Quantitative Measurement of Fiber Fractions of Cool- and Warm-Season Grass Herbage Using Cell-Wall-Degrading Enzymes†

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Quantitative *in vitro* methods using commercial cellulase (CEL), pectinase (PECT), and hemicellulase (HCEL) preparations were developed to simplify prediction of digestibility of cool- and warm-season forage grasses. Samples of orchardgrass (*Dactylis glomerata* L. var. Pennlate), tall fescue (*Festuca arundinacea* Schreb. var. KY 31) (cool-season grasses), big bluestem (*Andropogon gerardi* Vitman var. NY 1145), and switchgrass (*Panicum virgatum* L. var. KY 1625) (warm-season grasses) were taken at advancing stages of maturity. Isolated cell-wall material (CWM) was prepared by repetitively extracting lyophilized, ground tissue with aqueous and organic solvents. Relative solubilities of CWM digested individually with CEL, HCEL, or PECT were 100:30:40, respectively. When enzyme preparations were used sequentially, the influence of CEL, HCEL, and PECT on CWM solubility depended on the order in which the enzyme preparations occurred in the sequence. The fraction of CWM solubilized with CEL was similar to that obtained when CWM was digested with CEL, HCEL, and PECT simultaneously. Solubilities of cell-wall material in unfractionated tissues digested with CEL were comparable to those of corresponding CWM digested with CEL; thus, the laborious steps required for isolation of cell-wall material can be avoided.

Keywords: *Cool-season grass; warm-season grass; orchardgrass; Dactylis glomerata L.; tall fescue; Festuca arundinacea Schreb.; big bluestem; Andropogon gerardi Vitman; switchgrass; Panicum virgatum L.; fiber; digestibility; cell-wall-degrading enzymes; cellulase; pectinase; hemicellulase*

INTRODUCTION

Quantitative *in vitro* methods using cell-wall-degrading enzymes for assessing digestibility of forages have been in use since the original report of Donefer et al. (1963), and many variations have been proposed as more enzyme sources have become available commercially (Jones and Hayward, 1973, 1975; McQueen and Van Soest, 1975; Adegbola and Paladines, 1977; Goto and Minson, 1977; Roughan and Holland, 1977; McLeod and Minson, 1978, 1980). The thrust of the studies conducted by these researchers was to develop methods for predicting forage digestibility from small samples of material such as those from small-plot studies and selection or breeding programs. Most of the methods that have been developed are two-stage processes using either neutral detergent fiber (NDF) extraction or pepsin digestion followed by cellulase digestion, although Jarriage et al. (1970), Hartley et al. (1974), and Masaoka and Tarumoto (1979) used one-stage cellulase procedures. Cellulase digestion gave results correlated with those obtained when the forages were digested *in vitro* with rumen liquor, and Bughrara and Sleper (1986) found correlations between the *in vitro* cellulase dry matter digestibility techniques and *in vivo* dry matter digestibility to be highly significant.

The work described in this report is a series of investigations into the fiber components of cool- and warm-season grasses using gravimetric techniques similar to those used in the studies referred to above but incorporating pectinase and hemicellulase treatments developed from the studies of Talmadge et al. (1973) and Bauer et al. (1973). A procedure that permits rapid and convenient assessment of unfractionated tissue and gives results comparable to those obtained for corresponding isolated cell-wall material (CWM) has been developed.

MATERIALS AND METHODS

Plant Materials. Two cool-season grasses, orchardgrass (*Dactylis glomerata* L. var. Pennlate) and tall fescue (*Festuca arundinacea* Schreb. var. KY 31), and two warm-season grasses, big bluestem (*Andropogon gerardi* (L.) Vitman var. NY 1145) and switchgrass (*Panicum virgatum* L. var. KY 1625), were used. Samples were cut at different stages of growth from pure stands of the individual grasses maintained at the Rock Springs, PA, farm of the USDA, Agricultural Research Service, U.S. Regional Pasture Research Laboratory (Table 1). Cool-season grasses were harvested at ground level at approximately weekly intervals from mid-May until mid-June, during which time the plants progressed from the purely vegetative stage through the flowering stage to the seed-setting stage. Growth of warm-season grasses commenced later, and samples were taken at ground level beginning in late May when the plants were 23−30 cm high and vegetative and continuing through early- to mid-July when plants were 75−135 cm high and flowering. One sample of mature switchgrass was taken in September. Subsamples were either oven-dried at 100 °C or frozen immediately in liquid nitrogen and

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Table 1. Harvest Dates and Growth Stages of Cool- and Warm-Season Grass Herbage

harvest date	cool-season grasses		warm-season grasses	
	orchardgrass	tall fescue	big bluestem	switchgrass
1985				
May 6	vegetative, 23−30 cm	vegetative, 15−23 cm		
May 13	vegetative/early boot	vegetative		
May 20	vegetative, some inflorescences emerged	stemmy, inflorescences emerged		
May 23	>50% inflorescences emerged	ca. 90% inflorescences emerged	vegetative, 23−30 cm	vegetative, 23−30 cm
June 6	flowering, stemmy	flowering, stemmy	vegetative, 45−50 cm	vegetative, 45−50 cm
June 11	anthers withering on flowers, stemmy	seed set, stemmy	vegetative, 50−60 cm	vegetative, 50−60 cm
June 18		seeds mature, stemmy	vegetative, 60−75 cm	vegetative, 50−60 cm
June 24			75−90 cm, 10% shooting	vegetative, 60−75 cm
July 3			90−100 cm, 40% shooting	vegetative, 75−90 cm
July 9			≤135 cm, >50% shooting, some inflorescences visible	90−105 cm, 10% shooting
1986				
May 15	vegetative, 38−46 cm, some shooting			
May 29	>50% inflorescences emerged			
June 4	flowering, stemmy			vegetative, ≤30 cm
June 8	flowering, stemmy			
June 17	seed set, stemmy			
June 25				vegetative, ≤60 cm
July 1				vegetative, 64−76 cm
July 16				flowering, stemmy
July 28				seed set, stemmy
Sept. 11				seeds mature

maintained at −20 °C until lyophilized. After drying, plant tissue was ground to pass a 1 mm screen.

Preparation of Cell-Wall Material. Soluble material was removed from samples of ca. 30 g of lyophilized tissue by repeated treatment with aqueous and organic solvents using a modification of the method of Talmadge et al. (1973). Tissue was suspended in 600 mL of 0.1 M potassium phosphate buffer (pH 7.0), stirred intermittently for 1 h at room temperature (25 °C), and refrigerated overnight. Following centrifugation at 600*g* for 20 min, the buffer-soluble fraction was removed by aspiration of the supernate. The extraction, without the overnight incubation, was repeated sequentially using 600 mL volumes each of 0.1 M potassium phosphate buffer (pH 7.0), 0.5 M potassium phosphate buffer (pH 7.0) (twice), distilled water (four times), methanol (once), and 1:1 (v:v) chloroform: methanol (three times). After the final extraction, the insoluble residue was collected by filtration through a Büchner funnel, washed with acetone three times or until the filtrate was free of plant pigments, and air-dried at room temperature. This material was the CWM.

Enzyme Digestion of Plant Tissue. Samples were digested with cell-wall-degrading enzymes using modifications of procedures described by Bauer et al. (1973), Talmadge et al. (1973), and Keegstra et al. (1973). Samples (0.5 g) of air-dry unfractionated tissue or CWM were incubated in 50 mL Erlenmeyer flasks with digestive enzyme(s) contained in 20 mL of 0.025 M sodium acetate buffer (pH 5.0). A bulk sample of CWM prepared from mature switchgrass was used to test various commercially available enzyme preparations for their suitability for the proposed work and to determine appropriate enzyme concentrations and conditions for digestions. Six cellulase (CEL) preparations (Onozuka R-10 and Onozuka RS, Yakult Honsha Co., Ltd., Nishinomiya, Japan, Meicelase, Meiji Seika Kaisha, Ltd., Tokyo, Japan, and Cellulase Y-C, Seishin Pharmaceutical Co., Tokyo, Japan, all from *Trichoderma viride*; cellulase from *Penicillium funiculosum*, Sigma Chemical Co., St. Louis, MO; and cellulase from *Aspergillus niger*, Sigma) [Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion others that may also be suitable.], four hemicellulase (HCEL) preparations (Rhozyme HP150, Genencor, Inc., South San Francisco, CA; hemicellulase, Sigma; hemicellulase, Pflatz and Bauer, Inc., Waterbury, CT; and Novozyme SP249, Novo Laboratories, Wilton, CT, all from *Aspergillus niger*), and two pectinase (PECT) preparations (Pectolyase Y-23 from *Aspergillus japonicus*, Seishin Pharmaceutical Co., Ltd., Tokyo, Japan; and Macerozyme R-10 from *Rhizopus* spp., Yakult Honsha Co., Ltd.) were tested in various concentrations. With the exception of CEL from *Aspergillus* which was less effective than CEL from *Trichoderma* or *Penicillium* species, differences in effectiveness of enzymes from the various commercial sources were small. On the basis of these results, the following enzyme preparations and concentrations were used in this study: CEL, 2% (w/v) Onozuka RS; HCEL, 1% (w/v) Rhozyme HP150; and PECT, 0.1% (w/v) Pectolyase Y-23. These preparations contain mixtures of cell-wall-degrading enzymes, and thus abbreviations designate the major enzymatic activity associated with the preparation. Onozuka RS, for example, contains high xylanase (a hemicellulose-degrading enzyme) activity along with the cellulase activity generally associated with the extracellular enzyme product of *Trichoderma viride*.

Preliminary studies showed that, under the conditions of digestion, fungi and bacteria proliferated. Microbial growth was controlled by incorporating Thiram (tetramethylthiuram disulfide, 1.3 mg/mL; DuPont, Wilmington, DE), chloramphenicol (0.025 mg/mL; Sigma), and cycloheximide (0.025 mg/mL; Sigma) into the buffered enzyme solutions. These inhibitors did not disrupt digestion of cellulose paper.

Incubation mixtures were agitated continuously (140 rpm) on an orbital shaker for 20 h at 30 °C. Undigested material was collected by filtering mixtures through Gooch crucibles and rinsed with distilled water. Crucibles and their contents were dried at 95 °C, and weights of retentates were determined by difference. As blanks, 0.5 g samples were incubated with buffer only, and retentate weights were determined as above. The weight loss due to enzyme treatment was obtained by subtracting retentate weight from the weight of dry matter (DM) contained in the original 0.5 g air-dried material and correcting for weight loss due to buffer only. To ensure accurate DM weights, all weighings were done after transferring crucibles from the oven to a desiccator, evacuating, and readmitting air through tubes packed with dry silica gel. Dry matter determinations were done on all air-dried materials by drying samples at 95 °C overnight. Dry matter digested by the enzymes was expressed as a percentage of the dry starting material.

Sequential digestions of CWM were carried out with individual enzyme preparations. The retentate recovered after

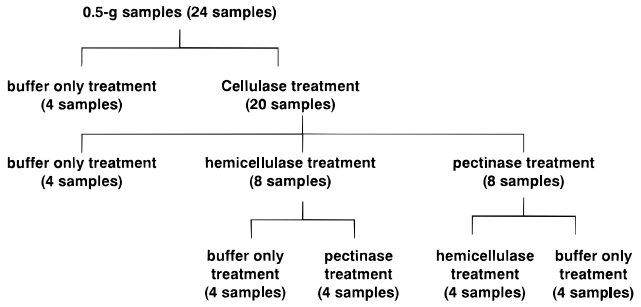

Figure 1. Example of the procedure used to investigate the effects of enzyme sequence on the digestion of cell-wall material.

digestion with the first enzyme was dried at 40 °C, weighed, and then digested with the next enzyme in the sequence. This procedure is illustrated in Figure 1 for CEL as the first enzyme treatment.

To determine whether digestibility analyses could be conducted on unfractionated tissue, dried samples were digested directly with CEL. The general conditions for these digestions were the same as for corresponding CWM; however, because results using CEL and CEL + HCEL + PECT were similar for CWM, only CEL was used in studies with unfractionated tissue samples. After incubation (with CEL or buffer) and filtration, retentates in Gooch crucibles were flushed with water to remove residual digestion medium and solubilized material and rinsed successively with 10 mL of 95% ethanol and 10 mL of acetone to remove lipids and other soluble constituents before drying and weighing. Cell-wall material solubilized by CEL ("CWM"S) was determined as the difference between the dry weights of the buffer-insoluble, alcohol- and acetone-rinsed residue ("CWM") and the CEL-insoluble, alcohol- and acetone-rinsed residue ("CWM"I).

Fiber Analyses. Standard procedures for forage fiber analysis were used to determine NDF and acid detergent fiber (ADF) (Goering and Van Soest, 1970) and permanganate lignin (Van Soest and Wine, 1968). Dry matter determinations were performed as described by the Association of Official Analytical Chemists (1980).

RESULTS

Digestion of CWM. Results obtained using all permutations of the three enzyme preparations (CEL, HCEL, and PECT) to digest CWM prepared from mature switchgrass are presented in Table 2. Removal of one component of the cell wall did not make other components more accessible for digestion. The degree of tissue digestion by an individual enzyme preparation depended on the order in which it occurred in the sequence, being highest when the enzyme was used first and lowest when it was used last. That enzyme preparations were not pure probably accounts for this observation. The sum of the dry matter solubilized by sequential digestion was comparable for all sequences in which CEL or PECT was the first enzyme used. When HCEL was used first, the total amount of dry matter solubilized was ca. 20% less.

Table 3. Digestive Powers (%)[a] of Cellulase, Hemicellulase, and Pectinase Preparations on Isolated Cell-Wall Material from Cool- and Warm-Season Grass Herbage Harvested in 1985

species	harvest date	enzyme treatment CEL	HCEL	PECT
cool-season				
orchardgrass	May 6	102.1	41.0	41.9
	May 13	97.6	32.5	37.5
tall fescue	May 6	100.8	40.3	43.4
	May 13	102.6	36.9	42.1
	May 23	102.1	36.8	49.7
	June 6	103.2	33.9	41.6
	June 18	94.8	32.1	ND
warm-season				
big bluestem	May 23	102.9	32.6	39.6
	June 6	100.3	ND	36.4
	June 18	95.9	29.3	42.2
	June 24	99.7	26.9	38.6
	July 9	97.8	25.1	ND
switchgrass	May 23	96.2	27.6	37.1
	June 6	96.9	33.6	40.7
	June 11	104.3	34.1	48.7
	June 18	101.0	31.1	40.1
	June 24	102.3	30.0	45.9
	July 3	97.1	24.5	40.6
	July 9	98.1	26.3	40.0
mean		99.8	31.9	41.5
SD		2.9	4.9	3.7

[a] Solubilities of CWM with individual enzyme preparations are normalized to results obtained when a mixture of the three enzyme preparations was used. CEL, 2% (w:v) cellulase Onozuka RS; HCEL, 1% (w:v) hemicellulase Rhozyme HP150; PECT, 0.1% (w:v) pectinase Pectolyase Y-23; ND, not determined.

Digestive powers of individual CEL, HCEL, and PECT preparations, relative to that for the mixture of the three preparations, were determined using CWM prepared for both cool- and warm-season grasses. Results, shown in Table 3, indicated that CEL alone was comparable to the CEL + HCEL + PECT combination in digesting CWM from orchardgrass, tall fescue, big bluestem, and switchgrass. Maturity of the plant tissues had little influence on the digestive power of CEL, probably because of activities of other cell-wall-degrading enzymes present in the CEL preparation. Averaged over all plant species and sampling dates, the digestive power of CEL was 99.8 ± 2.9% of that of the CEL + HCEL + PECT combination. The PECT preparation had ca. 40% of the digestive power of CEL or the combined enzyme preparations. The HCEL treatment was only about 30% as effective as the CEL or combined enzyme treatment in digesting the CWM.

Table 2. Dry Matter (DM) Solubilized from Isolated Cell-Wall Material from Mature Switchgrass by Sequential Treatment with Digestive Enzymes[a]

DM solubilized, mg (% of total)[e]			
enzyme treatment			
first[b]	second[c]	third[d]	sum
CEL, 38.6 ± 4.0 (68.4)	HCEL, 8.0 ± 4.7 (14.2)	PECT, 9.8 ± 2.1 (17.4)	56.4 (100)
CEL, 38.6 ± 4.0 (74.8)	PECT, 12.8 ± 2.7 (24.8)	HCEL, 0.2 ± 3.5 (0.4)	51.6 (100)
HCEL, 15.1 ± 2.5 (35.3)	PECT, 9.4 ± 5.6 (22.0)	CEL, 18.3 ± 3.9 (42.7)	42.8 (100)
HCEL, 15.1 ± 2.5 (34.5)	CEL, 24.8 ± 3.7 (56.8)	PECT, 3.8 ± 3.2 (8.7)	43.6 (100)
PECT, 25.3 ± 2.7 (44.4)	CEL, 26.2 ± 2.2 (46.0)	HCEL, 5.5 ± 2.7 (9.6)	57.0 (100)
PECT, 25.3 ± 2.7 (51.2)	HCEL, 5.3 ± 3.8 (10.7)	CEL, 18.8 ± 2.5 (38.1)	49.4 (100)

[a] CEL, cellulase, 2% (w:v) Onozuka RS; HCEL, hemicellulase, 1% (w:v) Rhozyme HP150; PECT, pectinase, 0.1% (w:v) Pectolyase Y-23. [b] Means ± SD of 20 replicates. [c] Means ± SD of eight replicates. [d] Means ± SD of four replicates. [e] Starting material = 480 mg.

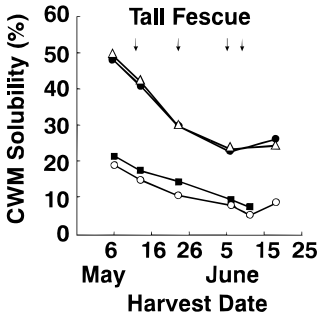

Figure 2. Solubilities of cell-wall preparations of tall fescue cut at different stages of maturity after treatment with different enzyme preparations. Enzyme treatments were cellulase [2% (w:v) Onozuka RS] (△), hemicellulase [1% (w:v) Rhozyme HP150] (○), pectinase [0.1% (w:v) Pectolyase Y-23] (■), and cellulase + hemicellulase + pectinase (●). Arrows indicate significant rainfall events. Solubilities are expressed as a percent of the initial dry weight (0.5 g) of isolated cell-wall material.

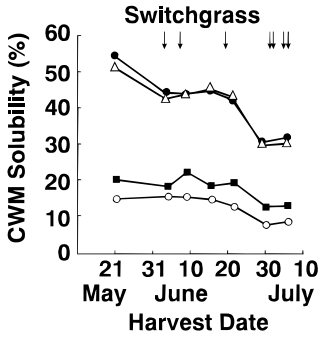

Figure 3. Solubilities of cell-wall preparations of switchgrass cut at different stages of maturity after treatment with different enzyme preparations. Enzyme treatments were cellulase [2% (w:v) Onozuka RS] (△), hemicellulase [1% (w:v) Rhozyme HP150] (○), pectinase [0.1% (w:v) Pectolyase Y-23] (■), and cellulase + hemicellulase + pectinase (●). Arrows indicate significant rainfall events. Solubilities are expressed as a percent of the initial dry weight (0.5 g) of isolated cell-wall material.

Solubilities of CWM from tall fescue and switchgrass decreased with advancing plant maturity (Figures 2 and 3). This trend was apparent, regardless of the enzyme preparation used to digest the CWM. In the case of tall fescue, almost 50% of the CWM was brought into solution by CEL or the mixture of enzyme preparations when the grass was at the vegetative stage (23−30 cm, May 6). Solubility decreased rapidly to <25% when the grass was stemmy and flowering (June 6). The CEL-soluble component of switchgrass CWM (CWMS-CEL), which was more than 50% on May 23 when the grass

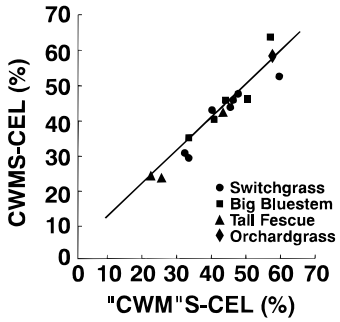

Figure 4. Comparison of solubilities of cell-wall components in isolated cell wall preparations (CWMS-CEL) and unfractionated tissue ("CWM"S-CEL) digested with 2% (w:v) cellulase Onozuka RS. For the regression equation, CWMS-CEL = 0.97-("CWM"S-CEL) + 3.03, $r = 0.959$ ($P < 0.0001$).

was 23−30 cm high and vegetative, decreased more slowly during the season than that in tall fescue and was still more than 40% of the CWM on June 24 and 30% of the CWM on July 3. Plants ranged in height from 50 to 60 cm on June 24 and from 75 to 90 cm on July 3 (Table 1). Inflorescences were not apparent on either date. The amount of CWM solubilized with CEL or the mixture of the three enzyme preparations was consistently higher than that solubilized with either PECT or HCEL. These results suggest that the CEL preparation contained sufficient HCEL and PECT activities to digest the hemicellulose and pectins in the CWM.

Digestion of Unfractionated Tissues with Enzymes. The method of preparation of CWM used in the studies described above is lengthy and laborious. Studies were therefore undertaken to determine whether similar digestibility results could be obtained using the corresponding unfractionated tissues. Insoluble material remaining after treatment of unfractionated plant tissues with buffer or CEL was rinsed with alcohol and acetone. Consequently, the buffer-insoluble, alcohol- and acetone-rinsed residue ("CWM") should be comparable to the CWM from the same source. The solubility of the "CWM" by CEL ("CWM"S-CEL), the difference between the "CWM" and the amount of CEL-insoluble "CWM" ("CWM"I-CEL), was highly correlated with the CEL solubility of the isolated CWM (CWMS-CEL) [$r = 0.959$ ($P < 0.0001$), CWMS-CEL = 0.97("CWM"S-CEL) + 3.0] for all of the species studied (Figure 4). Cellulose digestion results for unfractionated switchgrass samples are given in Table 4. As the grass matured, the "CWM" increased, ranging from 71.8% of the DM in samples collected on May 23 to 80.6% in samples collected on July 9. During the same period, "CWM"I-CEL increased

Table 4. Cellulase Solubilization of Cell-Wall Material in Unfractionated, Freeze-Dried Switchgrass Tissue[a]

harvest date, 1985	buffer-insoluble material ("CWM"),[b] % of DM	CEL-insoluble material ("CWM"I-CEL),[c] % of DM	"CWM"S-CEL,[d] % of DM	"CWM"S-CEL,[e] %
May 23	71.8 ± 1.0	30.5 ± 1.7	41.2 ± 1.9	57.4 ± 2.3
June 6	73.2 ± 0.7	41.5 ± 0.5	31.7 ± 0.6	43.3 ± 0.6
June 11	74.4 ± 1.0	40.7 ± 0.4	33.7 ± 1.0	45.3 ± 0.9
June 18	75.7 ± 0.3	42.3 ± 0.9	33.4 ± 0.9	44.1 ± 1.1
June 24	76.9 ± 1.1	47.9 ± 0.7	29.0 ± 0.6	37.7 ± 0.4
July 3	77.5 ± 0.5	53.4 ± 0.7	24.1 ± 0.2	31.1 ± 0.4
July 9	80.6 ± 0.5	56.4 ± 1.0	24.2 ± 0.6	30.0 ± 0.8

[a] Data are reported as means ± SD for five replicates. DM, dry matter. [b] "CWM", cell-wall material remaining after buffer treatment and ethanol and acetone rinses of unfractionated tissue, expressed as a percent of the DM of the starting sample. [c] "CWM"I-CEL, insoluble cell-wall material remaining after unfractionated tissue was digested with 2% (w:v) cellulase Onozuka RS and rinsed with ethanol and acetone, expressed as a percent of the DM of the starting sample. [d] Cell-wall material solubilized by treatment of unfractionated tissue with 2% (w:v) cellulase Onozuka RS, expressed as a percent of the DM of the starting sample ("CWM" − "CWM"I-CEL). [e] Cell-wall material solubilized by 2% (w:v) cellulase Onozuka RS treatment of unfractionated tissue, expressed as a percent of the buffer-insoluble material [("CWM" − "CWM"I-CEL)/"CWM"].

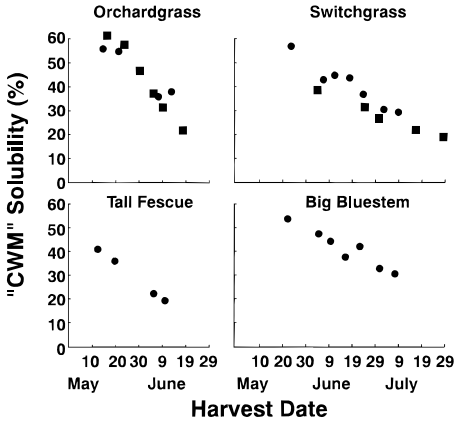

Figure 5. Cell-wall material in freeze-dried, unfractionated tissue solubilized by 2% (w:v) cellulase Onozuka RS. Plants were harvested at different stages of maturity in 1985 (●) and 1986 (■).

from 30.5% to 56.4% of the DM. Regression analysis of these data indicated a strong linear relationship between the "CWM" and the "CWM"I-CEL [$r = 0.945$ ($P < 0.01$), "CWM"I-CEL = 0.32("CWM") + 61.50] as the plants matured. A negative relationship existed between "CWM" and "CWM"S-CEL [$r = -0.910$ ($P < 0.01$), "CWM"S-CEL = −2.9("CWM") + 261.93]. Calculated values for "CWM"S-CEL, expressed as a percentage of the "CWM", are comparable to the corresponding results obtained using isolated CWM from the same source (Figure 3).

Results of the application of this technique for orchardgrass and switchgrass samples harvested in 1986 are plotted with 1985 data for all four grass species in Figure 5. Linear regression analyses, summarized in Table 5, indicate strong negative relationships between the extent of cell-wall degradation and plant maturity. Trends are similar for the 2 years, and the more rapid decline in solubility of CWM from cool-season grasses, compared to warm-season grasses (Figures 2 and 3), was evident when unfractionated tissue was subjected to cell-wall-degrading enzymes (Figure 5).

Estimates of fiber components in grass samples determined using conventional chemical procedures (NDF, ADF, and permanganate lignin) are compared with digestibility estimates obtained by CEL digestion of unfractionated tissues in Table 6. Fiber characteristics were similar for cool- and warm-season grasses at their respective vegetative and reproductive stages of development. In early June, cool-season grasses were well into their reproductive stages and contained higher concentrations of fiber components than the warm-season grasses which were still vegetative. Decreases in "CWM"S-CEL with advancing plant maturity corresponded with increases in concentrations of NDF, ADF, and lignin. The "CWM"I-CEL and ADF are similar fractions, and data reported for switchgrass in Tables 4 and 6 are consistent with this relationship. In addition, data reported in Tables 4 and 6 for "CWM" and NDF are consistent with the similarities in these fractions.

Table 6. Fiber and Digestibility Estimates for Cool- and Warm-Season Grass Herbage at Different Stages of Maturity[a]

	percent of DM			
	cool-season grasses		warm-season grasses	
fiber	tall fescue	orchard-grass	switch-grass	big bluestem
NDF				
May 13, 1985	54.4	51.8	ND	ND
May 20, 1985	59.8	52.6	55.4	55.8
June 6, 1985	65.6	62.8	60.3	59.8
June 11, 1985	68.2	62.1	57.9	59.9
June 18, 1985	65.3	ND	60.1	62.0
June 24, 1985	ND	ND	62.7	63.4
July 3, 1985	ND	ND	65.7	66.6
July 9, 1985	ND	ND	68.5	69.3
June 4, 1986	ND	65.5	63.5	ND
ADF				
May 13, 1985	29.9	28.0	ND	ND
May 20, 1985	31.9	29.0	27.6	28.3
June 6, 1985	39.2	36.8	29.1	31.0
June 11, 1985	41.4	36.2	29.2	32.3
June 18, 1985	39.2	ND	30.8	32.7
June 24, 1985	ND	ND	32.0	34.6
July 3, 1985	ND	ND	35.0	38.3
July 9, 1985	ND	ND	37.1	39.6
June 4, 1986	ND	37.6	30.7	ND
lignin				
May 13, 1985	2.3	3.3	ND	ND
May 20, 1985	2.7	3.4	2.7	2.4
June 6, 1985	5.0	5.2	2.9	3.0
June 11, 1985	5.5	4.9	2.8	3.2
June 18, 1985	5.1	ND	2.9	3.6
June 24, 1985	ND	ND	3.2	3.6
July 3, 1985	ND	ND	3.9	4.5
July 9, 1985	ND	ND	4.1	4.7
June 4, 1986	ND	5.9	3.4	ND
"CWM"S-CEL				
May 13, 1985	28.1	39.0	ND	ND
May 20, 1985	25.3	37.0	41.3	40.4
June 6, 1985	17.3	26.7	31.7	35.3
June 11, 1985	15.4	28.5	33.7	33.1
June 18, 1985	ND	ND	33.4	28.5
June 24, 1985	ND	ND	29.0	32.6
July 3, 1985	ND	ND	24.1	24.3
July 9, 1985	ND	ND	24.2	25.0
June 4, 1986	ND	27.6	28.9	ND

[a] ADF, acid detergent fiber; "CWM"S-CEL, solubility of cell-wall material in unfractionated tissue digested with 2% (w:v) cellulase Onozuka RS; DM, dry matter; ND, not determined; NDF, neutral detergent fiber.

Comparison of Dry Matter Solubilities (DMS) of Freeze-Dried and Oven-Dried Grass Herbage. Lyophilized and oven-dried, unfractionated tissues were

Table 5. Relationship between Day of the Year (x) and the Percent of Cell-Wall Material Solubilized by Cellulase Onozuka RS from Unfractionated, Freeze-Dried Tissue from Cool- and Warm-Season Grass Herbage (y)

species	year	n	r	regression equation
cool-season				
orchardgrass	1985	4	−0.953 ($P < 0.05$)	$y = -0.72x + 152.89$
	1986	6	−0.992 ($P < 0.001$)	$y = -1.30x + 239.80$
	1985 + 1986	10	−0.949 ($P < 0.001$)	$y = -1.05x + 200.78$
tall fescue	1985	4	−0.999 ($P < 0.001$)	$y = -0.74x + 139.32$
warm-season				
switchgrass	1985	7	−0.966 ($P < 0.001$)	$y = -0.56x + 136.34$
	1986	6	−0.956 ($P < 0.001$)	$y = -0.25x + 75.09$
	1985 + 1986	13	−0.906 ($P < 0.001$)	$y = -0.38x + 103.14$
big bluestem	1985	7	−0.961 ($P < 0.001$)	$y = -0.51x + 127.93$

Table 7. Cellulase and Buffer Solubilities of Freeze-Dried and Oven-Dried (100 °C) Unfractionated Tissue of Cool- and Warm-Season Grasses Harvested in 1985

	percent of DM[c]			
	DMS-C[a]		DMS-B[b]	
harvest date	freeze-dried	oven-dried	freeze-dried	oven-dried
orchardgrass				
May 13	68.4 ± 1.6	60.6 ± 0.4	29.4 ± 0.4	26.3 ± 0.8
May 20	69.7 ± 0.9	65.2 ± 0.9	32.7 ± 1.0	30.9 ± 0.6
June 6	52.7 ± 1.0	46.1 ± 0.6	26.0 ± 0.6	21.3 ± 0.5
June 11	53.8 ± 1.1	45.8 ± 0.9	25.3 ± 0.5	21.1 ± 0.8
tall fescue				
May 13	59.4 ± 1.7	53.5 ± 0.7	31.3 ± 0.3	28.3 ± 0.6
May 20	55.9 ± 1.0	52.5 ± 0.5	30.6 ± 0.7	28.6 ± 0.8
June 6	42.5 ± 0.6	38.6 ± 1.4	25.2 ± 0.8	27.6 ± 0.7
June 11	38.3 ± 0.9	36.4 ± 2.8	22.9 ± 0.4	22.2 ± 0.8
big bluestem				
May 23	65.9 ± 1.7	62.3 ± 0.5	25.5 ± 0.4	23.4 ± 0.7
June 6	62.1 ± 1.0	54.7 ± 0.9	26.8 ± 0.3	20.9 ± 0.3
June 11	58.7 ± 0.8	53.8 ± 0.9	25.6 ± 0.2	23.5 ± 0.5
June 18	54.0 ± 1.2	47.7 ± 0.7	25.5 ± 0.4	24.8 ± 0.9
switchgrass				
May 23	69.5 ± 1.5	61.4 ± 1.0	28.2 ± 0.9	23.0 ± 0.7
June 6	58.5 ± 0.4	51.4 ± 0.9	26.8 ± 0.7	19.0 ± 0.6
June 18	67.7 ± 0.4	52.5 ± 1.0	24.3 ± 0.3	24.3 ± 1.0
means	58.5	52.2	27.1	24.3

[a] DMS-C: dry matter solubility with 2% (w:v) cellulase Onozuka RS. [b] DMS-B: dry matter solubility with buffer only. [c] Data are means ± SD for five replicates.

Table 8. Relationship between the Percent of Cell-Wall Material Solubilized by 2% (w:v) Cellulase Onozuka RS from Freeze-Dried (*x*) and Oven-Dried (*y*) Unfractionated Tissue from Cool- and Warm-Season Grasses

species	*n*	*r*	regression equation
orchardgrass	4	0.986 ($P < 0.05$)	$y = 0.92x - 2.69$
tall fescue	4	0.995 ($P < 0.01$)	$y = 0.84x + 1.70$
switchgrass	3	0.968 ($P > 0.1$)	$y = 0.80x + 3.53$
big bluestem	4	0.998 ($P < 0.01$)	$y = 1.26x - 17.44$

treated with buffer and cellulase. In every case the DM solubilities of oven-dried samples with CEL were lower than those of the corresponding freeze-dried samples (Table 7). On the average, this difference was >5%. Linear regression analyses indicated a high correlation between CEL solubilities of freeze-dried and oven-dried samples for all species evaluated except switchgrass for which only three points were available (Table 8). All except one of the oven-dried samples exhibited a lower solubility with buffer treatment than the corresponding freeze-dried samples, the mean difference being about 3%.

DISCUSSION

Cell walls of plants are composed of cellulose, hemicellulose, lignin, and pectic substances. Changes in the composition of cell walls in response to environmental conditions and physiological development of plants are well documented (Akin et al., 1977; Wilson, 1982; Reid et al., 1988). These changes influence the digestibility, and thus the nutritive value, of forage materials. Degradation of cell-wall material *in vivo* is dependent upon the activities of microorganisms located in the rumen. Estimation of forage digestibility *in vitro* using rumen liquor (Tilley and Terry, 1963) provides results that are comparable to *in vivo* data, but this procedure requires fistulated animals and is complicated by variability in rumen liquor (Pace et al., 1984). Techniques involving fungal enzymes have received attention because they more closely parallel the ruminant digestive system than do chemical methods (Tilley and Terry, 1963; Goering and Van Soest, 1970) often used to predict DM digestibility and they overcome some of the difficulties associated with the Tilley and Terry (1963) approach.

Although others have assumed that inclusion of antibiotics in digestion media was not necessary (Donefer et al., 1963), these additives were essential to prevent bacterial and fungal growth that characterized digestion media prepared with the crude enzyme preparations we used. McQueen and Van Soest (1975) found that inclusion of toluene, merthiolate, sodium azide, penicillin, and streptomycin as microbial growth inhibitors did not affect the extent of solubilization. Chloramphenicol was included in digestion media used by Jones and Hayward (1973). Inhibitors incorporated in our digestion media did not interfere with the degradation of cellulose paper.

Forage samples are often oven-dried for subsequent analyses. Tilley and Terry (1963) reported that *in vitro* digestibility of freeze-dried herbage was similar to that for herbage dried at 40 or 100 °C, as long as oven drying at 100 °C was accomplished within 4 days. Noller et al. (1966), however, found that *in vitro* digestibility of freeze-dried forages was significantly higher than that of forages dried at 60 or 80 °C. Our data, presented in Table 7, indicate that drying tissues at a high temperature also reduces the amount of DM solubilized by CEL. A better estimate of the digestibility of fresh herbage is therefore obtained with lyophilized tissue.

Variations in the component enzyme activities occur in commercial cell-wall-degrading enzyme preparations (Gabrielsen, 1986); however, we were able to obtain similar results with the crude, multienzyme products from several different Japanese suppliers. Our experience with a variety of digestive enzymes for isolation of protoplasts provided the basis for the selection of the enzyme preparations and concentrations used in this study. In general, these preparations have proven to be more universally effective for grass species from which protoplasts are difficult to isolate, and variations in effectiveness among different batches of these enzyme preparations have not been noticeable. The significant variability in effectiveness of cellulase preparations reported by Bughrara et al. (1992) may be related to differences in the purity of the enzyme preparations used.

A synergistic influence of the various enzymes in microbial cellulase complexes has been demonstrated (Szakács-Dobozi et al., 1985). Other researchers (McQueen and Van Soest, 1975; Bughrara et al., 1992) have reported an increase in the amount of neutral detergent residue solubilized by a mixture of cellulase and hemicellulase, compared to the solubilization achieved with the cellulase preparation alone. Occurrence of multiple polysaccharide-degrading enzymes in the CEL preparation undoubtedly is responsible for our observation that the digestive power of CEL was similar to that of the mixture of CEL, PECT, and HCEL (Table 3). A mixture of enzymes in each of the preparations also accounts for the effectiveness of the individual enzyme preparations when they were used first in sequential digestions of CWM (Table 2). The lower total solubilities we observed when HCEL was the first enzyme in the sequence suggest that some factor associated with the HCEL preparation reduced the effectiveness of CEL and PECT. Deinum (1973) suggested that the weaving of

lignin, the indigestible cell-wall constituent, through cellulose and hemicellulase fibers limits the digestion of these other two cell-wall components. Greater enhancement of neutral detergent residue solubilization achieved with a mixture of CEL and HCEL for alfalfa, compared to red clover, was attributed to differences between the two plant species in the composition of the cell wall and in the association of hemicellulose with other cell-wall constituents (McQueen and Van Soest, 1975).

The solubility of CWM with CEL alone was comparable to that with the mixture of CEL, HCEL, and PECT (Table 3). However, when tissue was digested sequentially with the three enzyme preparations, the solubility of the CWM was greater than that with CEL alone (Table 2). Proteases are known contaminants in cell-wall-degrading enzyme preparations (Jones and Hayward, 1973), and these can reduce enzyme-catalyzed cell-wall degradation by hydrolyzing the digestive enzymes. The sequential approach to digestion of CWM takes advantage of the removal of undesirable components in one enzyme preparation before the remaining CWM is subjected to digestion by another enzyme preparation. In addition, inhibitors of cell-wall digestion in the enzyme preparations or products of the digestion process could have greater adverse effects when a mixture of the enzyme preparations is used.

This is the first report of the comparative effectiveness of CEL, HCEL, and PECT preparations for forage digestibility analysis. Relative to the digestive power of the enzyme mixture (CEL + HCEL + PECT), those of individual enzyme preparations were remarkably consistent with respect to grass type and maturity (Table 3). Further study is necessary to determine how results of the sequential approach relate to *in vivo* digestibility. Bughrara and Sleper (1986) found that CEL DM digestibility techniques provided information comparable to that obtained with *in vitro* digestibility techniques using rumen liquor, and correlations with *in vivo* DM digestibility were highly significant. Roughan and Holland (1977) concluded that DM digestibility assessment using CEL was limited by the same factors affecting *in vivo* DM digestibility. Microscopic examination of the process of tissue digestion by CEL indicated that degradation of the mesophyll occurred first followed by the epidermis and phloem, the same pattern that results from digestion by rumen liquor (Sleper and Roughan, 1984). Gabrielsen (1986) found that CEL solubility of warm- and cool-season grasses was highly correlated with rumen fermentation *in vitro* DM digestibility, and although values obtained with the CEL procedure were lower, Spearman rank-order correlations indicated similar sample rankings by the two analytical procedures.

Differences in digestibilities of tissue from various *Panicum* species were correlated with the photosynthetic type (C_3, C_4, C_3/C_4 intermediate), the mode of C_4 acid decarboxylation (NAD-malic enzyme, NADP-malic enzyme, phosphoenolpyruvate carboxykinase), and the mesophyll:bundle sheath ratio (Wilson and Hattersley, 1983). With our procedure for whole-plant samples, switchgrass, an NAD-malic enzyme type C_4 plant (Gutierrez et al., 1974), and big bluestem, an NADP-malic enzyme type C_4 plant (Gutierrez et al., 1974), exhibited similar trends in digestibility with advancing plant maturity. The solubility of the "CWM" was similar for the two species throughout a growing season and for switchgrass over two growing seasons (Figure 5). The high correlation between solubilities of isolated CWM and the "CWM" in corresponding unfractionated tissues (Figure 4), combined with the reproducibility of results (Table 4), means that digestibility assessments can be made without first isolating CWM. As a consequence, digestibility estimates using commercial cell-wall-degrading enzymes can be performed rapidly and conveniently by researchers who have limited plant material and do not have access to ruminally cannulated livestock. The assay is sufficiently sensitive to quantify changes related to maturation and environmental conditions.

ABBREVIATIONS USED

ADF, acid detergent fiber; CEL, cellulase; CWM, isolated cell-wall material; "CWM", cell-wall material in unfractionated tissue; "CWM"I, insoluble residue from enzyme digestion of unfractionated tissue; "CWM"I-CEL, insoluble residue from cellulase digestion of unfractionated tissue; CWMS, isolated cell-wall material solubilized by enzyme digestion; "CWM"S, cell-wall material in unfractionated tissue solubilized by enzyme digestion; CWMS-CEL, isolated cell-wall material solubilized by cellulase digestion; "CWM"S-CEL, cell-wall material in unfractionated tissue solubilized by cellulase digestion; DM, dry matter; DMS, dry matter solubility; HCEL, hemicellulase; NDF, neutral detergent fiber; PECT, pectinase.

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