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Characteristics of Cotton Cellulose Depolymerized by a Brown-Rot Fungus, by Acid, or by Chemical Oxidants[1)]

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Summary

Wood decay fungi of the brown-rot type destroy the strength of wood before significant weight loss occurs. This is due to extension depolymerization of the cellulose. Evidence indicates that enzymes cannot gain access to the cellulose in wood and that the depolymerizing agent might be oxidative. Our objectives here were to gain information about the nature of the agent by characterizing pure cellulose that had been depolymerized by a brown-rot fungus (BR) and to compare its characteristics with those of cellulose depolymerizcd by acid (A). by Fenton's reagent (Fe^{2+} + H_2O_2) (F), or by periodic acid/bromine/water (P). All four types of depolymerized cellulose exhibited molecular size characteristics indicating that depolymerization was due to cleavages within the noncrystalline regions. Carbonyl contents of the samples were similar, from 1.4 to 1.9 per cellulose molecule. Carboxyl contents were 0.44, 0.00, 0.24, and 7.38 per molecule for samples BR, A, F, and P, respectively; uronic acids were absent except for a trace in sample P. On complete acid hydrolysis the samples gave the expected amounts of glucose, except for sample P, which contained nonglucosyl moietics that did not contain carbonyl or carboxyl groups detected by our analytical procedures. With the glucose in the acid hydrolysates we found glyceric, crythronic, ararbonic, and gluconic acids in samples BR and F, and crythronic acid in sample P. Our results indicate that the brown-rotted cellulose resembles sample F more than samples A or P.

Introduction

The most destructive form of wood decay, brown rot, is caused by a relatively few species of higher fungi. Hyphae of these ubiquitous basidiomycetes invade wood cells and secrete agents that bring about a rapid depolymerization of the cellulose, often with very little reduction in total wood mass. The average number of glucosyl residues per cellulose molecule (degree of polymerization, DP) is reduced from about 10^4 (Goring and Timell 1962) to about 250 (Cowling 1961). The fragments apparently correspond to the size of the cellulose "crystallites." Similar depolymerization of cellulose to the "limit DP" (to the crystallites) is effected by acid hydrolysis (Battista 1950) and is brought about by cleavages in the amorphous regions. As a result of the initial attack by brown-rot fungi and the depolymerization of the cellulose, wood strength rapidly declines. How this rapid depolymerization occurs is a perplexing biochemical question. As recognized 20 years ago by Cowling and Brown (1969), only a very small percentage of the cell wall volume is accessible to even the smallest cellulases. Importantly, too, cellulases do not mimic the action of brown-rot fungi in generating cellulose crystallites (Chang *et al.* 1981).

In discussing this problem, Cowling and Brown (1969) noted that Halliwell (1965) had described the depolymerization of cellulose by Fenton's reagent (Fe^{2+} + H_2O_2), which generates hydroxyl radical or similarly powerful oxidant (Dunford 1987; Halliwell and Gutteridge 1988). Subsequently, Koenigs (1974) demonstrated that cellulose in wood can be depolymerized by Fenton's reagent, showed that brown-rot fungi produce extracellular H_2O_2, and demonstrated that wood contains enough iron to make the oxidant hypothesis reasonable. Support for an oxidative system was later provided by Highley (1977), who obtained evidence that brown-rotted cellulose contains carbonyl and carboxy1 groups. Later, Schmidt *et al.* (1981) demonstrated that oxalic acid, which is secreted by brown-rot fungi in liquid cultures (Takao 1965), can reduce Fe^{3+} to Fe^{2+} under certain conditions, and proposed that oxalic acid generates the Fe^{2+} for the Fenton system. Attemps to isolate a cell-free depolymerizing system have not been successful.

The problem has received relatively little research attention since these cited studies, and the actual

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biochemical system is not yet known. Although the fungi are good saprophytes, and are readily grown in simple chemically defined liquid media (Jennison 1952). the cellulose-depolymerizing system in such cultures has not been manifested. However, a solid substrate system for brown-rot fungal depolymerization of isolated cellulose has been described (Highley 1977).

The purpose of the present research was to gain insight into the biochemical mechanism of depolymerization by characterizing cellulose that had been degraded by a brown-rot fungus in the solid substrate system. We compared the fungus-depolymerized cellulose with acid-depolymerized cellulose, with cellulose oxidized and depolymerized by the Fenton reagent, and with cellulose oxidized and depolymerized by periodic acid and bromine water.

Materials and Methods

Cellulose

Cotton cellulose was purified Type A-600 from Holden Vale Manufacturing Co., Ltd. (Lancashire England).[3)]

Fungus-depolymerized cellulose

Cellulose was depolymerized by *Postia placento* (Fr.) M. Lars. et Lomb. (MAD-698) [syn: *Poria placento* (Fr.) (Cke.)] in chambers (1-liter jars) by procedures patterned after the standard ASTM soil-block procedure (ASTM, 1971). The cellulose was placed in chambers on previously inoculated and overgrown pine feeder strips (0.3 × 2.9 × 3.5 cm, long axis in the grain direction). After incubation for 6 weeks, the mycelium was manually separated from the residual cellulose, which was then shaken at 50°C in 0.1 N NaOH for 3 h in air, washed with 0.1 N HCl and then with water until neutral, and dried under vacuum at 60°C. These washings were to remove any fungal metabolites that might be present. Recovery was 70% of the original cellulose.

Acid-depoiymerized cellulose

Cellulose was boiled for 2.0 min in 20.2% HCl (azeotrope), washed with water until neutral, then washed with acetone, and dried as above (Wood *et al*.. 1986). Recovery was 85%.

Fenton-depoiymerized cellulose

Cellulose was shaken at 120 rpm in a solution of ferrous sulfate (0.44 mM; J.T. Baker Chemical Co., Phillipsburg. N.J.) and hydrogen peroxide (1%; Baker) in 0.1 M acetate buffer, pH 4.2, at 40°C (Koenigs 1972). After three days, the cellulose was recovered by filtration on a glass fiber filter, washed with NaOH, etc., as the brown-rotted sample. The pH did not change during the 3-day incubation. Recovery was 94%.

HIO_4/Br_2-depolymerized cellulose

Cellulose was oxidized for 1 h in 0.267 M HIO_4 at 24°C, filtered through a medium porosity sintered glass funnel, washed exhaustively with water, and dried (Jackson and Hudson 1937). To a stirred slurry of this cellulose (1.0 g) in water at 0°C containing 329 mg of calcium carbonate, 452 mg of bromine was added, and the mixture was shaken and stored at room temperature. After 2 days, nitrogen was bubbled through the slurry until it became colorless. The cellulose was recovered on a medium-porosity sintered glass funnel, washed with 0.05 M HCl and then exhaustively with water, and dried as above. Recovery was 94%.

[3)] The use of trade or firm names in this publication is for reader information and does not imply endorsement by the U.S. Department of Agriculture of any product or service.

Determination of molecular weight distribution, DP_n, DP_w, and polydispersity

Molecular weight distributions. number- and weight-average degrees of polymerization (DP_n and DP_w) and polydispersities were obtained by gel permeation chromatograph! of the tricarbanilate derivatives (Wood *et al*. 1986).

Determination of carboxryl and carbonyl contents

The carboxyl content was determined by titration (TAPPI 1988). Total uranic acid was determined after acid hydrolysis using the procedures of Scott (1979). Carbonyl content was determined by reducing the cellulose overnight with NaB_3H_4 in 0.1 mM NaOH (pH 10), then washing and drying the cellulose, and determining the ^3H content of a weighed subsample. The specific activity of the NaB^3H_4 was 116.8 mCi/mmol, determined from that of veratryl alcohol after reduction of veratraldehyde.

Glucose yield after acid hydrolysis

Glucose was determined by high pressure liquid chromatography after complete acid hydrolysis and neutralization with $Ba(OH)_2$ (Pettersen *et al.* 1984).

Identification of sugar acids after acid hydrolysates

Cellulose was completely acid-hydrolyzed and the hydrolysate neutralized as for glucose determination (Effland 1977). Solvents were evaporated and the residue dissolved in 0.1 M phosphate buffer, pH 8. This solution was applied to an ion exchange column (Dowex l-x8, 200-400 mesh; Bio·Rad Laboratories, Richmond, CA), which was then washed with warm water to remove the glucose. Acids were then eluted with 11.5 M formic acid. After evaporation of solvent, the samples were derivatized with pyridine: bis(trimethylsilyl)trifluoroacetanlide. (1:l. with 1% trimethylchlorosilane) (both from Aldrich Chemical Co., Milwaukee, WI), and analyzed by gas chromatography (GC) and GC/mass spectrometry (GC/MS) (Hyppänen *et al.* 1983).

The following standards were used: glyceric acid (Aldrich Chemical Co.), crythronic acid (from Dr. El Kadem, American University, Washington, DC), and arabonic acid (from Dr. O. Ramnäs, Chemical and Environmental Science, Chalmers University of Technology, Göteborg, Sweden), gluconic acid (Sigma Chemical Co., St. Louis. MO), xylitol (internal standard for gas chromatography) (Sigma Chemical Co.).

Results

The carbanilated cellulose samples all exhibited single peaks in their molecular size distribution curves. with small shoulders on the low molecular weight side (Fig. 1). These curves together with the calculated number- and weight-average molecular weights of the samples, and their polydispersities (Table 1), show that the samples differed in extent of depolymerization and in the size distribution of the fragments produced. The brown-rotted sample more closely resembled the sample depolymerized by acid than those depolymerized by the chemical oxidants. The sample oxidized with Fenton's reagent had been depolymerized to a somewhat lesser extent than the other samples, causing its DP_w, and consequently its polydispersity, to be higher. To study further the effect of the Fenton system on polymer properties, we oxidized another sample of cellulose more extensively by increasing the peroxide concentration to 2%, with other conditions as given

in Materials and Methods. This produced a depolymerized cellulose with DP_n = 59, DP_w = 271, and a polydispersity of 4.6.

The carbonyl contents of all the samples were between one and two per cellulose chain (Table 2). indicating that each contained internal carbonyl groups. In the cast of the acid-depolymerized sample, some dehydration with carbonyl group formation would be expected during the initial hydrolysis with hydrochloric acid. The sample oxidized with periodic acid/bromine water apparently contained some carbonyl groups generated by the periodic acid that had not been further oxidized by the bromine. The Fenton system and the fungus also oxidized small numbers of hydroxyl group to kotone groups.

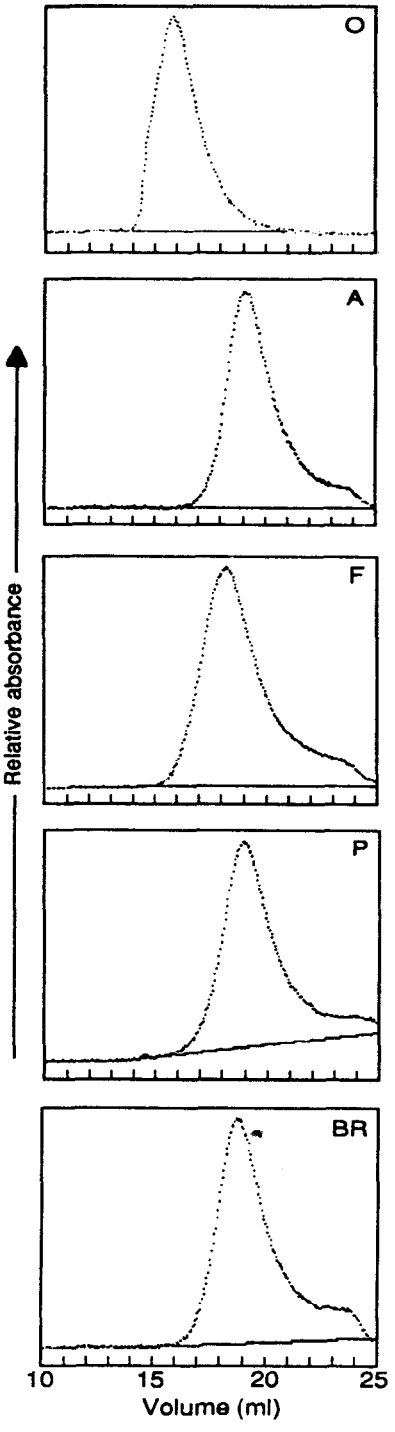

Fig. 1. Size exclusion chromatograms of carbanilated cellulose samples.
O: original: A: acid-depolymerized; F: Fenton-depolymerized; P: periodic acid/bromine water-depolymerized; BR: brown-rot fungus-depolymerized.

Table 1. Polymer properties of cellulose samples

Cellulose sample	DP_n	DP_w	Polydispersity
Original	1150	2111	1.8
Fungus-depolymerized	69	238	3.4
Acid-depolymerized	66	200	3.0
Fenton-depolymerized	74	371	5.0
HIO_4/Br_2-depolymerized	53	219	4.1

All samples except that hydrolyzed by acid contained carboxyl groups, from less than 0.5 per cellulose chain in the brown-rotted and Fenton samples to more than seven per chain in the periodic acid/bromine water sample (Table 2). This last sample, but not the others, contained a minor quantity of uranic acid residues; thus the majority of the carboxyl groups in the samples were at positions other than C_6. Using the Fenton-depolymerized sample, we found that the NaOH wash during sample preparation (see Materials and Methods) did not affect the carboxyl content, i.e., they were not labile to NaOH under the conditions used. Whether the NaOH wash affected the carboxyl content of the brown-rotted sample is not known. We identified small amounts of glyceric, erythronic, arabonic, and gluconic acids in the hydrolysates of the fungus- and Fenton-depolymerized samples. The periodic acid/bromine water-oxidized sample yielded erythronic acid; the other acids were not detected. Our quantification of the sugar acids recovered from the hydrolysates of the samples gave widely variable and nonreproducible values, probably because of the low yields of the individual sugar acids relative to the overwhelming excess of glucose and resulting losses of the acids during the work-up, which involved large volumes and large amounts of barium sulfate (Effland 1977). Similar difficulties have been reported by others (Nevell 1985). With the exception of the gluconic acid residues, which obviously were on the chain ends, we cannot conclude whether the other acid residues were internal or terminal.

The original cotton yielded 110.1% glucose on complete acid hydrolysis (Table 2), which is near the theoretical yield of 111%. The samples depolymerized by acid, by brown-rot, and by Fenton's reagent yielded only slightly less glucose than the original, indicating

Table 2. Analytical characteristics of cellulose samples

Sample	Glucose yield[1]	Carboxyl[2]	Uronic acid	Carbonyl
	(%)	(no. per cellulose chain)[3]		
Original	110.1	0.00	0.0	ND[4]
Fungus-depolymerized	108.1	0.44, 0.43	0.0	1.1
Acid-depolymerized	108.5	11.00	0.0	1.5
Fenton-depolymerized	107.6	0.23, .0.25	0.0	1.4
HIO_4/Br_2-depolymerized	86.9	7.27, 7.47	0.3	1.9

[1] Yield of glucose after complete acid hydrolysis based on cellulose. Theoretical yield from pure cellulose is 111%.
[2] Duplicate analyses were done for carboxyl-containing samples.
[3] Based on DP_n
[4] Not determined

that they contained approximately 1%, 1%, and 2% non-glucosyl residues, respectively. These values are in accord with the small numbers of keto-sugar and carboxyl-containing residues present in the samples. In contrast, the sample oxidized by periodic acid/ bromine water yielded only 86.9% glucose, and thus contained approximately 21% non-glucosyl components (100-[86.9/110.l·100]). This high content of non-glucosyl residues cannot be entirely accounted for by the carboxyl and carbonyl contents of the sample. Ester or lactone groups in the cellulose probably would not analyze as carbonyl or as acid with our assays. and can possibly account for the remaining non-glucosyl moieties.

Discussion

The only previous study of brown-rotted cellulose that we are aware of is one from this Laboratory (Highley 1977). In that study, cellulose depolymerized by *Postia placenta* was found to have increased reducing power: the copper number of 7.5 indicated approximately three carbonyl groups per cellulose chain. The copper number of the control, non-depolymerized cellulose was 0. The brown-rotted sample contained approximately 0.5 carboxyl groups per chain (control was 0). It was 31% soluble in 1% aqueous NaOH (control was 0), indicating the presence of considerable low-DP cellulose or fungal metabolites. Cellulose treated with iron/peroxide was found to give analytical values similar to those of the brown-rotted cellulose. The present study refines and extends the earlier work. We included two oxidized celluloses for comparison with the brown-rotted cellulose and examined several properties not examined earlier. Importantly, we purified the brown-rotted sample by extracting with NaOH solution to remove oxalic acid and other acids (or other base-soluble molecules) that might have been deposited as fungal metabolites (Takao 1965). The Fenton-oxidized sample was treated in the same way. The periodic acid/bromine water sample is known to be labile to base (Nevell 1985) and thus was not extracted with NaOH. To the extent that similar analyses were done, our results are in accord with the previous results, although the carbonyl content found earlier was about twice as high as found in our study.

Our data indicate that all four treatments depolymerized the cellulose by extensive cleavages in the noncrystalline regions. This conclusion is based on the long-recognized behavior of cellulose on acid hydrolysis (Battista 1950) and the fact that the other three samples exhibited polymer size properties broadly similar to those of the acid-depolymerized sample. As noted, the Fenton-treated sample had been less extensively depolymerized than the others, but this should not affect the basic conclusion concerning attack in noncrystalline regions. Notably, the properties of the brown-rot-fungus-depolymerized sample show that the attack by the agent that caused depolymerization differs markedly from that by isolated fungal cellulases (Chang *et al.* 1981) and from that by white-rot fungi, which are cellulolytic wood-decaying basidiomycetes that employ a classical cellulase system (Cowling 1961; Highley 1988).

With the exception of the polymer properties, the brown-rotted sample resembled the Fenton-oxidized sample more than it did the other two samples. The polymer properties indicated that the Fenton sample had been less extensively depolymerized than the others. Thus the Fenton system does not exactly duplicate the brown-rot system. One possible explanation for the difference between the Fenton- and fungus-depolymerized samples is that the fungus further degraded and consumed the smallest fragments. thus narrowing the polydispersity. Another explanation is that the two systems were quite different. [We considered the possibility that the brown-rot depolymerizing agent might be a Lewis acid catalyst rather than an oxidative agent. and that the depolymerized cellulosc was oxidized in reactions having nothing to do with depolymerization. However, we found that neither 0.4 mM nor 4 mM Fe^{2+} or Fe^{3+} in buffer at pH 4.2, in the absence of peroxide, significantly de-

polymerized the cotton cellulose.] Thus overall our results seem to be consistent with the brown-rot agent being oxidative and similar to the Fenton system.

We were unable to find a literature description of the chemistry of polysaccaride depolymerization by Fenton's reagent. Our results suggest that in both the Fenton and fungus depolymerizations, the cleavage reaction did not result directly in formation of carboxyl groups, because there was not one per chain. Those carboxyl values, however, do not rule out the possibility that the cleavage reaction formed some labile carboxyl groups that were lost subsequently; or that only some of the cleavage reactions resulted in carboxyl formation; or, in the case of the fungus-depolymerized sample, that some carboxyl-containing end residues of chain cleavage were subsequently cleaved off enzymatically.

The relatively small number of carbonyl groups in the Fenton-oxidized sample was surprising in view of past studies on the oxidation of soluble carbohydrates with Fenton reagent, which indicate that formation of carbonyl groups is a prominent reaction (Ahrgren and deBelder 1975; deBelder *et al.* 1963; Moody 1964; Uchida and Kawakishi 1988). The difference between our findings and past work could be due to the insolubility of the cellulose and inaccessibility to the oxidant except in the noncrystalline regions, or perhaps to differences in the concentrations or proportions of reactants used. Our results indicate in any event that the noncrystalline regions of the cellulose were much more reactive (accessible) to the Fenton system than were the crystalline regions.

Speculation that Fenton chemistry is involved in cellulose depolymerization by brown-rot fungi has biological precedent (Dunford 1987; Halliwell and Gutteridge 1988). A well-known case is the cleavage of DNA by bleomycin. Bleomycin is an organic iron complex of approximate MW 1300 that has some specificity for DNA and employs Fenton chemistry in DNA cleavage (Dunford 1987). We consider it likely that if the brown-rot agent is also of the Fenton type, then it too would be a specific organic iron complex. Fekete *et al.* (1989) demonstrated recently that iron-complexing agents - apparently siderophores - are produced by brown-rot fungi. including the species used here, but nothing is known yet about their chemistry.

Three other recent reports bear on a possible role for Fenton chemistry in cellulose degradation by fungi. Bratt *et al.* (1988) presented evidence that flax fiber degradation by *Botrytis cinerea,* which produces H_2O_2 but no cellulolytic enzymes, is caused by a Fenton-type system. Veness and Evans (1989) showed that sugar acids were formed from cellulose by a crude *Trichoderma reesei* cellulase system and that the same acids were also formed by Fenton's reagent. They proposed that H_2O_2-derived oxidizing radicals are formed as side reactions during cellulase action. In the most relevant study, Enoki *et al.* (1989) demonstrated one-electron oxidizing activity related to the degradation of cellulose by brown-rot fungi. The oxidizing activity in cultures of brown-rot fungi was detected by the production of ethylene from 2-ketothiobutyric acid.

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