escape were normal, showing relatively little skewness. These results suggested that conventional plant breeding techniques could be used to develop lines of red clover forage with improved protein utilization in ruminants.

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