Fermentation of a Bacterial Cellulose/Xylan Composite by Mixed Ruminal Microflora: Implications for the Role of Polysaccharide Matrix Interactions in Plant Cell Wall Biodegradability

Paul J. Weimer,*[,†,‡] John M. Hackney,[§] Hans-Joachim G. Jung,[||,⊥] and Ronald D. Hatfield[†]

U.S. Dairy Forage Research Center, Agricultural Research Service, U.S. Department of Agriculture, Madison, Wisconsin 53706; Plant Science Research Unit and U.S. Dairy Forage Research Center Cluster, St. Paul, Minnesota 55108; Departments of Bacteriology and Botany, University of Wisconsin—Madison, Madison, Wisconsin 53706; and Department of Agronomy, University of Minnesota, St. Paul, Minnesota 55108

Growth of the cellulose-synthesizing bacterium *Acetobacter xylinum* ATCC 53524 in media supplemented with 5% (w/v) glucose and 0.2% (w/v) of a water-soluble, nearly linear xylan from tobacco stalks resulted in the synthesis of a highly crystalline composite having a xylose/glucose ratio ranging from 0.06 to 0.24. The digestion of one composite (88% cellulose/12% xylan) by mixed ruminal microflora displayed kinetics of gas production similar to those of an unassociated mixture of the two components added in a xylan/cellulose ratio similar to that of the composite. The data suggest that intimate association of xylan and cellulose, as is typically found in secondary plant cell walls, does not inhibit the rate of digestion of the component polysaccharides.

Keywords: *Cellulose; kinetics; cell wall; ruminal bacteria; xylan*

INTRODUCTION

Ruminant animals are a central component of agricultural systems due to their ability to convert forages and other fibrous plant material to milk, meat, wool, and hides (Van Soest, 1994). Most of the digestion of plant material occurs within the rumen, a specialized organ in which microorganisms ferment feed materials to produce primarily volatile fatty acids and microbial cell proteins that are used by the animal as a source of energy and protein, respectively (Hungate, 1966). The slow rate of passage of feeds through the rumen enhances the extent of their digestion, whereas plant fiber—composed mostly of plant cell walls—is incompletely digested in the rumen.

Plant cell walls are complex structures that not only enclose the vital intracellular machinery of the plant cell but also provide the plant with its morphological structure and strength. Plant cell walls provide the living plant one means to resist invasion by pathogens and render the dead plant somewhat resistant to biodegradation in soil, sediment, and animal digestive tracts. Recent studies have emphasized that matrix interactions between polysaccharides and lignin, particularly those mediated by covalent cross-linkages involving ferulic and other phenolic acids, are the primary mechanism imparting recalcitrance of plant cell walls to biodegradation (Grabber et al., 1998; Ralph et al., 1998). Both chemical pretreatments of cell walls (Dale and Moreira, 1982; Gould, 1984) and genetic alterations of the plants themselves (Barnes et al., 1971; Hatfield et al., 1999a) have been shown to enhance plant cell wall biodegradability, largely via mechanisms that reduce either the amounts of lignin and phenolic acids or the degree of cross-linking of these materials to wall polysaccharides. It is not clear, however, which other cell wall structural features provide additional limitations to biodegradability. Because cellulose is found in intimate association with various hemicelluloses in plant cell walls (Hatfield, 1993) and because some plant cell walls are degraded without preferential loss or accumulation of specific component monosaccharides (Chesson, 1988), the interactions among the wall polysaccharides may have an effect on the degradation of individual component polysaccharides.

The purpose of this study was to determine if matrix interactions among the polysaccharides, independent of lignin, provide a secondary mechanism of recalcitrance that could restrict the rate of plant cell wall degradation below the rates of degradation of the pure polysaccharides. Direct studies of the effects of polysaccharide interactions on cell wall degradability are complicated by the great variety of polysaccharides present in plant cell walls and by the presence of lignin and phenolic acids in even very young plant tissue. Consequently, we took an alternative experimental approach in which we used the cellulose-synthesizing bacterium *Acetobacter xylinum* to prepare cellulose/xylan composites that were free of lignin and phenolic acids, and we then compared the kinetics of digestion of these composites by mixed ruminal microorganisms to those of the component polysaccharides and a nonassociated mixture of these polysaccharides.

* Address correspondence to this author at USDA-ARS-USDFRC, 1925 Linden Dr. W., Madison, WI 53706 [telephone (608) 264-5408; fax (608) 264-5147; e-mail pjweimer@facstaff.wisc.edu].

[†] U.S. Department of Agriculture.

[‡] Department of Bacteriology, University of Wisconsin—Madison.

[§] Department of Botany, University of Wisconsin—Madison.

[||] Plant Science Research Unit and U.S. Dairy Forage Research Center Cluster.

[⊥] Department of Agronomy, University of Minnesota.

10.1021/jf991372y CCC: $19.00
Published on Web 04/21/2000

MATERIALS AND METHODS

Preparation of Xylan. Tobacco stalk xylan was prepared according to a modification of the method of Eda et al. (1976). Flue-cured stalks of *Nicotiana tabacum* were broken with a hammer and ground through a Wiley mill having a 4-mm screen. Subsequent extraction steps were performed in a chemical hood, and all procedures involving water used deionized water. Ground stalks (25 g) were extracted three times (15 min each) with 600 mL of boiling toluene/ethanol (2:1, v/v). The solvent was removed by vacuum filtration through a 30-μm nylon screen, and the cake was washed successively with 0.5 L of ethanol, 0.5 L of 50% (v/v) ethanol, and 20 L of water. The cake was then soaked for 30 min in water (90 °C), vacuum filtered again, washed with 5 L of water, and resuspended in 900 mL of water. The solids were delignified by heating the suspended cake to 70 °C and then adding 5.4 mL of glacial acetic acid and 16.4 g of sodium chlorite (technical grade, ~70%). After further heating for 1 h (with the Parafilm-covered stopper loosely placed on the flask to permit venting of Cl_2), the spent chlorite solution was decanted and the delignification procedure repeated five additional times. The delignified solids were thoroughly washed with water until colorless and then stored overnight under N_2 at 5 °C.

Delignified solids were suspended in 800 mL of N_2-gassed 5% (w/v) KOH solution/0.1% (w/v) $NaBH_4$, and the suspension was incubated for 18 h at room temperature under N_2. The suspension was then filtered through a 30-μm nylon screen, and the filtrate was stored under N_2 at 5 °C. The remaining solids were re-extracted with 24% KOH/0.1% $NaBH_4$, as above, and the two KOH filtrates combined and neutralized with glacial acetic acid. Xylans were then precipitated from the pooled filtrate by the addition of 4 volumes of cold absolute ethanol. After the xylans had settled overnight (5 °C) and most of the liquid phase had been siphoned off, the xylans were resuspended in the remaining liquid, pelleted by centrifugation (10000*g*, 5 °C, 20 min), and then washed once with ethanol and twice with acetone. After vacuum removal of acetone, the pelleted xylans were resuspended in ice-cold water, placed in 1000 MWCO dialysis tubing, and dialyzed against water for 2 days at 5 °C, with frequent changes of water. The retained xylan solution was centrifuged (15000*g*, 4 °C, 30 min), and the supernatant was retained as tobacco stalk xylan fraction 1 (TSX1). The pellet was resuspended in 450 mL of water, warmed to 60 °C, and centrifuged (8000*g*, 60 min, room temperature), and the supernatant was saved as tobacco stalk xylan fraction 2 (TSX2). A small pellet (~0.2 g of dry weight) remained from the second centrifugation. The xylan fractions TSX1 and TSX2 were lyophilized and stored at room temperature. Yields of xylan fractions from three separate preparations, based on the dry tobacco stalks, averaged 17.9% (TSX1) and 9.7% (TSX2).

Preparation of Cellulose and Cellulose/Xylan Composites. Bacterial cellulose was purified from a culture of *A. xylinum* ATCC 53524 (Johnson and Neogi, 1989). Stock cultures (50 mL) of bacteria in their cellulose matrix were blended in a sterile Waring blender jar at high speed for 30 s, and 5 mL of the dispersed culture was aseptically pipetted into 1-L baffled flasks that contained 300 mL of the medium of Hestrin and Schramm (1954), which contained 5% (w/v) glucose as sole carbohydrate source. Flasks were incubated in a shaking water bath (30 °C, 250 rev/min) for 3 or 5 days prior to harvest. To recover the cellulose, replicate cultures were combined and vacuum filtered through a coarse sintered glass funnel. The filter cake was washed with methanol and then subjected to Soxhlet extraction with methanol (6 h) and then with chloroform/methanol (2:1, v/v, 16 h). The solids were washed with methanol, and solvent-exchanged with water. These solids were then (a) boiled twice with stirring in 1% (w/v) NaOH, with an intermediate washing with hot NaOH; (b) collected in a sintered glass funnel and washed with water; (c) refluxed three times (2 h each) in 1% NaOH/0.1% $NaBH_4$; (d) rinsed thoroughly with water in a sintered glass funnel; (e) incubated overnight in 900 mL of 0.6% (v/v) acetic acid/1.8% (w/v) sodium chlorite (technical grade, ~70%); (f) thoroughly rinsed with water; and (g) soaked overnight in 0.1% (w/v) diethylenetriaminepentaacetic acid. The final product was thoroughly rinsed with water and then lyophilized.

The bacterial cellulose/xylan composite was obtained in a similar fashion as the bacterial cellulose, except the glucose-containing Hestrin−Schramm growth medium was supplemented with 0.2% (w/v) of TSX 1 or TSX2. The composite will hereafter be referred to as BCX. At higher concentrations of the xylan fractions, the medium was more viscous, and both growth and polysaccharide production were visibly reduced.

Sigmacell 50 microcrystalline cellulose, a plant-derived cellulose used for comparison to bacterial cellulose, was obtained from Sigma (St. Louis, MO).

Characterization of Polymers. Neutral sugar composition of the tobacco stalk xylans was determined by the two-stage sulfuric acid hydrolysis method of Theander and Westerlund (1986), as modified by Jung and Russelle (1991). Briefly, an 80% (v/v) ethanol-insoluble residue was prepared for each xylan fraction and, after drying under N_2 gas, was solubilized with 12 M H_2SO_4 at 39 °C for 1 h. The acid solution was diluted to 0.4 M and autoclaved for 1 h to hydrolyze the xylans. The supernatant was then filtered through a glass fiber filter, neutralized with excess $BaCO_3$, and deionized by passing through cation and anion solid-phase extraction columns (Supelco, Inc., Bellefonte, PA). Neutral sugars were identified and quantified by HPLC using the Dionex BioLC system with a CarboPac PA1 column and pulsed amperometric detector (Dionex, Inc., Sunnyvale, CA). Sugars were eluted by pumping 0.02 M NaOH for 2.5 min, followed by a linear gradient to pure water over 0.5 min, and then water was pumped for an additional 27 min. Both solvents were pumped at 0.94 mL/min. The column temperature was maintained at 40 °C. Postcolumn, 300 mM NaOH was added prior to the detector. Inositol and deoxyglucose were added to the samples after hydrolysis as internal standards. Sugar yields were corrected for hydrolytic losses of standard sugars.

Polysaccharides (0.5−2.5 mg) were methylated using *n*-butyllithium and methyliodide (Carpita and Shea, 1989). The methylated polysaccharides were recovered according to the method of Harris et al. (1984) and were hydrolyzed with 2 M trifluoroacetic acid (TFA; 1.5 h, 120 °C). The partially methylated monosaccharides were converted to their alditol acetates according to the method of Blakeney et al. (1983). Partially methylated alditol acetates were analyzed by GLC on a 30 mm × 0.25 mm i.d. DB-1 capillary column (J&W Scientific, Folsom, CA) having a 1-μm film thickness. Samples were injected at a column temperature of 150 °C, which was increased at 4 °C min^{-1} to 250 °C and then held at this temperature for 25 min. Carrier gas was He (0.7 mL min^{-1}). The injector temperature was 225 °C, and the injector split ratio was 50:1. Peaks were detected with a Hewlett-Packard MSD 5790 mass selective detector.

X-ray diffraction was conducted in the transmittance mode as described previously (Weimer et al., 1995).

Fermentations. In vitro fermentations of the polysaccharides were conducted in serum vials (Wheaton Glass, Millville, NJ; nominal volume = 50 mL, each vial calibrated to 0.01 mL) placed in a chamber (39 °C) containing hypodermic needle-fitted pressure transducers that permitted determination of the kinetics of gas production from individual fermentation vials [Pell and Schofield (1993) as modified by Mertens and Weimer (1998)]. Vials contained 10−22 mg of substrate (weighed to 0.1 mg), 5.7 mL of McDougall buffer (McDougall, 1948), and 0.3 mL of reducing agent (6.5 mg each of cysteine·HCl and $Na_2S·9H_2O$ per mL of 0.04 N NaOH) under a CO_2 gas phase. Vials were inoculated with 4.0 mL of diluted ruminal fluid, prepared as described previously (Weimer et al., 1990), to provide a ruminal fluid concentration equivalent to 20% (v/v). Gas production was measured at 0.01 h intervals for the first ~0.6 h, then at 0.1 h intervals for ~10 h, and finally at 0.5 h intervals over the remainder of the 48-h incubation period. A total of 200−209 data points were collected for each sample.

Table 1. Models for Volume of Gas Produced by Mixed Ruminal Microflora during Fermentation of Polysaccharides

model	equation[a]
single-pool exponential	$V = A(1 - e^{k_1 z_1})$
dual-pool exponential	$V = A(1 - e^{k_1 z_1}) + B(1 - e^{k_2 z_2})$
single-pool logistic	$V = A[1 + e^{(2+[4\mu_{m1}(\lambda_1 - t)/A])}]^{-1}$
dual-pool logistic	$V = A[1 + e^{(2+[4\mu_{m1}(\lambda_1 - t)/A])}]^{-1} + B[1 + e^{(2+[4\mu_{m2}(\lambda_2 - t)/B])}]^{-1}$

[a] Terms: A = mL of gas (g of digestible organic matter)$^{-1}$, for single pool or for rapidly digesting pool (for dual pool); B = mL of gas (g of digestible organic matter)$^{-1}$, for slowly digesting pool (for dual pool); e = base of natural logarithms; k_1 = rate constant for single pool or for rapidly digesting pool (h^{-1}); k_2 = rate constant for slowly digesting pool (h^{-1}); L_1 = lag time for single pool or rapidly digesting pool (h); L_2 = lag time for slowly digesting pool (h); λ_1 = integration constant for single pool or rapidly digesting pool (h); λ_2 = integration constant for slowly digesting pool (h); μ_{m1} = logistic rate for single pool or rapidly digesting pool (mL of gas·h^{-1}); μ_{m2} = logistic rate for slowly digesting pool (mL of gas·h^{-1}); t = incubation time (h); V = total mL of gas (g of digestible organic matter)$^{-1}$; $z_1 = (L_1 - t - |t - L_1|/2$; $z_2 = (L_2 - t - |t - L_2|/2$. For dual pool, single lag models, a single L or λ term was used.

Table 2. Composition of Xylan Fractions Purified from Tobacco Stalks

	molar fraction[a]					
fraction	Xyl	Ara	Rha	Man	Gal	Glc
TSX1	0.814	0.019	0.039	0.033	0.032	0.062
TSX2	0.939	0.007	0.011	0.011	0.009	0.024

[a] Results are mean values of duplicate analyses. Xyl, xylose; Ara, arabinose; Rha, rhamnose; Man, mannose; Gal, galactose; Glc, glucose.

Following corrections for changes in ambient barometric pressure and for gas production in blank vials lacking added substrate, the data were fit to several models using the PROC NLIN routine of the SAS statistical analysis software package (SAS Institute, 1985). Because the substrates were lignin-free polysaccharides and were added at low concentrations (1.0−2.2 mg mL^{-1}) to maintain a final pH >6.4, all models assumed complete digestion of substrate (i.e., absence of an indigestible residue). The models used (Table 1) included the following: (a) a single-pool exponential model with a single discrete lag time (Mertens and Loften, 1980); (b) a dual-pool exponential model with two lag times; (c) a dual-pool exponential model with a single lag time; (d and e) single- or dual-pool logistic models with one or two lag times, respectively (Schofield et al., 1994); and (f) a dual-pool logistic model with a single lag time (Schofield et al., 1994). Within each model, convergence was assumed when the residual sum of squares did not change at the sixth significant figure for three successive iterations. The goodness of fit of each model was determined from the relationship [1 − (residual sum of squares/DF-corrected treatment sum of squares)]. Comparisons among means were performed with PROC ANOVA of SAS, using a Duncan−Waller t test at $p < 0.05$.

Nongaseous fermentation products were quantified by HPLC (Weimer et al., 1991b).

RESULTS

Characteristics of Substrates. The xylan fractions isolated from tobacco stalks were pure white in color and contained 6−19% (molar basis) of non-xylosyl residues (Table 2). Linkage analysis indicated that the xylose residues in both fractions were 1,4-linked, with relatively little branching (Table 3). On the basis of the content of terminal Xyl residues, the TSX1 and TSX2 xylans had approximate degrees of polymerization (DPs) of 45 and 100, respectively.

Table 3. Linkage Analysis of Xylan Fractions Purified from Tobacco Stalks

	molar fraction of linkages[a]			
fraction	1,4-Xyl	2,4- Xyl	terminal Xyl	terminal Ara
TSX1	0.887	0.075	0.021	0.016
TSX2	0.922	0.068	0.010	<0.001

[a] Calculated as a fraction of the sums of the peak areas of the detected components. Results are means of two separate analyses. Xyl, xylose; Ara, arabinose.

Table 4. Effect of Xylan Fraction and Incubation Time on Xylose/Glucose Ratio of Composites Produced by *A. xylinum* Grown in 5% (w/v) Glucose/0.2% (w/v) of the Indicated Xylan Fraction

xylan fraction	incubation time (days)	molar ratio of Xyl/Glc[a]
TSX1	3	0.059 ± 0.020
TSX1	5	0.238 ± 0.006
TSX2	3	0.200 ± 0.029
TSX2	5	0.152 ± 0.011

[a] Results are mean values of two analyses, plus or minus standard error of the mean. Xyl, xylose; Glc, glucose.

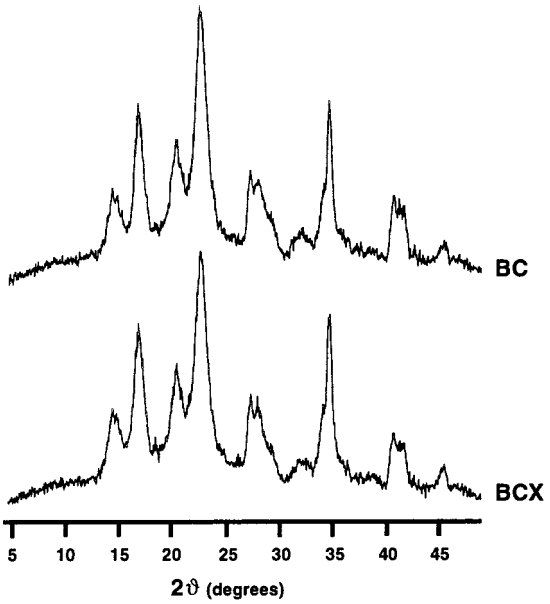

Figure 1. Transmission X-ray diffractograms of BC and BCX purified from cultures of *A. xylinum*.

The *A. xylinum* cultures fed glucose as sole carbohydrate source contained spherical or nearly spherical masses of cellulose (∼3−8-mm diameter) with included bacterial cells. Cultures fed both glucose and xylan contained consolidated but nonspherical masses of flocculent material. Neutral sugar analysis revealed that the cellulose/xylan composites purified from these cultures contained molar xylose/glucose ratios as high as 0.24 (∼18% xylose on a molar basis). The differences in composition observed among the polysaccharides isolated from cultures fed different xylan fractions for different incubation times were substantial (Table 4). Treatment of the composites with 2 N TFA for 90 min at 120 °C did not result in significant release of neutral sugars. X-ray diffraction patterns of the cellulose/xylan composite were virtually identical to those of the bacterial cellulose (Figure 1) and revealed that both the pure cellulose and the composite were highly crystalline.

Fermentation by Mixed Ruminal Microbes. Progress curves for gas production with time from the

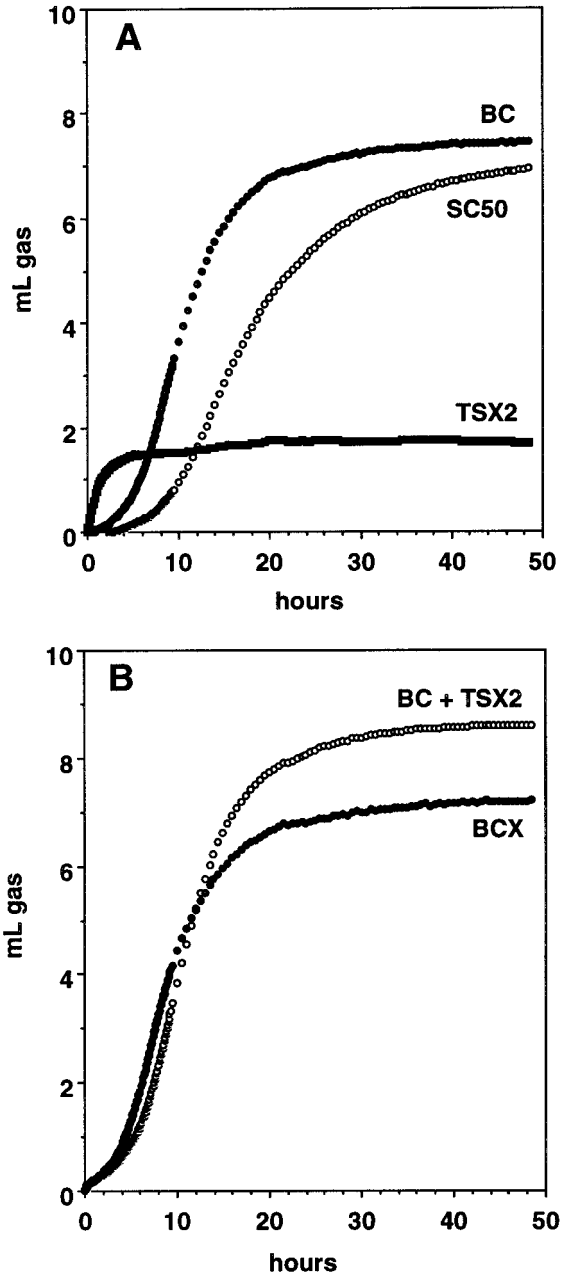

Figure 2. Time course profiles of gas production during fermentation of polysaccharides by mixed ruminal microorganisms in vitro. Data represent mean values of triplicate cultures for each substrate, following correction for gas production in control vials lacking added substrate. SC50, Sigmacell 50 microcrystalline cellulose (21 mg); BC, bacterial (*A. xylinum*) cellulose (21 mg); TSX2, tobacco stalk xylan fraction (10 mg); BC + TSX2, mixture of 20 mg of BC and 2 mg of TSX2; BCX, cellulose/xylan composite synthesized by *A. xylinum* (22 mg).

various substrates are shown in Figure 2. Gas production from the soluble TSX2 substrate proceeded almost immediately upon inoculation and was complete within 4 h (Figure 2), whereas gas production from the celluloses and composites proceeded more slowly and only after a substantial lag period.

Application of the data to a single-pool exponential model (Table 1) provided good fit of the data and allowed a quantitative comparison of the fermentation characteristics of the substrates. Using this model, gas production from TSX2 proceeded with a mean first-order rate constant of 0.52 h^{-1} and no discernible lag time (Table 5), in accord with the rapid fermentation of soluble

Table 5. Kinetic Parameters for Gas Production during in Vitro Fermentations of Polysaccharides and Their Mixtures by Mixed Ruminal Microflora (See Table 1 for Description of Model Terms)

Single-Pool Exponential Model with Discrete Lag Time

substrate[a]	*A* (mL of gas g^{-1})	*k* (h^{-1})	*L* (h)
SC50	377.7[d]	0.066[e]	7.23[c]
BC	413.8[cd]	0.131[d]	5.02[d]
TSX2	200.0[e]	0.524[c]	<0.01[e]
BC + TSX2	435.9[c]	0.120[d]	4.92[d]
BCX	405.4[c]	0.151[d]	4.07[d]

Dual-Pool Exponential Model with Two Discrete Lag Times

substrate[a]	4*A* (mL of gas g^{-1})	*B* (mL of gas g^{-1})	k_1 (h^{-1})	k_2 (h^{-1})	L_1 (h)	L_2 (h)
SC50	21.4[d]	341.6[c]	0.110[d]	0.074[c]	0.54[c]	7.99[c]
BC	80.3[cd]	274.3[cd]	0.140[d]	0.152[d]	0.3[c]	6.20[de]
TSX2	105.5[c]	67.5[e]	1.038[c]	0.190[d]	0.03[c]	<0.01[f]
BC + TSX2	90.2[cd]	286.0[cd]	0.125[d]	0.155[d]	0.66[c]	6.62[d]
BCX	108.3[c]	243.7[d]	0.120[d]	0.179[d]	0.39[c]	5.32[e]

Dual-Pool Logistic Model with a Single Lag Time

substrate[a]	*A* (mL of gas g^{-1})	*B* (mL of gas g^{-1})	SR_1[b] (h^{-1})	SR_2[b] (h^{-1})	λ[b]
SC50	136.1[cd]	195.6[c]	0.137[d]	0.046[c]	9.48[c]
BC	162.7[c]	158.2[c]	0.127[d]	0.043[c]	6.36[de]
TSX2	112.4[c]	61.6[d]	0.618[c]	0.097[c]	0.07[f]
BC + TSX2	176.2[c]	175.4[c]	0.130[d]	0.052[c]	6.38[de]
BCX	191.5[c]	141.3[c]	0.167[d]	0.055[c]	5.57[e]

[a] SC50, Sigmacell 50 microcrystalline cellulose; BC, bacterial (*A. xylinum*) cellulose; TSX2, tobacco stalk xylan fraction (see text); BC + TSX2, 10:1 mixture of BC and TSX2; BCX, cellulose/xylan composite synthesized by *A. xylinum* during growth on 5% glucose + 0.2% (w/v) of TSX2. [b] SR = specific rate of gas production, calculated as (μ_{m1} or μ_{m2})/(net mL gas per bottle), with μ_{m1} and μ_{m2} defined in Table 1. λ = integration constant, equivalent to lag time for a single lag model. See Schofield et al. (1994) for derivation of terms. [c–f] Results are mean values of triplicate cultures. Means having different superscripts within a column differ ($p < 0.05$).

polysaccharides such as pectin (Hatfield and Weimer, 1995). The rate constant for gas production from bacterial cellulose (BC) was greater than for Sigmacell 50 (SC50), a microcrystalline cellulose derived from cotton (0.130 versus 0.066 h^{-1}), and the corresponding lag time was shorter (5.02 versus 7.54 h). The total amount of gas produced was slightly greater from BC than from SC50, although the difference may be due in part to the different end product ratios that resulted from the two fermentations. Fermentation of BC resulted in a net ratio of acetate to propionate (A/P) that was 2.3-fold greater than that resulting from fermentation of SC50 (Table 6). Differences in fermentation pathways and in the resultant ratios of fermentation end products have been shown to affect gas yields per gram of carbohydrate consumed (Pell and Schofield 1993).

Gas production from an unassociated 10:1 (w/w) mixture of BC and TSX2 (BC + TSX2) was similar to that of the BC alone, except for a slightly elevated gas production earlier in the fermentation due to the relatively rapid fermentation of the small amount of TSX added to the mixture. The extent of gas production from BC + TSX2 was similar to the sum of these two components. The BCX composite and the unassociated mixture of the two components (BC + TSX2) displayed similar rate constants and lag times (Table 5) and similar A/P ratios (Table 6), although total gas production was slightly lower for the BCX composite (Figure 2).

To more closely examine the kinetics of digestion, the data were fit to a dual-pool exponential model (Table

Table 6. End Products of Polysaccharide Fermentations following Incubation with Mixed Ruminal Microflora[a]

substrate[b]	acetate (A) (mM)	propionate (P) (mM)	butyrate (B) (mM)	A + P + B (mM)	A/P ratio
SC50 (21 mg)	12.16 ± 0.84	7.93 ± 0.31	2.13 ± 0.30	22.21 ± 1.31	1.53 ± 0.09
BC (21 mg)	16.41 ± 0.98	3.97 ± 0.37	2.35 ± 0.58	22.74 ± 1.04	4.15 ± 0.18
TSX2 (10 mg)	7.87 ± 0.75	2.86 ± 0.17	1.06 ± 0.23	11.79 ± 1.19	2.75 ± 0.10
BC (20 mg) + TSX2 (2 mg)	17.98 ± 0.25	5.31 ± 0.27	2.48 ± 0.44	25.81 ± 0.28	3.40 ± 0.17
BCX (22 mg)	14.90 ± 2.52	4.45 ± 0.31	2.58 ± 0.70	21.93 ± 3.42	3.33 ± 0.39

[a] Net VFA concentrations and A/P ratio were determined following subtraction of VFA concentrations in blank vials lacking added substrate. Results are mean values of triplicate cultures, plus or minus standard error of the mean. [b] SC50, Sigmacell 50 microcrystalline cellulose; BC, bacterial (*A. xylinum*) cellulose; TSX2, tobacco stalk xylan fraction (see text); BCX, cellulose/xylan composite synthesized by *A. xylinum* during growth on 5% (w/v) glucose + 0.2% (w/v) of TSX2.

Table 7. Fit of Models to the Experimental Data (See Table 1 and Text for Descriptions of Models)[a]

	exponential			logistic		
substrate	1 pool, 1 lag	2 pools, 2 lags	2 pools, 1 lag	1 pool, 1 lag	2 pools, 2 lags	2 pools, 1 lag
SC50	0.9951	0.9975	0.9496	0.8816	0.9954	0.9961
BC	0.9936	0.9982	0.9721	0.9539	0.9936	0.9943
TSX2	0.9616	0.9964	0.9868	0.3606	nc[b]	0.9899
BC + TSX2	0.9901	0.9985	0.9887	0.9345	0.9978	0.9989
BCX	0.9890	0.9969	0.9938	0.9423	0.9770	0.9987

[a] Calculated as 1 − (residual sum of squares/total degree-of-freedom corrected sum of squares). [b] nc = not calculated. Residual sum of squares exceeded total degree-of-freedom-corrected sum of squares.

5), which provided a slightly better fit of the data than did the single-pool model (Table 7). This model suggests that the TSX2 was not a homogeneous substrate but instead contained two pools of similar size that are digested without an appreciable lag, but at greatly different rates.

Although gas production from SC50 fit the dual-pool exponential model slightly better than a single-pool exponential model, the rapidly digesting pool was essentially absent (<0.001% of the slowly digesting pool) in two of the three replicates. The slowly digesting pool displayed a rate constant for gas production of 0.07 h^{-1} and a lag time of 8.0 h, values similar to those obtained from the single-pool model. By contrast, BC contained two pools having different lag times but similar rate constants that were well above those for SC50 digestion, in accord with a more rapid rate of digestion suggested for BC by the single-pool exponential model. Both the BCX composite and the unassociated BC + TSX2 mixture closely resembled BC in the rates of gas production in the dual pool model.

Additional kinetic analyses were conducted using logistic models. The interpretation of kinetic parameters from logistic models is less straightforward than for exponential models and has been discussed in detail by Schofield et al. (1994). In general, the single-pool/single-lag and dual-pool/dual-lag logistic models provided poorer fits than did the corresponding exponential models (Table 7), and in a few cases the residual sum of squares exceeded the total degree-of-freedom-corrected sum of squares. In accord with the report of Schofield et al. (1994), the dual-pool model with a single lag provided better fits of cellulose digestion data than either single-pool/single lag or dual-pool/two-lag models. The kinetic parameters from the dual-pool/single-lag model followed the general pattern observed for the exponential models including (a) rapid fermentation of TSX2 without appreciable lag, (b) more rapid fermentation and/or shorter lag of BC than of SC50, and (c) similar rates and lags of BCX composite and the unassociated mixture BC + TSX2.

DISCUSSION

Although *A. xylinum* is well-known for its ability to synthesize crystalline cellulose during growth on glucose, this organism has also been reported to produce cellulose-containing composite structures when grown on glucose in the presence of water-soluble polysaccharides, including cellulose ethers (Haigler et al., 1982) and xyloglucan or glucomannan (Hackney et al., 1994). The incorporation of these polymers into the cellulose matrix results in minimal to substantial reductions in crystallinity and shifts in the relative proportion of cellulose Iα and Iβ crystalline lattices (Hackney et al., 1994). Several mechanisms have been proposed to explain the aggregation behavior of the polysaccharides in the composites, all of which propose an intimate relationship between the cellulose and the non-cellulose polysaccharide at the microfibrillar level or below. The intimacy of the relationship resembles somewhat the distribution of polysaccharides in the native plant cell wall, giving rise to hypotheses that hemicelluloses can control the aggregation behavior of cellulose at the site of cellulose synthesis and that the composites themselves can serve as model systems for the investigation of plant cell wall structure (Atalla et al., 1993; Hackney et al., 1994).

The results reported here indicate that *A. xylinum* can produce nearly pure composites of cellulose and xylan when grown in the presence of glucose and moderate amounts of a relatively pure and nearly linear xylan. The following observations suggest that xylan in the composites was fairly evenly distributed within a crystalline cellulose matrix, rather than being localized as a separate phase within the matrix: (a) xylosyl residues were stable to boiling in alkali (which normally solubilizes xylan) during purification of the composite; (b) prolonged treatment of the isolated composites with hot TFA did not release reducing sugars; and (c) the composites had X-ray diffraction patterns similar to those of crystalline bacterial cellulose. Compared to the somewhat less crystalline composites of cellulose and xyloglucan or of cellulose and glucomannan (Hackney et al., 1994), the cellulose/xylan composites prepared in this work display a lower fraction of incorporation of the hemicellulose component into the matrix, without a reduction in crystallinity of the composite relative to that of the pure cellulose. The reasons why xylan behaves differently from xyloglucan or glucomannan as a partner in composite assembly remain to be elucidated but may be due to the more linear structure of the xylan and its comparatively lower degree of polymerization. The linear structure would provide a means of maintaining an ordered placement of freshly extruded cellulose chains onto adjacent linear polysaccharide molecules, without the disordering effects of branching that would discourage this ordered place-

ment. Retention of crystallinity has been noted previously in composites of cellulose and sodium carboxymethylcellulose (NaCMC), in which the linearity of the CMC apparently permits hydrogen bonding of the CMC to the microfibrillar subunits, which in turn leads to retention of crystalline structure (Haigler et al., 1982). On the other hand, the low DP of the xylan (≤100, compared to 688 for glucomannan and ~5200 for xyloglucan; Hackney et al., 1994) might limit the extent of its incorporation because each separate incorporation event would emplace individual xylan chains having molecular masses that were only a small fraction of the molecular mass of a single molecule of cellulose (DP of ~2100−3700; Marx-Figini and Pion, 1974).

Although there were some differences in the fit of the kinetic models to the gas production data, these models generally yielded similar conclusions on the relative rates and lag times of digestion of the different substrates. BC was degraded substantially more rapidly than was the plant-derived SC50. These data are in agreement with the data of Schofield et al. (1994), who noted the more rapid degradation rate of BC relative to that of commercial α-cellulose derived from dilute acid hydrolysis of holocellulose. BC is known to contain both the Iα and Iβ forms of cellulose, in a ratio of ~2:1, whereas SC 50, derived from cotton, has the Iβ conformation typical of plant celluloses (Atalla and Van der Hart, 1984). On the basis of the degradation profiles of BC and SC50, it appears that the Iα form of cellulose was degraded more rapidly and with a shorter lag time. As the Iα and Iβ forms of cellulose are thought to differ in H-bonding patterns rather than in the dimensions of the crystallite (Atalla and Van der Hart, 1984), these data reinforce the conclusion that allomorphic form strongly affects the kinetics of cellulose digestion (Weimer et al., 1991a).

In addition to differences in digestion kinetics, bacterial and plant cellulose differed greatly in the ratio of A/P in fermentation end products, suggesting that different ruminal microbes predominate during growth on the two different forms of cellulose. The higher A/P ratio produced by fermentation of the BC suggests that this substrate selects for *Ruminococcus* species, which produce acetate as major fermentation product, rather than *Fibrobacter succinogenes*, which produces primarily succinate, the major ruminal precursor of propionate (Hungate, 1966). These observations extend a previous report that *F. succinogenes* and *R. flavefaciens* differ in their ability to utilize non-native crystalline allomorphs of cellulose (II, III_I, III_{II}, and IV_I; Weimer et al., 1991a). Fermentation of the BCX composite and an unassociated mixture of cellulose and xylan having a similar proportion of glucosyl and xylosyl residues yielded similar values of A/P that were intermediate between the A/P ratios obtained from BC and TSX2, suggesting that the cellulose and xylan were fermented as separate substrates by the microbial population.

The BCX composite was degraded at a rate similar to that of a mixture of its two nonassociated components. This suggests that the ruminal population is well adapted toward degrading cellulose-containing structures in intimate association with xylan. These results are consistent with observations that the lag time for degradation of cellulose in ground forages (materials of vastly more complicated architecture) is much shorter than for pure celluloses (Van Soest, 1973).

Use of the *A. xylinum* biosynthetic system allowed preparation of cellulose/xylan composites without lignin or phenolic acid contamination, thus permitting direct examination of the role of polysaccharide−polysaccharide interactions on digestion kinetics. Xylans and other hemicelluloses in secondary walls (the most abundant layer of mature plant cell walls) display considerable diversity in the degree of branching and substitution with other sugars and sugar acids, but in general this degree of branching and substitution is less than in less-abundant primary walls (Bacic et al., 1988; Carpita, 1984). On the basis of its recovery in abundance from tobacco stalks, the xylan fraction TSX2 that we used to prepare the composites for digestion kinetic studies were almost certainly derived from secondary cell walls. This fraction was relatively unbranched and contained a ratio of nonxylosyl to xylosyl residues of 0.06, a value that compares reasonably well with known sugar compositions of mature plant cell walls. For example, Hatfield et al. (1999b) determined the neutral sugar composition of five cell wall types in mature sorghum stems and found that the ratios of non-xylosyl to xylosyl residues (exclusive of glucosyl residues attributable mostly to cellulose) ranged from 0.06 to 0.10. Consequently, we believe that our biosynthetic composites are useful materials for the examination of the effect of polysaccharide−polysaccharide interactions on the kinetics of digestion of the component polysaccharides by ruminal microorganisms.

The fact that rate constants of digestion were not altered in the composite, in which linear cellulose and predominantly linear xylan interact via hydrogen bond formation (Stephen, 1983), makes it unlikely that digestion rates would be altered by the weaker matrix interactions between cellulose and more highly branched hemicelluloses also found in plant cell walls. Thus, although matrix interactions (e.g., hydrogen bonding) between cellulose and xylan may strengthen the plant cell wall, these interactions apparently do not inhibit the degradability of cellulose. On the basis of this observation, it is highly possible that sufficient removal of the primary constraints in plant cell wall degradation (cross-linking of polysaccharides to phenolics and lignin) will result in substantial increases in digestibility, without polysaccharide matrix interactions between cellulose and xylan—the two most abundant cell wall polysaccharides—providing secondary limitations to digestibility. Examination of interactions among less abundant cell wall polysaccharides may reveal whether the lack of inhibition observed due to cellulose/xylan interactions applies to other polysaccharide interactions as well.

ACKNOWLEDGMENT

We thank H. Spurr for providing the flue-cured tobacco stalks; C. Dietrich and C. L. Odt for technical assistance in purifying the cellulose/xylan composites; D. R. Mertens and R. E. Muck for helpful discussions; and P. Schofield and A. N. Pell for preliminary testing of our composites.

LITERATURE CITED

Atalla, R. H.; Van der Hart, D. L. Native cellulose: a composite of two distinct crystalline forms. *Science* **1984**, *223*, 283−285.

Atalla, R. H.; Hackney, J. M.; Uhlin, I.; Thompson, N. S. Hemicelluloses as structure regulators in the aggregation of native cellulose. *Int. J. Biol. Macromol.* **1993**, *15*, 109−112.

Bacic, A., Harris, P. J.; Stone, B. A. Structure and function of plant cell walls. In *The Biochemistry of Plants: A Comprehesive Treatise*; Preiss, J., Ed.; Academic Press: New York, 1988; Vol. 14, pp 297−371.

Barnes, R. F.; Muller, L. D.; Bauman, L. F.; Colenbrander, V. F. In vitro dry matter disappearance of brown mid-rib mutants of maize (*Zea mays* L.). *J. Anim. Sci.* **1971**, *33*, 881−884.

Blakeney, A. B.; Harris, P. J.; Henry, R. J.; Stone, B. A. A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydr. Res.* **1983**, *113*, 291−299.

Carpita, N. C. Fractionation of hemicelluloses from maize cell walls with increasing concentrations of alkali. *Phytochemistry* **1984**, *23*, 1089−1093.

Carpita, N. S.; Shea, E. M. Linkage structure of carbohydrates by gas chromatography−mass spectrometry (GC-MS) of partially methylated alditol acetates. In *Analysis of Carbohydrates by GLC and MS*; Bierman, C. J., McGinnis, G. D, Eds.; CRC Press: Boca Raton, FL, 1993; pp 157−216.

Chesson, A. Lignin-polysaccharide complexes of the plant cell wall and their effect on microbial degradation in the rumen. *Anim. Feed Sci. Technol.* **1988**, *21*, 219−228.

Dale, B. E.; Moreira, M. J. A freeze-explosion technique for increasing cellulose hydrolysis. *Biotechnol. Bioeng. Symp. Ser.* **1982**, *12*, 31−43.

Eda, S.; Ohnishi, A.; Kato, K. Xylan isolated from the stalk of *Nicotiana tabacum*. *Agric. Biol. Chem.* **1976**, *40*, 359−364.

Gould, J. M. Alkaline peroxide delignification of agricultural residues to enhance enzymatic saccharification. *Biotechnol. Bioeng.* **1984**, *26*, 46−52.

Grabber, J. H.; Ralph, J.; Hatfield, R. D. Ferulate cross-links limit the enzymatic degradation of synthetically lignified primary walls of maize. *J. Agric. Food Chem.* **1998**, *46*, 2609−2614.

Hackney, J. M.; Atalla, R. H.; Van der Hart, D. L. Modification of crystallinity and crystalline structure of *Acetobacter xylinum* cellulose in the presence of water-soluble β-1,4-linked polysaccharides: ^{13}C-NMR evidence. *Int. J. Biol. Macromol.* **1994**, *16*, 215−218.

Haigler, C. H.; White, A. R.; Brown, R. M., Jr.; Cooper, K. M. Alteration of in vivo cellulose ribbon assembly by carboxymethylcellulose and other cellulose derivatives. *J. Cell Biol.* **1982**, *94*, 64−69.

Harris, P. J.; Henry, R. J.; Blakeney, A. B.; Stone, B. A. An improved procedure for the methylation analysis of oligosaccharides and polysaccharides. *Carbohydr. Res.* **1984**, *129*, 57−73.

Hatfield, R. D. Cell wall polysaccharide interactions. In *Forage Cell Wall Structure and Digestibility*; Jung, H. G., Buxton, D. R., Hatfield, R. D., Ralph, J., Eds.; American Society for Agronomy, Soil Science Society of America, Crop Soil Science Society of America: Madison, WI, 1993; pp 285−313.

Hatfield, R. D.; Weimer, P. J. Degradation characteristics of isolated and *in situ* cell wall lucerne pectic polysaccharides by mixed ruminal microbes. *J. Sci. Food Agric.* **1995**, *69*, 185−196.

Hatfield, R. D.; Ralph, J.; Grabber, J. H. Cell wall structural foundations: molecular basis for improving forage digestibilities. *Crop Sci.* **1999a**, *39*, 27−37.

Hatfield, R. D.; Wilson, J. R.; Mertens, D. R. Composition of cell walls isolated from cell types of grain sorghum stems. *J. Sci. Food Agric.* **1999b**, *79*, 891−899.

Hestrin, S.; Schramm, M. Synthesis of cellulose by *Acetobacter xylinum*. 2. Preparation of freeze-dried cells capable of polymerizing glucose to cellulose. *Biochem. J.* **1954**, *58*, 345−352.

Hungate, R. E. *The Rumen and Its Microbes*; Academic Press: New York, 1966.

Johnson, D. C.; Neogi, A. N. Sheeted products formed from reticulated microbial cellulose. U.S. Patent 4,863,565, 1989.

Jung, H. G.; Russelle, M. P. Light source and nutrient regime effects on fiber composition and digestibility of forages. *Crop Sci.* **1991**, *31*, 1065−1070.

Marx-Figini, M.; Pion, B. G. Kinetic investigations on biosynthesis of cellulose by *Acetobacter xylinum*. *Biochim. Biophys. Acta* **1974**, *338*, 382−393.

McDougall, E. I. Studies on ruminant saliva. I. The composition and output of sheep's saliva. *Biochem. J.* **1948**, *43*, 99−109.

Mertens, D. R.; Loften, J. R. The effect of starch on forage fiber digestion kinetics in vitro. *J. Dairy Sci.* **1980**, *63*, 1437−1446.

Mertens, D. R.; Weimer, P. J. Method for measuring gas production kinetics. In *Occasional Publication 22, In Vitro Techniques for Measuring Nutrient Supply to Ruminants*; Deaville, E. R., Owen, E., Adesogan, A. T., Rymer, C., Huntington, J. A., Lawrence, T. L. J., Eds.; British Society of Animal Science: Edinburgh, U.K., 1998; pp 209−211.

Pell, A. N.; Schofield, P. Computerized monitoring of gas production to measure forage digestion in vitro. *J. Dairy Sci.* **1993**, *76*, 1063−1073.

Ralph, J.; Hatfield, R. D.; Grabber, J. H.; Jung, H. G.; Quideau, S.; Helm, R. F. Cell wall cross-linking in grasses by ferulates and diferulates. In *Lignin and Lignan Biosynthesis*; Lewis, N. G., Sarkanen, S., Eds.; American Chemical Society: Washington, DC, 1998; pp 209−236.

SAS Institute. *SAS User's Guide*, version 5; SAS Institute: Cary, NC, 1985.

Schofield, P.; Pitt, R. E.; Pell, A. N. Kinetics of fiber digestion from in vitro gas production. *J. Anim. Sci.* **1994**, *72*, 2980−2991.

Stephen, A. M. Other plant polysaccharides. In *The Polysaccharides*; Aspinall, G. O., Ed.; Academic Press: New York, 1983; Vol. 2, pp 97−193.

Theander, O.; Westerlund, E. A. Studies on dietary fiber: III. Improved procedures for analysis of dietary fiber. *J. Agric. Food Chem.* **1986**, *34*, 330−336.

Van Soest, P. J. The uniformity and nutritive availability of cellulose. *Fed. Proc.* **1973**, *32*, 1804−1808.

Van Soest, P. J. *Nutritional Ecology of the Ruminant*, 2nd ed.; Comstock Publishing: Ithaca, NY, 1994.

Weimer, P. J.; Lopez-Guisa, J. M.; French, A. D. Effect of cellulose fine structure on kinetics of its digestion by mixed ruminal microorganisms in vitro. *Appl. Environ. Microbiol.* **1990**, *56*, 2421−2429.

Weimer, P. J.; French, A. D.; Calamari, T. A., Jr. Differential fermentation of cellulose allomorphs by ruminal cellulolytic bacteria. *Appl. Environ. Microbiol.* **1991a**, *57*, 3101−3106.

Weimer, P. J.; Shi, Y.; Odt, C. L. A segmented gas/liquid delivery system for continuous culture of microorganisms on insoluble substrates and its use for growth of *Ruminococcus flavefaciens* on cellulose. *Appl. Microbiol. Biotechnol.* **1991b**, *36*, 178−183.

Weimer, P. J.; Hackney, J. M.; French, A. D. Effects of chemical treatments and heating on the crystallinity of cellulose, and their implications for evaluating the effect of crystallinity on cellulose biodegradation. *Biotechnol. Bioeng.* **1995**, *48*, 169−178.

Received for review December 15, 1999. Revised manuscript received March 8, 2000. Accepted March 8, 2000. This research was supported through Research Project CRIS 3555-31000-00-12D, by the Agricultural Research Service, U.S. Department of Agriculture. Mention of specific commercial products is for informational purposes and does not constitute an endorsement or warranty of said products over similar products that may be suitable.

JF991372Y