Determination of protein degradability of alfalfa hay via buffer or protease

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ABSTRACT
This study was conducted to determine the effect of different vegetative periods on protein fractions of alfalfa hay and to compare two different methods for estimation of its protein degradability. In this study, 44 alfalfa hay samples cut in late vegetative, late bud, early bloom and late bloom were used. Crude protein decreased with advancing maturity (p < .05), but neutral detergent insoluble nitrogen and acid detergent insoluble nitrogen were similar. Protein degradability of the samples was estimated using Streptomyces griseus protease or borate-phosphate buffer, kinetic model, as described in Cornell Net Carbohydrate Protein System (CNCPS). Alfalfa hay samples were subjected to proteolysis for 30 h with 0.115 U/mL or 0.230 U/mL of the protease at pH 6.8. There was a moderate correlation (0.66, 0.72) between enzymatic and buffer method estimates with protein degradation. As vegetation progresses, rapidly degradable fraction (A) decreased (p < .05) and undegradable fraction (C) increased (p < .05), while potentially degradable fraction (B) was unchanged. Rumen degradable protein content decreased (p < .05) with advancing maturity. The enzyme/duration limits should be standardised and investigated whether the protease enzyme alone is sufficient for all forages.

Introduction

Although the in situ method is probably accepted as the primary reference method for the determination of ruminal protein degradability, important variations in measured levels might be encountered between laboratories, or even within the same laboratory. Most of these variations depend on the used ration and the animal and its controlling are difficult. Besides this, the pore width of the bags, the particle size of the samples, the sample size, the type of the wash and the evaluation of the data are not uniform. Furthermore, as the quantities of the water-soluble substances and cellulose are high and the protein quantity is low in the roughage, the degradability might be wrong. Additionally, it is not a practical method regarding the commercial laboratories. In respect of the determination of the protein degradability, there are certain chemical methods, which use the reactions with the acidic solutions like trichloroacetic acid (TCA) and tungstic acid. With these chemical methods, the solubility properties of the forage in water, in the artificial rumen fluid, in various buffer solutions, at various pH levels and incubation periods, are determined. However, it was reported that the degradabilities determined with these methods might deviate from the in vivo or in situ methods in some forages and the usage of the enzymes might be more advisable (Aufrere et al. 1991).

In vitro studies were carried out with several enzymes, as there was a need to develop certain methods for the determination of the rumen protein degradability, which were cheap, easy to standardise and independent from the animal. For this purpose, protease obtained from the Streptomyces griseus had been used by several researchers (Krishnamoorthy et al. 1983; Mahadevan et al. 1987; Aufrere and Cartailler 1988; Aufrere et al. 1991; Terramoccia et al. 1992; Susmel et al. 1993; Cone et al. 1996; Licitra et al. 1996, 1998, 1999).

In the studies conducted to determine protein degradability, the pH of the buffer solutions varied between 4.8 and 8 (Krishnamoorthy et al. 1983; Licitra et al. 1996, 1998, 1999). Also, different concentrations of the enzymes were evaluated in these studies.
(usually 0.33 UE/mL) (Krishnamoorthy et al. 1983; Licitra et al. 1998, 1999). Cone et al. (1996) worked with a concentration of 4.6 U/mL equivalent to 20 mg/l and Aufrere and Cartailler (1988) with 2.6 U/50 mL. Licitra et al. (1999) experimented with enzyme concentrations of 0.33, 1.0, 2.4, 3.3 and 6.6 U/mL and reported that the optimal enzyme concentration was 1.5 U/mL. The duration of the enzyme reaction depended on the forage and it changed between 0.5 and 48 h in the studies (Krishnamoorthy et al. 1983; Aufrere et al. 1991; Licitra et al. 1998, 1999).

Edmunds et al. (2012) reported that the enzymatic method was more compatible with the in situ method. However, the enzymatic methods had two limitations. As also the proteolytic enzyme degraded itself, the enzyme activity diminished with the prolongation of the incubation period. Therefore, 1- to 2-hour incubation periods were more appropriate for. In the forages, there were protein fractions, which degrade in different times. The forage proteins degraded first fast then the degradation slowed down. Therefore, a longer treatment with the enzyme is required. The second limitation was that the degradation kinetics and enzyme activity did not match, which meant that enzyme activity level might restrict the degradation rate (Mahadevan et al. 1987).

In the studies, which were conducted to compare the protein degradability in enzyme and buffer solutions, it was demonstrated that enzyme solution had no advantage especially in mixed forages, there might be deviations in the incubation time of the roughages (DeBoever et al. 1997), there was a significant correlation between the borate–phosphate buffer solution \( r = 0.84 \), protease added to buffer solution \( r = 0.84 \) and the in situ degradability (Susmel et al. 1993).

Madsen and Hvelplund (1994) reported that there were significant differences between the laboratories and the pouches, filter paper and washing methods should be uniform and they should be standardised with the usage of standard forages. Mahadevan et al. (1987) found out that the protein degradability determined with the Streptomyces griseus protease and the protease, which was prepared from the rumen fluid, were different in the same forage types. It was also reported that the activity might differ between proteases obtained from Streptomyces griseus, Bacteroides amylophilus and rumen fluid (Stern et al. 1997). The first of two objectives of this study was to determine the effects of stage of maturity on protein fractions and rumen degradation characteristics in alfalfa samples and the second of them was to compare two methods for estimation of ruminal protein degradability.

Materials and methods

Sample collection

In this study, 44 samples of alfalfa hay were obtained from alfalfa growers in Konya \( (37°37′42.4″N, 33°46′40.2″E) \), Turkey. The alfalfa used was a local variety. It was sown at a seeding rate of 20 kg/h, fertilised with 150 kg/h of diammonium phosphate (18% nitrogen, 46% phosphorus content). It was irrigated one week before the harvesting.

Alfalfa hay samples were harvested by hand collection 5 cm stubble height at late vegetative, late bud, early bloom and late bloom stages using a square frame of \( 50 \times 50 \) cm. Fresh alfalfa samples were oven-dried at 65 °C. Then they were ground with 1-mm sieve in a laboratory mill (Retsch SM 100).

Chemical analysis

All analyses were carried out in duplicate. Crude protein (CP; AOAC 981.10) and dry matter (DM; AOAC 950.46) were determined in the alfalfa hay samples according to the standard methods in AOAC (2005). Neutral detergent fibre assayed with heat stable alpha amylase (aNDF) and acid detergent fibre (ADF) was determined with the ANKOM A200 Filter Bag technique (Ankom Technology, Fairport, NY) according to Van Soest et al. (1991). Neutral detergent insoluble CP (NDICP) and acid detergent insoluble CP (ADICP) were determined by the Kjeldahl-N analysis of the aNDF and ADF bag residues, respectively, as described by Licitra et al. (1996).

In all alfalfa hay samples, protein fractions were partitioned according to the NRC (2001). Values of protein soluble in the borate–phosphate buffer solution and trichloroacetic acid solution \( (\text{A} = \text{PNP}) \) and values of protein precipitable in TCA \( (B1) \) and NDIN and ADIN \( (C) \) values were used to calculate the \( B2 = (\text{CP-A + B1 + B3 + C}), B3 = (\text{NDIN-ADIN}) \) (Krishnamoorthy et al. 1982; NRC 2001).

Protein degradability

The protein degradability was determined with the in vitro enzymatic method using the \( S. \) griseus protease Type XIV (Sigma Chemical, Catalogue No. P5147, 4.9 units/mg) (Licitra et al. 1998, 1999). Approximately, duplicated 0.5 g alfalfa hays were weighed and transferred to a 100 mL capped plastic container. Forty mL of borate–phosphate buffer solution \( (\text{pH} = 6.8) \) was added and the container was placed in a water bath with an adjusted temperature of 39 °C for one hour. Then the freshly prepared enzyme solution, containing
2 units and 4 units of protease enzyme per mL, 2 or 3 mL were added according to the true protein level of the alfalfa (Licita et al. 1998), and shaken gently. Incubation fluid contained the concentration of enzyme 0.115 U/mL or 0.230 U/mL. These concentrations were equivalent to 13 and 26 units of enzyme for 1 g of true protein. From the results of the protein analysis, it was estimated that the alfalfa contained about 70% true protein. The samples, which were placed into the same environment for 30 hours, filtered through a Whatman 54 filter paper using a mild vacuum. The residue on the filter paper was washed with 250 mL cold water. The papers were folded with its content and transferred to the Kjeldahl protein tube and analysed for protein. The undegraded protein levels were determined and protein degradabilities were calculated (Licita et al. 1998):

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\text{CP Degradability, } \% = \frac{(\text{CP} - \text{undegraded CP})}{\text{CP}} \times 100
\]

**Statistical analysis**

Relative changes of parameters such as CP, NDICP, ADICP, RDP, DIP2, DIP4 and protein fractions were evaluated using 4 × 2 factorial design in alfalfa samples obtained from different vegetative periods. The obtained data were evaluated using one-way analysis of variance and Pearson’s correlation analysis method (SPSS v.22).

**Results**

The comparison of the analysis results, which were performed with total 44 alfalfa hay samples with different maturity stages, regarding CP, neutral detergent insoluble nitrogen, acid detergent insoluble nitrogen, acid detergent insoluble protein and RDP are shown in Table 1. The protein fractions, which were determined with the analyses using the buffer solution and trichloroacetic acid solutions and the rumen degradable protein ratios determined with the protease enzyme are shown in Table 2. The accepted Kp values (the passing rate of the forage through the rumen per hour) were 5.5 for the RDP calculation. As multipliers for the KdB1, KdB2 and KdB3 in the estimation of RDP, 260, 9.5 and 0.3 were used (NRC 2001). The rumen degradable protein (RDP) was between 68.1 and 70.7%. It was decreased with progression of maturity (p < .05). Similarly, rumen degradable protein (DIP) values determined by the enzymatic method (65.4–77.2%) were declined with maturity of alfalfa (p < .05). Differences and correlations (r = 0.51–0.53) between degradable protein rates obtained from the buffer solution or enzymatic methods are shown in Table 4.

### Table 1. Concentrations of CP and cell-wall bound protein in alfalfa hay samples (% DM).

| Maturity stage       | n | CP     | NDICP | ADICP |
|----------------------|---|--------|-------|-------|
| Late vegetative      | 11| 23.3a  | 2.03  | 1.29  |
| Late bud             | 11| 19.9bc | 1.88  | 1.17  |
| Early bloom          | 11| 20.6b  | 2.39  | 1.36  |
| Late bloom           | 11| 18.8bc | 2.17  | 1.36  |
| SEM                  |   | 0.35   | 0.09  | 0.04  |
| p                    |   | <.01   | .19   | .42   |

a, b, c: p < .05; CP: crude protein; NDICP: neutral detergent insoluble CP; ADICP: acid detergent insoluble CP.

### Table 2. Protein and protein fractions in alfalfa hay samples (% DM).

| Maturity stage       | n | A     | B1    | B2    | B3    | B    | C    |
|----------------------|---|-------|-------|-------|-------|------|------|
| Late vegetative      | 11| 33.7a | 1.9   | 55.5  | 3.3   | 60.7 | 5.6b |
| Late bud             | 11| 31.9ab| 3.0   | 55.6  | 3.6   | 62.9 | 3.9b |
| Early bloom          | 11| 30.5a | 4.1   | 53.7  | 5.0   | 62.8 | 6.7ab|
| Late bloom           | 11| 29.4a | 3.8   | 55.1  | 4.4   | 63.3 | 7.3a |
| SEM                  |   | 0.49  | 0.35  | 0.70  | 0.41  | 0.53 | 0.23 |
| p                    |   | <.01  | .10   | .76   | .48   | .33  | .04  |

a, b, c: p < .05; A: NPN; B: true protein; B1: protein precipitable in TCA; B2: protein precipitable in acetic acid according to the CNCPS (The Cornell Net Carbohydrate and Protein System) in NRC (2001) are listed in Table 2. According to the maturity stages of alfalfa, fraction A values were between 29.4% and 33.7%. There was a regular decline in A fraction as plant maturity advanced (p < .01). There was no difference in the fractions B1, B2 and B3 regarding the cutting maturity, also B fraction was not affected by maturity stage, nevertheless, it was increased slightly (from 60.7% to 63.3%) (Table 2). In the in situ method, the fraction A is highly influenced by the pore diameter of the bags, the type or the duration of washing. The protein fraction C was increased (5.6–7.3%) as alfalfa maturity advanced, as expected (p < .05).

Rumen degradable protein rates estimated from protein fractions determined with the borate–phosphate buffer and trichloroacetic acid solutions and the rumen degradable protein ratios determined with the protease enzyme are shown in Table 2. The accepted Kp values (the passing rate of the forage through the rumen per hour) were 5.5 for the RDP calculation. As multipliers for the KdB1, KdB2 and KdB3 in the estimation of RDP, 260, 9.5 and 0.3 were used (NRC 2001). The rumen degradable protein (RDP) was between 68.1 and 70.7%. It was decreased with progression of maturity (p < .05). Similarly, rumen degradable protein (DIP) values determined by the enzymatic method (65.4–77.2%) were declined with maturity of alfalfa (p < .05). Differences and correlations (r = 0.51–0.53) between degradable protein rates obtained from the buffer solution or enzymatic methods are shown in Table 4.
Discussion

As shown in Table 1, the CP content decreased significantly with the maturing of alfalfa plants. Crude protein was 23.1% DM for late vegetative stage. This value was in the range of 21.7–30.0% given in the NRC (2000) table for the alfalfa hay at vegetative stage. In the late bud stage, CP was significantly reduced to 19.9% DM. This value was slightly greater than values reported by Pop et al. (2010) and close to the values obtained by Yu et al. (2003) with alfalfa samples at same maturity stage. It was also within the range (19.5–22.0% DM) reported by Yari et al. (2012b). The protein levels may change day to day in the late bud stage as shown in the study of Pop et al. (2010). Besides, the factors such as the variety of alfalfa, irrigation, fertilisation, drying, sampling, etc., may affect the protein level of the plant. Regarding the CP level at early bloom stage, we obtained a greater CP level (20.6%) than those reported by Yu et al. (2003) and Yari et al. (2012b) or similar to those at feed tables in NRC (2000). Although Yu et al. (2003) reported a significant decline in CP after the transition from the bud to bloom stage, the same decline was insignificant in the present study.

In the contrary to the reports of Yu et al. (2003), Yari et al. (2012b), in this study, the NDICP was not changed with the advancing maturity. It was measured at late vegetative, late bud, early bloom and late bloom stages as 2.03%, 1.88%, 2.39% and 2.17%, respectively (Table 1). The greatest NDICP ratio was measured in early blooming. As known, NDIN is a protein bound to the cell wall, degraded slowly in the rumen and mostly digested in the intestinal tract. The NDICP levels were lower than the levels of NRC (2000) feed tables. The ADICP, which was also bound to the cell wall, damaged by the heat and may not be utilised by the animal, was measured between 1.17% and 1.36%. Although ADIN contributes to the increase of the bypass protein, its excess amount decreases the digestion of the proteins. Concentrations of ADICP were slightly lower than those in feed tables (NRC 2000).

The levels of fractions A, B1, B2, B3 and C, which were determined with the buffer solution in alfalfa hays by Gosselink et al. (2004) were similar to our study. However, different results might be obtained with the buffer solution. Fortina et al. (2003) determined the levels of fraction A, B and C as 22.8%, 66.1% and 11.3% and Gosselink et al. (2004) as 28.0%, 66.6% and 5.4%, respectively.

In the in situ method, it was reported a greater A fraction and lower B fraction (Aufrere et al. 1994; Coblelntz et al. 1999). In some of these studies (Aufrere et al. 1994; Coblenz et al. 1999), the C fraction was similar to our values and was consistent with our study. Using in situ method, Janicki and Stallings (1988) found values very close to our study (fraction A: 29.4% and fraction B: 61.6%).

The rumen degradable protein (RDP) was lower in this study than those of Coblenz et al. (1999), NRC (2000). However, RDP estimates were consistent with those of Yari et al. (2012a). There were a significant difference and a significant correlation ($r = 0.51–0.53$) between RDP which was estimated as in NRC (2001) model considering a 5.5% rate of passage and DIP2 or DIP4 which was determined using S. griseus protease. The RDP mean was higher than DIP2 which was determined using an enzyme concentration of 2 U/mL, but lower than RDP4 (twofold enzyme, 4 U/mL) ($p < .001$). Similarly, Licitra et al. (1998) also found the higher protein degradability (65.6% vs 58.2%) in alfalfa hay with the higher concentration of enzyme. They used a concentration of 24 U for 1 g true protein. In our study, enzyme concentrations for 1 g true protein were 13 and 26 units. Terramoccia et al. (1992) compared the enzymatic methods containing in sacco and S. griseus proteases and they found out that the degradabilities in alfalfa hays of 78% and 76%, respectively, as close to our values. Janicki and Stallings (1988) measured the RDP value of 73.7% after a treatment with the protease enzyme for 24 and 48 hours, which was close to the value in this study. In our study, the pH of the buffer solution was adjusted to 6.8.

Conclusions

Crude protein, protein fraction A and RDP decreased, but protein fraction C increased with advancing maturity in alfalfa hay.

An important correlation was determined between the compared buffer solution method and enzymatic method. As enzymes are expensive and should be freshly prepared, the buffer solution is more practical for the determination of the protein fractions. But, to estimate the RDP using buffer solution method it must be done with a few extra N analyses.
Due to various problems in in situ method, in vitro methods might be considered as more practical. Although several investigators reported that enzymatic method was more reliable, in our study, we used more enzyme than stated in the literature and the degradability was increased after using a double concentration of enzyme on the alfalfa hay incubated 30 hours long. Therefore, we believe that the enzyme/duration limits should be standardized and it should be determined whether the usage of only protease enzyme would be sufficient not only for alfalfa but also for all forages with different degradation kinetics.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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