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# CROP QUALITY & UTILIZATION

## Immunofluorescent Localization of RuBPCase in Degraded C<sub>4</sub> Grass Tissue

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### ABSTRACT

Digestion-resistant tissues found in C<sub>4</sub> grasses may allow soluble protein to escape rumen degradation. The objective of this study was to use immunofluorescent localization to follow loss of ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBPCase) from switchgrass (*Panicum virgatum* L.) and big bluestem (*Andropogon gerardii* Vitman) parenchyma bundle sheath cells (BSC) during rumen degradation. Fluorescent signal was observed from switchgrass and big bluestem BSC through 24 and 16 h in situ digestion, respectively, and from BSC associated with both intact tissue fragments, and isolated vascular bundles in omasal digesta and fecal material from steers (*Bos taurus* L.) grazing switchgrass. Immunofluorescent localization demonstrated that in certain C<sub>4</sub> grasses (i) parenchyma BSC can protect RuBPCase from degradation through 24-h in situ incubation, (ii) BSC containing RuBPCase can exit the rumen prior to degradation, and (iii) protein protected by BSC can escape degradation in the whole gastrointestinal tract and be excreted.

**P**ERENNIAL C<sub>4</sub> (warm-season) grasses often contain low concentrations of protein and proportionately high contents of tissues resistant to rumen degradation (Minson, 1990). However, performance of livestock grazing C<sub>4</sub> grasses is reportedly greater than would be predicted given characteristic C<sub>4</sub> leaf tissue composition (Abrams et al., 1983; Reid et al., 1988). Many studies of C<sub>4</sub> grass leaf degradation have used microscopic techniques to focus on description and comparison of relationships between location, amount, and disappearance rates of fibrous components (Akin, 1989). These studies have identified slow to partial degradation of parenchyma BSC as a major factor in reduced C<sub>4</sub> leaf blade digestibility. However, little information is available on the relationship between parenchyma BSC degradability and availability of soluble protein, or other sheath cell contents, in C<sub>4</sub> grass leaves.

Akin and Burdick (1977) used starch as a marker to demonstrate that the large amount of potential nutrients in the parenchyma bundle sheath of warm-season grasses may not be readily available because of slow degradation of the sheath cell by rumen bacteria. However, their study was conducted solely under in vitro conditions and loss of soluble protein was not followed through time. Mullahey et al. (1992) assessed ruminal escape protein using an in situ technique for switchgrass and smooth brome grass (*Bromus inermis* Leyss.) and hypothesized that RuBPCase and other enzymes localized within the

BSC of warm-season grasses may escape rumen degradation and pass intact to the lower gastrointestinal tract because they are physically protected from degradation by the sheath cells. Redfearn et al. (1995) followed forage grass leaf protein disappearance in situ using SDS-PAGE. These authors suggested that increasing proportions of protein fractions escaping ruminal degradation in C<sub>4</sub> compared with C<sub>3</sub> (cool-season) grass tissue strongly supported their hypothesis that a portion of C<sub>4</sub> proteins may be protected from rumen degradation by the BSC.

Akin and Burdick (1977) suggested that for clarification of the factors that limit nutritive value in forages, research should be focused on the role of plant anatomy in affecting availability of energy-rich constituents within cells, in addition to study of cell wall constituents per se. We believe that this suggestion also holds for the relationship between parenchyma BSC and soluble protein; however, characterization of this relationship is difficult. A major reason for this difficulty is that approaches which rely on analytical techniques for nitrogen or protein analysis can be confounded by rumen microbial protein that becomes intimately associated with forage cell walls as ruminal degradation proceeds (Olubobokun and Craig, 1990; Olubobokun et al., 1990).

Immunocytochemical techniques are available that allow investigation of presence or absence of specific plant proteins. Our hypothesis was that these techniques could be adapted to establish whether or not C<sub>4</sub> parenchyma BSC allow soluble protein to escape rumen degradation. The objective of this study was to follow soluble protein loss from switchgrass and big bluestem parenchyma bundle sheath cells by using ribulose 1,5-bisphosphate carboxylase/oxygenase as a marker for protein loss since this enzyme is compartmentalized in BSC of C<sub>4</sub> plants and comprises up to 230 g kg<sup>-1</sup> of total protein in leaves of C<sub>4</sub> plants (Ku et al., 1979). Results presented herein offer a unique application of immunocytochemical techniques to microscopic evaluation of the relationship between C<sub>4</sub> grass leaf anatomy during tissue degradation in the rumen and the possible fate of soluble plant protein in the ruminant gastrointestinal tract.

### MATERIALS AND METHODS

#### Enzyme Extraction/Purification

Tobacco (*Nicotiana tabacum* L. cv. 'Glurk') was grown from seed under greenhouse conditions. RuBPCase was isolated and crystallized from young fully expanded leaves according to

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**Abbreviations:** BSC, bundle sheath cell; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; PBS, phosphate buffered saline; RuBPCase, ribulose 1,5-bisphosphate carboxylase/oxygenase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

the protocol described by Kung et al. (1980). Crude protein extract was prepared by grinding 40 g of leaf lamina with 23 mL 2 M NaCl (4°C) plus 0.07 mL 2-mercaptoethanol with a mortar and pestle to break the chloroplasts and release proteins. The resulting slurry was squeezed through four layers of cheesecloth and one layer of Miracloth (CalBiochem, La Jolla, CA); this procedure yielded approximately 30 mL green filtrate. The filtrate was immediately heated in a 50°C water bath to 45°C to precipitate undesirable material, then rapidly cooled to 17°C in an ice bath. After cooling, 0.6 mL of a solution containing 100 mg mL<sup>-1</sup> Na<sub>2</sub> EDTA (ethylenediamine tetraacetate) was added and the pH adjusted to 7.5 with 1.0 M Tris [tris(hydroxymethyl)aminomethane] (about 1 mL). The suspension was centrifuged for 10 min at 48 000 × g then NaCl removed from supernatant by gel filtration at 4°C with a Sephadex G-50 (Aldrich Chemical, Milwaukee, WI) column equilibrated with 25 mM Tris-HCl buffer (pH 7.4) containing 0.2 mM EDTA (Tris-EDTA). The column bed-volume was approximately three times the volume of extract desalted. A flow rate of no more than 1 mL min<sup>-1</sup> cm<sup>-2</sup> of column cross-section was used. RuBPCase crystals formed in the column and were collected with the effluent by a fraction collector.

Enzyme recrystallization was repeated three times by collection of the crystalline enzyme after centrifugation (10 min at 5000 × g), suctioning off the supernatant, and washing the pellet twice with Tris-EDTA. The crystals were resuspended in a small volume of Tris-EDTA and solubilized by adding saturated NaCl. The solution was passed through the G-50 column and recrystallized RuBPCase collected.

### Antibody Production

Following solubilization of the crystalline RuBPCase in 0.15 M phosphate buffered saline (PBS), polyclonal antibodies were developed against the thrice-crystallized tobacco leaf protein in two rabbits (*Oryctolagus uniculus* L.) housed at the Monoclonal Antibody Core Facility, University of Nebraska-Lincoln (UNL). Antisera obtained were pooled; ELISA titer was 1:100 000.

Proteins in crude extracts of undegraded leaf tissue of switchgrass, big bluestem, and tobacco were prepared and separated electrophoretically (Laemmli, 1970). All tissues were first frozen (-80°C) then ground into a powder with a mortar and pestle. Protein extraction buffer (0.06 M Tris-HCl, 0.58 M SDS, 0.06 M glycerol, 0.05 mL L<sup>-1</sup> 2-mercaptoethanol, 0.002 M EDTA) was added to the frozen powdered tissue and grinding continued until intact tissue was no longer visible. These extracts were used to perform SDS-PAGE in duplicate 6.5- by 9-cm gels polymerized from 125 g kg<sup>-1</sup> acrylamide. Total protein content of the crude extracts was determined by the Bradford assay (Bradford, 1976). Extract aliquots that contained 2 µg protein were subjected to electrophoresis for one hr at 0.15 m<sup>2</sup> kg s<sup>-3</sup> A<sup>-1</sup>. The unstained gels were placed on sheets of nitrocellulose paper and electroblotted for 90 min at 0.20 m<sup>2</sup> kg s<sup>-3</sup> A<sup>-1</sup> (Towbin et al., 1979). Following the transfer, the nitrocellulose membranes were incubated for 1 h in a blocking buffer (20 mM Tris, 0.53 M NaCl, pH 7.5) containing 10 mg mL<sup>-1</sup> powdered milk to prevent nonspecific binding of antibodies to the transferred polypeptides. The nitrocellulose membranes were then incubated for 1 h at 25°C in two separate vessels of blocking buffer containing antibodies to tobacco RuBPCase. One membrane was incubated in a 1/10 000 (v/v) of antibodies developed to the most recently extracted tobacco RuBPCase. For comparison, the second nitrocellulose membrane was incubated in a 1/25 000 (v/v) of RuBPCase antibodies for which specific binding of the primary antibody had been previously verified (Dr. Raymond Chollet, 1991, personal communication). Following incubation, excess

antibody was removed from the membrane by a 5 min wash with blocking buffer followed by a wash with deionized water. Washed nitrocellulose membranes were then incubated for 1 h at 25°C in blocking buffer containing biotinylated goat antirabbit IgG at 1/1000 (v/v). After incubation, the unreacted antibodies were washed from the membrane as described previously. Specific binding of the primary antibodies was revealed on nitrocellulose membrane by adding a peroxidase substrate, 4-chloro-1-naphthol (Sigma), in Tris-saline buffer (0.6 mg mL<sup>-1</sup>) containing 50 mL L<sup>-1</sup> hydrogen peroxide. The reaction was allowed to proceed for 1 h in darkness.

### Plant Material Production, Degradation, and Sampling

Switchgrass (cv. 'Trailblazer') and big bluestem (cv. 'Kaw') were propagated vegetatively from sods in the greenhouse (32°C, full daylight, no fertilization) to yield 15 to 20 tillers in each of six 12-L containers filled with 1:1:1 soil:sand:peat moss mix. To provide plant tissue of contrasting protein contents and fiber maturity, all tillers were clipped to 10 cm and allowed to regrow for 10 wk. At that time, tillers in one-half of the containers were clipped again to generate 2-wk-old regrowth. At 12 wk, leaf blade midsections (0.5 cm long) from the four uppermost collared leaves on individual tillers of each grass at each age were randomly confined to dacron bags (5 by 12.5 cm; 53-µm mesh), and incubated in situ for 4, 8, 12, 16, 24, and 36 h in three rumen-fistulated steers fed a mixed warm-season grass hay. One dacron bag containing four leaf blade midsections from each species at each age was prepared for each incubation interval in each steer. Leaf tissue protein contents (Table 1) were characterized by both the Bradford assay for leaf extracts prepared as described previously for immunoblotting, and crude protein as calculated from total Kjeldahl N (AOAC, 1990).

Four leaf blade midsections from undegraded leaves (no rumen incubation) of each species were dissected at the start of the in situ trial to give sections 2 mm wide by 2.5 mm long. Undegraded sections were then vacuum-infiltrated with 0.025 M K-phosphate buffer (pH 7.3) containing 20 g L<sup>-1</sup> paraformaldehyde and fixed for 3 h at 25°C. Following each in situ incubation interval, degraded leaf blade sections were removed from the rumen, dissected, and fixed in the same manner described for undegraded leaf tissue. Each incubation interval yielded 12 degraded leaf blade midsections from each species at each age.

Omasal digesta and fecal samples were collected from rumen-fistulated steers grazing switchgrass or big bluestem monocultures at Mead, NE, in June 1992. Pastures had been burned in the spring. Omasal samples were obtained following total rumen evacuation of three animals on each pasture and samples stored in fixative (4°C). Digesta and fecal samples were washed with fresh fixative over four cheesecloth layers to isolate undigested fibrous material. Fifteen leaf fragments that had escaped complete reduction to fibers were recovered from fibrous omasal material from each animal, dissected, and fixed as described for leaf blade tissue incubated in situ. Fibers

**Table 1. Protein contents of C<sub>4</sub> grass leaves used for immunofluorescent localization of RuBPCase from in situ trial.**

Species	Tissue age	Soluble protein	Crude protein
		<div>g kg<sup>-1</sup></div>	
Switchgrass	2 wk	144	186
	12 wk	49.5	136
Big Bluestem	2 wk	89.3	180
	12 wk	43.3	146

recovered from omasal and fecal material were examined microscopically under low power. Forty individual vascular bundles with intact parenchyma BSC were identified and isolated from fibrous omasal material from each animal; eight to 10 vascular bundle-BSC tissues were isolated from fecal samples from each animal. After isolation, vascular bundle-BSC tissues were photographed, and stored in fixative at 4°C until embedded.

### Processing of Fixed Tissues

Undegraded and degraded leaf tissues, leaf fragments, and vascular fibers with attached bundle sheath cells were processed for immunocytochemical studies according to a modification of the method outlined by Reed and Chollet (1985). Tissues were removed from the fixative then rinsed in 0.1 M K-phosphate buffer (pH 7.3), dehydrated in a graded series of ethanol, then followed by two changes of propylene oxide (15 min each). Tissues were infiltrated and embedded in Araldite 502 (Luft) from Electron Microscopy Sciences (Fort Washington, PA). Undegraded and degraded leaf tissues, and omasal leaf fragments were oriented at both ends of flexible molds to obtain cross-sections. Between five and seven Araldite blocks were prepared for each age species incubated in each animal.

Isolated vascular fibers with intact BSC from omasal digesta and feces were embedded in Araldite using the flat embedding technique described by Raymond and Pickett-Heaps (1983). Fixed vascular bundle-BSC tissues were dehydrated as described for leaf blade tissues but embedded in a thin layer of Araldite between two microscope slides coated with liquid release agent (Electron Microscopy Sciences). After polymerization of the Araldite, the top slide was released and vascular bundle-BSC tissues located with a dissecting microscope. Small squares of the thin Araldite layer that contained longitudinally-oriented vascular bundle-BSC tissue were cut and attached with Super-Glue 5 (Loctite Corp., Cleveland, OH) to the ends of specially prepared Araldite stubs. This technique allowed preparation of between two and five Araldite blocks containing longitudinally-oriented vascular bundle-BSC tissues from omasal digesta and fecal material from each steer. From these blocks, longitudinal sections of the vascular fibers with attached BSC were obtained.

For all tissues, sections (2 µm thick) were mounted onto glass slides and heat-affixed for 30 min at 80°C. At least four slides with 15- to 25 2-µm sections were prepared from each Araldite block. Thus, at least 65 sections were examined from each block. Resin was removed from all sections according to the method of Mayor et al. (1961).

### Immunofluorescent Localization

For immunofluorescent localization, the indirect method described by Reed and Chollet (1985) was used. Nonspecific adsorption of FITC-conjugated secondary goat (*Capra hircus* L.) antibody was minimized by tissue preincubation in a 100 mL L<sup>-1</sup> solution of normal goat serum in PBS (pH 7.3) for 2 h at 25°C. Following a 30 s rinse in distilled water, separate slides that contained sections of each tissue were incubated for 60 h at 4°C in humid chambers in either normal rabbit serum (1 slide; 15–25 sections) or anti-RuBPCase IgG (3 slides; 45–75 sections) diluted to 0.3 mg mL<sup>-1</sup> protein with PBS (pH 8.4). Sections were washed for three periods of 10 min each in PBS, then incubated 45 min (25°C, dark) in FITC-conjugated goat anti-rabbit IgG (U.S. Biochemical Corp., Cleveland, OH). Sections were again washed for two periods of 10 min each in PBS (pH 7.3). Tissue autofluorescence was minimized by immersing the slides (15 min) in PBS

(pH 7.3) containing 0.096 M Evan's Blue (Aldrich). Sections were rinsed (10 s) in distilled water, and mounted in PBS-glycerol 1/4 (v/v) containing 0.01 M *p*-phenylenediamine (Aldrich) to retard fading of fluorescence during microscopy (Johnson and Nogueira-Araujo, 1981).

Observations were made and representative sections photographed with a Nikon 'Optiphot' epifluorescence photomicroscopy system (Garden City, NY) with an excitation band of 410 to 485 nm, a 505-nm dichroic mirror, and a 515- to 560-nm barrier filter.

## RESULTS AND DISCUSSION

### Greenhouse-Grown Tissues

Progressive examination of switchgrass (Fig. 1A–1D) and big bluestem (Fig. 1E–1H) leaf blade tissues incubated in-situ revealed degradation patterns consistent with those reported in previous light and electron microscopy studies (Akin et al., 1983; Hastert et al., 1983; Wilson and Hattersley, 1983; Twidwell et al., 1990). In general, mesophyll tissues were degraded more rapidly than parenchyma BSC associated with large vascular bundles, and lignified vascular tissue resisted degradation throughout the longest incubation period. Parenchyma BSC associated with large and small vascular bundles of undegraded (0-h) switchgrass (arrow, Fig. 1A) and big bluestem (Fig. 1E) leaf sections incubated in normal rabbit serum displayed neither specific fluorescence nor marked autofluorescence. Localization patterns observed for RuBPCase in undegraded switchgrass (Fig. 1B) and big bluestem (Fig. 1F) exposed to rabbit anti-RuBPCase IgG were as expected for C<sub>4</sub> grasses: Signal was detected from BSC only (Fig. 1B, arrow); fluorescence was absent in mesophyll cells. There was no difference between 2- and 12-wk-old tissues of either grass in sequence of tissue degradation or RuBPCase localization characteristics throughout the in situ trial. Also, results for degraded switchgrass and big bluestem leaf sections incubated in normal rabbit serum were identical to undegraded leaf sections: No specific fluorescence was displayed. Thus, figures presented are the results for 2-wk-old degraded leaf sections incubated in anti-RuBPCase IgG only.

Switchgrass and big bluestem mesophyll and BSC remained intact through 12 h of incubation; strong signals were present from RuBPCase in BSC of both grasses (figure not shown). Although extensive degradation of switchgrass mesophyll occurred by 16 h, BSC associated with large and small (Fig. 1C) vascular bundles remained intact and the RuBPCase signal strong. Degradation of big bluestem mesophyll was not as extensive as that observed for switchgrass mesophyll at 16 h. However, BSC associated with large vascular bundles of big bluestem (Fig. 1G, right) also resisted degradation until 16 h, while BSC associated with small vascular bundles (Fig. 1G, arrow) were disrupted and signal was lost by 16 h. Hastert et al. (1983) also observed that digestion of small vascular bundles and associated BSC in big bluestem leaf blades proceeded more quickly than mesophyll or BSC associated with large vascular bundles. By 24 h, switchgrass BSC began to show evidence of disruption although occasional signals were still detected from cell

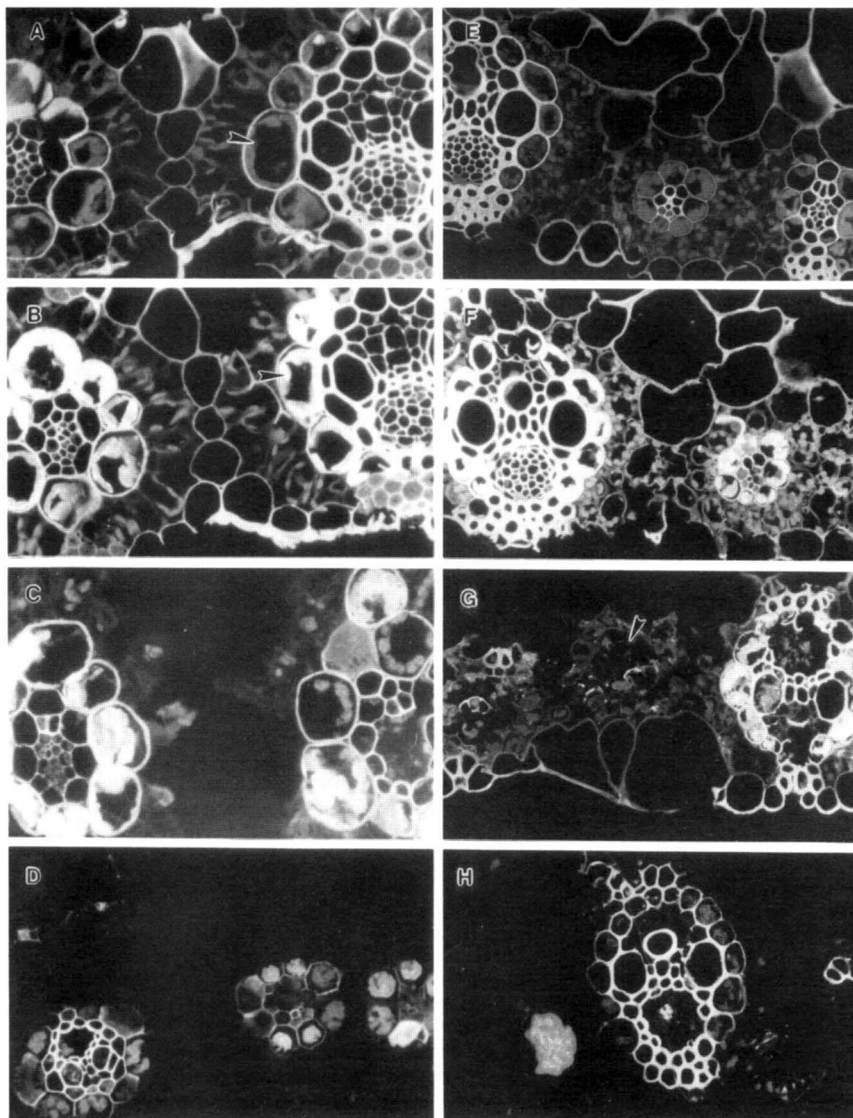


Fig. 1. Fluorescent microscopy of grass leaf blade cross sections. A: Undegraded (0 h) switchgrass incubated in normal rabbit serum, parenchyma bundle sheath of large vascular bundle (arrow)  $\times 364$ ; B: undegraded switchgrass incubated in anti-RuBPCase IgG, parenchyma bundle sheath of large vascular bundle (arrow)  $\times 384$ ; C: switchgrass after 16 h of rumen degradation  $\times 415$ ; D: switchgrass after 24 hr of rumen degradation  $\times 350$ . E: Undegraded (0 h) big bluestem incubated in normal rabbit serum  $\times 283$ ; F: undegraded big bluestem incubated in antiRuBPCase IgG  $\times 283$ ; G: big bluestem after 16 h rumen degradation, loss of small bundle sheath cell and associated protein (arrow)  $\times 283$ ; H: big bluestem after 24 h of rumen degradation  $\times 272$ .

contents (Fig. 1D). In contrast, signals were not detected from large vascular bundles of big bluestem leaf blades at 24 h (Fig. 1H). After 36 h of incubation, vascular tissue of both grasses retained mainly heavily lignified components (figure not shown).

### Omasal Tissues

Neither degraded leaf fragments from the omasum nor vascular fibers with intact BSC from omasal or fecal samples were found in fibrous material from steers grazing big bluestem. This is consistent with results from the dacron bag study which indicated that for big bluestem, few small vascular bundles remain in the rumen past 16 h and BSC associated with large vascular bundles are degraded by 24 h. In contrast, numerous leaf fragments that escaped complete reduction to fibers before

leaving the rumen were found in omasal samples from steers grazing switchgrass. Sections from switchgrass leaf fragments from the omasum incubated in normal rabbit serum did not display fluorescence (figure not shown), while signal was observed from BSC of those incubated with anti-RuBPCase IgG (Fig. 2A).

Switchgrass vascular fibers with attached BSC were abundant and easily sampled in omasal digesta from all three steers (Fig. 2B, 2C). Zoosporengia of rumen anaerobic fungi were consistently found associated with these vascular fiber-BSC complexes (Fig. 2B, arrow). Zoosporengia were also found associated with vascular fibers from big bluestem omasal digesta even though BSC were no longer attached (figure not shown). No specific fluorescence was displayed from longitudinal sections of BSC associated with vascular fibers isolated



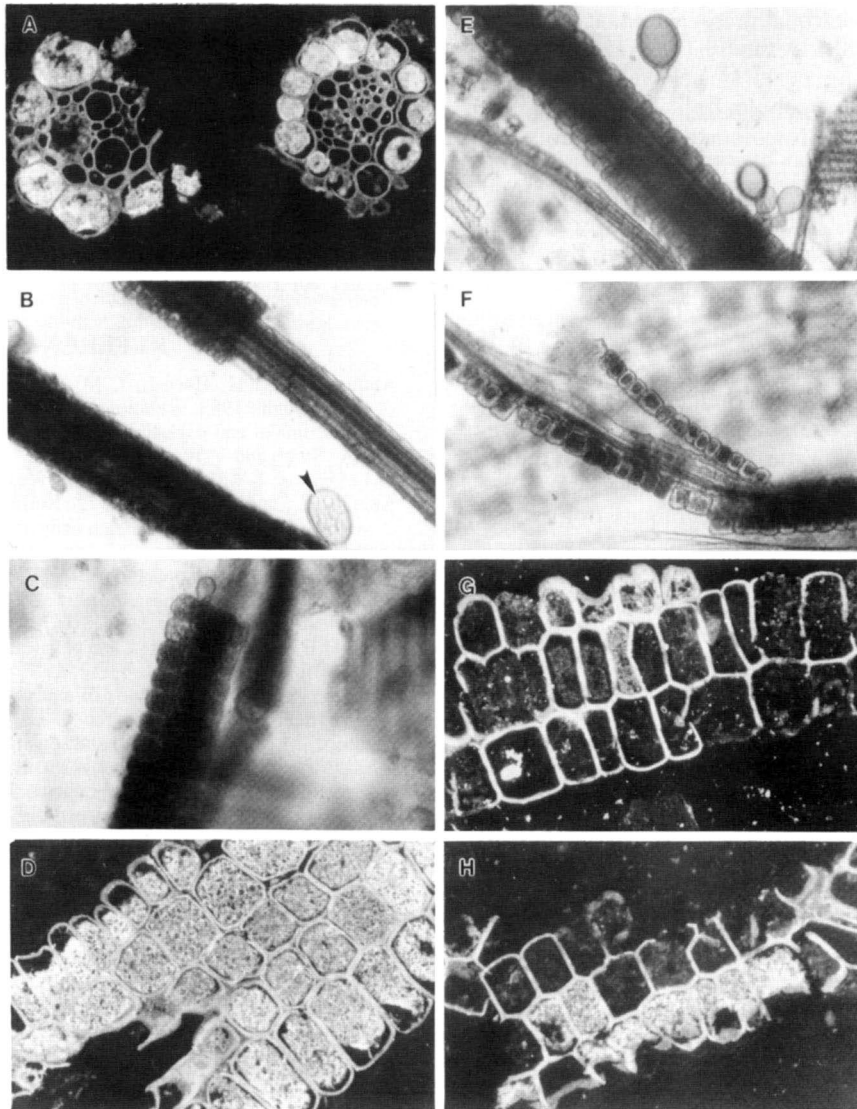


Fig. 2. Switchgrass parenchyma bundle sheath cells isolated from omasal digesta (A-D) and fecal material (E-H). A: Cross sections from leaf fragments incubated with anti-RuBPCase IgG  $\times 300$ ; B-C: BSC associated with vascular bundles and zoosporangia (arrow) of rumen anaerobic fungi  $\times 300$ . D: flat-embedded section incubated with anti-RuBPCase IgG  $\times 295$ ; E-F: BSC associated with vascular bundles and zoosporangia  $\times 350$  (E),  $\times 300$  (F); G: flat-imbedded section incubated with normal rabbit serum  $\times 300$ ; H: flat-imbedded section incubated with anti-RuBPCase IgG  $\times 300$ .

from switchgrass omasal digesta and incubated with normal rabbit serum (figure not shown). Signal was consistently obtained from BSC associated with vascular fibers found in omasal digesta from steers grazing switchgrass (Fig. 2D).

Vascular fibers with associated BSC were also found in various stages of degradation in fecal samples (Fig. 2E, 2F) from steers grazing switchgrass, although these were more difficult to find than was the case in omasal digesta. Zoosporangia of rumen anaerobic fungi were observed associated with fecal vascular fibers and associated BSC (Fig. 2E). Specific fluorescence was not observed from longitudinal sections of BSC recovered from fecal material when incubated with normal rabbit serum (Fig. 2G). Occasional switchgrass bundle sheath cells that displayed specific fluorescence when incubated with antiRuPBCase IgG were found in fecal material (Fig. 1H).

Wilson et al. (1989) reported that BSC remained attached to fine fibers in green panic (*Panicum maximum* var. *trichoglume* Jacq.) leaves following mastication and rumen incubation for 24 h. They postulated that external roughness of typical  $C_4$  fiber particles caused by attached BSC would increase resistance to independent flow from the fiber raft in the rumen and thus prolong residence time in the rumen. Results from observations on omasal samples in this study indicated that for some  $C_4$  species, e.g., switchgrass, vascular fibers with associated BSC can escape the fiber raft prior to disruption and degradation.

Bacterial colonization of the outer tangential walls of switchgrass BSC was demonstrated by Wilson and Hattersley (1983) to be extremely low. Rumen anaerobic fungi may play a role in disruption of BSC in both grasses studied since these fungi produce a wide range of enzymes that can lower the tensile strength and digest

the major structural carbohydrates of plant cell walls (Orpin and Joblin, 1988). Although the exact mechanism by which anaerobic fungi penetrate cell walls is unknown, rumen fungi are also proteolytic, unlike the rumen cellulolytic bacteria and their proteolytic action probably facilitates the penetration of the proteinaceous cell membrane by rhizoids (Ho et al., 1988; Orpin and Joblin, 1988). Dehority (1993) suggested that the rumen fungi act synergistically in the digestion of forages by physically disrupting lignified tissue and allowing rumen microbes greater access to plant cell contents. Thus, fungal colonization of vascular fibers with associated BSC in the rumen could affect subsequent release of protein in the lower gastrointestinal tract. Whether or not observed protection of protein occurs at a sufficient level to offer a significant advantage for ruminant performance remains to be determined.

Use of immunocytochemical techniques in this study allowed demonstration that in certain  $C_4$  grass species (i) bundle sheath cells can protect protein from ruminal degradation through at least 24 h of in situ incubation, (ii) bundle sheath cells with associated RuBPCase can leave the rumen prior to protein degradation and loss, and (iii) a portion of protein protected by bundle sheath cells can completely escape degradation in the ruminant gastrointestinal tract and be excreted. The immunocytochemical technique used was easily adapted to study of rumen-degraded  $C_4$  grass leaf tissue. When used for determination of presence or absence of RuBPCase, immunofluorescent localization has advantages over other molecular techniques such as SDS-PAGE that require tissue extraction prior to protein separation or analysis. One advantage is that protease action is eliminated by immediate fixation of tissue protein in situ following incubation in the gastrointestinal tract. For techniques that require tissue extraction, especially where extracts must be stored before analysis, the extent of the impact that proteases may have on protein detection cannot be definitely known. Another important advantage of immunofluorescent localization is that it allows observation of the cell wall-cell protein relationship in situ during tissue degradation. Information about this relationship is lost when tissues are ground during extraction processes.

A drawback of immunofluorescent localization is that quantification of RuBPCase remaining in  $C_4$  tissues would be difficult because distribution of this enzyme in the BSC follows chloroplast distribution, which is characteristically near the cell wall. Other immunocytochemical techniques such as radial immunodiffusion and ELISA could be adapted to quantify RuBPCase at various locations in the ruminant gastrointestinal tract. These techniques, in combination with immunofluorescent localization, show promise for use in research designed to elucidate the role of plant anatomy in availability of proteinaceous components of  $C_4$  grass cells.

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