Initial Rates of Degradation of Protein Fractions from Fresh, Wilted, and Ensiled Alfalfa

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ABSTRACT

Initial rates of in vitro degradation of alfalfa proteins were studied. Fresh, 24-h wilted, and ensiled forages were homogenized before analysis for total proteins. Some of the homogenates were fractionated by differential solubility in 10 and 40% ammonium sulfate, followed by ultrafiltration of the 40% salt-saturated solution. The protein fractions obtained were chloroplast membrane proteins (fraction 3), soluble proteins from plant cell cytoplasm and the chloroplast (fraction 2), and proteins remaining soluble in the extracted 40% salt-saturated solution (fraction 2B), respectively. Total and fraction 3 silage proteins were degraded faster than the respective fresh and wilted proteins. There were no treatment effects on the rates of degradation of the soluble proteins of fractions 2 and 2B. Protein fractions from fresh and 24-h wilted alfalfa degraded, from greatest to least, in the following order: fractions 2, 2B, and 3. Degradation rates for fractions 2 and 2B of ensiled forages were similar but greater than that of fraction 3. Alfalfa proteins were degraded rapidly in the rumen, and soluble proteins were degraded faster than the chloroplast membrane proteins. Ensiling of alfalfa increased the rate of degradation of chloroplast membrane proteins, but neither wilting nor ensiling affected degradation rates of the soluble protein fractions 2 and 2B.

(Key words: degradation rate, alfalfa, protein fractions)

Abbreviation key: F = fraction (used with number).

INTRODUCTION

Degradation of digestible protein in excess of microbial needs results in excessive N loss in the rumen. Consequently, current feeding systems evaluate dietary protein for its resistance to ruminal microbial proteases. The extent of ruminal protein degradation is determined by the rate of digesta passage and by the rate of ruminal degradation. Protein sources that are resistant to ruminal microbial degradation increase the amount of dietary proteins that enter the small intestines. Thus, the influence that the rate of protein degradation has on dietary protein degradation in the rumen is of primary concern and provides valuable information for ration formulation.

Forages contain a variety of proteins that probably degrade at different rates in the rumen (13). Previous forage protein degradation studies focused on fraction (F) 1 (ribulose-1,5-biphosphate carboxylase oxygenase enzyme) leaf protein from fresh herbage (6, 18).
The practice of forage wilting and fermentation to silage undoubtedly alters the conformation of forage proteins. Little information is available comparing the degradation behavior of the proteins in fresh and conserved forages. A modified fractionation procedure (12) was used to group numerous forage proteins according to their differential solubility in ammonium sulfate. The objectives were to determine the initial rates of degradation of alfalfa proteins in vitro and to determine the effect of wilting and ensiling on the initial rates of degradation of the alfalfa proteins.

MATERIALS AND METHODS

A second-cutting prebloom alfalfa (Medicago sativa L. cv., 'Pioneer') was wilted for 24 h before ensiling 500 kg (in duplicate) in 1-m diameter x 2 m high plastic-lined (cardboard concrete forming) tubes. Samples for fractionation were collected at three stages of preservation: before wilting, immediately after wilting, and 60 d after ensiling. All forages were immediately cooled on ice and then frozen in liquid N before storage at -20°C.

About 250 g of fresh and 24-h wilted alfalfa and 500 g of ensiled alfalfa were homogenized in a buffer at 4°C before analysis for total proteins. The homogenizing buffer contained .05 M phosphate buffer at pH 7.4, a chelator, antioxidant, and enzyme inhibitors (12). Fractionation procedure for the in vitro studies reported here did not employ polyvinylpyrrolidone to remove phenolics. About one-fourth of the homogenate was dialyzed and lyophilized; the remainder was fractionated by differential solubility in 10, 24, and 40% ammonium sulfate concentrations, followed by ultrafiltration of the extracted solution. The protein fractions from the fractional salt precipitation were designated as chloroplast membranes (F3), ribulose-1,5-biphosphate carboxylase oxygenase (F1), and mixtures of soluble proteins originating from both chloroplasts and cytoplasm (F2 and F2B). The F1 protein was not used in this study.

The following analyses were made on the protein samples: protein (21), lipid (2), chlorophyll pigments (15), and ashing at 500°C overnight. The method of Mahadevan et al. (10) was used to extract microbial protease from digesta obtained from the rumen of a heifer that was adapted to and maintained on alfalfa hay and cubes. Protease activity was determined by hydrolyzing 5 mg of casein with increasing concentrations of the protease for 1 h. Specific activity was expressed based on the fact that 1.2 mg of protease hydrolyzed 1 mg of casein in 1 h.

In vitro degradations were carried out according to methods of Mahadevan et al. (10). Incubation intervals of 0, .5, 1, 2, 4, and 6 h were used. Incubations were carried out in 250-ml Erlenmeyer flasks using 1 M potassium phosphate buffer at pH 6.8. The medium was shaken gently and continuously in a controlled environment, shaker incubator (New Brunswick Scientific Co., Inc., Edison, NJ) maintained at 39°C. To correct for substrate and protease autolysis during digestion, three incubations were carried out for each run: 1) medium and protease, 2) medium and substrate, and 3) medium, substrate, and protease. Aliquots of the incubation medium were withdrawn, and the reaction was stopped using a solution of ice-cold 10% TCA solution. After setting on ice for 30 min, the TCA-precipitated protein was centrifuged, and the hydrolyzed protein products were estimated by the modified ninhydrin method (18). Absorbances at 570 nm were calculated as

\[
\text{absorbance} = \text{tube 3} - (\text{tube 2 + tube 1})
\]

where tube 3 had medium, substrate, and protease; tube 2 had medium and substrate; and tube 1 had medium and protease.

The ninhydrin-positive materials were quantitated as glycine equivalent micromoles of AA (10). The initial rates of protein degradation then were estimated in the 1st h of hydrolysis using regressions to determine the slopes.

Statistical Analyses

Data were analyzed as a split-plot design replicated three times. The forage treatments were the main plots, tested by main plot error, and protein fractions were the subplots. The general linear models (19) procedure was employed for the model:

\[
Y_{ijk} = \mu + \alpha_i + \beta_j + \epsilon_{ij} + \tau_k + (\beta\tau)_{jk} + \epsilon_{ijk}
\]

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\[
\begin{align*}
\mu &= \text{general mean effect}, \\
\alpha_i &= \text{replicate effect (i = 1, 2, and 3)}, \\
\beta_j &= \text{forage treatment effect (j = 1, 2, and 3)}, \\
\epsilon_{ij} &= \text{main plot error}, \\
\tau_k &= \text{protein fraction effect (k = 1, 2, \ldots, 4)}, \\
(\beta \tau)_k &= \text{interaction effect of forage and protein}, \\
\epsilon_{ijk} &= \text{split-plot error},
\end{align*}
\]

Significance was set at 5%. Means of forage treatments and protein fractions were compared by Student-Neuman-Keuls test.

RESULTS AND DISCUSSION

Table 1 shows the chemical composition of the forage samples homogenized for this study. The forage was harvested at 24% DM and wilted to 64% DM before ensiling. Results of the partial chemical composition of the protein fractions employed for the in vitro study are given in Table 2. The protein estimates are similar to those reported by others (5, 13). The estimates of percentage protein indicated that the protein precipitation procedure used did not yield pure protein fractions. However, the data are typical of the forage fluid released by ruminants during mastication (14). Protein percentages were higher in the soluble proteins F2 and in F2B versus F3. The F3 is chloroplast membranes containing about 18% lipid of which 65 to 70% is unsaturated (11, 13). The decrease in extractable lipid after ensiling was probably a consequence of lipid peroxidation (11). High chlorophyll concentrations of F3 partly explained the high ash content of F3 versus both F2 and F2B because Mg$^{2+}$ forms the core of the chlorophyll porphyrin ring (13, 22). Because F3 was recovered by 10% ammonium sulfate and initial centrifugation of the homogenate, high ash could also be due to sedimentation of forage debris.

Figure 1 illustrates the curvilinear in vitro degradation profiles of the total proteins, F2, F2B, and F3, for three forage treatments: fresh, wilted, and ensiled. Linear initial degradations during the 1st h are illustrated. Except for F3 proteins (Figure 1D), which continued to be degraded slowly, maximum velocity was attained within 2 to 3 h of incubation. The magnitude of the intercepts indicated that substrate and protease preparations were adequate because, initially, all fractions had ninhydrin-positive materials below 0.05 absorbance units. High blank or initial impurities compromised reaction rates (10).

The rate of degradation of protein (milligrams per 5 h) is shown in Table 3. Substantial forage protein is usually degraded within the first 5 h in the rumen (14). Total fresh and wilted proteins were degraded similarly and had significantly lower degradation rates than did total silage protein (Table 3). Although not significant, the wilted total protein tended to degrade faster than fresh protein. This trend was not unusual for silage protein (3, 4, 22).

### Table 1. Forage chemical composition.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fresh</th>
<th>Wilted</th>
<th>Silage</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM, %</td>
<td>23.6</td>
<td>64.2</td>
<td>65.2</td>
</tr>
<tr>
<td>CP, % of DM</td>
<td>21.3</td>
<td>19.9</td>
<td>20.3</td>
</tr>
<tr>
<td>HWIN, % of DM</td>
<td>19.6</td>
<td>17.5</td>
<td>6.4</td>
</tr>
<tr>
<td>ADF, % of DM</td>
<td>31.1</td>
<td>34.1</td>
<td>37.5</td>
</tr>
<tr>
<td>NDF, % of DM</td>
<td>43.9</td>
<td>44.3</td>
<td>47.9</td>
</tr>
<tr>
<td>Ash, % of DM</td>
<td>10.7</td>
<td>10.5</td>
<td>11.1</td>
</tr>
</tbody>
</table>

1HWIN = Hot water-insoluble N.

### Table 2. Mean chemical composition of alfalfa fractionated protein samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Parameter</th>
<th>Protein 1</th>
<th>Lipid 1</th>
<th>Ash 1 (%) of lyophilized protein fraction DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total fresh</td>
<td>43.7</td>
<td>10.1</td>
<td>9.3</td>
<td></td>
</tr>
<tr>
<td>Total wilted</td>
<td>45.5</td>
<td>13.5</td>
<td>10.9</td>
<td></td>
</tr>
<tr>
<td>Total silage</td>
<td>40.5</td>
<td>1.8</td>
<td>12.9</td>
<td></td>
</tr>
<tr>
<td>Fresh F3</td>
<td>42.7</td>
<td>16.0</td>
<td>11.6</td>
<td></td>
</tr>
<tr>
<td>Wilted F3</td>
<td>37.0</td>
<td>19.3</td>
<td>11.9</td>
<td></td>
</tr>
<tr>
<td>Silage F3</td>
<td>36.0</td>
<td>8.2</td>
<td>13.8</td>
<td></td>
</tr>
<tr>
<td>Fresh F2</td>
<td>67.7</td>
<td>4.9</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>Wilted F2</td>
<td>69.0</td>
<td>5.1</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>Silage F2</td>
<td>62.4</td>
<td>5.0</td>
<td>7.0</td>
<td></td>
</tr>
<tr>
<td>Fresh F2B</td>
<td>57.4</td>
<td>ND 4</td>
<td>6.4</td>
<td></td>
</tr>
<tr>
<td>Wilted F2B</td>
<td>53.2</td>
<td>ND 4</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>Silage F2B</td>
<td>49.4</td>
<td>ND 4</td>
<td>1.6</td>
<td></td>
</tr>
</tbody>
</table>

1Protein fractions were precipitated at 10 (F3) or 40% (F2) or recovered from the extracted 40% ammonium sulfate solution (F2B).
2Protein assayed by bicinchoninic acid.
3Corrected for chlorophyll contamination.
4Not determined.

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From the point of cutting the forage through wilting to silage, definite biochemical changes resulted from loss of cell compartments (23). For example, the changes in the proteins resulted in loss of structural conformation (denaturation), which increased peptide bond cleavage by proteases (3). Proteins with little ordered structure were hydrolyzed more rapidly than those that possessed tertiary and quaternary structures (1). Similar but faster rates of degradation of F2 and F2B have been reported previously in bloat studies (7). In our study, degradation of F1 was not tested because the proteolysis was rapid even when F1 was stored at -20°C. Thus, little can be inferred from rates of proteolysis of F1 compared with those of F2. Previous work (7, 8) suggests that F1 degradation rate exceeds that of F2. However, the soluble proteins, F2 and F2B, were degraded faster than the insoluble F3. For insoluble proteins of F3, solubility or protease access may be a factor that limits degradation. Also, F3, which is mostly membrane lipoproteins, can sterically protect bonds that are susceptible to hydrolysis through hydrophobicity. Enzymatic activity in F2 is considerable (22). Whether this activity augmented the faster rate of degradation in vitro was not evident; no increase in autolysis occurred in substrate blank tubes. The F2 is a composite of chloroplast enzymes and other cytoplasmic-soluble proteins that constitute about 25% of total leaf protein (13). Thus, the presented rates for both F2 and 2B were pooled rates. The constituent proteins individually contribute little to the bulk of dietary protein, but different soluble proteins degrade at different rates (9, 16). Hence, the components of these soluble fractions, albeit small, may contribute less to bypass protein.

There were no treatment differences in F2 proteins. Regardless of treatment, forage F2
TABLE 3. Rates of alfalfa protein degradation.

<table>
<thead>
<tr>
<th>Fraction (F)</th>
<th>Forage treatment</th>
<th>Rate (mg of proteins degraded/5 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh</td>
<td>Wilted</td>
</tr>
<tr>
<td></td>
<td>Rate SE</td>
<td>Rate SE</td>
</tr>
<tr>
<td>Total sample</td>
<td>2.6b .3</td>
<td>3.3b .5</td>
</tr>
<tr>
<td>F3</td>
<td>1.7b .z</td>
<td>2.0b .z</td>
</tr>
<tr>
<td>F2</td>
<td>6.3a .4</td>
<td>5.9a .3</td>
</tr>
<tr>
<td>F2B</td>
<td>4.6a .5</td>
<td>5.0a .2</td>
</tr>
</tbody>
</table>

*Means within forage treatment (rows) indicate difference (P < .05).
*Means within protein fraction (columns) indicate difference (P < .05).

Linear regressions were derived from three incubations in triplicate for 0, .5, and 1 h. Each mean of three determinations is multiplied by 5. Hydrolysis: 1.2 mg of protease could hydrolyze 1 mg of casein/h.

was degraded faster than F2B proteins. Research from other plant varieties revealed that, during wilting, loss of cellular organization often led to complexation of the soluble proteins to glycosides and to phenolic compounds such as rutin and chlorogenic acids (1, 5). Indeed, F2 proteins from wilted and ensiled forages were darker brown than the fresh proteins. The higher rates of degradation of F2 proteins probably are a consequence of noncovalent phenolic attachment to these proteins (1, 5).

Similarly, no differences were found among fresh, wilted, or ensiled proteins of F2B. De Jong and Lam (5) reported a protein fraction that was similar to F2B. In their report, F2B consisted of euglobulins that constituted about 5% of the total leaf protein. Soluble F2B was recovered by ultrafiltration of the brown solution that remained after extraction of all other protein fractions. This brown solution contained phenolic compounds (8) that could have bound the remaining proteins and rendered them less susceptible to proteolysis.

Fraction 3 constituted 40% of chloroplast protein and was made up of light-harvesting protein complexes, CPI and CPII (13). From this study, these proteins degraded very slowly, and their breakdown after wilting or ensiling tended to be similar to that of the total protein fraction. The total protein fraction was likely to be largely F3 proteins (12), which explains why the nutritional value of total leaf protein was rather low (13). The slow degradation implied that F3 provides more bypass protein. However, this inference may not be correct under in vivo conditions because previous workers (14) showed that chloroplasts and their membrane fragments were engulfed rapidly by ruminal protozoa. No differences were observed in the degradation rates of F3 proteins from fresh and wilted forages, but the silage F3 degraded faster. Fresh and wilted alfalfa F3 contained about 18% lipids and nearly all of the chlorophyll pigments (Table 2). Peroxidation of the lipid fraction during wilting and ensiling might have altered the conformation of these hydrophobic lipoproteins, resulting in increased rates of proteolysis (11).

Comparisons of the degradation rates of protein fractions from fresh and wilted forages showed similar patterns when degradation rates followed the decreasing order: F2, F2B, and F3 and, for silages, F2 equal to F2B and both greater than F3. The present study placed more emphasis on the choice of protease with a broader specificity on forage proteins (10). The absolute breakdown of any given protein in the rumen varies with diet and intake. Other workers (17) observed that a change of diet from hay and crushed oats to fresh alfalfa increased three- and ninefold the rate of breakdown of F1 and bovine serum albumin, respectively. The rates obtained in our study probably would have been greater if the heifer had been fed a fresh forage diet.

CONCLUSIONS

Silage chloroplast membrane proteins were degraded faster than those in fresh or wilted alfalfa. Very little alfalfa silage protein was
likely to have survived degradation in the rumen. In addition, soluble alfalfa proteins were degraded faster than the insoluble structural proteins. Faster rates of passage from the rumen are probably the only way to ensure greater bypass of the soluble protein fractions in alfalfa. This conclusion is particularly true for F2. Chloroplast membrane proteins likely constitute a significant bypass fraction in defaunated ruminants fed high alfalfa diets. Postharvest research could be aimed at preserving cell compartments to reduce proteolysis and optimizing microbial production in ruminants fed alfalfa silage.

REFERENCES