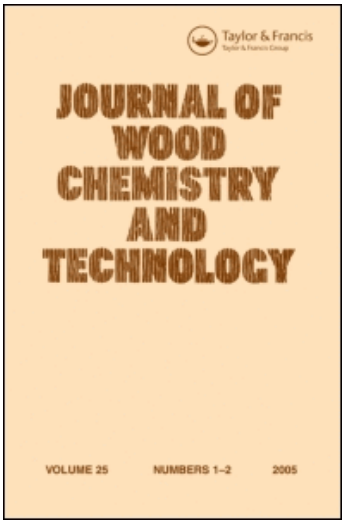

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EFFECTS OF C_α-OXIDATION IN THE FUNGAL METABOLISM OF LIGNIN

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ABSTRACT

C_α-Oxidation (benzyl alcohol oxidation) is a prominent reaction in the degradation of lignin by white-rot fungi. This study showed that such oxidation markedly retards metabolism of a non-phenolic β-O-4 model compound, 1-(3-methoxy-4-ethoxyphenyl)-2-(o-methoxyphenoxy)propane-1,3-diol, by cultures of Phanerochaete chrysosporium Burds. Surprisingly, however, selective chemical C_α-oxidation of spruce lignins enhanced their depolymerization by the cultures. Thus the decrease in intrinsic degradability of substructures is more than compensated by another effect of C_α-oxidation in lignin. One possibility is that the oxidation increases the accessibility of the lignin to enzymes by decreasing its steric complexity. This study also revealed that the β-O-4 model, like lignin in wood, is degraded in part via C_α-oxidation by P. chrysosporium. Reduction of the α-carbonyl groups thus formed does not occur. Addition of L-glutamate to ligninolytic cultures completely suppresses their competence to degrade the model compound, as it does their ability to oxidize lignin to CO_2. This result strengthens past evidence indicating that substructure models are metabolized by the same enzyme system as lignin.

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INTRODUCTION

Present knowledge of the chemistry of lignin degradation by white-rot fungi is based largely on characterizations of partially degraded lignin polymers,[3 8] and on the identification of low molecular weight degradation fragments of lignin.[9,10] Those studies revealed a number of degradative reactions that include, prominently, C_α-oxidation (i.e., oxidation of the benzyl alcohol groups to α-carbonyl groups (Fig. 1)).

Because C_α-oxidation is prominent in lignin degradation, it was surprising to observe that chemical C_α-oxidation greatly slows metabolism of substructure models of the β-1 (1,2-diarylpropane-1,3-diol) type by the white-rot fungus Phanerochaete chrysosporium Burds.[11,12] In the absence of C_α-oxidation, these models are rapidly degraded in ligninolytic cultures via an initial cleavage between C_α and C_β.[11,12] This cleavage, not only in β-1 substructures, but also in the quantitatively more important β-O-4 substructures, is probably important in the fungal depolymerization of lignin.[13]

There is some evidence that C_α-oxidation in lignin retards C_α-C_β cleavage. Several low molecular weight products formed on the degradation of lignin in spruce wood by P. chrysosporium contained intact side chains, most of which had α-carbonyl groups.[10]

These results with lignin and β-1 models suggested to us that C_α-oxidation might have substantial effects on lignin degradation, retarding C_α-C_β cleavage and consequently depolymerization, and in effect "directing" the degradation to the aromatic rings. Lignins after partial degradation by white-rot fungi contain both ring cleavage fragments and α-carbonyl groups.[6-8]

The purpose of this study was to examine the effect of C_α-oxidation in the degradation of lignin by P. chrysosporium. We first studied the effect of C_α-oxidation on metabolism of a β-O-4 model, which represents the dominant substructure in lignins.[14] We then examined its effect on fungal depolymerization of lignin. Previous work has shown that depolymerization of lignin occurs rapidly following addition of the lignin to ligninolytic cultures of P. chrysosporium.[15,16]

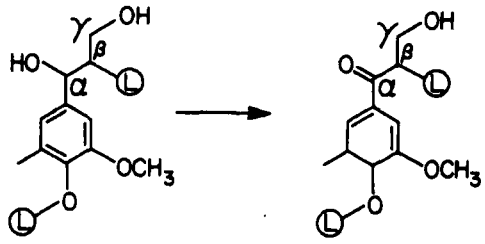
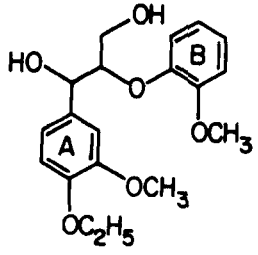


FIGURE 1. C_α-oxidation is a prominent reaction in the degradation of lignin by white-rot fungi.[1-10] Circled L = continuation of lignin.

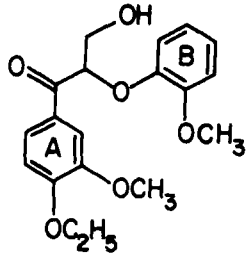
EXPERIMENTAL

Approach

Model compound I and the corresponding C_α-oxidized model II were ^{14}C-labeled in either rings A or B. Comparative studies with these four compounds (A- and B-ring labeled I, and A- and B-ring labeled II) determined the influence of C_α-oxidation on (a) the rate of metabolism, (b) the rate of conversion to other products, and (c) the nature of the other products, in ligninolytic cultures of P. chrysosporium.



I



II

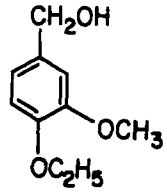
Investigations with spruce lignins (Brauns' and milled wood lignins) then assessed the effect of selective chemical C_α-oxidation on its depolymerization in the cultures. Each lignin was

first reduced with $NaBH_4$ to remove the small number of α-carbonyl groups already present, and then it was 4-O-methylated with $^{14}CH_3I$ to permit accurate and sensitive assessment of its molecular size distribution using gel permeation chromatography and scintillation spectrometry. A sample of the lignin was then C_α-oxidized for comparative studies with the non-oxidized sample.

Preparation of Model Compounds and Lignins

The phenolic β-O-4 compound 3-methoxy-4-hydroxy-α-(2-methoxyphenoxy)-β-hydroxypropiophenone, labeled uniformly in either the A ring (sp. act. 7.67×10^8 dpm/mmole) or the B ring (sp. act. 6.56×10^7 dpm/mmole), was synthesized as described previously.[17] The compounds were ethylated with excess C_2H_5I/K_2CO_3 in N,N-dimethylformamide (DMF) at room temperature. The products (model II, A- or B-ring labeled) were purified by thin layer chromatography (TLC) using silica gel with CH_2Cl_2:MeOH (97:3 v/v) as solvent; yields were approximately 90%. Reduction of these models with $NaBH_4$ in 95% ethanol afforded A- and B-labeled model I [1-(3-methoxy-4-ethoxyphenyl)-2-(o-methoxyphenoxy)propane-1,3-diol]. The products were purified by silica gel TLC using ethyl acetate:hexanes (1:1 v/v) as solvent. The structure of compound I was confirmed by ^1H-NMR using an unlabeled sample. TLC and scintillation spectrometry revealed that the labeled compounds were >99% radiochemically pure.

3-Methoxy-4-ethoxybenzyl alcohol (III) was prepared by ethylating vanillyl alcohol with C_2H_5I/K_2CO_3 in DMF.



III

A lignin preparation (15 mg) of the "Brauns' native lignin" type, which had been isolated from Engelmann spruce wood and characterized earlier, ("Lignin 3"[6,18]), was treated with 15 mg of $NaBH_4$ in 2 ml of dioxane:water 7:3, on ice. After 10 min., the temperature was raised to ~25°C, and the sample stirred for 4 h. The sample was then acidified with glacial acetic acid, and solvents removed by vacuum evaporation to near dryness. The precipitated lignin was recovered by centrifugation, washed with 500 µl of H_2O, and again recovered by centrifugation. The moist sample was dissolved in 500 µl of DMF and purified by passage through a 1.0 x 16.0 cm column of Sephadex LH-20 in DMF.[19] Fractions (0.5 ml) containing the lignin that was excluded from the gel (fractions 16-20; monitored by UV) were pooled and the lignin (7 mg) recovered by evaporation of the DMF. This sample was methylated overnight at room temperature with $^{14}CH_3I$ (ICN, Irvine, Calif.) in a stirred mixture of DMF/K_2CO_3. Excess unlabeled CH_3I was then added and stirring continued for 3 h. Insoluble K_2CO_3 was removed by filtration, and the solvent volume reduced to ~0.5 ml. Following purification by Sephadex LH-20 chromatography as above, the sample (5 mg) was recovered by evaporation of the DMF. The specific activity, determined in a dioxane-based liquid scintillation fluid,[20] was 6.5 x 10^7 dpm/mg. Two mg of this sample were retained as the reduced ("α-carbinol") sample. The remaining portion was C_α-oxidized to give the "α-carbonyl" sample. For this, it was dissolved in 2 ml of purified dioxane containing one drop of methanol, and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ, 3 mg) was added.[21] The solution was stirred overnight at room temperature in the dark, and the solvents removed by vacuum evaporation. The oxidized lignin was isolated by Sephadex LH-20 chromatography as above; 1.4 mg were recovered, as determined from the radioactivity. UV spectra of the reduced and oxidized samples were recorded, using dioxane:water, 7:3, as solvent. From the difference in absorption at 310 nm between the reduced and oxidized materials (Fig. 2), the α-carbonyl content of the latter was calculated to be 0.29/C_9-unit.[21,22] Calculations were based

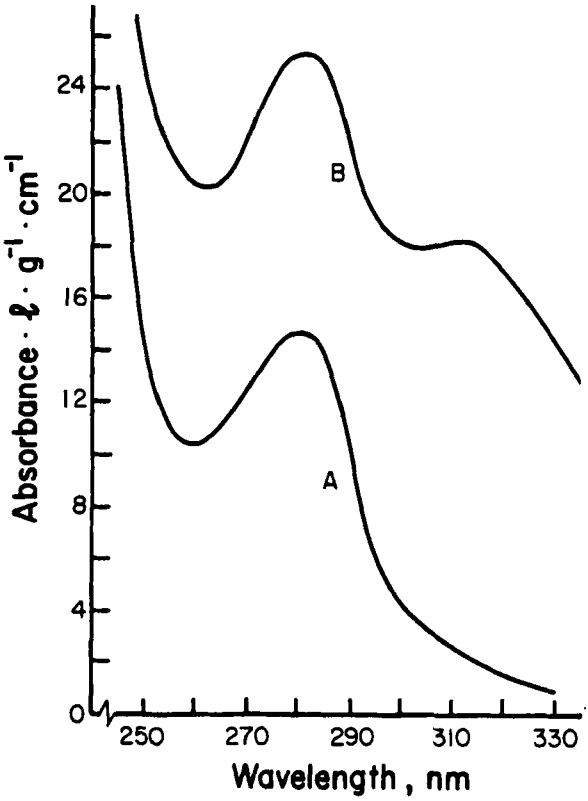


FIGURE 2. UV spectra of a spruce lignin preparation after reduction and methylation (A), and after further treatment with DDQ to oxidize α-carbinol groups (B).

on a C_9-unit weight of 193,[18] and a molar extinction coefficient of 1×10^4 for the α-carbonyl chromophore.

A milled wood lignin (MWL) sample from the same spruce wood ("Lignin 1"[6,18]), was treated exactly as above. Its specific activity was 4.1×10^7 dpm/mg, and its C_α-carbonyl content after DDQ oxidation was $0.19/C_9$-unit.

A sample of synthetic [ring-U-^{14}C]lignin (DHP)[20,23] (9×10^5 dpm/mg) was oxidized with DDQ as above, without prior reduction or methylation. Its α-carbonyl content after oxidation was approximately $0.04/C_9$-unit.

Organism and Culture Conditions

A single basidiospore isolate of Phanerochaete chrysosporium Burds., strain ME-446 (ATCC 35541), was used. This isolate (ME-446-6) was selected for its more rapid degradation of sweetgum sapwood than the parent isolate.[24] Cultures (10 ml in 125-ml Erlenmeyer flasks) were grown from conidial suspensions as described previously, without agitation, at 39°C under oxygen.[23,25,26] The culture medium contained 10 g D-glucose, 0.2 g ammonium tartrate, 2.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$, 1 mg thiamine·HCl and 10 ml of a trace element/mineral solution[25] per liter. The medium was buffered with (sodium) polyacrylic acid (pH 4.5, 10 mM in carboxyl).[27] Cultures become ligninolytic (i.e. capable of converting ^{14}C-labeled lignins to $^{14}CO_2$) 3 to 4 days following spore inoculation.[23,25] The ligninolytic system appears constitutively as a part of idiophasic metabolism, initiated in this medium by nitrogen depletion.[23]

Degradation of Models in Cultures

Models were dissolved in DMF and these solutions added to sterile water to give solutions for addition to 6.5-day-old cultures. Each culture received 0.5 ml of solution containing <25 µl of DMF. After incubation for the desired time under O_2 at 39°C, whole cultures were extracted with chloroform:acetone (1:1, v/v).[26] Extracts were examined by TLC using aluminum-backed silica gel plates (Merck 60_{F-254}, Darmstadt FRG), and ethyl acetate:hexanes (1:1, v/v) as solvent. Products were identified by co-elution with known standards and by spectroscopic procedures as described. Products were quantified by liquid scintillation spectrometry: bands on the plates were located with UV light, cut out with scissors, and the plate pieces put directly in the dioxane-based liquid scintillation fluid.[9] Counting efficiency, determined with an internal standard of ^{14}C-toulene (Packard, Downers Grove, Ill.), was near 85%.

The effect of pre-incubating cultures with 7.8 mM L-glutamate on metabolism of I was determined.[28] The amino acid, in 1.0 ml of

H_2O per culture, was added to triplicate 6-day-old cultures; triplicate control cultures received only H_2O. Cultures were flushed with oxygen and incubated at 39°C for 16 h. Model I was then added, cultures flushed with O_2 and incubated for an additional 6 h, extracted, and the extracts examined by TLC and liquid scintillation spectrometry as above.

Degradation of Lignins in Cultures

The lignin samples were each dissolved in 1 ml of DMF, and 80 µl added to 4.0 ml of sterile distilled water with vigorous stirring. Each 10 ml culture received 0.5 ml of the resulting suspensions. Six cultures were set up with the oxidized (α-carbonyl) and reduced (α-carbinol) samples (12 cultures total). Cultures were flushed with oxygen and incubated at 39°C. After 6 and 24 h, triplicate cultures with each lignin sample were killed by addition of 10 ml of dioxane. After standing overnight at room temperature, the triplicate cultures were pooled and the mycelia removed by centrifugation. Radioactivity in the mycelia and in the supernatants was determined.[15] To each supernatant solution, 20 ml of DMF was added, and the total solvent volumes reduced to ~10 ml by vacuum evaporation. Approximately 1 ml of each was then filtered through glass wool and analyzed for molecular size distribution with a 1.0 x 22 cm column of Sephadex LH-20 in DMF.[19] Fractions (0.5 ml) were collected directly in the dioxane-based scintillation fluid, and radioactivity determined.

Instrumentation

The following instruments were used: Packard Instruments 3330 liquid scintillation spectrometer (Packard, Downers Grove, Ill.); Cary 14 UV spectrophotometer (Varian, Palo Alto, Calif); Bruker 250 MHz NMR spectrometer (Bruker, Billerica, Mass.).

RESULTS

Metabolism of Model Compounds

C_α-Oxidation strongly influenced the rate of metabolism of the β-O-4 model. C_α-carbinol model I was degraded immediately on addi-

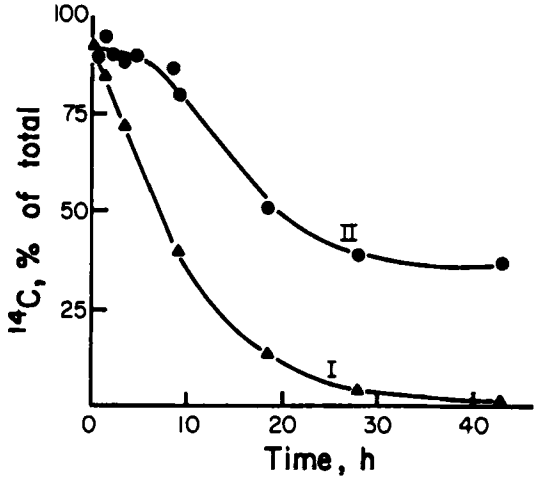


FIGURE 3. Depletion of models I and II following addition to ligninolytic cultures. Residual I or II (A-ring-labeled) was isolated by TLC and measured by scintillation spectrometry. Cultures initially contained 2.7×10^5 dpm of I (124 μg) or 3.5×10^5 dpm of compound II (158 μg). Triplicate cultures were pooled for extraction and analysis.

tion to ligninolytic cultures; the model had a half-life of about 7 h. In contrast, C_α-carbonyl model II was degraded only sluggishly for about 6 h after addition to cultures, and then at a rate less than half that of model I (Fig. 3).

Conversion of the aromatic carbons to CO_2 followed the same pattern as disappearance of the models. Both aromatic rings in model I were degraded at least twice as fast as the corresponding rings in model II (Fig. 4). The ether-linked ring (ring B) was degraded 4 to 5 times faster than the A ring in both models. In accord with the rate of disappearance of model II, degradation of both rings proceeded only sluggishly for approximately the first 6 h after addition to cultures.

Pre-incubation of cultures with L-glutamate completely suppressed the ability of the cultures to degrade model I. All radioactivity was still associated with model I 6 h after its addition to glutamate-amended cultures.

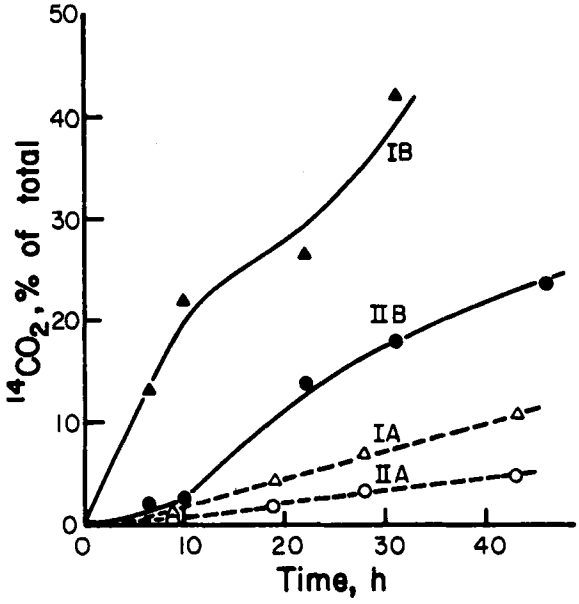


FIGURE 4. Oxidation of aromatic carbons to $^{14}CO_2$ on incubation of models I and II (A- or B-ring-labeled) in ligninolytic cultures. Values are means for 2 or 3 replicate cultures. (Variation = ±10% of values shown). Amounts of models added per culture: I (A-ring):2.7 x 10^5 dpm; I (B-ring):4 x 10^4 dpm; II (A-ring):3.5 x 10^5 dpm; II (B-ring):3.4 x 10^4 dpm.

Degradation of both models I and II resulted in formation of vanillyl alcohol ethyl ether (III), which was isolated by TLC and identified by co-elution with standard on TLC plates using two different solvents.[9] This product was metabolized relatively slowly, and accumulated to a maximum of 20 mol-% from both models (Fig. 5). The product began accumulating immediately upon introduction of model I to cultures, but accumulated only after a lag of several hours after introduction of model II (Fig. 5). Significant amounts of other products were not detected in the extracts of cultures to which compound II (A- or B-ring labeled) had been added.

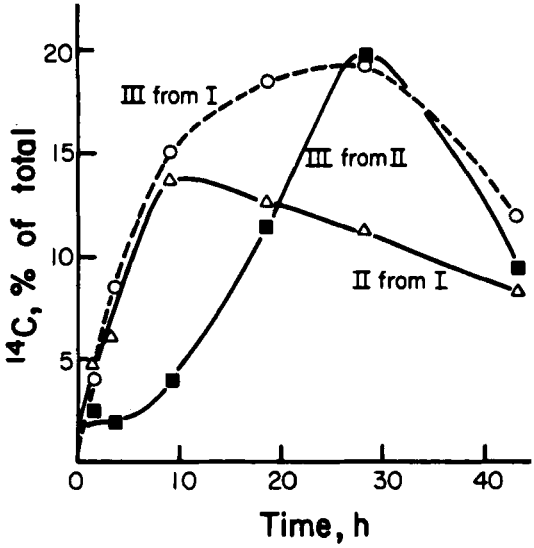


FIGURE 5. Products formed from A-ring-labeled models I and II during 43 h in ligninolytic cultures. Amounts added: I (2.7×10^5 dpm) and II (3.5×10^5 dpm).

A second major product, in addition to III, was formed from model I, however. It was isolated and identified from its ^{1}H-NMR and UV spectra as compound II, with which it co-eluted on TLC plates. Product II reached a maximum concentration of 13 mol-%, and then exhibited a net decrease (Fig. 5). Reduction of II back to I was not observed, even with a sensitive isotope-trapping technique.[9] Prior incubation of II in cultures (to induce a reductase system if required) did not result in reduction.

Depolymerization of Lignins

Both C_α-oxidized and non-oxidized Brauns' lignins were depolymerized in ligninolytic cultures (Fig. 6A,B). Depolymerization was apparent after 6 h, and was extensive after 24 h. Surprisingly, the C_α-oxidized lignin was depolymerized more rapidly than the non-oxidized sample. This difference was clear after 6 h, and was marked after 24 h.

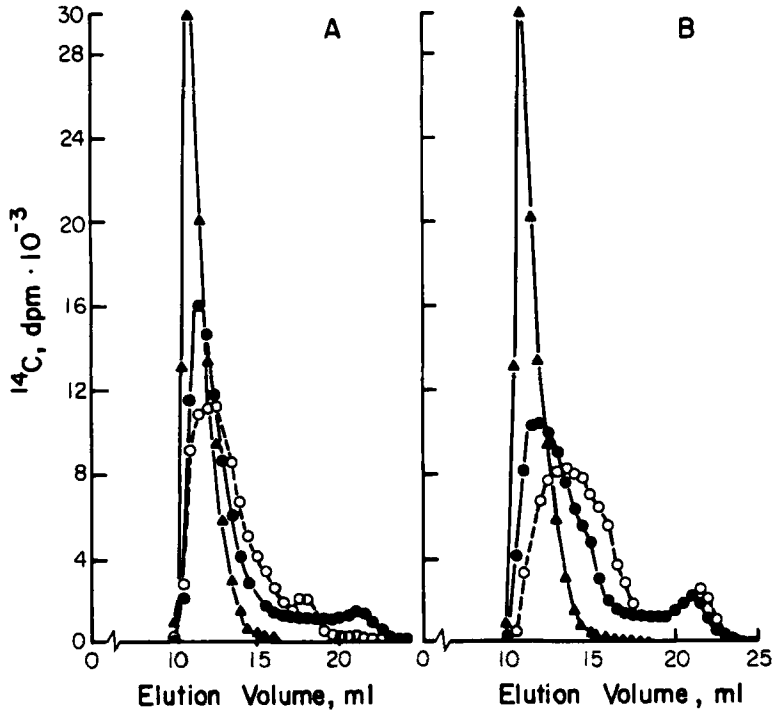


FIGURE 6. Molecular size distribution of Brauns' lignin before and after incubation in ligninolytic cultures. Each culture received 1.3 x 10^6 dpm (20 μg) of the reduced/methylated lignin, or 9.1 x 10^5 dpm (14 μg) of the reduced/methylated/C_α-oxidized lignin. A: 6 h; B: 24 h. Lignin samples: ▲ non-degraded control lignins; reduced/methylated, fungus-degraded (-●-); and reduced/methylated/C_α-oxidized, fungus-degraded (-o-). All samples have been normalized to the same total 14C. Each curve is from the lignin from pooled triplicate cultures.

[In these experiments, 83 and 88% of the added ^{14}C was recovered as dioxane/water-solubles after 24 h from the non-oxidized and oxidized samples, respectively. Mycelium-associated ^{14}C, 7 and 8%, and $^{14}CO_2$, 8 and 6%, accounted for the rest. After 6 h lignin incubation, CO_2 production was nil; 97% of the ^{14}C was in the dioxane-water solubles, and the remainder was in the mycelium in the cases of both oxidized and non-oxidized lignins.]

We also examined the effect of DDQ oxidation on depolymerization of the spruce MWL and of the synthetic [ring-U-^{14}C] lignin. The effect on depolymerization of the MWL was the same as observed with the Brauns' lignin, but the difference between oxidized and non-oxidized samples was not as great. Although the synthetic lignin samples were readily depolymerized in cultures (cf.[15]), no significant differences were observed between oxidized and non-oxidized samples.

DISCUSSION

Our results demonstrate that C_α-oxidation increases the rate of depolymerization of lignin by P. chrysosporium. The correlation between α-carbonyl content following DDQ oxidation and magnitude of enhanced fungal depolymerization provides good evidence that the enhancement was in fact due to C_α-oxidation. Thus the DDQ-oxidized synthetic lignin contained very few α-carbonyl groups, and it was not depolymerized faster than the non-oxidized sample.[29] Also, the oxidized MWL contained fewer α-carbonyl groups than the oxidized Brauns' lignin, and the effect of C_α-oxidation on the depolymerization of the former was not as great. Adler et al.[21] showed that the major effect of DDQ on lignin is C_α-oxidation.

The basis for the enhanced depolymerization rate caused by C_α-oxidation is not clear. Results with the β-O-4 models here, and with β-1 models in previous studies,[11,12] show clearly that C_α-oxidation decreases the rate of their metabolism. Thus faster depolymerization of lignin occurs despite a decrease in the intrinsic degradability of the oxidized substructures. Clearly C_α-oxidation has another effect on the lignin that offsets the

retarding effect on substructure degradability. One possibility is that C_α-oxidation increases the accessibility of lignin to the depolymerizing enzymes. C_α-oxidation directly removes one center of asymmetry (at C_α) and it also relieves the steric rigidity at C_β through enolization.[30] Thus C_α-oxidation would be expected to simplify the steric complexity of lignin, creating a more open and accessible polymeric structure for enzyme attack.

A second possibility is that DDQ-oxidation increases the susceptibility of the interunit linkage between the α-carbonyl-conjugated aromatic ring and the adjacent unit (i.e. the linkage to the lower "L" in Fig. 1). We consider this possibility less likely than the one based on altered stereochemistry.

Interestingly, the oxidized lignin was partially depolymerized in 6 h, whereas the C_α-oxidized model (II) was only slightly degraded during the first 6 h in cultures. This result suggests that the initial attack on the polymer leading to the early depolymerization was probably not in β-O-4 substructures related to model II.

[Deductions such as the foregoing, based in part on studies with model compounds, are premised on the assumption that the models are metabolized by the same enzyme system as lignin. Evidence that β-O-4 models such as I are metabolized by the ligninolytic system was provided by Weinstein et al.,[31] who showed that the culture factors affecting the metabolism of lignin to CO_2 have the same influence on metabolism of a β-O-4 model. Our results here demonstrating that L-glutamate completely suppresses metabolism of model I provide additional support for the contention that the ligninolytic system is responsible for degradation of the model. Our past work has shown that glutamate strongly represses the ligninolytic system in P. chrysosporium.[28]]

The 6-h lag in metabolism of model II suggests that enzyme induction was required. We suspected that this might be induction of a reductase system, which would convert II to I. Indeed, the possibility that reduction to I might be the first step in its degradation was suggested by the fact that both models are degraded via product III, pointing to a common pathway. Strong evidence was

obtained, however, that II is not reduced to I, suggesting that different pathways are in fact involved in metabolism of I and II.

Although reduction of II to I is not observed, oxidation of I to II is a prominent reaction, as it is in lignin itself. Curiously, C_α-oxidation in β-O-4 models has not been reported in any of several previous studies of degradation of such models by P. chrysosporium.[32-35] C_α-oxidation in a β-1 model by P. chrysosporium has been observed, but it is a minor reaction.[12]

Recent results[36] have shown that C_α-oxidation in model I is catalyzed by an enzyme in the extracellular fluid of ligninolytic cultures of P. chrysosporium. Action of the purified enzyme on model I results in ~15% conversion to II, and ~85% cleavage between C_α and C_β.[36] It is probable, therefore, that oxidation of a portion of I to II in the cultures here was catalyzed by this enzyme. The C_α-C_β cleavage catalyzed by the enzyme produces from the C_α moiety an aromatic aldehyde, which cultures rapidly reduce to the corresponding alcohol.[12,26] The enzyme's action, therefore, probably accounts for the formation of product III. It seems probable that the newly discovered enzyme plays a major role in degradation of I in cultures. The enzyme, an oxygenase, requires H_2O_2 as well as molecular oxygen for its activity. Extracellular H_2O_2 is found in the cultures.[37] The enzyme partially depolymerizes lignins,[13] probably by C_α-C_β cleavage.

The C_α-C_β cleavage activity of the enzyme in model I results in release of the β-ether-linked aryl moiety (guaiacol), probably via spontaneous degradation of a hemiacetal, α-hydroxy-α-(2-methoxyphenoxy)ethanol, thought to be the initial cleavage product.[36] It is probable, therefore, that a substantial percentage of the B-rings are released as guaiacol from compound I. This phenolic product is probably degraded more rapidly than compound III, explaining the more rapid conversion of B-ring carbons to CO_2 than A-ring carbons in the case of model I. In the case of model II also, the B-ring carbons are oxidized to CO_2 more rapidly than the A-ring carbons, but whether guaiacol is an intermediate is not clear. The B ring in model II might be oxidatively cleaved

while still β-ether-linked, with further degradation releasing ring carbons without cleavage of the β-ether linkage. Indeed, this pattern of degradation in lignin itself is suggested by the identities of six products of spruce lignin degradation by P. chrysosporium;[10] the products contain intact side chains linked to fragments of aromatic ring cleavage, and five of the six contain α-carbonyl groups.[10] After partial degradation by white-rot fungi, lignins contain ring cleavage fragments as well as α-carbonyl groups.[6-8] Our model study here and the previous ones[11,12] indicate that C_α-oxidation makes the side chains more resistant to degradation. All of these findings, therefore, suggest that C_α-oxidation does in fact direct the degradative attack away from side chains, as we originally suspected. A surprising effect in the polymer, however, is to increase the rate of depolymerization.

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