LIGNINOLYTIC ACTIVITIES OF THE BIOPULPING FUNGUS *CERIPORIOPSIS SUBVERMISPORA* IN SOLID STATE CULTURES

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ABSTRACT

Lignin peroxidases are generally considered the primary catalysts for the fungal cleavage of nonphenolic lignin structures. However, some white-rot fungi, such as *Ceriporiopsis subvermispora*, efficiently degrade nonphenolic lignin without expressing any detectable lignin peroxidsse activity. Since *C. subvermispora* is a likely candidate for the biological pretreatment of wood chips (biopulping) on an industrial scale, a deeper knowledge of its ligninolytic system is desirable. To study the mechanism of lignin depolymerization by *C. subvermispora*, cellulose block cultures of the fungus were given a polymeric lignin model compound in which the β-O-4-1inked substructure of lignin is covalently attached to polyethylene glycol. The C_α sidechain carbon of the model was labeled with ^{14}C to enable the detection of structural changes following fungal attack. Chromatographic and mass spectrometric methods were used to identify the products. The new models proved to be valuable tools to elucidate the major ligninolytic reactions of *C. subvermispora*. C_α-C_β and C_β-O- aryl cleavage were shown to be major pathways of lignin model cleavage in this fungus despite its evident lack of lignin peroxidase.

INTRODUCTION

Lignin is an aromatic biopolymer that functions as a barrier to microbial attack on cellulose and hemicellulose in woody tissues. White rot fungi possess a highly efficient mechanism to degrade lignin, and therefore play an essential role in the carbon cycle on earth. The lignin-degrading systems of these fungi also have great potential for application in pulp and paper manufacture. Treatment of wood chips with lignin-degrading fungi, a process called "biopulping," results in at least 30% energy savings during refining in mechanical pulping (1, 2). Among many species tested, *Ceriporiopsis subvermispora* has proven to be the most efficient biopulping fungus (3, 4).

Although the technology has already approached industrial scale (5), very little is known about the lignin-degrading mechanism of *C. subvermispora*. Previous work indicates that this fungus does not produce any lignin peroxidsse (LiP) (6), an enzyme generally considered the primary catalyst for the depolymerization of lignin during white rot (7). Lip. degrades lignin by catalyzing the oxidation of nonphenolic structures to aryl cation radicals, which results in subsequent C_α -C_β and C_β-O-aryl cleavage of the aromatic interunit linkages (8).

However, it has been shown that *C. subvermispora* does degrade nonphenolic lignin structures efficiently (9). A preliminary investigation has provided some evidence for C_α-C_β cleavage, and thus for an aryl cation radical mechanism (10), in this fungus, but most of the cleavage products were not identified. Moreover, the data do not rule out the possibility that the formation of aryl cation radicals is quantitatively insignificant in *C. subvermispora* when it degrades lignin under natural conditions in wood or during the biopulping process.

To addsess this question, we have cultivated *C. subvermispora* under conditions that mimic those in wood, and which lend themselves to subsequent analytical procedures. Using a ^{14}C-labeled polymeric lignin model compound, C_α -C_β and C_β-O-aryl cleavage were shown in fact to be major pathways of nonphenolic lignin model cleavage in *C. subvermispora*, despite its evident lack of LiP.

MATERIALS AND METHODS

Organisms and Reagents

C. subvermispora (FP-90031) and *P. chrysosporium* (ATCC 24725) were obtained from the Center for Forest Mycology, USDA Forest Products Laboratory.

The polymeric lignin model **I,** ^{14}C-labeled at C_α (9.5×10^{-6} mCi mg^{-1}, 0.11 mCi $mmol^{-1}$ of attached dimer), was synthesized as described previously (11).

Substrate Preparation and Growth Conditions

Solid state cultures were set up as shown in Fig. 1. The cellulose blocks, prepared from sheets of a highly bleached wood pulp (Lenzing AG, Austria), were autoclaved and then dried under vacuum in a dessicator jar. Four replicate blocks were infiltrated with 1.3 ml of a solution of 16 mg (3.3×10^5 dpm) of model **I** in methanol. After evaporation of the methanol under vacuum, the cellulose blocks were placed in 125-ml flasks and inoculated with 1.6 ml of a blended suspension of *C. subvermispora* mycelium or a conidiospore suspension of *P. chrysosporium* in basal medium that contained 0.1% glucose. The pH of the medium was adjusted to 5.0 for *C. subvermispora* and 4.5 for *P. chrysosporium*. The flasks were fitted with gassing manifolds and incubated at 29°C (*C. subvermispora*) or 39°C (*P. chrysosporium*). The $^{14}CO_2$ formed from model **I** mineralization was tripped into a scintillation cocktail by periodically flushing the headspaces of the flasks with air (12, 13).

Culture Workup and Product Analysis

The cultures were harvested after 6-7 days and extracted with methanol. The concentrated extract was fraactionated by gel permeation chromatography (GPC) on a column (1.9 x 33 cm) of Sephadex LH-20 in *N,N* -dimethylformamide. A portion (0.2 ml) of each collected fraction (1.5 ml) was assayed for ^{14}C by scintillation counting. Fractions 42 to 48 were pooled evaporated to dryness and taken up in 0.5 ml methanol. A portion (20 µl) was then further analyzed by reversed-phase HPLC. Fractions (1.0 ml) were collected and assayed for ^{14}C by scintillation counting.

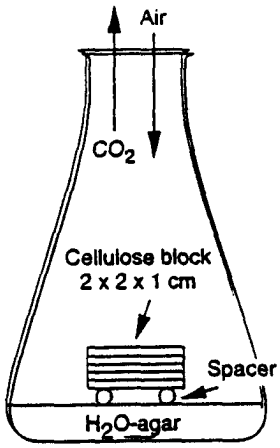

Fig. 1. Setup for the degradation of α-^{14}C-labeled model **I** by *P. chrysosporium* and *C. subvermispora* in solid state cultures.

HPLC peak fractions that contained the metabolizes depicted in Fig. 4 were pooled and analyzed by gas chromatography-electron impact mass spectrometry (GC-MS) after trimethylsilylation. The structures of the metabolites were confirmed by comparing their mass spectra with those of authentic standards. Quantitative analysis was done by chemically converting the metabolites as follows. Compound **V** was oxidized to compound **VI** with 2,3-dichloro-5,6-dicyanobenzoquinone, compound **VI** was reduced to compound **V** with $NaBH_4$, and compound **IV** was hydrolyzed to compound III with NaOH. A more detailed description of the analytical procedures cart be found elsewhere (10, 12).

LiP Assay

The cultures (8 replicates) were harvested after 6-7 days and extracted with 100 ml water by shaking the pulp blocks for 2 hours at room temperature. The suspension was then filtered dialyzed against 20 mM sodium acetate (pH 5), and concentrated to 1 ml by ultrafiltration. The procedure was then repeated with 1 M NaCl and 1% Triton X-100. Samples (0.5 ml) were spectrophotometrically assayed for LiP according to Tien and Kirk (14). None of the extracts and concentrates contained any detectable activity.

RESULTS AND DISCUSSION

The degradation of nonphenolic lignin structures is generally believed to be a consequence of LiP-catalyzed aryl cation radical formation (8). However, it has been shown that the apparently LiP-negative fungus *C. subvermispora* and the Lip producer *P. chrysosporium* both degrade lignin in wood at similar rates, regardless of whether the lignin structures are phenolic or nonphenolic (9). The nonphenolic β-O-4 linked lignin model **I** was also very rapidly degraded by *C. subvermispora* on wood, which further demonstrates that its ligninolytic system was fully expressed under these conditions (10). Despite that, LiP could not be detected in these cultures.

However, it is difficult to assay for LiP in wood and, therefore, in that earlier work we attempted to analyze the degradation products formed from model **I** to determine whether reactions diagnostic for one-electron oxidation occurred during cleavage. One-electron oxidation of ring A and ring B would result in C_α-C_β and C_β-O-aryl cleavage, respectively, of the model **I** propyl sidechain (8, 15, 16).

A complex mixture of degradation products was obtained and only a small portion could be identified as C_α-C_β cleavage products unless a large amount of isotope trap was added to the cultures. C_β-O-aryl cleavage products, which arc also indicative for an aryl cation radical mechanism were not identified. Thus, the data did not prove that aryl cation radical formation was quantitatively significant in model **I** degradation by *C. subvermispora.*

The object of this work was to develop a new *C. subvermispora* culture system in which high molecular weight products of ligninolytic metabolism can be characterized by techniques such as nuclear magnetic resonance (NMR) spectroscopy. Macromolecular products, unlike small ones, are likely to persist in fungal cultures, which should make it possible to detect diagnostic functional group changes in the lignin models. In this preliminary study, we report on the performance of the new system when it degrades model **I,** a substrate we used previously to assess ligninolytic activity in wood block and liquid cultures of *C. subvermispora*

The new system employs cellulose block cultures rather than wood block cultures because wood blocks contain extractives that interfere with product analysis. Moreover, the workup is greatly facilitated and metabolizes from model **I** degradation are easily extractable in high yields wheat cellulose blocks are used. Initial studies showed that, under the conditions described in Materials and Methods, cellulose block cultures of *C. subvermispora* and *P. chrysosporium* mineralized model **I** at high rates (Fig. 2) which were comparable to those obtained in wood blocks (10). To minimize the accumulation of ^{14}C metabolites derived from further metabolism of model **I** cleavage products initially formed, the cultures were harvested and worked up as soon as the mineralization rates became linear (Fig. 2).

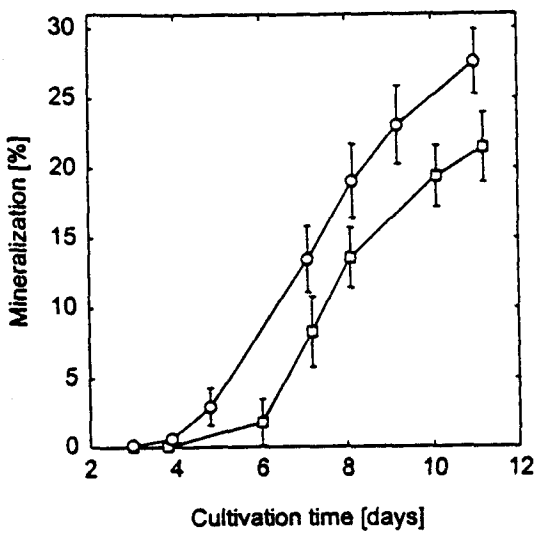

Fig 2. Mineralization of α-^{14}C-labeled model I by *P. chrysosporium* (□) and *C. subvermispora* (○) in solid state cultures. Error bars show 1 standard deviation of the sample.

Gel permeation chromatography of the culture extract showed that model **I** was depolymerized by both fungi to give low molecular weight ^{14}C-labeled products (Fig. 3). However, in *P. chrysosporium* the pool of low molecular weight products was much larger than in *C. subvermispora.* HPLC analysis showed that this was due to the accumulation of compound **II,** which arose via C_α-C_β cleavage of model **I** and accounted for 50% of the total low molecular weight material. In *C. subvermispora,* by contrast, only trace amounts of compound II could be identified by GC-MS. The most plausible explanation for this discrepancy is that *P. chrysosporium* mineralizes compound **II** much more slowly than *C. subvermispora* does. This conclusion is supported by the fact that veratryl alcohol, a structural analog to compound **II,** is biosynthesized by *P. chrysosporium* (17) and accumulates in the culture medium, whereas it does not in *C. subvermispora* (result not shown). Significant amounts of compound **IV** (5% of total low molecular weight material), which is also derived from C_α-C_β cleavage of model **I,** could be identified in *C. subvermispora,* which suggests that C_α-C_β cleavage is involed in model **I** degradation by this fungus. Compound IV is probably formed intracellularly by methylation of compound **III,** which was also identified in trace amnounts by GC-MS (Figs. 3 and 4).

P. chrysosporium and *C. subvermispora* both accumulated approximately the same amounts of another product, compound **V,** which was found as a mixture of its *threo* and *erythro* isomers. In *C. subvermispora,* this product accounted for 45% of the total ^{14}C-labeled low molecular weight material. The formation of compound **V** is highly diagnostic for β-O-aryl cleavage of model **I** Significant amounts (7% of the total low molecular weight material) of compound **VI,** which is the oxidized form of compound **V,** could also be identified in *C. subvermispora* (Figs. 3 and 4).

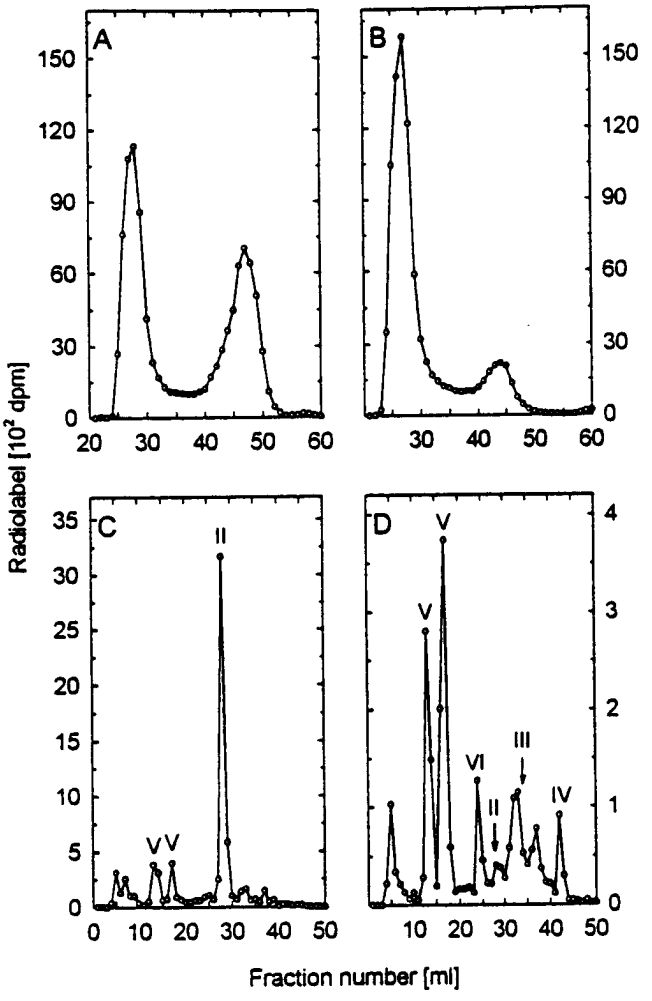

Fig. 3. GPC analysis (A, B) and reversed-phase HPLC analysis of the low molecular weight GPC fractions (C, D) of metabolizes produced from α-^{14}C-labeled model **I** by *P. chrysosporium* (A, C) and *C. subvermispora* (B, D). Roman numerals refer to the chemical structures depicted in Fig. 4. Arrows indicate positions of compounds found only in trace amounts.

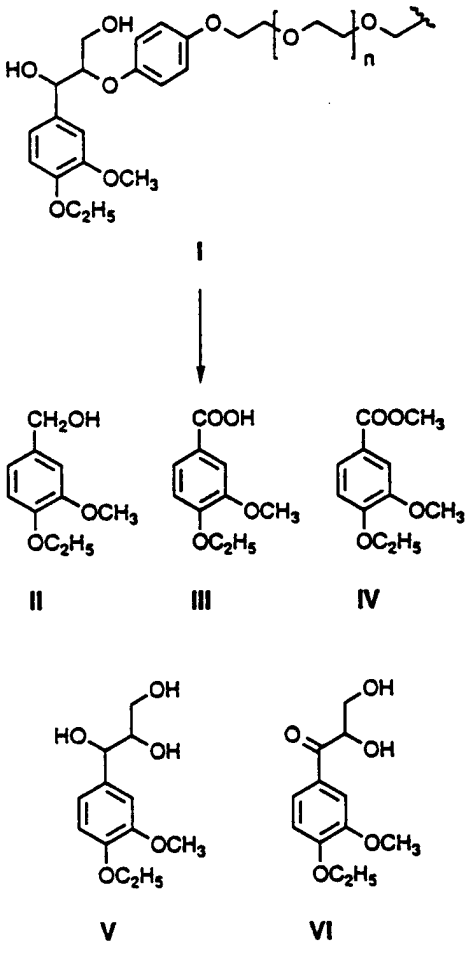

Fig. 4. Products obtained when α-^{14}C-1sbeled model **I** was degraded in solid state cultures by *P. chrysosporium* and *C. subvermispora.*

C_α-C_β and C_β-O-aryl cleavage products from model **I** degradation accounted for at least 57% of the total ^{14}C found in the low molecular weight fractions of the *C. subvermispora* culture extract, strongly suggesting that one-electron oxidation is a significant pathway of model **I** degradation in this fungus. In subsequent NMR work with ^{13}C-labeled models related to model **I,** we have recently shown that one-electron oxidation is in fact the major ligninolytic pathway in this fungus (12).

LiP activity, which could accomplish the one-electron oxidation of nonphenolic lignin structures, was not found in the cellulose block cultures of *C. subvermispora* (see Materials and Methods) but this does not rule out the possibility that LiP is bound to hyphal structures or cellulose in solid state cultures. Alternatively, manganese peroxidase and laccase, acting in concert with co-oxidants and mediators (10, 18-21) may also be able to degrade nonphenolic lignin by one-electron oxidation. Further research is needed to distinguish between these possibilities. However, it is clear from the results presented here that *C. subvermispora* is able to degrade nonphenolic lignin structures efficiently via one-electron oxidation. Therefore, new ligninolytic mechanisms need not be hypothesized for this biopulping fungus.

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