Changes in Molecular Size Distribution of Cellulose during Attack by White Rot and Brown Rot Fungi

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The kinetics of cotton cellulose depolymerization by the brown rot fungus Postia placenta and the white rot fungus Phanerochaete chrysosporium were investigated with solid-state cultures. The degree of polymerization (DP; the average number of glucosyl residues per cellulose molecule) of cellulose removed from soil-block cultures during degradation by P. placenta was first determined viscosimetrically. Changes in molecular size distribution of cellulose attacked by either fungus were then determined by size exclusion chromatography as the tricarbanilate derivative. The first study with P. placenta revealed two phases of depolymerization: a rapid decrease to a DP of approximately 800 and then a slower decrease to a DP of approximately 250. Almost all depolymerization occurred before weight loss. Determination of the molecular size distribution of cellulose during attack by the brown rot fungus revealed single major peaks centered over progressively lower DPs. Cellulose attacked by P. chrysosporium was continuously consumed and showed a different pattern of change in molecular size distribution than cellulose attacked by P. placenta. At first, a broad peak which shifted at a slightly lower average DP appeared, but as attack progressed the peak narrowed and the average DP increased slowly. From these results, it is apparent that the mechanism of cellulose degradation differs fundamentally between brown and white rot fungi, as represented by the species studied here. We conclude that the brown rot fungus cleaved completely through the amorphous regions of the cellulose microfibrils, whereas the white rot fungus attacked the surfaces of the microfibrils, resulting in a progressive erosion.

Brown rot fungi comprise a limited number of wood-decaying basidiomycetes that damage wood by rapidly depolymerizing the cellulose component. Generally, depolymerization occurs before significant loss in the weight of wood. At advanced stages of decay, structural polysaccharides are quantitatively removed, and a modified lignin residue remains (4, 14).

The biochemistry of the cellulytic system of brown rot fungi has received little attention, despite the apparent uniqueness of the mechanism involved and the economic importance of brown rot. Because pores in sound wood are too small to allow cellulytic enzymes to penetrate, it seems probable that the agent responsible for initial cellulose depolymerization is not a classical cellulase (5). Flournoy et al. (8) recently studied changes in the pore size of wood during decay by the brown rot fungus Postia placenta. They concluded that the agent responsible for cellulose depolymerization has a molecular radius of less than 38 Å (3.8 nm) and probably not greater than 15 Å (1.5 nm).

In discussing the nature of the cellulose-depolymerizing agent, Cowling and Brown (5) noted that G. Halliwell (10) had described the depolymerization of cellulose by Fenton's reagent (Fe³⁺ + H₂O₂, which generates a hydroxyl radical or similar oxidant [9]). Subsequently, Koenigs (16, 17) demonstrated that cellulose in wood is depolymerized by Fenton's reagent, that brown rot fungi produce extracellular H₂O₂, and that wood contains enough Fe (Fe³⁺) to make the hypothesis reasonable. Support for the hypothesis of an oxidative system was later provided by Highley (11) and Kirk et al. (15), who obtained evidence that cellulose subjected to brown rot fungi is oxidized. Schmidt et al. (19) demonstrated that oxalic acid, which is secreted by brown rot fungi in liquid cultures (21), reduces Fe³⁺ to Fe²⁺ under certain conditions.

Recently, Shimada et al. (20) proposed an alternate role for oxalic acid in cellulose depolymerization by brown rot fungi. They reported that oxalic acid (1% [pH 1.3], 35°C, 4 weeks) alone reduces the viscosity of wood pulp to 60% of the original and therefore may be directly involved in the cellulose-depolymerizing process.

Enoki et al. (6) suggested that iron-containing glycopeptides which are able to oxidize 2-keto-4-thiomethylbutyric acid to ethylene in the presence of H₂O₂ are somehow involved in wood degradation by brown rot fungi. However, it has yet to be established whether these glycoproteins are involved in cellulose depolymerization.

Although brown rot fungi grow well in chemically defined media, liquid culture conditions in which the depolymerizing system is demonstrable have not been found. However, Highley has shown that the system is demonstrable in solid-state (soil-block) cultures (11) and in cultures over an agar medium (12).

Research on the cellulytic system of brown rot fungi lags behind research on that of the wood-degrading white rot fungi. The cellulytic system of the latter group is a classical one comprised of endo- and exoglucanases and β-glucosidases. Several cellulse components of white rot fungi have been isolated and characterized, and the respective genes have been cloned and sequenced previously (7). Most brown rot fungi possess endoglucanase and β-glucosidase activities but not exoglucanase activity (7, 12, 18).

The purpose of our study was to compare the kinetics of cellulose depolymerization by brown and white rot fungi in the solid-state system. Our ultimate goal is to understand the...
chemistry and biochemistry involved in cellulose depolymerization by brown rot fungi.

MATERIALS AND METHODS

Fungal strains, maintenance, and inoculum preparation. P. placenta (Fr.) M. Lars. et Lomb. (MAD-698; ATCC no. 11538) was maintained at 27°C on malt agar slants. Bits of mycelium-covered agar from 3- to 4-week-old slants were used to seed petri plates of the same medium. Phanerochaete chrysosporium (BKM-F-1767; ATCC no. 11538) was maintained at 30°C on yeast-malt-peptone-glucose agar. Mycelium-covered agar from 7- to 10-day-old slants was used to seed petri plates of malt agar. Ten-millimeter-square sections from the petri dish cultures (<3 weeks old) were used to start the experimental soil-block cultures with both fungi.

Cellulose. Cotton cellulose was purified Type A-600 from Holden Vale Manufacturing Co., Ltd. (Lancaster, England). After incubation with the fungus, cellulose samples were ground in a Wiley mill to pass a 30-mesh screen.

Cellulose depolymerization studies. Cellulose was exposed to the fungi in soil-block bottles (1) that were incubated at 27°C with 70% relative humidity. Weighed balls of cellulose (approximately 100 mg [dry weight]) were placed directly on mycelium-covered wood feeder strips, which in turn rested on sterile, moist soil in French square bottles. The cotton balls were rapidly invaded by the hyphae. Triplicate balls were removed after various incubation times, and the surface mycelia were removed, and the residual cellulose was dried at 60°C for 48 h before weighing.

Cellose analyses. Solubility in 1% sodium hydroxide was determined as described in Technical Association of the Pulp and Paper Industry Standard T212 om-83 (22). In the initial experiment, the average degree of polymerization (DP; the average number of glucosyl residues per cellulose molecule) was measured viscosimetrically (2) after cellulose solubilization in cupriethylenediamine (GFS Chemicals, Columbus, Ohio). In subsequent experiments, molecular size distributions of the samples were determined by size exclusion chromatography (SEC) of the tricarbanilate derivatives dissolved in tetrahydrofuran (24). Increased amounts of derivatizing reagent [5.0 ml of pyridine plus 1.5 ml of phenylisocyanate] per 5.0 mg of cotton] were used to derivatize cellulose exposed to P. chrysosporium because of the excess amount of fungal material present in the samples. The number-, weight-, and viscosity-average degrees of polymerization (DPn, DPw, and DPv, respectively) were calculated from the SEC data. (The SEC analysis of the cellulose sample most excessively attacked by the white rot fungus was complicated because the sample did not completely dissolve during the workup. This was most likely because of the presence of fungal mycelia, which were likely dispersed with the cellulose and could not be removed completely. The presence of an insoluble residue was also observed when the mycelia alone were analyzed as a control.)

RESULTS

Initial experiments determined the depolymerization of cellulose by the brown rot fungus P. placenta in the soil-block cultures as a function of time. The DP was measured by the viscosimetric procedure. Residual cellulose was further characterized by its solubility in alkali. In subsequent experiments with both P. placenta and P. chrysosporium, changes in the molecular size distribution of cellulose were determined.

Kinetics of cellulose depolymerization. Depolymerization of cellulose by P. placenta (Fig. 1) occurred in two phases as seen by the viscosimetric assay: a phase in which a rapid decrease in the DP from 2,200 to approximately 800 (0 to 5 days) occurred and a slower phase lasting approximately 15 days during which the DP decreased to about 250. Further decrease in the DP was negligible. The solubility of the residual cellulose in 1% sodium hydroxide was not biphasic; rather, it increased in a roughly linear manner throughout the incubation period and reached approximately 33% by the termination of the experiment. Almost all depolymerization occurred before any weight loss occurred. The first loss in weight was observed at day 16, and only 20% of the cellulose was consumed after 42 days.

In a similar experiment with P. chrysosporium, the DP of cellulose determined by SEC remained relatively high (DPw in Table 1). Also, in contrast to results with the brown rot fungus, a continuous weight loss of the cellulose was observed; a maximum of 50% loss was seen after 24 days (Table 1). Solubility in 1% NaOH was not determined but

![FIG. 1. Time course study of cellulose depolymerization by P. placenta in soil-block cultures. Three samples were removed every 4 days, and the DP (○) was determined viscosimetrically. The solubility of the remaining cellulose in 1% NaOH (○) was also evaluated. The arrow indicates the onset of weight loss in the cellulose, and the vertical lines are the standard deviations.](http://aem.asm.org/)

<p>| TABLE 1. Changes in molecular size distribution of cotton cellulose degraded by brown and white rot fungi |
|-------------------|------------------|-------------------|-------------------|-------------------|-------------------|</p>
<table>
<thead>
<tr>
<th>Incubation time (days)</th>
<th>Weight loss (%)</th>
<th>DPn</th>
<th>DPw</th>
<th>Dpv</th>
<th>Polydispersity (DPw/DPn)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Brown rot fungus</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>0</td>
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<td>1,150</td>
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<tr>
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<td>754</td>
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<td>20</td>
<td>65</td>
<td>293</td>
<td>273</td>
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<tr>
<td><strong>White rot fungus</strong></td>
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<td></td>
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<td></td>
</tr>
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<td>50</td>
<td>984</td>
<td>1,570</td>
<td>1,511</td>
<td>1.60</td>
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</tbody>
</table>
had been shown earlier by Highley (12) to increase very slightly during decay.

**Molecular size distribution of cellulose decayed by *P. placentae***. Cellulose depolymerization by the brown rot fungus was investigated in detail by examining the change in molecular size distributions during the course of degradation (Fig. 2). Single major peaks were resolved for each sample, irrespective of the length of time of incubation. With time, the peaks became broader and were centered at progressively lower DP values. In addition, by day 14 a shoulder representing low-molecular-size components (DP < 60) appeared; its contribution increased with time, although it was never a major component. A gradual increase in the polydispersity of the samples (DPw/DPn, which is an indication of the range of molecular sizes) was observed and is explained by the increase in the proportion of lower-molecular-size components (Table 1). Both DPw (DP) and DPn decreased progressively with time (Table 1; Fig. 2).

**Molecular size distribution of cellulose decayed by *P. chrysosporium***. The changes in the patterns of molecular size distribution during attack by *P. chrysosporium* (Fig. 3) differed greatly from those observed for *P. placentae*. The molecular-size-distribution curve broadened initially but narrowed as the cellulose was attacked. Single major peaks were resolved for each sample and were centered at a slightly lower average DP than that of starting material. Unlike the observations with the brown rot fungus, both DPw (DP) and DPn decreased by day 8 but then gradually increased to near control values thereafter (Table 1; Fig. 2). The polydispersity of the residual cellulose changed in a similar manner.

**DISCUSSION**

Our study of cellulose depolymerization by *P. placentae* shows that the DP decreased substantially before any weight loss (utilization) occurred. Cowling (4) also observed this phenomenon in experiments with wood blocks from which holocellulose was isolated during decay by this fungus (which was classified at the time as *Poria monticola* Murr.).

In addition, Highley (12) reported that a number of brown rot fungi depolymerized cotton before any weight loss occurred. Depolymerization before utilization suggests that the system responsible for utilization (i) acts only on low-molecular-size fragments produced late during depolymerization, (ii) is inhibited during initial depolymerization, (iii) is induced by late-depolymerization products, or (iv) is simply inefficient. The fact that the solubility of the cellulose in 1% NaOH increased in an approximately linear manner during attack by the brown rot fungus indicates that small (soluble) fragments were produced and accumulated during depolymerization; thus, possibilities (ii) and (iv) are more likely than (i) or (iii). Cowling (4) showed that 1% NaOH solubility of holocellulose isolated from wood attacked by brown rot fungi also increased during degradation. Likewise, Highley observed an increase in alkali solubility of cotton cellulose attacked by several brown rot fungi (12). However, our results show that alkali solubility of cellulose decayed by *P. placentae* does not directly correlate with depolymerization and consequently cannot be used as a simple assay for depolymerization.

The kinetic curve of cellulose depolymerization by the brown rot fungus revealed two phases of depolymerization, resulting in an eventual drop in the DP to approximately 250. This may be interpreted as a biphasic mode of depolymerization, but a linear relationship is observed when the log (DP – 250) is plotted against time, suggesting that depolymerization is simply first order.

Cessation at a DP of 250 suggests that extensive cleavages occurred within the noncrystalline (amorphous) regions, producing crystallites. This is analogous to the “leveling off” DP of cellulose observed upon acid hydrolysis in which crystallites are released (3). Highley et al. (13) showed that the overall crystallinity of residual cellulose increases during depolymerization by brown rot fungi. Apparently, the depolymerizing agent is able to cleave through the amorphous regions of the cellulose but not through the cellulose crystallites (4, 15). (Although the DP value of approximately 250 was near the detection limit of our viscosimetric assay [DP =
merization study with several white rot fungi in solid-state cultures. Changes in the molecular size distribution of cellulose in our study during decay by P. chrysosporium suggest that initial attack involved the generation of fragments several hundred glucose units long. With time, these were consumed without accumulation of a substantial proportion of fragments of lower DP values. Removal of low-molecular-weight material resulted in low polydispersity values as decay occurred. This suggests that the white rot fungus preferentially consumed the small molecules before generating more.

From these results, it is apparent that the mechanism of cellulose degradation differs markedly between P. placenta and P. chrysosporium. The brown rot fungus cleaves entirely through the cellulose microfibrils, presumably in the amorphous regions, before utilizing the cellulose, whereas the white rot fungus attacks the surfaces of the microfibrils, consuming the cellulose as it is degraded. These two modes of degradation are illustrated in Fig. 4.

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REFERENCES


FIG. 4. Illustration of the apparently different modes of degradation of cellulose by P. placenta and P. chrysosporium. Wt., weight.


