Physical Degradation of Lignified Stem Tissues by Ruminal Fungi

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Ruminal bacteria or fungi were selected by the addition of cycloheximide or streptomycin and penicillin, respectively, to ruminal fluid, and the weakening and degradation of lignified tissues in alfalfa and Bermuda grass stems by these treatments and whole ruminal fluid were evaluated in vitro. Dry weight loss in alfalfa was similar for whole ruminal fluid and streptomycin-penicillin treatment, whereas that with streptomycin-penicillin treatment was significantly higher (P ≤ 0.05) than that with cycloheximide treatment. In Bermuda grass, dry weight loss was significantly higher with streptomycin-penicillin than that with whole ruminal fluid and cycloheximide treatment, which were equal. Both peak load (Newtons) and peak stress were less (P ≤ 0.05) for streptomycin-penicillin treatment than with other treatments in both forages. Fungi colonized the lignified ring in alfalfa and tended to reduce the width of cell walls in this tissue, but a large number of fungal penetrations through cell walls was not observed. In contrast, fungal rhizoids frequently penetrated into and through cell walls in the lignified ring of Bermuda grass, often expanding the pit fields between the cells. Ruminal fungi disrupt lignified tissues in stems, and their activity results in a weakened residue more amenable to physical degradation. This weakening may allow plant digesta to be more easily broken apart during animal’s rumination and thus facilitate digesta flow and fiber utilization.

It has been well established that anaerobic fungi are a significant part of the population of fiber-digesting ruminal microorganisms (5, 9, 22). Furthermore, it is now clear that ruminal fungi produce cellulases and xylanase, which are active against forage cell wall carbohydrates (20, 23).

In the first in-depth studies of ruminal fungal colonization of plants, Bauchop (9) reported that sporangia were produced on lignocellulosic plant tissues. Further studies on specific plant tissues (4) showed that the leaf blade sclerenchyma was extensively degraded, whereas the more resistant xylem cells were only partially degraded. Despite this ability to colonize and degrade the lignocellulosic tissues, ruminal fungi have recently been shown to solubilize phenolic compounds from plant fiber but not to catabolize the phenolic compounds to CH₄ and CO₂ (3). In studies of grass leaves, often the dry weight loss attributed to the fungal population was similar or less extensive than that by the mixed bacterial population (5). However, even when dry weight loss was less extensive, ruminal fungi were shown to erode portions of the xylem cells, resulting in a weakened residue.

Plant anatomy and structural characteristics related to physical breakdown and reduction in particle size that occur during mastication are thought to be important in forage quality, since they influence fiber passage through the digestive tract (12, 18). Stems have an anatomy that results in a highly lignified residue after digestion by ruminal microorganisms (13, 27) and appear to be especially resistant to digestion and physical destruction. There is presently little quantitative information on the ability of the ruminal fungi or bacteria to weaken the plant fiber or on the effect that microbial digestion might have on subsequent physical forces that reduce structural limitations. We have previously added antibiotics to ruminal fluid and selected for bacteria (cycloheximide) or fungi (streptomycin and penicillin) that degrade fiber (3). Our purpose in this study was to utilize an objective procedure to quantitatively evaluate in vitro the potential for ruminal fungi and bacteria to alter the texture of stem fragments of two forages.

MATERIALS AND METHODS

Plant substrate. Coastal Bermuda grass (Cynodon dactylon (L.) Pers), grown in well-managed plots near Athens, Ga., was harvested after a 6-week regrowth. Alfalfa (Medicago sativa L.), grown in well-managed plots near the University of Minnesota, was from a 4-week regrowth. Whole plants of each forage were frozen immediately after harvest and maintained at −10°C until used. Stem segments were cut at 1-cm lengths for dry weight loss and fracture tests and at 3-mm lengths for microscopy. Bermuda grass stems were cut from the fourth or fifth internodes from the apex, and alfalfa stems were from internodes midway of the plants. Stem segments were collected from several plants and mixed together, and ten segments were placed into each Hungate tube. For dry weight loss and fracture tests, sections were freeze-dried and weighed before incubation. Three-millimeter segments were not freeze-dried.

In vitro incubation with microbial groups. Hungate tubes with stem segments were prepared with the basal medium of Caldwell and Bryant (11) as described previously (1). Antibiotics were added to autoclaved tubes to select for microbial groups as follows: (i) whole ruminal fluid (WRF) without antibiotics to evaluate the mixed microbial population, (ii) SP, 130 U of streptomycin (S) plus 2,000 U of penicillin (P) per ml of medium to inhibit bacteria and select for anaerobic fungi, (iii) 0.5 mg of cycloheximide (C) per ml of medium to inhibit fungi and select for bacteria, and (iv) all three antibiotics (SPC) to inhibit all microbial groups. Five tubes with 1-cm stem segments and one tube with 3-mm stem segments for each of the antibiotic treatments were inoculated with 0.4 ml of strained ruminal fluid from a canulated steer; a similar set of tubes was left uninoculated to serve as controls and to assess washout of soluble material. The diet of the steer was Bermuda grass hay plus 4.6 kg of a supplement (37% corn, 37% soybean hulls, 23% soybean
meal, and 3% trace minerals and limestone) per day plus Bovatec at 0.05% of the supplement. Tubes were incubated at 39°C for 72 h.

**Dry weight loss determination.** After incubation with the various treatments for 72 h, 1-cm stem segments from triplicate tubes for each treatment were retrieved, freeze-dried, and weighed, and the loss was calculated as a percentage of the original weight. The stems were then treated with acid pepsin for 48 h as described previously (2) and freeze-dried, and the corrected loss was calculated as a percentage of the original weight.

**Textural properties.** Stem segments in duplicate tubes incubated for 72 h were evaluated for objective textural properties by using an Instron Universal Measuring Instrument equipped with a single, dull blade Allo Kramer unit. Stems were held in water until the tests were carried out. The diameters of individual stems were measured with a pair of calipers. Each stem segment was centered over a 3.2-mm-wide slot, and a single, 3.0-mm-wide blade contacted the stem and compressed it until a fracture occurred as noted by a load reduction. The single blade was attached to a 100-kg load cell mounted on a model 1122 Intron equipped with a computer and printer. Stem segments were evaluated at room temperature for texture by measuring the following characteristics: (i) peak load of force (Newtons) necessary to break the stem, (ii) peak energy required (joules) to break the stem, and (iii) stress load applied divided by the stem cross-sectional width (Newtons per centimeter). The test conditions for the instrument were a cross-head speed of 50 mm/min and a chart speed of 500 mm/min. The total cross-head travel (i.e., the downstroke) per sample ranged from 9 to 12 mm. Twenty individual stems (10 for each of two tubes) were measured for textural properties for each treatment.

**Preparation for electron microscopy.** Three-millimeter stem segments were placed into 4% buffered (0.1 M cacodylate [pH 7.4]) glutaraldehyde for several hours and then postfixed for 4 h in buffered 1.5% OsO₄. After fixation, stem segments were dehydrated in a graded ethanol series and prepared for scanning electron microscopy (SEM) or transmission electron microscopy (TEM) as described previously (4).

**Microscopic evaluation of cell wall degradation.** Transmission electron micrographs were prepared at precise enlargement (total magnification, ×6,960) for three sites for each of two stems per treatment. Cell wall widths were then measured for the lignified ring and for the parenchyma (6, 14, 27). Additionally, the number of breaks or partial breaks due to microbes through the thick-walled cells of the lignified ring were counted within a thin section for each of two stems per treatment. The microbial breaks were characterized as follows: (i) partial invagination by microbial structures into a cell wall, (ii) complete penetration by microbial structure through the secondary wall to the middle lamella, and (iii) transverse invagination into contiguous cells and penetration across the middle lamella. Tissue was evaluated within at least 20 sites for each thin section.

**Statistical analysis.** Data for percent dry weight loss were treated within forages by a two-way analysis of variance (26). Data on cell wall widths were treated by a one-way analysis of variance within treatments for a forage. Data for energy and forces to break stems were treated within forages by a two-way analysis of variance with duplicate test tubes nested within treatments, and stem diameter was fitted as a covariant. Data for peak stress were treated by a two-way analysis of variance but without stem diameter fitted as a covariant because peak stress is a measure of the force necessary to deform the sample divided the diameter of the stem. Difference means and adjusted means were determined by using Scheffe's multiple comparison as described by Kleinbaum and Kupper (17).

### RESULTS

**In vitro dry weight loss of stems.** In alfalfa, dry weight loss was highest for WRF and SP (38 and 41%, respectively) compared with other treatments (Table 1). Loss due to the bacteria inoculum (i.e., C treatment) tended to be less (but was not significant; P > 0.05) than that with WRF. The loss due to solubilization of about 20% in the uninoculated control was not different from loss in SPC treatment, showing that the combination of antibiotics effectively prevented fiber degradation. For Bermuda grass, the greatest loss occurred with SP, whereas WRF and C treatments were similar (Table 1). Losses in SPC treatment and controls were similar and lower (P ≤ 0.05) than those with other treatments.

**SEM of microbial colonization of fiber in stems.** SEM of alfalfa and Bermuda grass (Fig. 1 through 3) provided information on the microorganisms degrading the fiber of stems. Losses were not apparent in SPC treatment (Fig. 1). The C treatment resulted in heavy colonization by ruminal bacteria for both alfalfa and Bermuda grass (Fig. 2) and lack of activity by ruminal eucaryotes (i.e., protozoa and fungi). Loss of tissue by bacteria in alfalfa was slight, showing degradation of the innermost parenchyma cells but resistance of the lignified ring. In Bermuda grass, the ruminal bacteria in C treatment resulted in loss of most of the parenchyma with a residue of lignified epidermis, lignified ring, and vascular bundle cells (Fig. 2). With SP treatments, ruminal fungi were the predominant microorganisms degrading fiber. Colonization by sporangia and rhizoids of the lignified ring in alfalfa and Bermuda grass (Fig. 3) was extensive, but little to no colonization of the parenchyma cells occurred and this tissue was not extensively degraded as had occurred in the bacterial treatment. No evidence of ruminal protozoal degradation, which had been observed by others in the parenchyma (8), was found in our study.

In comparing the stems of the two plants, both dry weight loss and SEM of individual tissue degradation indicated that Bermuda grass fiber was more digestible than alfalfa regardless of the treatment (Table 1). The SP treatment resulted in a significantly higher degradation in Bermuda grass compared with that with other treatments, whereas in alfalfa the SP treatment was similar to WRF treatment. These data indicate that the anaerobic fungi were better able to degrade or solubilize material in Bermuda grass, although the visible loss of tissues was not great.

### TABLE 1. Dry weight loss of stems incubated with ruminal fluid in the presence of antibiotics

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Alfalfa</th>
<th>Bermuda grass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninoculated control</td>
<td>19.9a</td>
<td>21.1a</td>
</tr>
<tr>
<td>Whole rumen fluid</td>
<td>38.4bc</td>
<td>47.2b</td>
</tr>
<tr>
<td>Fluid plus C</td>
<td>35.7c</td>
<td>46.4b</td>
</tr>
<tr>
<td>Fluid plus SP</td>
<td>40.8b</td>
<td>53.8c</td>
</tr>
<tr>
<td>Fluid plus SPC</td>
<td>22.4a</td>
<td>19.3a</td>
</tr>
</tbody>
</table>

* The standard errors of the mean were 0.8% for alfalfa and 0.9% for Bermuda grass. Values within columns followed by different letters differ at P < 0.05.
Physical characteristics. Preliminary work was undertaken to evaluate physical characteristics as measured objectively with the Instron. The use of metal blades to effect shear stress was chosen as the action most closely related to rumination of plant digesta. Initial tests comparing multiple blades or a single blade indicated that the use of the single blade provided a more uniform system for measuring physical characteristics of stem segments 1 cm in length. Further preliminary work on undigested alfalfa and Bermuda grass stem segments indicated that differences in physical characteristics could be measured by using wet or dry samples. The procedure for testing included wet samples maintained in water after incubation to maintain plant segments in a form similar to that in the digesta and the use of a single blade contacting the stem segment at the midpoint to simulate shearing forces of the ruminant’s teeth.

In alfalfa, incubation of stem segments with SP resulted in a weaker \( P \leq 0.05 \) residual segment based on lower peak load and peak stress values compared with other treatments, which were similar to each other (Table 2). Energy to compress stems also tended \( P > 0.05 \) to be lower for the SP treatment. The C treatment resulted in higher \( P \leq 0.05 \) values for energy, peak load, and peak stress than those for WRF. In Bermuda grass, SP also resulted in residues with lower \( P \leq 0.05 \) values for peak load and peak stress and a tendency for a lower energy value (Table 2) compared with those for all other treatments. The C treatment resulted in values that were not different \( P > 0.05 \) from those with WRF treatment but tended to be lower.

For both plants, SPC treatment resulted in values similar to that of control stems, further confirming gravimetric values that SPC effectively inhibited fiber degradation. In comparing the stem segments of the two plants, energy and peak stress values were similar in uninoculated stems, but the peak load value was higher for alfalfa.

Interaction of microorganisms with plant cell walls. The association with and degradation of plant cell walls by ruminal microorganisms was shown by TEM. In alfalfa, cell walls in the lignified ring after treatment with SPC were compared with those incubated with C or SP. For SPC treatment, ruminal microorganisms were absent, and the pit fields of adjacent cells were seen. For C treatment, bacteria with a Bacteroides morphology (19) closely adhered to and caused localized erosion in the cell wall. In SP treatment, ruminal fungi were prevalent, whereas bacteria were scarce. A general thinning of the cell walls was evident for cells in the lignified ring. A comparison of wall width in lignified ring and parenchyma cells indicated that those in the SP treat-
TABLE 2. Physical characteristics of stems incubated with ruminal fluid in the presence of antibiotics

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Physical characteristicsa</th>
<th>Physical characteristicsa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alfalfa</td>
<td>Bermuda grass</td>
</tr>
<tr>
<td></td>
<td>Energy (J)b</td>
<td>Energy (J)</td>
</tr>
<tr>
<td></td>
<td>Peak load (N)c</td>
<td>Peak load (N)</td>
</tr>
<tr>
<td></td>
<td>Peak stress (N/cm)d</td>
<td>Peak stress (N/cm)</td>
</tr>
<tr>
<td>None (control)</td>
<td>0.043a</td>
<td>68.7a</td>
</tr>
<tr>
<td>Whole rumen fluid</td>
<td>0.036ab</td>
<td>56.7bc</td>
</tr>
<tr>
<td>Fluid plus C</td>
<td>0.053a</td>
<td>49.2b</td>
</tr>
<tr>
<td>Fluid plus SP</td>
<td>0.025b</td>
<td>29.7d</td>
</tr>
<tr>
<td>Fluid plus SPC</td>
<td>0.043a</td>
<td>64.6ac</td>
</tr>
</tbody>
</table>

a Standard errors of the mean were as follows: for alfalfa, 0.007 J, 11.1 N, and 19.8 N/cm; for Bermuda grass, 0.009 J, 4.1 N, and 49.2 N/cm. Values within columns followed by different letters differ at P < 0.05.

b Work done when the point of application of 1 N is displaced 1 m in the direction of force.

c The force that gives to a mass of 1 kg an acceleration of 1 m/s².

d The force producing deformation measured by the force applied per unit area.

ment tended to be thinner, but widths were not significantly different (Table 3). In Bermuda grass cell walls and pit fields in lignified ring tissue of control stems (Fig. 4) were compared with those in stems incubated with C or SP treatments (Fig. 5 and 6). In C treatment, ruminal bacteria eroded small pits in the cell wall and occasionally expanded the pit fields (Fig. 5). In SP treatment, ruminal fungi were prevalent, and in some sites multiple pit fields were expanded (Fig. 6) with rhizoids transversing the middle lamella regions (Fig. 6, inset). Further, in SP treatment fungi eroded areas in the lignified xylem cell walls of vascular tissue, resulting in a weakened structure. Cell walls in the lignified ring were not different in width, but parenchyma cell walls were thinner (P ≤ 0.05) with C and SP treatments compared with those with other treatments.

The lignified rings in alfalfa and Bermuda grass were the tissues most resistant to degradation. In alfalfa, the only recognizable difference in C and SP treatments was a trend for thinner walls after SP treatment. In contrast, in Bermuda grass the cell walls were penetrated to a greater extent by fungi than by bacteria. In comparing sites within thin sections for degradation of plant walls (Fig. 5 and 6) the following values give the extent of microbial penetrations of walls per site for bacteria and fungi, respectively: partial penetration, 0.27 and 0.83; complete penetration, 1.36 and 1.75; and penetration across two cells, 0 and 0.13.

DISCUSSION

The production of volatile fatty acids as energy sources and single-cell protein by ruminal microbial fermentation of forages is well established (16). However, the effects of the interaction between plants and microbes, in terms of forage quality, are complex and pertain to physical and biochemical parameters. The time of retention and passage of fiber from the rumen controls various characteristics of forage quality (e.g., intake, digestibility, protein metabolism) (30). Physical attributes of fiber associated with size and specific gravity of particles, in turn, affect the passage of fiber out of the rumen (30). Recently, it was reported that changes in specific gravity of fiber were affected by the initial particle size in alfalfa and timothy hays (21). Reduction in particle size is frequently cited as an important factor in forage utilization, and a threshold size for particles to escape the rumen (1.0 to 2.0 mm for sheep and 2.0 to 4.0 mm for cattle) has been reported (28). The major causes for reduction in particle size are eating and ruminating by the animal, with reduction due to microorganisms being less pronounced (28, 31).

Investigations by microscopy of various plants have shown that extreme rupture of lignified tissues occurs during feed ingestion, and a potential result is that surface areas are increased for microbial colonization and degradation (24). Research involving masticated leaf blades placed in nylon bags within the rumen showed that the microbial population did not reduce lignified fiber bundles in length but that the

TABLE 3. Width of cell walls in stems after incubation with ruminal fluid in the presence of antibiotics

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Width (μm) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lignified ring</td>
</tr>
<tr>
<td>Whole rumen fluid</td>
<td>1.3</td>
</tr>
<tr>
<td>Fluid plus C</td>
<td>1.4</td>
</tr>
<tr>
<td>Fluid plus SP</td>
<td>1.1</td>
</tr>
<tr>
<td>Fluid plus SPC</td>
<td>1.7</td>
</tr>
</tbody>
</table>

a Values represent averages of four (lignified ring) or three (parenchyma) measurements for each of three sites in two stems. Values within columns followed by different letters differ at P ≤ 0.05. Standard errors of the mean were 0.1 μm throughout.

FIG. 4. TEM of Bermuda grass stem incubated for 72 h with ruminal fluid plus SPC showing no microorganisms present and no loss of cell wall material in the lignified ring. Pit fields (arrows) are present between cells. Bar, 2 μm.
width of leaves was reduced by microorganisms (31). The lignified fibers in grass leaves have been reported to account for 90 to 95% of the longitudinal stiffness (29). In this present work, none of the stem segments were broken apart by action in vitro of either the bacteria or the fungi.

Previous work (4, 5) has shown that differences occur between ruminal bacteria and fungi in the site and pattern of tissue degradation. Ruminal fungi extensively degrade the lignified sclerenchyma cells in grass leaves and, to a lesser extent, cause erosion zones in the xylem cells. In contrast, ruminal bacteria are able to degrade the periphery of the sclerenchyma but cannot degrade the xylem. It has been suggested that digestive weakening of tissues, although not directly responsible for particle size reduction, may play an indirect role in enhancing fiber breakdown during rumination (31).

Residues in mature stems of both legumes and grasses after digestion are comprised of a prominent, cylindrical band of lignified cells, and in grasses the parenchyma is also present. In extremely mature grasses, virtually all stem tissues resist microbial degradation (6, 14, 27). Before the present study, no work had been done to compare the activity of ruminal bacteria and fungi on specific tissues in stems. Our data showed that the mixed fungal population that was selected with SP readily colonized and partially degraded the most resistant tissues in the stem at times leaving digestible tissues undegraded, apparently because of selected sites of colonization. Ruminal bacteria caused only slight tissue loss in the most resistant tissues. As observed in other studies (7), ruminal protozoa were not present as primary fiber degraders in this system, although they do participate in other systems (8). The pattern of attack by the fungi varied with the type of forage. Fungi exerted more erosion zones within Bermuda grass tissues than did bacteria, whereas fungi reduced the cell wall width in alfalfa compared with that reduced by bacteria. In Bermuda grass, often rhizoids grew in the pit fields, with a expansion of this region and penetration across cell wall boundaries. The ability of fungi to penetrate deeply into tissues usually resistant or only partially available to bacteria is an important characteristic (10), and some researchers have reported specialized ruminal fungal structures for penetration, including appressorium-like structures and penetration pegs that caused narrow canals in plant tissues (15). Our results suggest that pit fields may be particularly amendable for colonization and growth by ruminal fungal rhizoids.

Regardless of the pattern of fungal attack on the lignified cells, the resulting residue was weaker than that produced by other treatments when tested objectively. It is likely that a weaker residue would require less chewing by the ruminant to modify the fiber for passage from the rumen. The effect of the fungi on the prominent structural barrier in stems would appear to be particularly important for forage utilization.

Further work is needed to verify the interaction of fungal degradation of lignified tissues and forage intake, but studies with grass leaves suggested that such a relationship occurred in sheep (4, 25).

The weaker residue after fungal degradation compared with that by the whole population occurred whether dry weight loss of the forage was higher or not. These data, along with those from other studies where fungi degraded less fiber components than did bacteria (3), suggest that the percentage of dry weight loss may not be a valid indicator of the contribution of fungi to feed utilization and indicate that a measurement of the physical modification of the plant is required. To this end, the Instron equipped with the Allo-Kramer single, blunt blade to simulate compression and shearing by ruminants' teeth is useful.

Although the stem residue was significantly weakened by the fungi, the lack of this weakening by WRF indicated that the fungi did not perform to their potential in the mixed population. Other research has indicated that sporangial numbers from ruminal fluid inocula are highest when bacteria are suppressed with antibiotics (5). A strategy to enhance fungal growth in the presence of other microorganisms and
to weaken recalcitrant tissues should be explored to increase the efficiency of fiber utilization by ruminants.

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LITERATURE CITED


