Effect of Progressive Maturity of Timothy on Digestibility by Fungal Cellulases†

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An *in vitro* method for the determination of digestibility of grass hay was developed by using a combination of three commercial cellulases (*Aspergillus niger, Trichoderma viride,* and *Penicillium funiculosum*) showing activities of β-1,4-glucanase, β-1,4-xylanase, β-1,3-glucanase, mixed linkage enzymes, and esterases hydrolyzing ferulic acid and *p*-coumaric acid esterified to methyl arabinofuranoside. For individual enzymes, hydrolysis of glucose from mature timothy was proportional to their β-1,4-glucanase activities, but the hydrolysis of xylose was limited by the phenolic acid esterase activity. Percent of acid-labile xylose hydrolyzed by the combination of cellulases from two varieties of timothy, each harvested at three stages of maturity (preheaded to 7 days past full bloom), was highly correlated (R^2 = 0.965) with the *in vivo* digestibility of energy. The energy digestibility was also well related to the hydrolysis of acid-labile glucose (R^2 = 0.930) and alkali-labile *p*-coumaric acid (R^2 = 0.912).

INTRODUCTION

Digestibility of forages is an important criterion in defining their value for ruminant animals. Van Soest (1967) showed that the cellular-contents fraction of forages is completely digestible, whereas the nutritional availability of the other fraction, the plant cell wall, varies considerably between forages. As cellulose and hemicellulose are the major components of plant cell walls, a number of studies using enzymes from cellulolytic fungi have been conducted [reviewed by Martin and Barnes (1980)] with a view to establish some *in vitro* enzymatic method for measuring forage digestibility. In these studies, solubilization of dry matter (DM) was measured, but the linkages that had to be hydrolyzed to yield maximum tissue hydrolysis were not considered. Since then, a large number of enzymes in the rumen ecosystem which are necessary for the degradation of forages have been enumerated (Forsberg and Cheng, 1990) and considerable details about the physicochemical nature of the forage cell wall elucidated [for a review, see Hatfield (1989)].

Although isolated cell wall polysaccharides are completely degraded by the rumen microorganisms or enzyme fractions isolated from such organisms (Dehority, 1973; Smith *et al.*, 1973; Williams and Withers, 1982), the polysaccharides of the intact cell wall matrix are degraded to varying degrees depending upon plant tissue, species, and stage of maturity (Morrison, 1979). The interaction between phenolics and carbohydrates in the cell wall is considered to be the primary obstacle to the degradation of the cell walls (Van Soest, 1981). However, it is becoming evident that rumen microorganisms can partly degrade the linkages of phenolics and cell wall polysaccharides. Among the fibrolytic bacteria found in the rumen, *Fibrobacter succinogenes* generally degrades plant cell walls to the greatest extent (Miron *et al.*, 1989) and has been reported to possess a feruloyl esterase activity (McDermid *et al.*, 1990). Also, fibrolytic fungi are universally present in the rumen and have been reported to account for as much as 8% of the microbial mass (Orpin and Joblin, 1988). Five of these fungi were found to contain *trans*-feruloyl and *trans*-coumaroyl esterases (Borneman *et al.*, 1990).

In the present study, we used a combination of three commercial fungal cellulases to ensure the presence of various enzyme activities necessary for hydrolyzing the type of linkages which seem to be accessible to rumen microorganisms. Optimal conditions for this assay system were established, and hydrolysis of major monosaccharides and phenolic acids of two varieties of hay-type hexaploid timothy (*Phleum pratense*) harvested at three stages of maturity were correlated with the *in vivo* digestibility of energy.

MATERIALS AND METHODS

Fungal enzymes were purchased from Sigma Chemical Co., St. Louis, MO. Chemicals and substrates were purchased from Sigma or Fisher Scientific, Ottawa, and were of analytical grade.

Enzymes. Commercial impure cellulases [1,4-(1,3; 1,4)-β-D-glucan 4-glucanohydrolase; EC 3.2.1.4] from *Aspergillus niger, Trichoderma viride, Penicillium funiculosum,* and *Basidiomycetes* (Driselase) were extensively dialyzed against a 25 mM citrate–phosphate buffer, pH 5.0, using a Minitan tangential flow concentrator (Millipore Ltd.) to eliminate particles less than 10 000 molecular weight.

Hydrolysis of Standard Substrates. *Polysaccharide Substrates.* Milled filter paper (MFP), Whatman No. I filter paper was dissolved by ball milling. Low-viscosity (carboxymethyl)-cellulose (CMC), oat glucan, oat xylan, and laminarin were dissolved by autoclaving for 30 min at 121 °C. Final concentrations in assay mixtures were 2.5 mg/mL for laminarin and 10 mg/mL for the others.

Enzyme Assays. Appropriate substrate and enzyme combinations in 0.5 mL of 25 mM citrate–phosphate buffer, pH 5.0, containing 0.025% sodium azide as an antimicrobial agent were incubated for 30 min. The reaction was terminated by adding 0.5 mL of Nelson's copper reagent and boiling for 20 min. Reducing sugars released were determined colorimetrically using arsenomolybdate reagent and glucose standard (Nelson, 1944).

Synthesis of (5-O-Feruloyl)- and (5-O-p-Coumaroyl)methyl α-L-Arabinofuranosides. The arabinofuranosyl derivatives of ferulic acid (FA Me.Ara.) and *p*-coumaric acid (pCA Me.Ara.) were synthesized using the methods of Hatfield *et al.* (1991) and Helm *et al.* (1992), with the following modifications. Cleanup of the synthetic intermediates involving silica gel column chromatography was eliminated to avoid loss of material by binding to the silica. Batches of material were prepared without

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purification of the intermediates; purification of final products was achieved using an ODS-2 semipreparative HPLC column (0.94 cm i.d. × 25 cm, Chromatography Sciences Co., Inc.) under FPLC conditions, with a mobile phase of 30% methanol/water (v/v), flow rate of 1.5 mL/min, and ultraviolet detection at 280 nm. The identity of the pure compounds was confirmed by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy.

Spectrophotometric Assay. Basically, the method of Hatfield *et al.* (1991) was used, and 1.5 mM solutions of FA Me.Ara. or *p*CA Me.Ara. in 25 mM citrate phosphate buffer, pH 7.0, were incubated with enzymes at room temperature. The change in absorbance was measured at 323 nm (Okamura and Watanabe, 1982) for several minutes. Complete hydrolysis of 127.2 and 65.16 nmol/0.5 mL of assay volume of FA Me.Ara. and *p*CA Me.Ara., respectively, showed a decrease of 1.0 OD.

Hydrolysis of Timothy Hay. *Hay Samples.* Forced air dried samples of Drummond (Dru) and Quebec common (QC) varieties of timothy harvested at three stages (S_1, S_2, and S_3) of maturity were kindly provided by Dr. D. P. Heaney of our Research Centre and have been described earlier (Heaney *et al.*, 1966). Samples of Dru timothy cut on May 23, June 18, and July 11, 1963, i.e., S_1, S_2, and S_3, respectively, were preheaded, 5% headed, and in full bloom. The QC timothy samples cut on these dates were preheaded, 80% headed, and 7 days past full bloom, respectively. These samples were further ground in a cyclotron mill and dried at 60 °C. Total amount of acid-labile monosaccharides (Sloneker, 1971), alkali-labile phenolic acids (Borneman *et al.*, 1990), and 72% sulfuric acid insoluble lignin (AOAC, 1980) in hay samples were quantitated.

Hydrolysis by Fungal Cellulases. Hay samples (25 mg) in duplicates or triplicates were incubated in 25 mM citrate–phosphate buffer, pH 5.0, including 0.025% sodium azide and filter (0.22 μM) sterilized enzyme preparations, for 16 h at 50 °C. The reaction was terminated by autoclaving the samples.

Determinations of Hydrolyzed Products by HPLC. The sugars solubilized by the fungal enzymes were separated by using a Dionex Carbopack PAI column with a PA guard column. The solvent used was 15 mM NaOH for 18 min (1 mL/min). The sugars were quantitated using a Dionex pulsed amperometric detector (PAD, Dionex Canada Ltd.) with a gold PAD cell filled with 300 mM NaOH. Standard sugars (20 nmol each of arabinose, galactose, glucose, xylose, fructose, xylobiose, and cellobiose) were injected in 10-μL volume. Phenolic acids were separated by using a Spherisorb-ODSI column (5 μm, 4.6 × 250 mm operated at 50 °C) and detected by a multiwavelength detector using a Maxplot feature to scan for peaks at 308, 322, and 375 nm to detect *p*-coumaric, ferulic, and sinapic acids, respectively. The standard (2, 2, and 10 nmol, in the above order of phenolic acids) was injected in 10 μL. The solvent used was 2.5% 1-butanol and 0.3% acetic acid in deionized water at a flow rate of 2 mL/min.

RESULTS

Methods Development. In time course preliminary studies, it became evident that the initially hydrolyzed monosaccharides (by the fungal cellulases) were further slowly metabolized by some contaminating enzymes or microbes. This situation was controlled by filter sterilization (0.22-μM Millipore filters) of enzymes, autoclaving of substrates before adding enzyme, and assaying at 50 °C instead of 39 °C.

The activities of the three fungal cellulases of *A. niger*, *T. viride*, and *P. funiculosum*, as well as those of Driselase, a mixed enzyme preparation of *Basidiomycetes*, toward a number of substrates are shown in Table I. Each of the four cellulases showed β-1,4-glucanase activity with MFP and CMC as the substrates, mixed-linkage glucanase activity measured using oat glucan, β-1,4-xylanase activity with xylan, and β-1,3-glucanase activity with laminarin. Finally, phenolic esterase activities determined with synthetic 5-*O*-feruloyl- and 5-*O*-(*p*-coumaroyl)methyl α-L-arabinofuranosides as the substrates were present in three cellulases, but Driselase lacked these activities. The specific activity of β-1,4-xylanase was highest [25 234 μmol (mg of protein)$^{-1}$ h^{-1}] in *T. viride*, but all other enzyme-specific activities were highest for *A. niger*. Phenolic acid esterase specific activities were about 40–130-fold higher for *A. niger* as compared to those for *T. viride* or *P. funiculosum*.

Table I. Relative Activities of Fungal Enzymes[a]

substrate[b]	enzyme activity	μmol/h *A. niger*	*T. viride*	*P. funiculosum*	Driselase
MFP	β-1,4-glucanase	60	60	60	60
CMC	β-1,4-glucanase	145	193	333	213
oat glucan	mixed linkage	213	82	271	229
xylan	β-1,4-xylanase	779	2034	424	386
laminarin	β-1,3-glucanase	22	19.5	36	109
FAARA	FA esterase	1.82	0.003	0.006	0.000
*p*CAARA	*p*CA esterase	3	0.006	0.003	0.000

[a] Equalized for 1 μmol of reducing sugar/min from milled filter paper. These activities were expressed by 54.65, 80.59, 472.68, and 172.82, 25 μg of protein, respectively, for *A. niger*, *T. viride*, *P. fumiculosum*, and Driselase. [b] MFP, milled filter paper; CMC, (carboxymethyl)cellulose; FAARA, (5-*O*-feruloyl)methyl α-L-arabinofuranoside; *p*CAARA, (5-*O*-*p*-coumaroyl)methyl α-L-arabinofuranoside.

As Driselase lacked phenolic acid esterase activity, only the other three enzymes were compared in subsequent experiments. The concentrations of each enzyme were equalized [on the basis of β-glucanase activity with MFP so that 1 mL of each enzyme would produce 25 000 μg of reducing sugar/h (2.5 μmol/min)].

Comparison of a series of pH points using sodium acetate buffer (pH 4.5–5.75) and potassium phosphate buffer (pH 6.0–6.8) for the release of total reducing sugars in 30 min showed that the cellulase of *A. niger* has a pH optimum at pH 6.25. Similarly, the pH optima for *T. viride* and *P. funiculosum* enzymes were 5.0 and <4.5, respectively. Using citrate–phosphate buffer, pH 4.0–6.5, showed the pH optima of all three enzymes to be in the range pH 4.0–5.5 with a broad plateau over this range; thus, citrate–phosphate buffer, pH 5.0, was chosen for further studies.

Because the initial autoclaving may solubilize certain oligomers from the polysaccharides of plant cell walls and the fungal enzymes might hydrolyze these oligomers more readily than the original polysaccharides, the autoclaving method for sterilization of timothy hay sample was compared with azide treatment. At both pH 5.0 and 6.8, a combination of *A. niger* and *T. viride* released higher amounts of arabinose, glucose, and xylose from the autoclaved samples than for azide treatment (Table II). The azide addition was used in the standard assay, to avoid any artifact of autoclaving. It may be noted that the amounts of various monosaccharides released from timothy were higher for assays at pH 5 than at pH 6.8.

A comparison of two assay volumes (2 vs 5 mL) using a combination of three cellulase enzymes (*A. niger*, *T. viride*, and *P. funiculosum*) showed no difference in the amounts of various monosaccharides released from mature timothy hay (Table III). It was inferred that there was no product inhibition in the 2-mL assay volume, and this was adapted as a standard technique. Incubation time course studies using the same amount of enzyme as in the above experiment showed that release of glucose, xylose, and arabinose from a 25-mg timothy sample was maximized in 6–8 h and further incubation had no effect. A 16-h assay time was adapted to ensure maximal release of sugar.

Hydrolysis of Timothy Hay of Progressive Maturity. Use of enzymes equalized for the release of reducing sugars from MFP showed that from the mature timothy grass *P. funiculosum* released the highest amount of glucose, followed by *T. viride* and then *A. niger* (Table

Table II. Comparison of Sterilization Treatments on Sugar Release from Drummond Timothy by a Mixture of *A. niger* and *T. viride*[a]

pH	treatment		amount of monosaccharide[b] released				
			ARA (μmol/25 mg of forage)	GLC (μmol/25 mg of forage)	XYL (μmol/25 mg of forage)	FRU (μmol/25 mg of forage)	DMD[c] (%)
6.8	autoclaved	mean	3.46	15.42	10.72	2.10	58.76
		SD[d]	0.18	0.35	0.21	0.49	1.13
	azide	mean	2.79	12.61	8.01	2.68	52.40
		SD	0.13	0.92	0.51	0.02	0.79
5.0	autoclaved	mean	4.88	17.33	12.09	2.17	49.56
		SD	0.44	3.74	1.34	0.38	2.32
	azide	mean	4.19	15.74	9.82	3.05	50.41
		SD	0.15	0.51	0.29	0.33	2.60

[a] Each assay contained 27.33 and 40.30 μg of protein for *A. niger* and *T. viride*, respectively. Incubation at 50 °C for 16 h. [b] ARA, arabinose; GLC, glucose; XYL, xylose; FRU, fructose. [c] DMD, dry matter disappearance. [d] SD, standard deviation for duplicate assays.

Table III. Effect of Assay Volume on Hydrolysis of Drummond Timothy by Fungal Enzymes[a]

assay volume (mL)		μmol/25 mg of sample				
		ARA[b]	GAL[b]	GLC[b]	XYL[b]	FRU[b]
2	mean	3.08	0.84	19.40	9.74	3.47
	SD	0.14	0.03	0.55	0.13	0.22
5	mean	3.27	0.79	19.18	9.54	3.07
	SD	0.09	0.03	0.59	0.32	0.45

[a] Each assay contained 27.33, 40.30, and 236.34 μg of protein for *A. niger*, *T. viride*, and *P. funiculosum*, respectively. Incubation at 50 °C for 16 h. [b] Abbreviations as in Table II.

IV). This order is a reflection of β-1,4-glucanase activities with CMC as a substrate. In spite of very high β-1,4-xylanase activity in *T. viride* (Table I), the release of xylose from both varieties of timothy (only data for Dru timothy is shown in Table IV) was highest for *A. niger*, followed by *P. funiculosum* and then *T. viride*. This might suggest some limitations caused by phenolic esters, and activities of phenolic acid esterase may be important in this aspect. Release of arabinose in general was parallel to that of xylose.

When *A. niger* and *T. viride* enzymes were used together (50% of each used individually), each of the monosaccharides released was more than the amounts released by the individual enzymes, indicating some synergistic effect. An addition of *P. funiculosum* to the combination of the other two enzymes (each of the three enzymes at 50% of that used individually) increased the release of monosaccharides, particularly that of arabinose and xylose (Table IV). It seems that maximal hydrolyzable monosaccharides have been released by the use of the mixture of three enzymes, because the total hydrolytic capacity of these three enzymes was much higher than the actual release of various monosaccharides.

Acid hydrolysis of timothy hay harvested at three stages of maturity showed that the order of abundance of monosaccharides at each of these stages and for both varieties was glucose, xylose, arabinose, and galactose (Table V). Glucose and xylose content increased markedly as timothy matured from preheaded to headed stage, i.e., from S_1 to S_2, whereas there was only a minor change in arabinose content and a small but significant ($p < 0.05$) decrease in galactose content. Further maturity to full bloom (S_3) stage caused no change in the concentration of any monosaccharides. On the other hand, the alkali-hydrolyzable ferulic acid (FA) was of maximal concentration at the S_2 stage and decreased thereafter. The alkali-hydrolyzable *p*-coumaric acid (*p*CA) increased about 4-fold from preheaded (S_1) to 5% headed stage for Dru (S_2) and to 80% headed stage for QC (S_2) variety. But on further maturity *p*CA significantly ($p < 0.05$) increased in Dru timothy only as it came to full bloom (S_3), whereas for maturity of QC from 80% headed (S_2) to 7 days past full bloom (S_3), there was a significant ($p < 0.05$) decrease in *p*CA. For each variety, the ratio of *p*CA/FA, as well as lignin content, increased progressively with the stage of maturity, although for QC timothy the increase in lignin content was not significant ($p > 0.05$) as it matured from 80% headed to 7 days past full bloom.

For the three stages of maturity, a combination of the three fungal enzymes released (micromoles per 100 mg) 108.3, 102.4, and 71.7 units of glucose for Dru timothy and 106.4, 103.4 and 77.4 units of glucose for QC timothy. The corresponding values for xylose were 50.6, 58.8, and 46.1 units for Dru and 44.5, 55.1, and 36.1 units for QC timothy. Expressed as percent of acid-hydrolyzable monosaccharides, hydrolyses of glucose and xylose from Dru polysaccharides were very comparable at three stages of maturity (Table VI). On the other hand, for QC variety, hydrolysis of xylose was about 8–10 units less than that of glucose. For both varieties, hydrolyses of glucose, xylose, and arabinose were maximal at the preheading (S_1) stage, whereas that of galactose was maximal at S_2 of maturity, i.e., headed stage. Hydrolysis of alkali-labile FA was very high and that of *p*CA was comparatively low, particularly at advanced stages (S_2 and S_3) of maturity (Table VI).

In vivo energy digestibilities of the timothy hay determined by Heaney *et al.* (1966) with sheep are also noted in Table VI. Although xylose hydrolysis is much lower than the *in vivo* energy digestibility, the orders for these sets of data were similar, even when two varieties were considered together. Regression analysis of *in vivo* energy digestibility (IVED) *vs* enzymatic hydrolysis of xylose showed a very high correlation coefficient (0.965), intercept of 28.2, and slope of 0.789. The correlation coefficients of IVED were also high for hydrolysis of glucose (0.930) and *p*CA (0.912). Interestingly the correlation coefficient for IVED *vs* DM disappearance in enzymatic hydrolysis (0.872) was much less than those noted above. Thus, the superiority of a technique based on enzymatic hydrolysis of acid-labile sugars compared to solubilization of DM has been demonstrated.

DISCUSSION

The commonly used *in vitro* method for the estimation of DM digestibility of forages involves incubations with rumen fluid followed by pepsin digestion (Tilley and Terry, 1963). Its several drawbacks such as variability due to rumen inoculum, lack of information about linkages hydrolyzed, and the necessity to maintain fistulated animals to obtain rumen inoculum were emphasized in a review paper by Martin and Barnes (1980). Recently, Cherney *et al.* (1993) elucidated the variations due to diets

Table IV. Enzymatic Hydrolysis of Mature Drummond Timothy[a]

enzyme[b]	μmol/25 mg of sample						
	ARA[c]	GAL[c]	GLC[c]	XYL[c]	FRU[c]	FA[c]	pCA[c]
A. niger	2.50[b]		11.27[d]	7.96[b]	3.34	0.16[b]	0.12[ab]
T. viride	1.36[d]		12.70[c]	3.57[c]	3.02	0.09[c]	0.07[c]
P. funiculosum	1.81[c]	0.38	15.63[b]	4.49[c]	2.84	0.14[b]	0.10[b]
A. niger + *T. viride*	2.53[b]		15.28[b]	8.54[b]	2.81	0.18[a]	0.14[a]
A. niger + *T. viride* + *P. funiculosum*	3.17[a]	0.88	17.92[a]	11.52[a]	2.75	0.19[a]	0.14[a]
SEM	0.05	0.06	0.20	0.32	0.23	0.004	0.006

[a] Averaged values of duplicate assays. Different superscripts in a column indicate significant difference ($p < 0.05$). [b] Each enzyme used singularly would hydrolyze 1 μmol of glucose/min from MFP as in Table I. For combination of enzymes each enzyme was used at 50% of that level. [c] GAL, galactose; FA, ferulic acid; pCA, *p*-coumaric acid; other abbreviations as in footnote *b* of Table II.

Table V. Acid Hydrolysis of Monosaccharides, Alkaline Hydrolysis of Phenolic Acids, and Lignin Content of Timothy Hay[a]

variety and maturity	μmol/100 mg						pCA/FA ratio	lignin (%)
	ARA[b]	GAL[b]	GLC[b]	XYL[b]	pCA[b]	FA[b]		
Drummond								
S_1	25.86	10.36[ab]	168.66[b]	76.95[b]	0.46[e]	1.07[e]	0.43[e]	2.790[cd]
S_2	26.86	7.12[c]	211.76[a]	129.10[a]	1.98[b]	1.70[b]	1.17[c]	4.663[bc]
S_3	26.03	6.48[c]	211.89[a]	131.38[a]	2.16[a]	1.44[c]	1.50[a]	7.482[a]
Quebec common								
S_1	22.50	11.36[a]	153.40[c]	73.70[b]	0.48[e]	1.22[d]	0.39[e]	2.400[d]
S_2	25.16	7.60[bc]	205.90[a]	129.16[a]	1.85[c]	1.85[a]	1.00[d]	5.057[b]
S_3	23.84	6.80[c]	209.10[a]	130.15[a]	1.70[d]	1.35[cd]	1.26[b]	6.358[ab]
SEM	1.49	0.93	1.97	3.16	0.04	0.05	0.02	0.642

[a] 72% sulfuric acid and 1 N sodium hydroxide were used as acid and alkali, respectively. Forage samples (Heaney *et al.*, 1966) were harvested at preheaded (S_1), 5% headed (S_2), and full bloom (S_3) for Drummond timothy; for Quebec common timothy, the three respective stages were preheaded, 80% headed, and 7 days past full bloom. Different superscripts in a column indicate significant difference ($p < 0.05$) for triplicate determinations. [b] Abbreviations as in Tables II and IV.

Table VI. Hydrolysis of Monosaccharides and Phenolic Acids of Timothy Hay by Fungal Enzymes[a]

variety and maturity	%		% of acid labile				% of alkali labile	
	IVED[b]	DMD[b]	ARA[b]	GAL[b]	GLU[b]	XYL[b]	pCA[b]	FA[b]
Drummond								
S_1	78.1	73.8[a]	69.4[b]	68.6[bc]	64.3[b]	65.7[a]	85.1[a]	92.7[a]
S_2	65.0	55.7[c]	57.3[c]	80.1[a]	48.4[c]	45.6[b]	42.9[c]	64.3[c]
S_3	53.6	48.6[e]	48.8[d]	54.0[de]	33.9[e]	35.1[c]	24.7[d]	51.1[d]
Quebec common								
S_1	76.5	69.5[b]	79.6[a]	59.9[cd]	69.4[a]	60.4[a]	87.5[a]	91.5[a]
S_2	65.4	52.4[d]	67.6[b]	69.3[b]	50.2[c]	42.67[b]	53.9[b]	91.0[a]
S_3	49.2	48.2[e]	50.2[d]	50.0[e]	37.0[d]	27.7[d]	29.8[d]	76.3[b]
SEM[b]		0.53	2.05	2.75	0.99	1.9	2.28	2.73
R^2 with IVED[b]		0.872	0.584	0.289	0.930	0.966	0.912	0.465

[a] A combination of three enzymes (as in Table III) was used for 16-h assays at pH 5.0. Stages of maturity stated in footnote *a*, Table V. Different superscripts in a column indicate significant difference ($p < 0.05$) for triplicate assays. [b] IVED, *in vivo* energy digestibility [reproduced from Heaney *et al.* (1966)]; SEM, standard error of means; R^2, correlation coefficients of IVED to the products of enzymic hydrolysis; other abbreviations as in Tables II and IV.

fed to fistulated cows providing rumen inocula on *in vitro* DM digestibility of a number of typical forages.

Use of fungal enzymes to replace the Tilley and Terry method has been studied by a number of researchers since Donefer *et al.* (1963) reported on this technique. Although different sources of carbohydrate hydrolyzing enzymes from fungi were used either singly or in combination [reviewed by Martin and Barnes (1980)], early studies measured only DM disappearance. In the present study, we used a mixture of three fungal enzymes showing activities of β-1,4-glucanase, β-1,4-xylanase, β-1,3-glucanase, mixed linkage enzymes, and esterases capable of hydrolyzing FA and pCA esterified to methyl arabinofuranoside to ensure the hydrolysis of the type of linkages which seem to be accessible to rumen microorganisms. Optimal assay conditions were established and the method validated by quantitating the monosaccharides and phenolic acids released from timothy hay of two varieties harvested at three stages of maturity ranging from preheaded to 7 days past full bloom.

We found that the ratio of acid-labile xylose to arabinose in both varieties of timothy increased with maturity, particularly from preheaded (S_1) to headed (S_2) stage (Table V). These data are supported by Morrison (1980), who observed a similar increase in hemicellulose fractions of both leaf and stem tissue for 10 grass varieties, including Scots timothy grown in row trials, as plants matured from vegetative to 50% ear emergence. In grasses most of the xylose and arabinose would be present in hemicelluloses because pectic polysaccharides are very low (Hatfield, 1989). We also observed that compared to Dru timothy, the QC timothy had a higher xylose to arabinose ratio (Table V) and a lower release of xylose by fungal enzymes (Table VI). Morrison (1980) observed that varieties with higher xylose to arabinose ratios showed higher lignin content. At each of the three stages of maturity, in our study, lignin contents of the two varieties were not significantly ($p > 0.05$) different.

It is well accepted that the physicochemical effects of lignin on plant cell walls are a major limitation of forage digestibility by the rumen microorganisms. Chesson *et al.* (1983) reported that the extent of alkali-labile substituents at the C-5 position of arabinose in a variety of gramineae species was highly correlated with the amount

of lignin present. But in our study, the concentrations of FA for both varieties and that of *p*CA for QC timothy were lower at S_3 as compared to S_2 (Table V), in spite of the marked increase in lignin content of timothy on maturity from S_2 to S_3, i.e., heading to full bloom (Table V). Thus, the deduction of Chesson *et al.* (1983) would be limited to only early growth stages of monocots, possibly prior to full bloom. Consistent with the observations of Burritt *et al.* (1984) for a number of grasses, we found that for each variety of timothy the *p*CA/FA ratio increased with progressive maturity (Table V). Because *p*CA is rarely detected in the primary cell wall of monocots, which is characterized by the presence of FA only (Gordon *et al.*, 1985) and the appearance of *p*CA occurs in concert with lignification (Chesson, 1988), the *p*CA/FA ratio is simply an empirical expression of the extent of early lignification. This may explain the high degree of correlation between the *p*CA/FA ratio and digestibility of grasses observed by Hartley (1972) and Burritt *et al.* (1984) and in our present study. We obtained a correlation coefficient of 0.843 for *in vivo* energy digestibility (Heaney *et al.*, 1966) *vs* *p*CA/FA ratio (Table V) for the two varieties of timothy, each harvested at three stages of maturity. It improved to 0.963, if the sample of highest maturity (7 days past full bloom of QC timothy) was not included in the comparison. Thus, the empirical relationship between digestibility and *p*CA/FA ratio does not hold for excessively mature plant material.

In our study the best correlation of *in vivo* energy digestibility was with the proportion of acid-labile xylose hydrolyzed ($R^2 = 0.965$) by the mixture of three enzymes (Table VI); those for acid-labile glucose hydrolyzed ($R^2 = 0.930$) and alkali-labile *p*CA hydrolyzed ($R^2 = 0.912$) by the same enzyme combination were also very impressive. Of the fungal cellulases tested, *T. viride* enzyme contained the highest β-1,4-xylanase activity (Table I), yet from mature timothy it released the minimum amount of xylose (Table IV). This seems to reflect the low phenolic acid esterase activity of *T. viride* enzyme. Brice and Morrison (1982) reported that hemicellulase isolated from ovine rumen fluid hydrolyzed lignin–hemicellulose complexes prepared by potassium hydroxide extraction from rye grass or Scots timothy to a much smaller extent than the corresponding hemicelluloses prepared by the chlorite method. Chesson (1988) reported that after 48 h of incubation of ryegrass in rumen fluid, the xylose content of the residue was higher than that of the original material (13.8 *vs* 9.7%), whereas the glucose content decreased slightly (36.9 *vs* 39.0%). This showed that xylose hydrolysis was the limiting factor affecting energy digestibility in the rumen environment. The *in vitro* assay system using fungal cellulases reported here leads to a similar conclusion that the extent of xylose hydrolysis is best related to the energy digestibility of maturing timothy varieties. Thus, our enzymatic method is capable of elucidating the chemical linkages hydrolyzed as well as generating data congruent with the rumen environment. Enzymatic release of xylose, glucose, and *p*CA, in that order, from timothy of two varieties harvested at different stages of maturity commonly used for forage harvesting, i.e., preheaded to 7 days past full bloom, was highly correlated with the *in vivo* digestibility of energy.

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