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Effect of Cellulose Fine Structure on Kinetics of Its Digestion by Mixed Ruminal Microorganisms In Vitro

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The digestion kinetics of a variety of pure celluloses were examined by using an in vitro assay employing mixed ruminal microflora and a modified detergent extraction procedure to recover residual cellulose. Digestion of all of the celluloses was described by a discontinuous first-order rate equation to yield digestion rate constants and discrete lag times. These kinetic parameters were compared with the relative crystallinity indices and estimated accessible surface areas of the celluloses. For type I celluloses having similar crystallinities and simple nonaggregating particle morphologies, the fermentation rate constants displayed a strong positive correlation ($r^2 = 0.978$) with gross specific surface area; lag time exhibited a weaker, negative correlation ($r^2 = 0.930$) with gross specific surface area. Crystallinity was shown to have a relatively minor effect on the digestion rate and lag time. Swelling of microcrystalline cellulose with 72 to 77% phosphoric acid yielded substrates which were fermented slightly more rapidly than the original material. However, treatment with higher concentrations of phosphoric acid resulted in a more slowly fermented substrate, despite a decrease in crystallinity and an increase in pore volume. This reduced fermentation rate was apparently due to the partial conversion of the cellulose from the type I to the type II allomorph, since mercerized (type II) cellulose was also fermented more slowly, and only after a much longer lag period. The results are consistent with earlier evidence for the cell-associated nature of cellulolytic enzymes of ruminal bacteria and suggest that ruminal microflora do not rapidly adapt to utilization of celluloses with altered unit cell structures.

Cellulose is the most abundant biopolymer on earth and is the chief component of plant biomass, including forages (6, 36). In the rumen, pure cellulose is degraded rather rapidly, and cellulose in forage is thought to be degraded to the extent that it is accessible within the matrix containing other structural biopolymers such as hemicelluloses and lignin (3, 5, 7, 19).

Interpretation of the available literature data on the digestibility of different celluloses by pure and mixed cultures of ruminal bacteria is complicated by two facts. (i) Most digestibility data have been obtained at fixed incubation times, without regard to measurement of kinetic parameters such as rates or lag times, and (ii) only a few incompletely characterized substrates have been compared in previous experiments. For example, Halliwell (16) has shown that the extent of digestion of excess amounts of different celluloses by mixed ruminal microflora after 7 to 10 days of incubation proceeded in the following order: H_3PO_4-swollen cellulose > Whatman cellulose powder > HCl-treated cellulose powder > cotton fiber. Halliwell and Bryant (17) demonstrated that different strains of ruminal bacteria exhibited different abilities to hydrolyze cellulose powder and cotton fibers. Smith et al. (33) reported that the rate of digestion of different celluloses by cell extracts of the ruminal cellulolytic bacterium *Ruminococcus albus* followed the following order: pebble-milled filter paper > never-dried crystalline cellulose from cotton > Sigmacell (type not specified). No attempt was made in these studies to quantitatively measure any fine structural features of the substrates, thus precluding determination of structure-digestibility relationships.

Studies with pure cultures and cellulase enzymes from nonruminal cellulolytic microorganisms have revealed that fine structural features such as allomorphic form (28), crystallinity (9, 11, 12, 29, 31, 37), surface area (11, 28, 34), and pore volume (35, 37) are strong determinants of degradability of pure celluloses. It might thus be expected that such fine structural features would affect cellulose digestibility in the rumen. However, no systematic examination of such effects has been described previously. We report here on the effect of cellulose fine structure on the kinetics of cellulose digestion by mixed ruminal microflora in an in vitro digestibility assay.

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MATERIALS AND METHODS

Celluloses. Eight commercial pure celluloses were used. Sigmacell 20 (SC20), Sigmacell 50 (SC50), Sigmacell 100 (SC100), α-cellulose, microgranular cellulose (CC31), medium fibrous cellulose (CF11), and long fibrous cellulose (CF1) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Avicel PH101 (FMC Corp., Philadelphia, Pa.) was purchased from Fluka AG (Hauppauge, N.Y.). The celluloses included a variety of physical forms (microcrystalline, fibrous, and microgranular) which varied in certain fine structural features (see Results).

In addition, one of the substrates (SC50) was used to prepare a series of six different phosphoric acid-swollen celluloses. Swelling of celluloses was carried out by adding 20 g of SC50 powder to 800 g of cold (0°C) phosphoric acid (72.0, 75.5, 77.0, 78.0, or 80.0% wt/wt) with rapid stirring

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with a plastic rod. All samples were stirred for 1 h in an ice bath. At that time, each cellulose was diluted with 2 liters of cold water, thoroughly mixed, and allowed to settle, after which the overlying liquid was removed by siphoning; this washing procedure was repeated several times to reduce the acid content. The cellulose slurries were then neutralized with solid $NaHCO_3$, rinsed, decanted as above, and then secured inside bags formed from nylon-reinforced paper toweling. These bags were filled with ~1 liter of distilled water, and the excess liquid was squeezed off; this process was repeated 20 times. The bags were then sealed, suspended in buckets containing 5 liters of cold deionized water, and dialyzed for 10 days, with frequent changes of water; prior to each change of water, the bags were tightly hand squeezed to facilitate removal of the equilibrated solutions. After completion of dialysis (when the phosphate content [2] of the dialyzate reached <1 μg/liter), the celluloses were removed from their bags and lyophilized. These celluloses were designated PAS/x, where x equals the concentration of phosphoric acid (PAS) used (e.g., PAS/77.0).

Cellulose II was prepared as follows. Two 10-g batches of SC50 were each suspended in 500 ml of ice-cold 20% NaOH and stirred in an ice bath for 1 h. The slurries were vacuum filtered through nylon-reinforced paper towels and washed with 400 ml of water and the wet cakes were transferred to 1-liter beakers of distilled water. The celluloses were mixed, allowed to settle, and washed by alternately siphoning off the overlying liquid and refilling with 900 ml of distilled water; this process was repeated 10 times. The celluloses were next suspended in 10% glacial acetic acid, stirred for 30 min at room temperature, and vacuum filtered as described above. The wet cakes were rinsed with 10 liters of distilled water (i.e., until the pH of the filtrate was 6.5 to 6.8), combined, and dialyzed against 8 liters of distilled water (three times for 24 h each time at 8°C) prior to lyophilization. The yield of the resulting material (SC50/II) was 41%, based on the weight of the original SC50.

Separation of celluloses by particle size was accomplished by shaking them for 3 h through a series of standard Tyler sieves (150-, 90-, 75-, 63-, and 37-μm-mesh size).

Characterization of celluloses. Allomorphic form (i.e., the geometry of the unit cell of the cellulose crystallite) and relative crystallinity indices (i.e., the relative extent of hydrogen bonding among adjacent cellulose molecules within the microfibrils) were determined by the X-ray diffraction method of Segal et al. (32). A modification (37) of the acid hydrolysis kinetics method of Phillip et al. (26) was used to determine the first-order rate constants for acid hydrolysis, as well as to provide additional measurements of the relative crystallinity index.

Estimation of accessible surface area. Because the cellulolytic enzymes of ruminal bacteria appear to be predominantly cell bound (14, 25) and cellulolysis is mediated primarily by attached bacteria (1, 4), determination of accessible surface area required a technique which measured only the gross surface area available to the microbial cells, rather than the far larger area contributed by the micropores and capillaries which would be available to extracellular cellulolytic enzymes (but not to the cells themselves). Since no technique is available for directly measuring these gross surface areas, they were estimated from calculations based on measured particle size and assumed, idealized geometries of the cellulose particles.

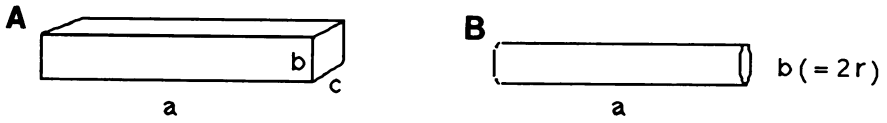
Because of the variability of shape of the cellulose particles, estimations of particle size and its distribution could not be accurately achieved by sieving. Consequently, particle size was determined by microscopic measurements of random subsamples of each cellulose with a Carl Zeiss Axioskop microscope fitted with an ocular micrometer. The celluloses were first swollen in water overnight, and the dimensions (length and width) of 300 random particles were determined for each of the substrates. These measurements were then applied to each of two structural models of the cellulose fiber (Fig. 1): (i) a box model, in which the fiber is shaped as a rectangular parallelepiped with a length a, a width b, and an unmeasurable third dimension c (depth of the box) assumed to equal b, and (ii) a rod model, in which the fiber has a length a and a diameter equal to the measured width b. These two models correspond to the fibrillar arrangements proposed for wood and cotton celluloses, respectively (6, 10). The geometric features of the idealized particles for each of the two models are summarized in Fig. 1. Inspection of Fig. 1 reveals that although the surface area and volume of a given cellulose particle varies depending on the model, the estimated accessible surface area per unit of mass (gross specific surface area [GSSA]) is equal in both cases. The GSSA values for each of the individual cellulose particles within a type were averaged to obtain a mean GSSA for that cellulose type.

Preparation of mixed ruminal microflora inoculum. Rumen contents were collected 10 h postfeeding from a fistulated, nonlactating Jersey cow maintained on a 100% alfalfa hay diet. The rumen contents were squeezed through two layers of cheesecloth until ~1.0 liter of filtrate had been collected under a blanket of CO_2. An equal volume of CO_2-gassed McDougall buffer (23) was poured (in four stages of about one-quarter volume each) over the remaining solids, and the mixture was tightly squeezed each time to obtain additional filtrates. All filtrates were combined and squeezed through two layers of cheesecloth. The resulting filtrate was held at 39°C under a blanket of CO_2 and was used within 0.5 h of preparation.

In vitro fermentations. The in vitro fermentations with mixed ruminal microflora were designed in such a way as to minimize differences among celluloses with regard to rehydration rate and with regard to potential differences in microbial colonization of substrate resulting from differences in the settling rate of cellulose that would occur in unshaken cultures. Fermentations were carried out in 158-ml serum vials (Wheaton Glass, Vineland, N.J.) sealed with butyl stoppers and aluminum crimp seals. Vials contained 250 mg (±2 mg, weighed to the nearest 0.1 mg) of cellulose and 20 ml of McDougall buffer supplemented with 10 mM NH_4Cl and 1 mg of resazurin per liter under an atmosphere of O_2-free CO_2. Vials were allowed to stand for 12 to 16 h to ensure complete hydration of the celluloses (20, 34), warmed to 39°C, reduced with 0.2 ml of reducing agent (1.25% [wt/vol] each of cysteine hydrochloride and $Na_2S \cdot 9H_2O$), and inoculated with 4.8 ml of fresh inoculum (see above).

Vials were incubated at 39°C with shaking at 100 rpm. At time zero and at nine other times (e.g., 8, 12, 15, 18, 21, 24, 30, 36, and 48 h), paired vials of each substrate were removed, chilled in ice, and uncapped. A 50-ml portion of neutral detergent solution (12) was added to each vial, and the vials were frozen at −20°C prior to analysis.

Analysis of residual cellulose. Residual cellulose was determined by a modification of the neutral detergent fiber procedure (13). Fermentation vials (previously amended with neutral detergent and frozen as described above) were thawed, sealed with flanged rubber stoppers, crimp sealed, and autoclaved for 45 min at 125°C in a 10-cm-deep pan. The hot vials were then vented and decapped, and their contents



$A = 4\,ab + 2\,bc = 4\,ab + 2\,b^2$

$V = abc = ab^2$

$A = (2\,\pi\,r\,a) + (2\,\pi\,r^2) = \pi\,(ab + 0.5\,b^2)$

$V = \pi\,r^2\,a = \pi\,(0.25\,ab^2)$

Both models:

$m = D\,V$

$D = [(D_c)\,(RCI) + (D_a)\,(100 - RCI)] / 100$

$GSSA = (4a + 2b) / D\,a\,b$

FIG. 1. Box (A) and rod (B) structural models of cellulose fibers and mensuration formulas used for estimating GSSA. a, Length; b, width; c, depth (unmeasurable by light microscopy but assumed to equal b); r, radius; A, gross surface area; V, gross volume; m, mass; D, density; D_c, density of crystalline regions (equal to 1.61 g/cm^3); D_a, density of amorphous regions (equal to 1.27 g/cm^3); RCI, relative crystallinity index (determined by acid hydrolysis kinetics). The celluloses are assumed to fit a two-phase model having purely crystalline and purely amorphous regions, a considerable oversimplification of the known order distribution (18, 22).

were immediately vacuum filtered through preweighed 47-mm Gelman GF/D glass fiber filters (nominal pore size, 2.7 μm), with liberal hot-water washing. The filters were dried at 105°C for 3 days and weighed after equilibration to room temperature in a vacuum desiccator. Net cellulose dry weight was determined by subtraction. Fractional dry weight losses at each time point were calculated after subtracting the weight of inocula (from similarly inoculated, incubated, and neutral detergent-treated vials lacking substrate) and after correcting for the moisture content of each of the fresh celluloses (which varied from 4.28 to 7.76% by weight, depending on the substrate, determined from the time zero data). With two test substrates (SC50 and α-cellulose) amended with a rumen fluid inoculum and immediately processed to recover neutral detergent fiber, the above procedure gave results that were essentially identical ($P < 0.01$) to those of the standard method, while avoiding the severe bumping behavior that characterizes the standard method when used with finely divided particulate celluloses.

Determination of kinetic parameters. The weight loss data were fitted by linear regression analysis to a discontinuous first-order kinetic equation (24): when $t \leq t_1$, $\ln y = \ln 1$, and when $t > t_1$, $\ln y = -k(t - t_1)$, where y is the fraction of original cellulose remaining, k is the first-order rate constant, t is the incubation time (in hours), and t_1 is the discrete lag time (in hours). In fitting the data to the model, the rate was calculated first by regressing ($\ln y$) against t (Fig. 2). The lag time t_1 was then calculated by solving the regression equation for time when $\ln y = \ln 1$. Mathematically, this reduces the following algebraic expression: $t_1 = [(y\text{-intercept of the regression equation})]/k$. Datum points at times $t < t_1$ (i.e., all time zero data and, where necessary, some data from the 8- and 12-h time points) were excluded from the analysis, and the regression was repeated. For each substrate, two rate equations were obtained (corresponding to the paired vials taken at each time and randomly assigned to separate groups).

Because of the number of celluloses ($n = 18$) used in the data set and the number of samples ($n = 20$) used for each kinetic determination, it was not practical to compare all samples with one another directly. Consequently, the celluloses were divided into four groups of two to eight substrates, and kinetic parameters for each substrate were normalized to those of SC50, which was used in all experiments. The use of two dimensionless terms, a normalized rate ($k_{cellulose}/k_{SC50}$) and a normalized lag (lag time on cellulose divided by lag time on SC50) permitted the comparison of data from different experiments in which kinetic parameters varied as a result of different fresh inocula being used.

RESULTS

Characterization of celluloses. The fine structural characteristics of the different commercial celluloses are summarized in Table 1. X-ray diffraction revealed that all eight of the celluloses were of the natural type I allomorph. Six of the eight displayed similar crystallinities but varied considerably in mean particle size (and thus in GSSA). It is important to note that, owing to the large variation in the sizes of individual particles within each type of cellulose (Fig. 3), the standard errors of the dimensions a and b were very large (similar to the mean values of a and b themselves). However, successive iteration of individual particle size measurements and calculated GSSAs yielded relatively constant values for these parameters, even though the relative standard errors were not reduced by measuring large numbers of particles (Table 2), as would be expected if particle selection for measurement purposes were random. This implies that the dimensions and their derived GSSA values were useful in

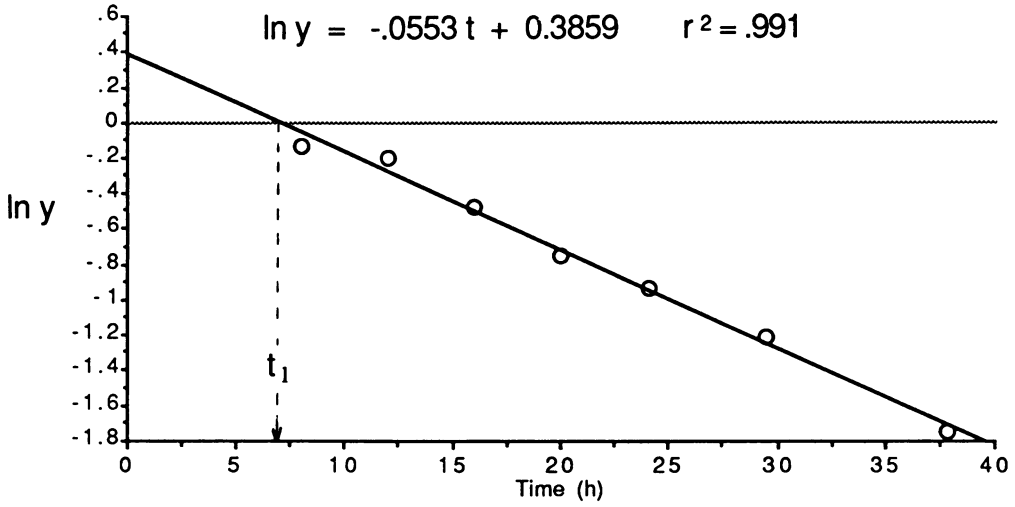


FIG. 2. Demonstration of the fitting of a sample data set to the discontinous first-order kinetic equation. The data were first applied to linear regression of ln y against time (t) to generate a first-order rate constant (viz., 0.0553 h^{-1}). The lag time t_1 was then calculated as the time at which the regression line gave a value of $y = 1$ (viz., ln 1 = $-0.0533\ t_1 + 0.3859$, which reduces to t_1 = [(ln 1) $-0.3859]/-0.0533$ or 0.3859/0.0533 = 6.98 h). See reference 24 for a more detailed discussion of the model and its application.

describing the population of cellulose particles but would be of no use in predicting the size (or GSSA) of an individual, randomly selected cellulose particle within a given population.

Microscopic examination revealed that two of the eight celluloses (SC50 and PH101) exhibited complex particle aggregation patterns characterized by geometrically irregular conglomerations of several to many discrete fibers. The resulting complexity of the surface topologies of these two substrates precluded estimation of their surface areas by the methods described above.

Treatment of SC50 with increasing concentrations of phosphoric acid yielded a series of swollen celluloses with progressively lower bulk densities and lower crystallinities. At higher acid concentrations, some conversion of the SC50 crystalline lattice from the original native type I lattice to the alternate type II allomorph was noted (Fig. 4). This conversion was not expected, in light of a report that similar treatments of cotton fabric did not cause conversion from the type I allomorph (27).

In vitro fermentation of commercial celluloses. At the cellulose concentrations tested (10 mg/ml), all eight of the commercial celluloses were completely digested in the in vitro system within 48 h. No weight loss occurred in uninoculated control cultures. Light and phase-contrast microscopic observations revealed that by the earliest time point (8 h), all of the celluloses were completely colonized by bacteria with coccus and coccobacillus morphologies resembling those of *Ruminococcus* sp. and *Fibrobacter (Bacteroides) succinogenes*. Virtually no protozoa or fungi were observed after the first 8 h of incubation.

We did not observe the strong inhibition of cellulolysis reported by Halliwell (15) resulting from agitation of the fermentation vials, except in rare cases in which the culture medium was incompletely reduced (as evidenced by the pink color of the redox indicator resazurin).

The kinetic data from the in vitro fermentations are summarized in Tables 3 through 6. The discontinuous first-order kinetic model was found to accurately describe the observed digestion of all of the celluloses (regression coef-

TABLE 1. Physical characteristics of commercial celluloses

Cellulose	Physical form[a]	k_{ah}[b]	RCI_{ah}[c]	RCI_{xrd}[d]	Mean particle dimensions[e] (μm)		GSSA[f] (m^2/g)
					Length	Width	
SC50	Microcrystalline	0.142	90.1	94.9	56	23	ND[g]
PH101	Microcrystalline	0.123	89.3	97.5	65	31	ND
SC20	Microcrystalline	0.148	86.3	96.6	36	15	0.301
SC100	Noncrystalline	0.176	64.8	78.9	43	15	0.310
α	Fibrous	0.150	72.4	92.2	105	20	0.177
CC31	Microgranular	0.051	90.8	98.4	61	20	0.228
CF11	Fibrous	0.049	88.2	99.4	173	20	0.163
CF1	Fibrous	0.050	87.1	97.4	182	20	0.158

[a] Vendor descriptions.
[b] First-order rate constant for hydrolysis of crystalline component of each cellulose by 6 N HCl at 100°C.
[c] Relative crystallinity index as determined by acid hydrolysis kinetics.
[d] Relative crystallinity index as determined by X-ray diffraction.
[e] Means of 300 measured particles.
[f] Means of 300 separate calculated GSSA values. See Fig. 1 legend for description of dimensions and GSSA.
[g] ND, Not determinable because of extensive particle aggregation.

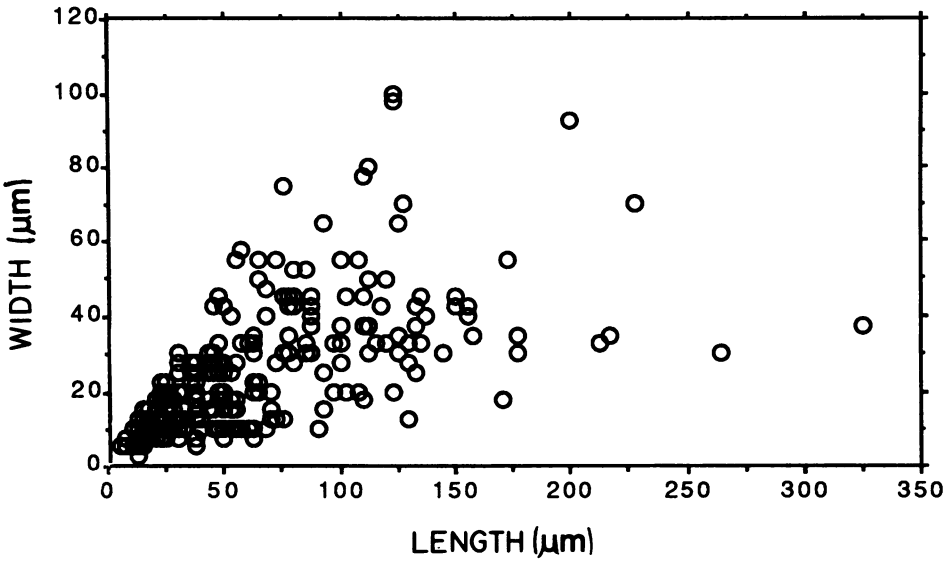


FIG. 3. Particle dimension measurement distribution for SC50. Note the large variability in the sizes of individual particles, a feature common to all of the celluloses used in this study.

ficients (r^2) of 0.945 to 0.999 [mean, 0.986]). Considerable differences in fermentation rate and lag time were observed among different runs for the single common substrate (unsieved SC50) used in all experiments; this was apparently due to differences in inocula, since replicate runs within the same experiment showed very close agreement (Table 3). In order to allow meaningful interpretation of the data from different experiments, the data from all the celluloses within an individual experiment were normalized to this standard, unsieved SC50 substrate.

Data from two separate experiments with the different commercial celluloses are summarized in Table 4. These celluloses displayed substantial (approximately twofold) differences in the first-order rate constants of digestion and slight but significant differences in normalized lag time (i.e., lag time on cellulose divided by lag time on SC50). Linear regression of the mean rate constant obtained for each substrate in the first experiment showed a strong correlation (r^2 = 0.968) with the mean rate constant for the homologous substrate obtained in the second experiment; correlations in lag time between the two experiments were somewhat weaker (r^2 = 0.916). By contrast, regressions of rate constants on lag times for homologous substrate within experiments showed little correlation (r^2 of 0.558 and 0.386 for experiments I and II, respectively). There was no correlation (r^2 = 0.068) between the normalized rates for microbial

TABLE 2. Measured particle sizes and calculated GSSAs for CF1 cellulose demonstrating convergence of data to constant mean values despite large standard error terms

Cumulative no. of particles counted	Length (μm)		Width (μm)		GSSA (m^2/g)	
	Mean	SE[a]	Mean	SE	Mean	SE
30	146.7	74.5	18.0	4.0	0.166	0.051
60	151.7	78.4	19.0	6.0	0.162	0.056
90	170.4	104.7	19.4	6.2	0.158	0.056
120	178.7	109.1	19.5	6.0	0.157	0.056
150	179.3	107.6	19.8	6.4	0.156	0.056
180	177.9	108.1	19.7	6.7	0.158	0.057
210	179.4	119.8	19.6	6.7	0.158	0.058
240	178.3	126.8	19.5	6.7	0.160	0.059
270	181.8	128.8	19.8	6.9	0.158	0.059
300	181.8	130.5	19.7	6.8	0.158	0.059

[a] SE, Standard error.

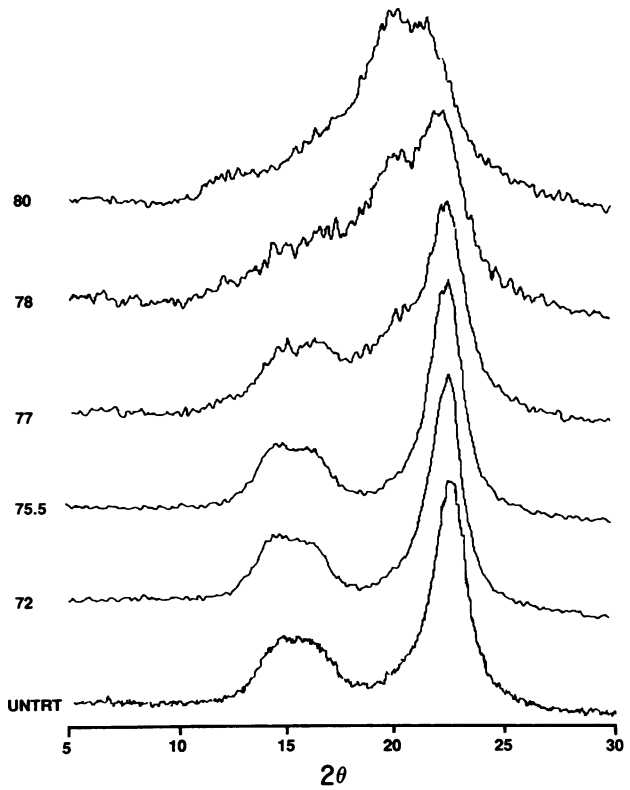


FIG. 4. X-ray diffractograms of untreated (UNTRT) SC50 (a type I cellulose) and SC50 treated with various concentrations of phosphoric acid. Note the increasing extent of conversion from the type I to the type II allomorph at acid concentrations of >77%. θ, Diffraction angle.

TABLE 3. Kinetic parameters for the digestion of SC50 microcrystalline cellulose by mixed ruminal microflora in five different experiments

Cellulose class	Table reference no.	k (h^{-1})		t_l (h)	
		Replicates	Mean	Replicates	Mean
Commercial 1	4	0.0862, 0.0856	0.0859	9.81, 9.70	9.76
Commercial 2	4	0.0612, 0.0581	0.0596	8.64, 8.00	8.32
Particle sized	5	0.0989, 0.1003	0.0996	10.07, 10.14	10.11
Phosphoric acid swollen	6	0.0769, 0.0745	0.0757	7.31, 6.67	6.99
Allomorphs	6	0.0681, 0.0642	0.0661	7.49, 6.53	7.01

fermentation of the celluloses and the rate constants of acid hydrolysis of the crystalline region of the homologous substrate (k_{ah}).

Linear regression analysis revealed that the kinetic parameters for the four nonaggregated isocrystalline celluloses (SC20, CC31, CF11, and CF1) were strongly dependent on microbially available GSSA (Fig. 5): [normalized rate = 0.068 + 2.845 (GSSA), (r^2 = 0.978)] and [normalized lag = 1.751 − 3.014 (GSSA), (r^2 = 0.930)]. However, the effects of crystallinity could be demonstrated by including the kinetic data from the two less crystalline celluloses (SC100 and α-cellulose in the regression analysis. Under these conditions, the correlations were somewhat poorer: [normalized rate = 0.232 + 2.324 (GSSA), (r^2 = 0.676)] and [normalized lag = 1.564 − 1.998 (GSSA), (r^2 = 0.658)]. Nevertheless, crystallinity did not appear to be a major determinant of digestion rate, since SC20 and SC100, two celluloses with similar GSSAs but widely different crystallinities (Table 1), were digested at similar rates (Table 4).

The effects of surface area could also be observed, though less dramatically, by comparing the kinetic parameters of similar celluloses having different particle sizes. One relatively crystalline cellulose (SC50) and one less crystalline cellulose (SC100) were fractionated into different particle size classes, and two fractions of each substrate (particle size ranges, 37 to 63 μm and 90 to 150 μm) were used for further kinetic studies. In vitro digestion data of these substrates are summarized in Table 5. For both the SC50 and SC100 substrates, the lag times were slightly, but not significantly, shorter for the smaller particles than for the larger particles. As expected, the rate of digestion of the smaller particles exceeded that of the larger particles for SC50; however, SC100 displayed the opposite effect. At equivalent particle sizes, the SC100 was actually digested at a slower rate than the more crystalline SC50, suggesting again that crystallinity itself is not a major determinant of the rate of cellulose digestion by mixed ruminal microflora.

TABLE 5. In vitro digestion kinetics of SC50 and SC100 for two different particle size ranges

Cellulose	Particle size (μm)	Normalized rate[a]	Normalized lag[b]
SC50	Unfractionated	1.00 (0.01)	1.00 (0)
	37–63	1.03 (0.01)	0.98 (0.03)
	90–150	0.94 (0.06)	1.03 (0.04)
SC100	37–63	0.86 (0.04)	0.96 (0.05)
	90–150	0.98 (0.03)	1.06 (0.02)

[a] Normalized rate is equal to $k_{cellulose}/k_{SC50}$. Coefficients of variation (standard deviation between paired k values for a given cellulose, divided by the mean rate constant for that cellulose) are noted in parentheses.

[b] Normalized lag is equal to lag time on cellulose divided by lag time on SC50. Coefficients of variation (standard deviation between paired lag times for a given cellulose, divided by the mean lag time for that cellulose) are noted in parentheses.

In vitro fermentation of treated celluloses. The phosphoric acid-swollen celluloses displayed a somewhat unexpected pattern of kinetic behavior (Table 6). Maximal rates of substrate disappearance were noted in substrates treated with an intermediate concentration of phosphoric acid. Higher treatment concentrations resulted in more slowly degraded substrates, despite a decrease in crystallinity and a presumed increase in pore volume (35). Because of the aggregated nature of these cellulose particles, the GSSA could not be estimated from particle dimensions. However, a simple measurement of particle dimensions revealed that after the phosphoric acid treatment, the average cellulose particle was actually smaller than the original starting material.

The decreased rate constants in the celluloses swollen with the higher concentrations of phosphoric acid coincided with a significant degree of conversion from the type I to the type II allomorph (Fig. 4). Consequently, type II cellulose was prepared by alkali treatment of SC50, and the conversion to the type II allomorph was confirmed by X-ray diffraction (Fig. 6). Despite a reduced particle size, this SC50/II substrate was digested considerably more slowly than the standard type I substrate (Table 5), following a lag period that was twice as long.

TABLE 4. In vitro digestion kinetics of commercial cellulose powders by mixed ruminal microflora

Cellulose	Normalized rate[a]			Normalized lag[b]		
	Expt 1	Expt 2	Mean	Expt 1	Expt 2	Mean
SC50	1.00 (0.01)	1.00 (0.03)	1.00	1.00 (0.01)	1.00 (0.05)	1.00
PH101	1.04 (0.07)	1.11 (0.01)	1.07	1.03 (0.04)	1.03 (0.06)	1.03
SC20	0.95 (0.09)	0.93 (0.01)	0.94	0.85 (0.10)	0.78 (0.03)	0.81
SC100	0.95 (0.05)	0.97 (0.12)	0.96	1.03 (0.04)	1.15 (0.07)	1.09
α	0.81 (0.01)	0.88 (0.02)	0.85	1.13 (0)	1.29 (0.03)	1.21
CC31	0.63 (0.01)	0.73 (0.01)	0.68	1.06 (0)	1.20 (0)	1.13
CF11	0.53 (0.02)	0.60 (0)	0.57	1.21 (0.05)	1.33 (0)	1.27
CF1	0.47 (0.01)	0.54 (0.02)	0.51	1.18 (0.01)	1.26 (0.03)	1.22

[a] Normalized rate is equal to $k_{cellulose}/k_{SC50}$. Coefficients of variation (standard deviation between paired k values for a given cellulose, divided by the mean rate constant for that cellulose) are noted in parentheses.

[b] Normalized lag is equal to lag time on cellulose divided by lag time on SC50. Coefficients of variation (standard deviation between paired lag times for a given cellulose, divided by the mean lag time for that cellulose) are noted in parentheses.

TABLE 6. Kinetics of in vitro digestion of chemically treated microcrystalline celluloses by mixed ruminal microflora

Cellulose	RCI[a]	Particle dimensions[b] (μm)		Normalized rate[c]	Normalized lag[d]
		Length	Width		
SC50	94.9	56	23	1.00 (0.02)	1.00 (0.06)
PAS/72.0	96.7	41	14	1.03 (0.07)	0.91 (0.08)
PAS/75.5	97.1	46	17	1.16 (0.05)	1.07 (0.06)
PAS/77.0	82.2	45	25	1.08 (0.08)	1.18 (0.06)
PAS/78.0	77.1	33	15	0.85 (0.01)	1.24 (0.02)
PAS/80.0	54.2	32	16	0.88 (0.05)	1.34 (0.06)
SC50[e]	94.9	56	23	1.00 (0.04)	1.00 (0.09)
SC50/II[e]	ND[f]	32	17	0.57 (0.01)	1.92 (0.01)

[a] RCI, Relative crystallinity index, as determined by X-ray diffraction.
[b] Each value is the mean of 210 measured particles.
[c] Normalized rate is $k_{cellulose}/k_{SC50}$. Coefficients of variation (standard deviation between paired k values for a given cellulose, divided by the mean rate constant for that cellulose) are noted in parentheses.
[d] Normalized lag is lag time on cellulose divided by lag time on SC50. Coefficients of variation (standard deviation between paired lag times for a given cellulose, divided by the mean lag time for that cellulose) are noted in parentheses.
[e] Kinetic data from a separate experiment.
[f] ND, Not determined.

DISCUSSION

The results of this investigation suggest that available surface area is an important determinant of the rate of digestion of pure type I celluloses by mixed rumen microflora. Different celluloses of similar crystallinity showed an increased rate of digestion and decreased lag time with increasing estimated GSSA. The dependence of these kinetic parameters on gross surface area (which excludes the vast network of capillaries and pores accessible to cellulolytic enzymes but not available to whole microbial cells) is consistent with both electron microscopic observations of the importance of adherent microbial cells in rumen cellulolysis (1, 4) and enzymatic evidence that cellulolytic activity in pure cultures of several ruminal bacteria is localized with the bacterial cells rather than in the culture medium (14, 25).

The relationship between the observed kinetic effects and gross surface area is related to, but distinct from, the relationship between kinetic parameters and particle size. It is well documented that the rate and extent of in vitro digestion of forage particles by mixed rumen microflora are related to particle size (8). Interpretation of data from the literature on this relationship is complicated by the morphological heterogeneity of forage particles (i.e., particles are of nonuniform shape but are usually sized by sieving) and by the experimental methods used to obtain kinetic data. Particle size-related differences in digestion kinetics observed in previous in vitro studies may have been due to the variable behavior of cellulose particles in liquid slurries. For example, larger particles settle more rapidly than do smaller ones and thus might be expected to be more poorly colonized by the nonmotile, slower-settling ruminal bacteria if incubated in unshaken culture. However, the experiments reported here were performed with shaken culture to eliminate the effects of settling rate on digestion. Consequently, the differences we observed in fermentation kinetics must be due to other particle size-related factors such as available surface area. It is likely that the greater specific surface area of the particles permits both more rapid microbial colonization (due to a greater probability of an encounter between microbe and substrate) and more rapid degradation of the colonized substrate due to a greater total number of adherent cellulolytic bacteria.

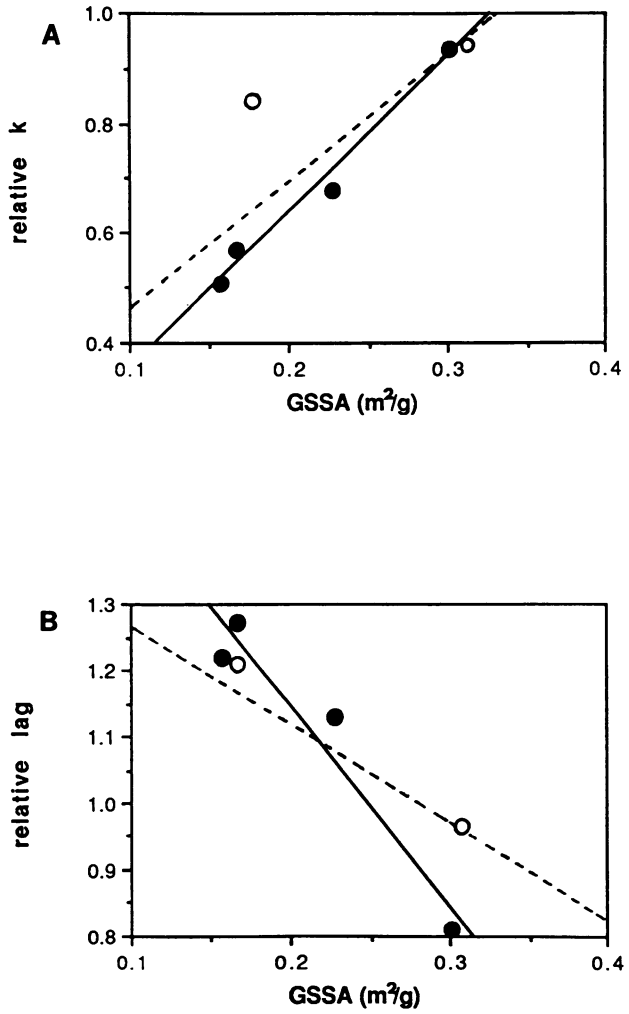


FIG. 5. Relationship between digestion kinetic parameters and the estimated GSSA of the nonaggregated commercial cellulose powders. (A) Relationship between normalized rate (relative k) and GSSA; (B) relationship between normalized lag (relative lag) and GSSA. Solid lines incorporate data for isocrystalline celluloses SC20, CC31, CF1, and CF11 (●); broken lines incorporate data for the isocrystalline celluloses plus data for the less crystalline celluloses SC100 and α (○).

Our kinetic data are in general accord with the data of Van Soest (36), who has reported that four purified celluloses (surgical cotton, Whatman 41, and celluloses isolated from alfalfa and orchard grass) are essentially completely digestible by mixed rumen microflora in vitro and that digestion proceeds at different rates following similar lag periods of ~15 h. However, Van Soest attributed the kinetic differences he found in part to presumed but unverified differences in cellulose crystallinity.

Our conclusion that crystallinity appears to be of only minor importance in determining rate of digestion of natural celluloses by mixed ruminal microflora is somewhat surprising in view of its greater importance in determining cellulolytic rate in bacterial monocultures (9) and cellulolytic enzyme complexes (11, 12, 35, 37). This disparity may be due to the synergistic activities of the different cellulolytic species which make up the ruminal microflora.

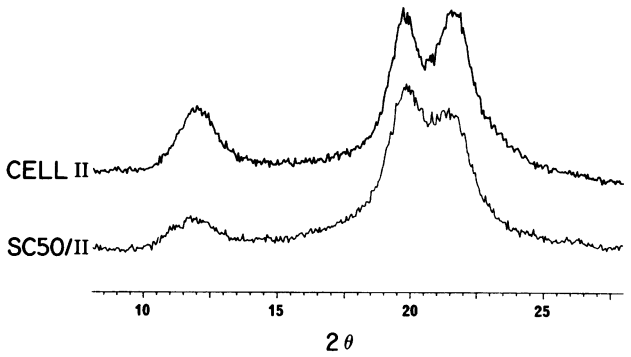


FIG. 6. X-ray diffractograms of authentic type II cellulose (CELL II) and SC50/II obtained by alkali treatment of SC50. θ, Diffraction angle.

This study did not include an investigation of the micropore structure of the substrates, a characteristic which has been shown to be an important determinant in the degradation rate of cellulosic substrates by cell-free cellulase complexes (11, 35, 37). However, our observation that digestion rate was not enhanced by treatment of SC50 with phosphoric acid at concentrations which have been shown to increase pore volumes fourfold is consistent with the data of Lin et al. (21), who have reported that micropore structure is not an important determinant of the digestibility of SolkaFloc BW300 cellulose by mixed ruminal microflora.

Relatively little information is available on the biodegradability of different allomorphic forms of cellulose. Rautela and King (28) have demonstrated that the cellulase enzymes from the fungus *Trichoderma viride* are most active on the allomorphic form of cellulose used as substrate for enzyme synthesis. In our experiments with mixed ruminal microflora, the bulk of cell growth and enzyme production occurred during the experiment itself (i.e., in the presence of the test substrate) rather than having been supplied with the inoculum. The considerably longer lag time prior to digestion of type II cellulose suggests that the ruminal microflora may synthesize a separate set of cellulolytic enzymes for this substrate, as has been suggested for the aerobic *Trichoderma* fungus (28). However, unlike *Trichoderma* species, the enzymes which are produced display an inherently reduced ability to digest the type II substrate. This lower rate may be due to any of several modifications of unit cell structure, including the presence of intersheet hydrogen bonds or the proposed antiparallel packing of adjacent cellulose chains within the microfibrils (30).

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LITERATURE CITED

1. **Akin, D. E.** 1986. Interaction of ruminal bacteria and fungi with southern forages. J. Anim. Sci. **63**:962–977.
2. **American Public Health Association.** 1980. Standard methods for the analysis of water and wastewater, 15th ed., p. 417–420. American Public Health Association, Washington, D.C.
3. **Beveridge, R. J., and G. N. Richards.** 1975. Investigation of the digestion of cell-wall components of spear grass and of cotton cellulose by viscometry and by x-ray diffraction. Carbohydr. Res. **43**:163–172.
4. **Cheng, K.-J., J. P. Fay, R. E. Howarth, and J. W. Costerton.** 1980. Sequence of events in the digestion of fresh legume leaves by rumen bacteria. Appl. Environ. Microbiol. **40**:613–625.
5. **Chesson, A.** 1981. Effect of sodium hydroxide on cereal straws in relation to the enhanced degradation of structural polysaccharides by rumen organisms. J. Sci. Food Agric. **32**:745–758.
6. **Cowling, E. B.** 1975. Physical and chemical constraints in the hydrolysis of cellulose and lignocellulosic materials. Biotechnol. Bioeng. Symp. **5**:163–181.
7. **Darcy, B. K., and R. Belyea.** 1980. Effect of delignification upon in vitro digestion of forage cellulose. J. Anim. Sci. **51**:798–803.
8. **Dehority, B. A., and R. R. Johnson.** 1961. The effect of particle size upon the in vitro cellulose digestibility of forages by rumen bacteria. J. Dairy Sci. **45**:508–512.
9. **Dermoun, Z., and J. P. Belaich.** 1985. Microcalorimetric study of cellulose degradation by *Cellulomonas uda*. Biotechnol. Bioeng. **27**:1005–1011.
10. **Fan, L. T., Y. H. Lee, and D. R. Beardmore.** 1980. Major chemical and physical features of cellulosic materials as substrates for enzymatic hydrolysis. Adv. Biochem. Eng. **14**: 101–117.
11. **Fan, L. T., Y. H. Lee, and D. R. Beardmore.** 1981. The influence of major structural features of cellulose on rate of enzymatic hydrolysis. Biotechnol. Bioeng. **23**:419–424.
12. **Focher, B., A. Marzetti, M. Cattaneo, P. L. Beltrame, and P. Carniti.** 1981. Effect of structural features of cotton cellulose on enzymatic hydrolysis. J. Appl. Polym. Sci. **26**:1989–1999.
13. **Goering, H. K., and P. J. Van Soest.** 1970. Forage fiber analysis. Agriculture handbook no. 379. U.S. Department of Agriculture, Washington, D.C.
14. **Groleau, D., and C. W. Forsberg.** 1981. Cellulolytic activity of the rumen bacterium *Bacteroides succinogenes*. Can. J. Microbiol. **27**:517–530.
15. **Halliwell, G.** 1957. Cellulolysis by rumen micro-organisms. J. Gen. Microbiol. **17**:153–165.
16. **Halliwell, G.** 1957. Cellulolytic preparations from micro-organisms of the rumen and from *Myrothecium verrucaria*. J. Gen. Microbiol. **17**:166–183.
17. **Halliwell, G., and M. P. Bryant.** 1963. The cellulolytic activity of pure strains of bacteria from the rumen of cattle. J. Gen. Microbiol. **32**:441–448.
18. **Hearle, J. W. S.** 1958. A fringed fibril theory of structure in crystalline polymers. J. Polym. Sci. **28**:432–435.
19. **Kerley, M. S., G. C. Fahey, J. M. Gould, and E. L. Ianotti.** 1988. Effect of lignification, cellulose crystallinity, and enzyme accessible space on the digestibility of plant cell wall carbohydrate by the ruminant. Food Microstruct. **7**:59–65.
20. **Lee, Y. H., and L. T. Fan.** Kinetic studies of enzymatic hydrolysis of insoluble cellulose. II. Analysis of extended fermentation times. Biotechnol. Bioeng. **25**:939–966.
21. **Lin, K. W., M. R. Ladisch, M. Voloch, J. A. Patterson, and C. H. Noller.** 1985. Effect of pretreatments and fermentation on pore size in cellulosic materials. Biotechnol. Bioeng. **27**: 1427–1433.
22. **Marchessault, R. H., and J. A. Howsmon.** 1957. Experimental evaluation of the lateral order distribution in cellulose. Textile Res. J. **27**:30–41.
23. **McDougall, E. I.** 1948. Studies on ruminant saliva. I. The composition and output of sheep's saliva. Biochem. J. **43**: 99–109.
24. **Mertens, D. R.** 1977. Dietary fiber components: relationship to the rate and extent of ruminal digestion. Fed. Proc. **36**:187–192.
25. **Pettipher, G. L., and M. J. Latham.** 1979. Production of enzymes degrading plant cell walls and fermentation of cellobiose by *Ruminococcus flavefaciens* in batch and continuous culture. J. Gen. Microbiol. **110**:29–38.
26. **Philipp, H. J., M. L. Nelson, and H. M. Ziifle.** 1947. Crystallinity of cellulose fibers as determined by acid hydrolysis. Textile Res. J. **17**:585–596.
27. **Porter, B. R., and M. L. Rollins.** 1972. Changes in porosity of

treated lint cotton fibers. I. Purification and swelling treatments. J. Appl. Polym. Sci. **16**:217–236.
28. **Rautela, G. S., and K. W. King.** 1968. Significance of the crystal structure of cellulose on the production and action of cellulase. Arch. Biochem. Biophys. **123**:589–601.
29. **Ryu, D. D. Y., S. B. Lee, T. Tassinari, and C. Macy.** 1982. Effect of compression milling on cellulose structure and on enzymatic hydrolysis kinetics. Biotechnol. Bioeng. **24**:1047–1067.
30. **Sarko, A.** 1986. Recent x-ray crystallographic studies of cellulose, p. 29–49. *In* R. A. Young and R. M. Rowell (ed.), Cellulose: structure, modification, and hydrolysis. John Wiley & Sons, Inc., New York.
31. **Sasaki, T., T. Tanaka, N. Nanbu, Y. Sato, and K. Kainuma.** 1979. Correlation between X-ray diffraction measurements of cellulose crystallinity and the susceptibility to microbial cellulase. Biotechnol. Bioeng. **21**:1031–1042.
32. **Segal, L., J. J. Creely, A. E. Martin, Jr., and C. M. Conrad.** 1959. An empirical method for estimating the degree of crystallinity of native cellulose using the x-ray diffractometer. Textile Res. J. **27**:30–41.
33. **Smith, W. R., I. Yu, and R. E. Hungate.** 1973. Factors affecting cellulolysis by *Ruminococcus albus*. J. Bacteriol. **114**:729–733.
34. **Stone, J. E., and A. M. Scallan.** 1968. A structural model for the cell wall of water-swollen wood pulp fibres based on their accessibility to macromolecules. Cellulose Chem. Technol. **3**: 343–358.
35. **Stone, J. E., A. M. Scallan, E. Donefer, and E. Ahlgren.** 1969. Digestibility as a simple function of a molecule of similar size to a cellulase enzyme. Adv. Chem. Ser. **95**:219–241.
36. **Van Soest, P. J.** 1973. The uniformity and availability of cellulose. Fed. Proc. **32**:1804–1808.
37. **Weimer, P. J., and W. M. Weston.** 1985. Relationship between the fine structure of native cellulose and cellulose degradability by the cellulase complexes of *Trichoderma reesei* and *Clostridium thermocellum*. Biotechnol. Bioeng. **27**:1540–1547.