Scanning Electron Microscopy of Coastal Bermuda and Kentucky-31 Tall Fescue Extracted with Neutral and Acid Detergents

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Leaf sections of Coastal bermudagrass (*Cynodon dactylon* (L.) Pers.) and Kentucky-31 tall fescue (*Festuca arundinacea* Schreb.) were extracted with neutral detergent reagent for 60 min and with acid detergent reagent for 10, 30, and 60 min. Microanatomical characteristics of the residues were identified using scanning electron microscopy and related to the percentage fiber content. Neutral detergent residues consisted of intact cell walls comparable to water-extracted, control samples. Extraction with acid detergent for 10 and 30 min yielded residues in which the tissues most digestible by rumen microorganisms were removed first. After extraction for 60 min, acid detergent residues consisted mostly of rigid, lignified tissue. Tissues in tall fescue were extracted faster and to a greater extent than those in Coastal. Observations indicated that the acid detergent residue may contain any lignified cell wall constituent.

Van Soest (1963a; Van Soest and Wine, 1967) reported that sodium lauryl sulfate (SLS) in neutral or slightly alkaline aqueous solutions and cetyltrimethylammonium bromide (CTAB) in strong acid showed promise in yielding fibrous residues useful in estimating the nutritive value of forages. The residue from the SLS treatment, called neutral detergent fiber (NDF), consisted of the cell wall constituents of the forage while the acid detergent fiber (ADF) represented the highly lignified and "probably represents the more indigestible portion of the fiber" for ruminants (Van Soest, 1963a). The ADF and lignin obtained via ADF have been widely used to estimate the nutritive value of various forages (Duble et al., 1971; Mowat et al., 1969; Utley et al., 1971; Van Soest, 1963b; Wilkinson et al., 1969; Wurster et al., 1971).

Results of analyses of ADF residues have not revealed a consistent chemical composition for this residue in all forages. Colburn and Evans (1967) reported that the ADF of temperate grasses and alfalfa contained almost all the cellulose and lignin of the whole plant. Bailey and Ulyatt (1970) stated that, while ADF contained most of the plant cellulose, this fraction also contained large amounts of hemicellulose and pectic substances. These authors indicated that attempts to relate ADF to digestibility or leaf strength have more merit than correlations with the chemical constituents in the ADF residue. McLeod and Minson (1972) reported higher residual standard deviations using ADF than using the in vitro procedure (with rumen microorganisms) to estimate digestibility and have suggested modifications of acid strength and hydrolysis time to optimize the empirical procedures for individual species.

Scanning electron microscope (SEM) observations of forage tissues after incubation with rumen microorganisms have revealed that grass tissues differ in ease of digestibility and that nonlignified tissue can influence the rate of total leaf degradation in some forages (Akin et al., 1973). However, information is not available which identifies the NDF and ADF residues with specific plant tissues in forages. The objective of this study was to determine the microanatomy of plant residues after neutral and acid detergent extractions in leaves of two grasses with different digestibilities. Extracted leaf sections were examined using the SEM and specific tissues related to the NDF and ADF of intact fresh-frozen leaves and whole, milled forage samples.

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EXPERIMENTAL SECTION

Preparation of Grass Samples. Coastal bermudagrass (*Cynodon dactylon* (L.) Pers.) and Kentucky-31 (Ky-31), tall fescue (*Festuca arundinacea* Schreb.) were collected after 4 weeks of summer regrowth. Samples were frozen after harvesting and maintained at −30° until used. Intact leaf sections, 2 to 5 mm long, were cut from the frozen leaf blades so that sections near the apex or base were avoided. The leaf section samples were subdivided with one portion used for NDF and ADF determinations and the other portion for SEM observations of detergent extracted residues. Samples of whole forage (leaf and stem as harvested) were freeze-dried and ground in a Wiley Mill to pass a 20 mesh screen for NDF and ADF determinations.

NDF and ADF Dry Matter Determinations. Neutral and acid detergent reagents were prepared according to the procedures of Van Soest (1963a; Van Soest and Wine, 1967). The glassware was modified from that used in the published procedures in that a 250-ml 14/20 standard taper round-bottomed flask was fitted with a 70 mm i.d. 14/20 standard taper Dewar condenser filled with Dry Ice–2-propanol as coolant. Residues from whole, ground forages were filtered through sintered glass crucibles, washed according to Van Soest procedures, and dried at 100° in a forced air-draft oven overnight before weighing.

Identical procedures were used to determine the NDF and ADF residues of intact fresh-frozen leaves except that 1.0 g of 2 to 5 mm leaf sections replaced 1.0 g of whole, ground forage. Determinations were made at least in triplicate for all samples.

Table I. Percent Residue of NDF and ADF from Whole, Wiley-Milled Forage and Intact Leaf Samples of Coastal Bermudagrass and Ky-31 Tall Fescue

	Residue			
	NDF		ADF	
Grass	Whole[a]	Leaf[b]	Whole[a]	Leaf[b]
Coastal Bermudagrass	59.4 ± 0.3	78.3 ± 1.6	29.1 ± 0.8	25.3 ± 0.7
Ky-31 Tall Fescue	50.7 ± 0.6	79.1 ± 2.3	28.6 ± 0.2	27.8 ± 1.3

[a] Average of 12 determinations plus standard deviation for whole, ground samples. [b] Average of 3 determinations plus standard deviation for leaf samples.

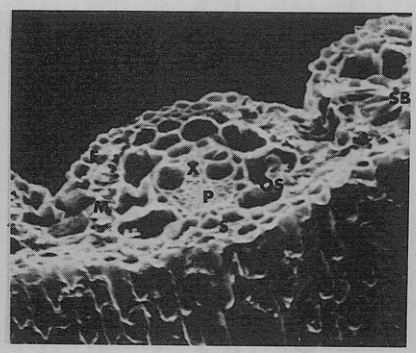

Figure 1. Cross-section of Coastal bermudagrass control leaf boiled in water for 60 min. All cell walls of tissues are intact and have maintained structural integrity: mesophyll (M); phloem (P); xylem (X); outer bundle sheath (OS); epidermis (E); sclerenchyma (S); small vascular bundle (SB); ×240.

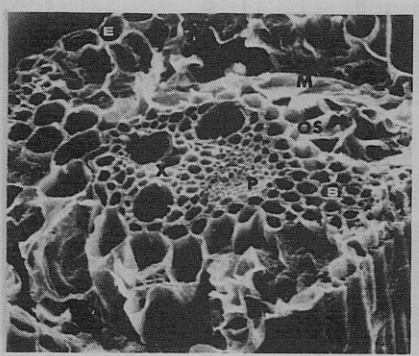

Figure 2. Cross-section of Ky-31 tall fescue control leaf boiled in water for 60 min. Cells of the outer sheath (OS), phloem (P), xylem (X), sclerenchyma (S), and epidermis (E) are structurally intact, but the mesophyll (M) has collapsed although the tissue has not been removed from the cross-section; ×240.

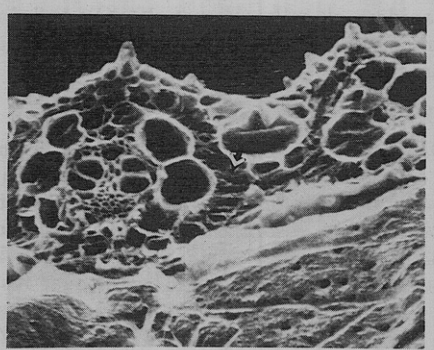

Figure 3. Cross-section of Coastal bermudagrass leaf extracted for 60 min with neutral detergent reagent. Tissues, including the mesophyll (arrow), are intact as in the control sections; ×240.

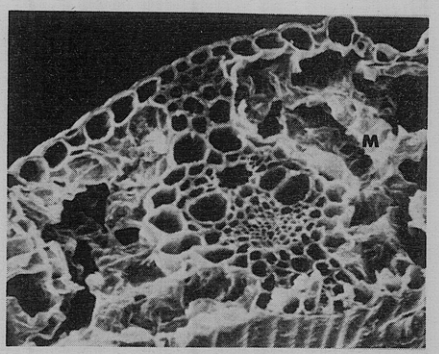

Figure 4. Cross-section of Ky-31 tall fescue leaf extracted for 60 min with neutral detergent reagent. Tissues are similar to those in control samples; all are intact except for the mesophyll (M); ×224.

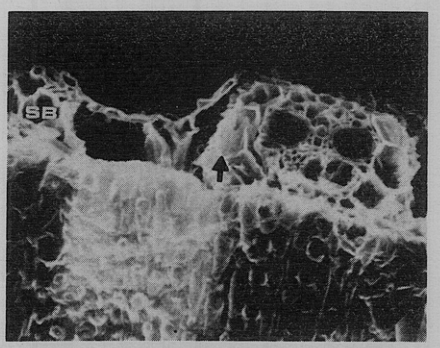

Figure 5. Cross-section of Coastal bermudagrass leaf extracted for 10 min with acid detergent reagent. The outer bundle sheath (arrow) and small vascular bundles (SB) are distorted, and the mesophyll and phloem are beginning to be removed; ×256.

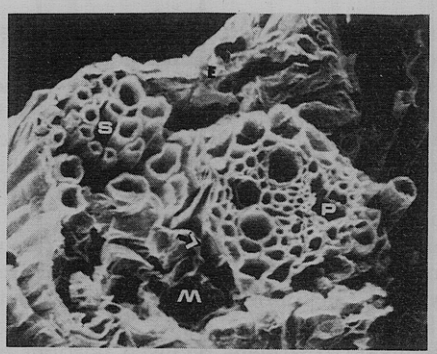

Figure 6. Cross-section of Ky-31 tall fescue extracted for 10 min with acid detergent reagent. The outer bundle sheath (arrow), phloem (P), mesophyll (M), and epidermis (E) are in the initial stage of removal. The sclerenchyma (S) is being separated into individual cells; ×224.

NDF and ADF Preparations for SEM. Intact leaf sections were placed in flasks, 100 ml of either NDF or ADF reagents or of water (control) was added, and the flasks were brought to reflux. Sections were removed from the NDF and control flasks after 60 min and after 10, 30, and 60 min from the ADF flasks. Leaf sections from these flasks were placed in a 250-ml beaker with 200 ml of hot (≥98°) deionized, distilled water and washed for 3–5 min prior to washing in 100 ml of acetone for 5 min. Excess acetone was removed with absorbent paper and the leaf sections were placed in vials of 4% glutaraldehyde in 0.1 *M* cacodylate buffer at pH 7.0 for 18–20 hr. Particular care was taken to include tissues which had separated from the intact leaves in 60-min ADF solutions. Sections were postfixed in buffered 1.5% osmium tetroxide for 4 hr and then adhered to aluminum specimen stubs and frozen in a Dry Ice–2-propanol bath. Frozen samples were placed in a vacuum evaporator and coated with gold–palladium alloy wire (60:40) for conductive purposes. Leaf sections were observed in a field emission SEM at about 15 kV. Trials were run in duplicate for both species.

RESULTS

The NDF and ADF values for whole, ground samples and intact leaf sections (identical with those observed by SEM) are shown in Table I. The NDF value appeared to be higher in Coastal bermudagrass than in Ky-31 tall fescue for the whole, ground grass samples. However, the NDF values of intact leaf sections, similar in both species, were higher than the values for the whole, ground samples in either species. The ADF values for both intact leaf sections and whole, ground grasses were similar in fescue, but values

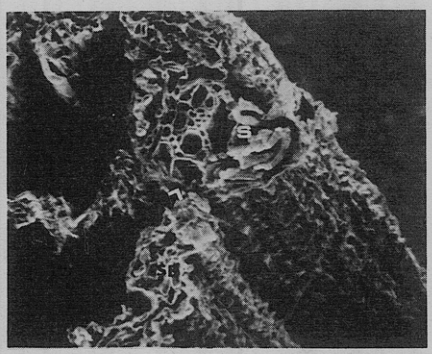

Figure 7. Cross-section of Coastal bermudagrass extracted for 30 min with acid detergent reagent. The outer bundle sheath (arrow) and small bundles (SB) are being removed. The phloem, mesophyll, and noncutinized epidermis appear to be degraded; ×240.

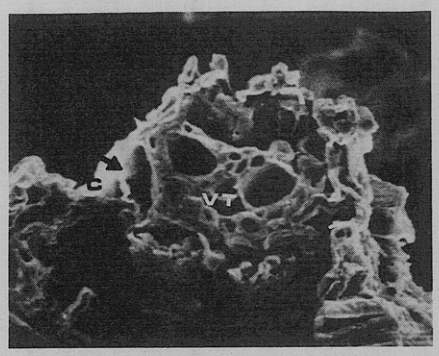

Figure 9. Cross-section of Coastal bermudagrass extracted for 60 min with acid detergent reagent. Lignified vascular tissue (VT) and cutinized epidermis (C) remain. Portions of the outer bundle sheath (arrow) are infrequently observed to remain; ×256.

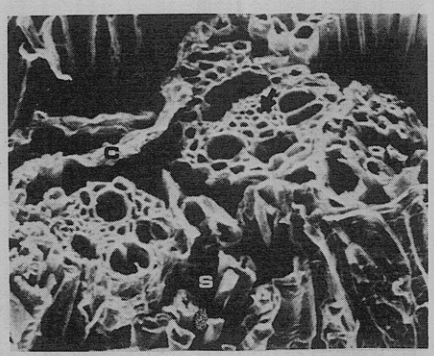

Figure 8. Cross-section of Ky-31 tall fescue extracted for 30 min with acid detergent reagent. Lignified vascular tissue (arrow) and cutinized epidermis (C) remain; sclerenchymal tissue (S) has been separated into individual cells; ×208.

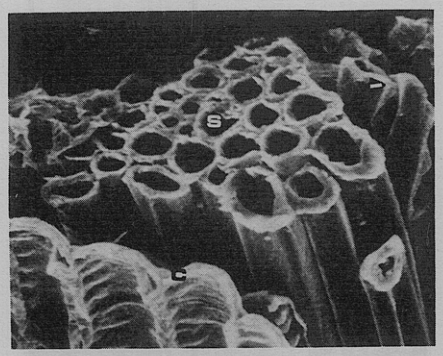

Figure 10. Portions of tissues remaining in Ky-31 tall fescue after 60 min extraction with acid detergent reagent. Cuticular layers of epidermis (C) and sclerenchyma (S) separated into individual cells (arrow) are seen; ×650.

for leaf samples were slightly lower than those for the whole, ground forages in Coastal bermudagrass.

Scanning electron micrographs of water-extracted, control samples of Coastal bermudagrass and Ky-31 tall fescue indicated that none of the cell walls were removed from the leaf lamina (Figures 1 and 2). Mesophyll cell walls in fescue were distorted and collapsed whereas those of Coastal appeared to maintain structural integrity.

The cell walls of the leaves of Coastal and fescue extracted with neutral detergent solutions for 60 min were intact and resembled the control cross-sections of both forages (Figures 3 and 4).

Acid detergent treatment removed portions of the leaf tissues of both species. After the 10-min incubation, phloem and mesophyll cells were removed in both Coastal (Figure 5) and fescue (Figure 6). The outer bundle sheath (Figure 5, arrow) and the small vascular bundles (Figure 5, SB) were distorted in some samples of Coastal and the outer bundle sheaths (Figure 6, arrow) removed in some fescue leaves. Furthermore, sclerenchymal cells (Figure 6, S) in fescue were beginning to be separated and the epidermis (Figure 6, E) was partially removed.

After incubation of Coastal with acid detergent for 30 min, some outer bundle sheath tissue (Figure 7, arrow) was removed and small vascular bundles (Figure 7, SB) were partially removed; lignified vascular tissue was intact but sclerenchymal cells were separating. In fescue the epidermis and outer sheaths were removed and sclerenchymal cells (Figure 8, S) were separating. After 60-min extractions of Coastal with acid detergent, outer bundle sheath cells were found only in a few leaf cross sections (Figure 9, arrow). Other nonlignified tissues were removed and, at times, break-up of sclerenchymal tissue into individual cells was evident (not shown) while the lignified portions of the vascular tissue (Figure 9, VT) were intact. In fescue, intact lignified tissue was rarely found, but the cuticular layers of epidermal cells (Figure 10, C) and individual sclerenchymal cells (Figure 10, arrow) were evident after 60-min extractions.

DISCUSSION

The NDF residues of both Coastal bermuda and Ky-31 tall fescue as observed with the SEM appeared to have intact cell walls and support Van Soest's statement of "cell wall constituents in essentially undegraded form" (Van Soest, 1963a). The higher NDF values (Table I) of the whole, ground grass samples for Coastal over fescue support other data that tropical grasses generally have more cell walls than temperate species (Deinum and Dirven, 1971; Van Soest, 1973). However, the NDF values for intact leaf sections, about equal in both grasses, were higher than those for the whole, ground grass samples (Table I). Since microscopic observations (Figures 3 and 4) showed that cell walls were not ruptured by the NDF reagent, unextracted cell contents apparently accounted for the higher NDF values of the intact leaf samples.

Close agreement between the ADF values for intact leaf sections and whole, ground samples (Table I) indicated that residues can be obtained without grinding and, furthermore, indicated that the residues observed with the SEM were comparable to the fibrous residues of the analytical determinations. The ADF values for samples of Coastal bermudagrass intact leaf sections were lower than those for the whole, ground grass samples reflecting the higher expected ADF (Mowat et al., 1969) because of the stem content in whole harvested forage after 4-week regrowth (Burton and Prine, 1956). Since fescue harvested at 4-week regrowth appeared to be almost all leaf, one would expect the values for whole and leaf samples to be about equal as is shown in Table I. Our values are in agreement

with ADF values in the literature for Coastal bermudagrass (Duble et al., 1971) and Ky-31 tall fescue (Allinson, 1971) at similar digestibilities.

In Coastal bermudagrass the tissues remaining after treatment with acid detergent for 60 min are comparable for the most part with tissues resisting in vitro degradation by rumen microorganisms for 72 hr; however, in microbial digestions all outer bundle sheath cells appeared to be degraded (Akin et al., 1973). In fescue the lignified vascular tissue, remaining after rumen microbial degradation for 72 hr (Akin et al., 1973), were rarely found after 60-min acid detergent extraction. That the mesophyll cell walls of Coastal maintained a more rigid nature than those in fescue suggested inherent structural differences between these two species. Differences in cell walls are further indicated by the faster removal of outer sheath and epidermal cells in fescue upon treatment with acid detergent. Furthermore, the tissues in fescue appeared to be degraded to a greater extent by treatment with acid detergent for 60 min than those in Coastal with the sclerenchyma degraded into individual cells and only isolated portions of the cuticular layers remaining.

McLeod and Minson (1972) reported that the residual standard deviations for acid detergent extractions from 1 to 6 hr were not consistent among certain grass species. Our results indicated that tissues common to both species were removed at a slower rate and to a lesser extent in the less digestible, tropical bermudagrass than in the temperate Ky-31 tall fescue. All tissue digested by rumen microorganisms after 72 hr (Akin et al., 1973) appeared to be removed after 30 min of acid detergent hydrolysis in fescue; in Coastal, some nonlignified cell walls (i.e., outer bundle sheath cells) were intact even after 60 min but would probably be removed after longer hydrolysis times.

Cellulose is defined as a linear array of D-glucopyranose units linked by β-(1→4)-glycosidic bonds with a degree of polymerization (DP) of about 300 to more than 2500 glucose residues (Mahler and Cordes, 1971). Cellulose is known to be a component of almost all plant cell walls (Esau, 1965) and, therefore, cellulose and glucans with a DP less than 300 would be expected to be removed with the removal of mesophyll, phloem, bundle sheath, and epidermal cell walls. The removal by acid detergent of large numbers of intact cell walls is not consistent with reports in which Colburn and Evans (1967) found that ADF contained 92% of the plant cellulose and Bailey and Ulyatt (1970) found that essentially all plant cellulose in the original ground forage appeared in the ADF residues. Possibly, the empirically defined "cellulose" is actually the polysaccharide fraction removed by 72% sulfuric acid treatment or remaining after acetic–nitric acid treatment and is not necessarily only the β-(1→4)-D-glucopyranosyl polymer. Furthermore, the cells in the residual tissues after acid detergent extraction appeared to have intact walls (as observed by the SEM). Of the 25.3 to 27.8% leaf ADF (Table I), only about 16% of this material is lignin (unpublished data). In addition to lignin, ash, and cellulose, the ADF could contain hemicellulose and pectin (bound in or between cell walls) (Esau, 1965) and, perhaps, any cell wall constituent normally present in these tissues. Therefore, it is not surprising that pectic substances and hemicellulose were found in the ADF of ryegrasses by Bailey and Ulyatt (1970).

Our observations indicated that the thick-walled outer sheath and epidermal cells require longer extraction times than the mesophyll. Differences were noted in the rates of extraction between similar tissues of these two grass species which correspond generally to the patterns of degradation by rumen microorganisms.

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