Cellulosic Materials: Structure and Enzymatic Hydrolysis Relationships

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Synopsis

Structural and morphological features of four different cellulosic materials have been deeped by X-ray, CP–MAS NMR, water retention, and specific surface area analysis. Hydrolysis time courses of two of these celluloses were followed by employing an enzymatic system consisting of a cellulase from *Trichoderma viride* and a cellobiase from *Aspergillus niger*. Experimental results were rationalized on the basis of a mathematical model previously verified on the other two substrates. All the celluloses presented the same mechanistic framework involving product inhibitions. The most efficient pretreatment was found to be the dissolution of cellulosic material in the dimethyl sulfoxide–paraformaldehyde system and regeneration with ammonia. This treatment cancelled the memory of the initial structural order.

INTRODUCTION

In previous works we pointed out that the susceptibility of cellulosic materials to enzymatic attack depends significantly on their structural and morphological features and that a correlation exists between kinetic and structural parameters.[1–3]

To get insight into the mechanism of hydrolysis reaction taking into account the complexity of both the cellulase system and the substrate, a mathematical model has been developed[4,5] and verified on differently pretreated cellulosic substrates. This model provides the possibility of different kinds of end-product inhibition (noncompetitive, competitive, and anticompetitive) for endoglucanase and cellobiohydrolase enzymes, and the inhibition by end product and substrate for cellobiase enzyme.

In the present work this mathematical model has been applied to the study of the hydrolysis kinetics of two cellulosic substrates, whose structural and morphological characterization has been depeened.

MATERIALS AND METHODS

Substrates

Textile cotton wastes were washed in acid and alkaline media and sieved: the 200–400 mesh fraction was collected (cellulose A). Portions of this material underwent one of the following pretreatments:

(i) Zinc Chloride–HCl Treatment. The cotton was treated with aqueous

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70% (w/w) $ZnCl_2$ solution and regenerated with 0.05*N* HCl, as previously reported (cellulose B).

(ii) Zinc Chloride–Acetone Treatment. The sample, after aqueous 70% $ZnCl_2$ treatment at 20±1°C for 6 h, under slow stirring, was regenerated with acetone and washed with it up to a negative test for Zn^{2+} (cellulose C).

Prehydrolyzed sulfate cellulose pulp (cellulose D_0) dissolved in dimethyl sulfoxide–paraformaldehyde solution[6] (kindly supplied by Snia Viscosa Laboratories) was precipitated with acetone and then demethylolated with ammonia solution before enzymatic hydrolysis (cellulose D).

Substrate Characterization

Degree of Polymerization ($\overline{DP}_v$). It was determined by viscosimetric measurements in cuproethylendiamine (CED),[7] at 25.0±0.1°C.

X-Ray Diffraction Analysis. A Siemens D500 diffractometer, equipped with a scintillation counter and a linear amplifier, was utilized in the conditions previously reported.[1]

Moisture Regain. It was measured at 20±1°C and 65% relative humidity, as previously reported.[1]

Specific Surface Area. The measurements were carried out with a Sorptomatic Carlo Erba apparatus. The surface area and the pore size distribution were calculated by the BET gas–adsorption–desorption procedure,[8] after outgassing at 40°C and 10^{-2} Torr for at least 24 h.

Water Retention Value. 0.5 g of dry material were mixed with 100 mL of isopropylic alcohol; the alcohol excess was filtered away under vacuum and the sample centrifugated at 400 rpm for 40 min. The sample was weighed and then dried at 105±2°C up to a constant weight. The water retention value was calculated as:

$$\frac{W_1 - W_2}{0.786\ W_2} \times 100$$

where W_1 is the sample weight after centrifugation, W_2 is the weight of dry sample, and 0.786 is the relative density of isopropylic alcohol.

Solubility in Sodium–Iron Tartrate (FeTNa). The dissolution value was determined as previously reported.[1]

Nuclear Magnetic Resonance. The magic angle sample spinning and cross polarization techniques were used to achieve high resolution and sensitivity for the study of solid celluloses.

The spectra of celluloses A, B, and D were recorded on a Brucker CPX 200 Spectrometer operating at 50.33 MHz.

The instrument settings were as follows: 90° pulse width, 3s; spectra width, 0.033s; scan number, 1400; contact time, 1 ms; time delay between scans, 4s.

The samples were hand packed into deuterated poly(methyl methacrylate) rotors which were spun at approximately 4 kHz.

Enzymes

A crude commercial cellulase complex from *Trichoderma viride* (Novo Enzyme Ind.) was used: Its activity was 190 IU/g toward cellulose powder (Whatman CC31) and 3160 U/g toward carboxymethylcellulose (molecular weight, 80,000; degree of substitution, 0.77).

Known amounts of crude cellobiase from *Aspergillus niger* (Novo Enzyme Ind.), with a declared activity of 250 IU/g towards cellobiose, were added to the enzymatic complex to prevent cellobiose inhibition.

Kinetics

The hydrolysis reaction progress was followed in the temperature range 26–50°C. Runs were performed in a thermostated reactor, with mechanical stirring (450 rpm) to eliminate external mass transfer control, by using 0.05*M* acetate buffer (pH 4.8). Samples (2 mL), drawn from the reactor at different times up to 24 h, were filtered and frozen to quench the enzymatic reaction. The samples were analyzed for reducing sugars, cellobiose, and glucose concentrations.

Sugar Analysis

Reducing sugars and glucose were determined by the ferricyanide method[9] and the Glucoquant test (Boehringer), respectively, using glucose for calibration. Cellobiose was determined as glucose, after its complete hydrolysis by cellobiase.

RESULTS

Characterization of the Substrates

The polymerization degree values of samples are listed in Table I. The difference of depolymerization extent between the two $ZnCl_2$-treated celluloses seems to be likely due more to the $ZnCl_2$-treatment dishomogeneity than to the different regeneration step.[1]

The fairly low $\overline{DP}_v$ value of cellulose D depends more on the low value of this parameter in the starting material (D_0) than on degradative effects of dissolution treatment.

Generally both chemical and physical pretreatments of native cellulose

TABLE I
Physicochemical Parameters of Different Celluloses

Samples	$\overline{DP}_v$	Moisture regain (% w/w)	BET surface area(m^2/g)	Pore volume (mL/g)	Water retention (% w/w)
Cellulose A	2010	5.93	1.0	0.11	13.1
Cellulose B	1850	8.17	6.0	0.09	25.3
Cellulose C	1440	9.50	5.0	0.13	39.5
Cellulose D[a]	410	8.50	12.0	0.10	560.0

[a] Cellulose D was prepared starting from a native cellulose (D_0) with $\overline{DP}_v$ = 410, moisture regain = 7.60, water retention = 18.2.

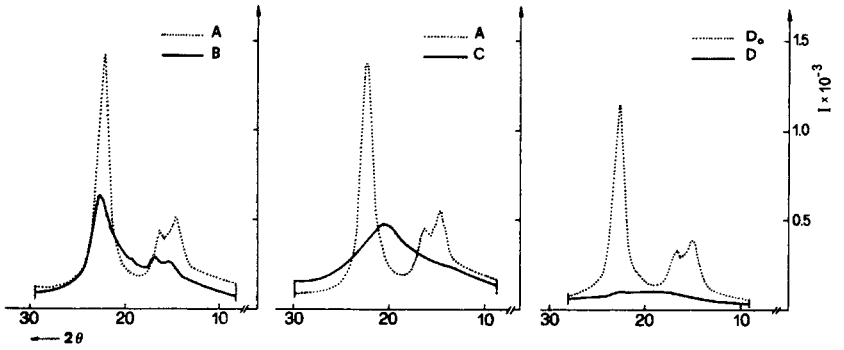

Fig. 1. X-ray diffractograms of untreated (A and D_0) and treated celluloses (B, C, D).

lead to modifications of fibrous architecture of the material, in particular to the disappearance of the supermolecular structure, as pointed out by X-ray analysis (Fig. 1).

The $ZnCl_2$ pretreatments involved a remarkable decrystallization of the materials, more significant in the sample regenerated with acetone. The precipitation with organic solvent of the solution of cellulose D determined the complete disappearance of the native structure.

Supermolecular structure differences of cellulosic materials can be also pointed out by moisture regain measurements, whose results are reported in Table I. We observe an increase of moisture regain in going from cellulose A to cellulose C as well as from the native to the regenerated cellulose D.

Fibrous material permeability to chemical agents or enzymes can be evaluated by measurements of the total specific surface area and of the pore volume accessible to molecules with different diameters. These measurements ought to be carried out removing water in such a way to preserve the internal structure of porous solid.[10]

In the present work the specific surface area has been determined on samples in which the water was removed by solvent exchange. The $ZnCl_2$ pretreatment involved remarkable variations of specific surface area while the different regeneration gave no significant differences (Table I).

Cellulose D showed the highest surface area value, due to the dissolution treatment and probably to the different origin of the starting material.

Pore size distribution (Fig. 2) of cellulose A is very broad, with a maximum around 450 Å; pretreated celluloses B and C show a greater number of pores with a small diameter (maximum 40–50 Å), but large enough to allow the penetration of cellulase macromolecules.[11] A maximum in the same range, but with a broader pore distribution, appears in the case of cellulose D. No significant differences in the pore volume values were found for the examined celluloses (Table I).

The BET surface area values are rather low in comparison with those obtained by measurements on fibers saturated with water.[10] This suggests that the solvent exchange procedure is only partially successful for measurement of the total pore volume.

The water retention determinations (Table I) provide values related to the surface area that are not affected by any shrinkage of the material and

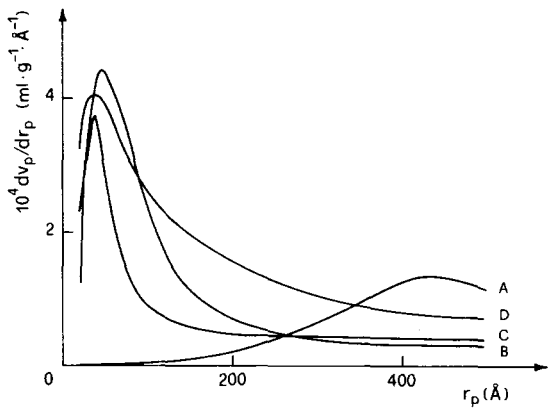

Fig. 2. Pore size distribution of celluloses A, B, C, and D.

reflects its physical state during the enzymatic attack. In our case, the water retention determination allows a better discrimination among the samples than the BET surface area measurements. In particular, cellulose D presents a water retention value significantly higher than the other ones.

The FeTNa solubility data of the samples (Fig. 3) are consistent with $\overline{DP}_v$ data and crystallinity as estimated by X-ray analysis. The samples with low $\overline{DP}_v$ values and with a very modified supermolecular structure showed a greater solubility in all the examined FeTNa solutions, as previously observed.[1]

CP–MAS NMR analysis provides further insight into the structure of cellulosic materials. Recent studies[12,13] showed a relation between the carbon chemical shifts and the polymorphic forms of cellulose.

The spectra of cellulose A, B, and D, reported in Figure 4, are in a close agreement with literature data.[12–16] The split peak in the 105 ppm region was assigned to the C-1 carbon and the peaks in the 70–80 ppm region to C-2, C-3, and C-5 carbons. The narrower peak at 89 ppm and the broader one at 84 ppm were assigned to C-4, while the narrower peak at 66 ppm and the broader one at 63 ppm to the C-6 carbon. Figure 4 shows that the

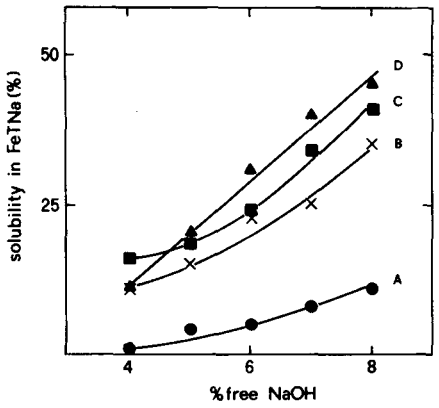

Fig. 3. Solubility in FeTNa of celluloses A, B, C, and D.

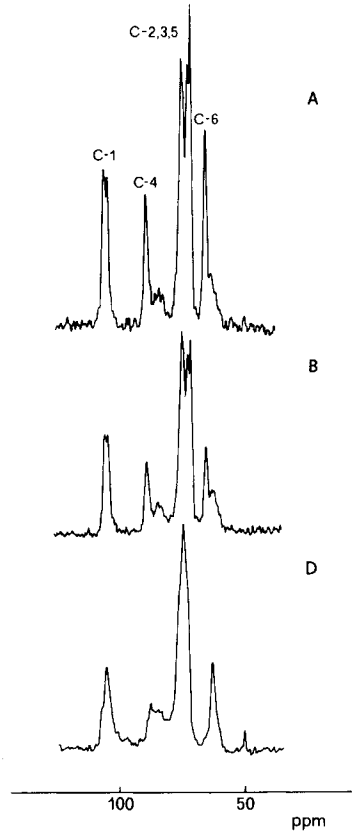

Fig. 4. CP–MAS ^{13}CNMR spectra of celluloses A, B, and D.

relative intensities of the broader peaks at 84 and 63 ppm progressively increase in going from cellulose A to decrystallized celluloses B and D while those of the narrower peaks at 89 and 66 ppm remarkably decrease. According to some authors,[12–16] the narrower peaks could be due to either highly ordered or internal regions of the cellulose fibrils, while the broader peaks could be due to either less ordered or external regions.

Moreover, for the C-1 anomeric carbon a decrease of 105.8 ppm peak intensity was observed in going from the highly ordered cellulose A to the less ordered celluloses B and D. Also this peak could be correlated with the presence of highly ordered environments in the sample.

The comparison between NMR spectra of celluloses A and B shows no differences in the chemical shifts, suggesting the absence of any polymorphic transitions. For cellulose D the chemical shifts occur at lower fields for C-2, C-3, and C-5 carbons and at higher fields for C-6.

In Figure 5 is reported the spectrum of cellulose D obtained by resolution enhancement using 40-Hz Gaussian multiplication. This figure shows a structural reality more complex than the one of Figure 4, but its understanding has not been achieved yet.

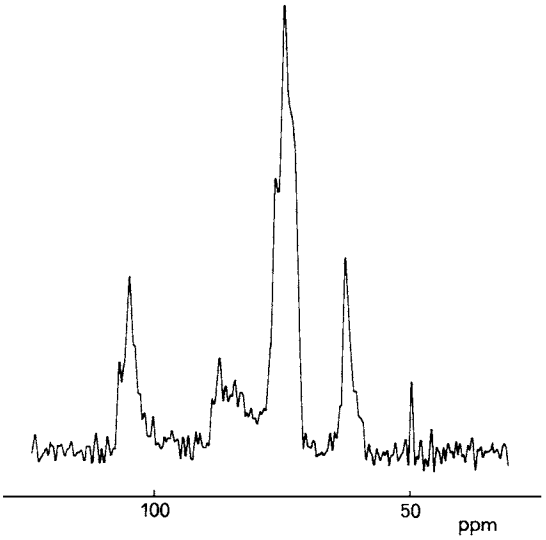

Fig. 5. Resolution enhanced ^{13}C NMR spectrum of cellulose D.

Enzymatic Hydrolysis

In a previous work,[5] we reported the time courses of the hydrolysis reactions of celluloses B and D, in terms of reducing sugars, cellobiose, and glucose production. These kinetic results were rationalized on the basis of the mechanistic model drawn in the following scheme, which holds for the action of both endoglucanase (E_1) and cellobiohydrolase (E_2).

$$\begin{array}{ccccc} EG & \underset{K'_G}{\overset{+G}{\rightleftharpoons}} & E & \underset{K'_C}{\overset{+C}{\rightleftharpoons}} & EC \\ & & +G_i \uparrow\downarrow & & \\ EG_iG & \underset{K''_G}{\overset{+G}{\rightleftharpoons}} & EG_i & \underset{K''_C}{\overset{+C}{\rightleftharpoons}} & EG_iC \\ & & \downarrow & & \\ & & E + G_j + G_{i-j} & & \end{array}$$

where G_i is a polysaccharide molecule ($i \geqslant 3$); G and C refer to glucose and cellobiose, respectively; K terms represent inhibition constants.

A good agreements was observed between experimental and calculated values, in the considered temperature range (26–50°C).

In the present work the kinetic study has been extended, with the same mathematical approach, to celluloses A and C. Their hydrolysis time courses, measured at different temperatures, are reported in the Figure 6.

The cellulose pretreatment with 70% $ZnCl_2$ and the regeneration with acetone (giving cellulose C) have determined a remarkable increase of hydrolysis yield at all the considered temperatures with respect to the untreated cellulose A.

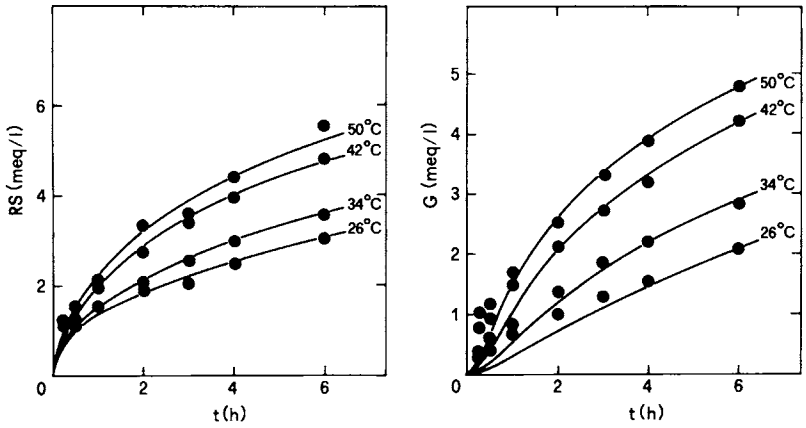

Fig. 6. Hydrolysis time courses of cellulose A (RS-reducing sugars; G-glucose).

The curves of Figures 6 and 7 show the employed mathematical model gave a good fitting of the experimental data. Table II shows the optimized kinetic and inhibition constant values for the endoglucanase (E_1) and cellobiohydrolase (E_2) components of the cellulase complex. For the E_1 component, the differences among the substrates seem to affect more the K_m and the term $1/K''_G$ than the V_{max} values. For the E_2 component, the V_{max} and $1/K'_G$ values were found to be quite close to each other for all the substrates, likely due to the endwise attack of cellobiohydrolase, as already noticed.[5] Thus, mean values are reported in Table II and employed for calculations. Furthermore, the glucose inhibition term appeared independent of temperature too. On the other hand, the K_{m2} values reveal rather significant differences among the substrates.

In Figure 8 are reported the calculated hydrolysis time courses at 50°C and 34°C for all the examined substrates. The hydroysis yield of cellulose B is higher than the one of cellulose C at 50°C and 34°C. At both temperature, cellulose D shows the highest reaction rates and hydrolysis yields in line with its structural and morphological features, which reflect a deep modification of the initial order.

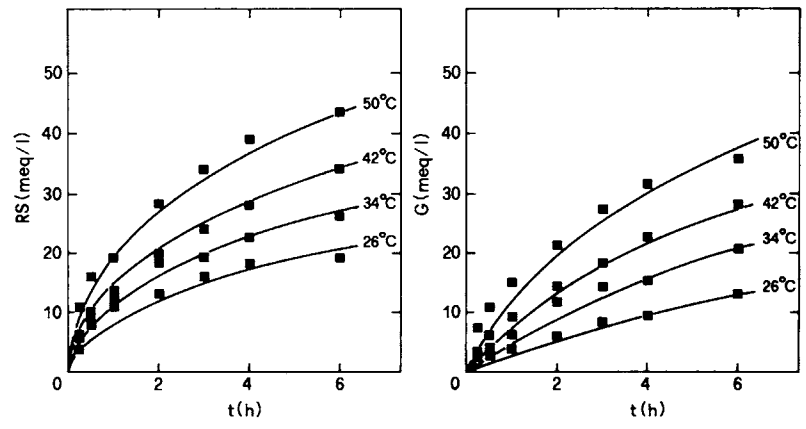

Fig. 7. Hydrolysis time courses of cellulose C (RS-reducing sugars; G-glucose).

TABLE II
Kinetic Parameters[a] for the Enzymatic Hydrolysis Reaction of Cellulose Substrates A, B, C, and D

Temperature (°C)	V_{max1}	K_{m1}	$1/K''_{1G}$	V_{max2}	K_{m2}	$1/K'_{2G}$
Cellulose A						
26	19.4	326	262	1.32	8.18	0.289
34	38.6	508	381	5.56	17.3	0.289
42	73.8	774	544	21.6	35.0	0.289
50	138	1160	766	79.5	68.9	0.289
Cellulose B[b]						
26	22.6	33.9	2.03	1.32	3.78	0.289
34	40.1	40.3	2.03	5.56	7.55	0.289
42	68.7	47.5	2.03	21.6	14.5	0.289
50	115	55.5	2.03	79.5	27.1	0.289
Cellulose C						
26	14.6	7.93	1.44	1.32	5.30	0.289
34	28.7	11.7	1.78	5.56	10.4	0.289
42	54.0	16.9	2.18	21.6	19.8	0.289
50	99.6	24.1	2.65	79.5	36.7	0.289
Cellulose D[b]						
26	22.9	158	0.591	1.32	1.16	0.289
34	40.8	94.4	0.806	5.56	2.59	0.289
42	70.2	58.2	1.08	21.6	5.53	0.289
50	118	36.6	1.43	79.5	11.5	0.289

[a] Units: V_{max1} = mM RS/h; V_{max2} = mM cellobiose/h; K_{m1} = mM bond; K_{m2} = mM RS; K_G = mM glucose.
[b] Ref. 5.

CONCLUSIONS

The characterization of the celluloses shows that the most efficient pretreatment is the one that has cancelled the memory of the initial order. This occurs in the case of cellulose D obtained by dissolution of prehydrolized sulfate cellulose pulp in the dimethyl sulfoxide–paraformaldehyde system and regeneration with ammonia.

The structural order is described by crystallinity index, water retention value, and specific surface area. New insights are provided by the CP–MAS

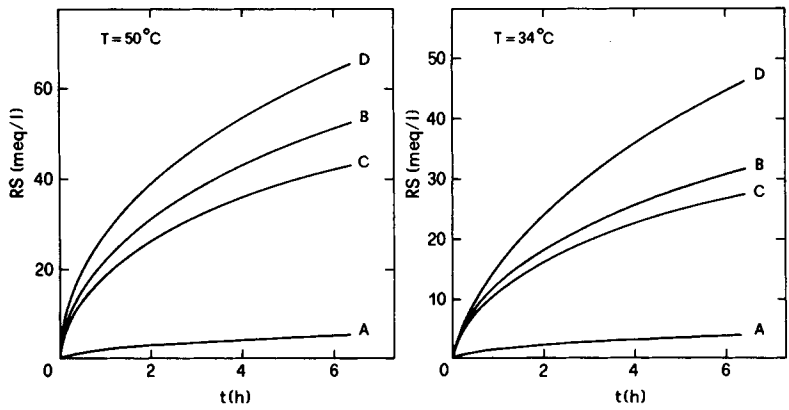

Fig. 8. Calculated hydrolysis time courses of celluloses A, B, C, and D (RS-reducing sugars).

NMR technique that appears to be suitable to discriminate the different substrates at structural as well as morphological level.

Hydrolysis reaction time courses are in a good agreement with morphological and structural parameters.

All the substrates presented the same mechanistic framework involving anticompetitive and competitive glucose inhibitions on E_1 and E_2, respectively. The different reactivity of the substrates in signed mainly by the K_m values and, to a lesser extent, by the anticompetitive inhibition constante for E_1.

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