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Rapid polyether cleavage via extracellular one-electron oxidation by a brown-rot basidiomycete

ZOHAR KEREM, WULI BAO*, AND KENNETH E. HAMMEL†

Institute for Microbial and Biochemical Technology, U.S. Department of Agriculture Forest Products Laboratory, Madison, WI 53705

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ABSTRACT **Fungi that cause brown rot of wood are essential biomass recyclers and also the principal agents of decay in wooden structures, but the extracellular mechanisms by which they degrade lignocellulose remain unknown. To test the hypothesis that brown-rot fungi use extracellular free radical oxidants as biodegradative tools, *Gloeophyllum trabeum* was examined for its ability to depolymerize an environmentally recalcitrant polyether, poly(ethylene oxide) (PEO), that cannot penetrate cell membranes. Analyses of degraded PEOs by gel permeation chromatography showed that the fungus cleaved PEO rapidly by an endo route. ^{13}C NMR analyses of unlabeled and perdeuterated PEOs recovered from *G. trabeum* cultures showed that a major route for depolymerization was oxidative C—C bond cleavage, a reaction diagnostic for hydrogen abstraction from a PEO methylene group by a radical oxidant. Fenton reagent (Fe(II)/H_2O_2) oxidized PEO by the same route *in vitro* and therefore might account for PEO biodegradation if it is produced by the fungus, but the data do not rule out involvement of less reactive radicals. The reactivity and extrahyphal location of this PEO-degrading system suggest that its natural function is to participate in the brown rot of wood and that it may enable brown-rot fungi to degrade recalcitrant organopollutants.**

Lignocellulose, the predominant form of terrestrial fixed carbon, is degraded efficiently only by certain filamentous fungi. Among the least-understood of these are the brown-rot basidiomycetes, which are essential contributors to biomass recycling and soil fertility in coniferous forest ecosystems (1, 2). Brown-rot fungi are also responsible for the most destructive type of decay in wooden structures. It has been estimated that ≈10% of all trees cut in the United States go to replace wood that decays in service, largely because of brown rot (3).

Brown-rot fungi have the singular ability to digest wood cellulose without removing the lignin that encases and normally protects it from microbial attack (4). Instead, the lignin is modified chemically by reactions that include side chain oxidation and cleavage of ether linkages (5, 6). These reactions are unusual because lignin and other polyethers are relatively unreactive and resist oxidation by most biochemical mechanisms (4, 7, 8). The cellulose in wood also is oxidized and depolymerized rapidly during brown rot, which may indicate cleavage of its acetal linkages by the same species that oxidizes lignin (9, 10).

Because sound wood is impermeable to enzymes (11, 12), it has been proposed that the agent responsible for brown rot is a low molecular weight one-electron oxidant such as the hydroxyl radical (·OH) or Fenton reagent (Fe(II)/H_2O_2) (13–19). These extremely reactive oxidants attack ethers and acetals by similar pathways that include β-scission of aliphatic C—C bonds (20, 21). However, the chemical changes that occur in lignin and cellulose during brown rot are complex and might reflect processes other than one-electron oxidation. Moreover, although brown-rot fungi do produce one-electron oxidants of some type (13–16, 18), it never has been demonstrated that these agents are sufficiently reactive to degrade ethers or that they have the extrahyphal location necessary for a role in lignocellulose breakdown.

To address these problems, we have examined the ability of the brown-rot fungus *Gloeophyllum trabeum* to degrade a structurally simple polyether by using extracellular reactions. The model substrate we used, poly(ethylene) oxide (PEO), meets several important criteria: It undergoes diagnostic cleavage reactions when oxidized by one electron (22–24), it is inert to hydrolysis under physiological conditions (8), and it is unable to penetrate cell membranes (25–27). PEOs resist biodegradation and have become widespread, persistent environmental contaminants (8). Our results show that *G. trabeum* produces an extracellular one-electron oxidant that cleaves PEO rapidly via β-scission reactions.

MATERIALS AND METHODS

Chemicals. PEOs with M_rs under 5×10^4 generally are called poly(ethylene glycols), but we use the former term throughout for brevity. Unlabeled PEOs were obtained from Polysciences. PEO labeled with ^{14}C in its terminal hydroxyethyl positions [19.2 mCi·g^{-1} (1 Ci = 37 GBq); M_r stated as 4×10^3] was from Amersham, and poly(ethylene-$^2H_4$ oxide) (99.8% perdeuterated, $M_r = 1.4 \times 10^4$) was from CDN Isotopes (Quebec, Canada). Formate ester-terminated PEO was obtained by refluxing PEO overnight in 96% formic acid and was purified by gel permeation chromatography (GPC) on a 3 × 36-cm column of Sephadex G-25 (Pharmacia) in ethanol:water (1:1). This procedure resulted in ≈20% formylation. All other chemicals were reagent grade.

Culture Conditions. *G. trabeum* (ATCC 11539) was grown at 30°C under air in 125-ml Erlenmeyer flasks that contained 2.5 g of perlite and 15 ml of basal growth medium with 10 g/liter glucose as the carbon source (28). The cultures were inoculated at a rate of 1% with homogenized potato dextrose agar plates of the fungus.

In experiments to measure PEO degradation, the polymer was added to 7-day-old cultures at a final concentration of 3.3 g/liter in 1.0 ml of sterile H_2O. This initial concentration of PEO was high enough to ensure that the proportion of polymer mineralized was negligible (3% in 9 days), leaving most of the degradation products as oligomers for analysis. When GPC of the degraded PEO was done, unlabeled PEO with a M_r of 4×10^3 was used in combination with [^{14}C]PEO [1.1×10^5 dpm per culture (1 Bq = 60 dpm)]. For ^{13}C NMR experiments, unla-

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Abbreviations: GPC, gel permeation chromatography; PEO, poly(ethylene) oxide.

*Present address: School of Pharmacy, University of Wisconsin, Madison, WI 53706.
†To whom reprint requests should be addressed. e-mail: kehammel@facstaff.wisc.edu.

beled PEO with a M_r of 2×10^5 or perdeuterated PEO with a M_r of 1.4×10^4 was used.

In experiments to measure glucose mineralization, 1.1×10^5 dpm of 6-[^{14}C]glucose (45 mCi·mmol^{-1}; Research Products International) was added with the unlabeled glucose at the time of culture inoculation. The $^{14}CO_2$ evolved by these cultures and by cultures that received [^{14}C]PEO was determined as described (29).

M_r Analysis of Degraded PEO. Cultures were harvested in triplicate 0, 5, 7, and 9 days after [^{14}C]PEO was added. Each culture was extracted by shaking it for 1 h with 15 ml of ethanol, and the mycelium was removed by filtration through a 0.45-μm pore-size nylon membrane. This procedure gave >90% recovery of the ^{14}C present in each culture. The extracts from each set of triplicates then were pooled, and 1.5 ml of the total was fractionated by GPC on a 1.3×24.5-cm column of Sephadex LH-60 in ethanol:water (1:1). Fractions (0.9 ml) were collected and analyzed for ^{14}C by scintillation counting. PEO standards with M_rs of 7.5×10^3, 3.4×10^3, 1.5×10^3, 1.0×10^3, 4.0×10^2, and 1.5×10^2 were used to calibrate the GPC column and were detected in the eluate by refractometry. Number-average M_r (M_n) values and weight-average M_r (M_w) values were calculated by using the standard equations (30).

The rate of PEO scission was calculated from the time course of M_n decrease in PEO recovered from the cultures. The number of times a polymer is cleaved is one less than the ratio of its M_n before cleavage to its M_n after cleavage (31). Accordingly, the rate of PEO cleavage, r, was calculated from the equation

$$r = \frac{\left[\frac{M_n^0}{M_n^t} - 1\right]c}{t},$$

where M_n^0 equals the M_n of the polymer at time zero, M_n^t equals its M_n at time t, and c equals the concentration of polymer chains at time zero. Values for M_n and t used in the calculations are given in the legend to Fig. 1. The value for c (0.67 mM) was based on an initial M_n of 5.0×10^3.

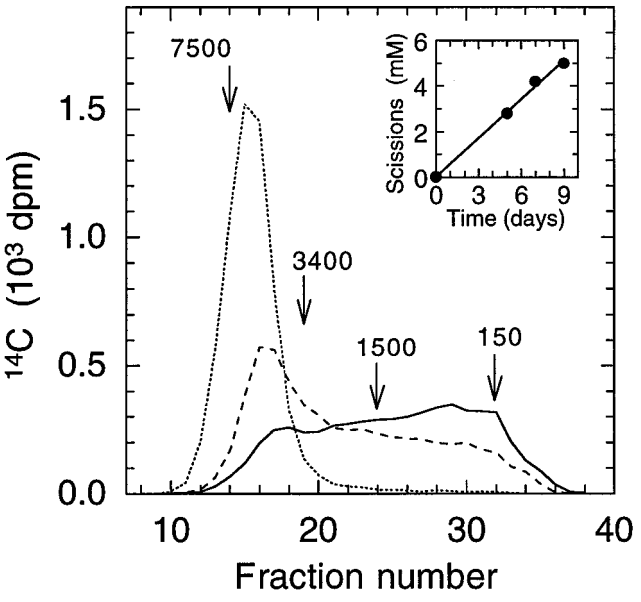

FIG. 1. GPC analyses of [^{14}C]PEO degraded by *G. trabeum*. Day 0 after PEO addition (····): $M_n = 5.0 \times 10^3$, $M_w = 6.2 \times 10^3$. Day 5 (- - -): $M_n = 9.7 \times 10^2$, $M_w = 3.0 \times 10^3$. Day 7 (not shown): $M_n = 6.9 \times 10^2$, $M_w = 2.3 \times 10^3$. Day 9 (—): $M_n = 5.9 \times 10^2$, $M_w = 1.9 \times 10^3$. M_r values and elution positions are shown for some of the PEO M_r standards used to calibrate the GPC column. (*Inset*) Time course of PEO scission as calculated in *Materials and Methods*.

^{13}C NMR Spectrometry of Degraded PEO. Cultures (five replicates) were harvested 13 days after the polymer was added. The pooled medium and mycelium were stirred for 30 min with two volumes of water, the mycelium was removed by filtration through a 0.45-μm pore-size nylon membrane, and the combined aqueous extracts were extracted four times with 0.25 volume of CH_2Cl_2. The organic phase was dried over Na_2SO_4, was evaporated under reduced pressure to dryness, was redissolved in 10 ml of ethanol:water (1:1), and was fractionated by GPC on a 3×36-cm column of Sephadex G-25 in ethanol:water (1:1). The excluded portion of the eluate ($M_r > 5 \times 10^2$) was collected and evaporated to dryness under reduced pressure. The residue, containing 60–90 mg of PEO, was redissolved in ≈0.5 ml of C^2HCl_3 and was transferred to a 5-mm NMR tube.

^{13}C NMR analyses were obtained in C^2HCl_3 at 62.9 MHz and 300 K on a Bruker DPX250 spectrometer (Bruker, Billerica, MA) with a 5-mm conventional geometry four-nucleus probe. Proton-decoupled ^{13}C spectra were acquired with a 30° pulse and a relaxation delay of 1.0 s for a total of at least 2×10^4 scans. Chemical shifts were referenced to internal tetramethylsilane. Semiquantitative signal integrations were based on the assumptions that the relaxation times for all PEO methylenes were equivalent and that the relaxation times for PEO formate carbonyls and aldehyde carbonyls were equivalent.

Distortionless enhancement with polarization transfer (DEPT) spectra (1.4×10^4 scans with a 135° editing pulse) were obtained with a standard Bruker microprogram. The times for relaxation delay and polarization transfer were 0.5 s and 3.57 ms, respectively, resulting in a coupling constant of 140 Hz.

Inverse z-gradient-selected two-dimensional heteronuclear multiple bond correlation experiments (g-HMBC) and heteronuclear single quantum coherence experiments (g-HSQC) were obtained using standard Bruker microprograms. Coupling delays for the formate ester-terminated PEO were optimized such that the one-bond and three-bond coupling constants ($^1J_{CH}$ and $^3J_{CH}$) between the formate ester proton and neighboring carbons were 205 Hz and 5 Hz, respectively. All two-dimensional experiments consisted of 256 increments of 64 scans each and were acquired by using a relaxation delay of 1.5 s.

Fenton Oxidation of PEO. PEO (400 mg; $M_r = 2 \times 10^5$) and $FeCl_2 \cdot 4H_2O$ (200 mg) were dissolved in 250 ml of H_2O. H_2O_2 (5 mmol) then was added dropwise with stirring over 30 min. The final pH of the reaction mixture was ≈2. The products were extracted into CH_2Cl_2 and were fractionated by GPC as described for PEO from *G. trabeum* cultures.

RESULTS AND DISCUSSION

Rate of PEO Cleavage. GPC analyses of [^{14}C]PEO recovered from *G. trabeum* cultures showed that the fungus cleaved the polymer rapidly. The cultures mineralized their carbon source, glucose, at a linear rate of 1.6 mM per day (data not shown) and cleaved [^{14}C]PEO at a linear rate of 0.6 mM scissions per day (Fig. 1 *Inset*). This PEO scission rate is an underestimate because it neglects contributions from PEO fragments with M_rs <150, which were not resolved by our GPC procedure. High performance liquid chromatography of the metabolized PEO confirmed that it contained at least two such low-M_r products, ethylene glycol and diethylene glycol (data not shown).

Possible Routes for PEO Oxidation. The M_r distribution of [^{14}C]PEO in *G. trabeum* cultures spread unimodally to lower values as degradation progressed (Fig. 1). Because the PEO was labeled with ^{14}C only at its termini, this result establishes that depolymerization followed an endo rather than an exo route. That is, exo degradation would have released all of the

^{14}C from the PEO as low-M_r material at the outset of degradation. There are two likely routes for endo PEO oxidation by *G. trabeum*: oxygen insertion between carbon and hydrogen in a methylene group or hydrogen abstraction from a methylene carbon. These two mechanisms are expected to yield different product profiles.

Oxygen insertion into a PEO methylene group would yield a hemiacetal, which would undergo acid-catalyzed C—O cleavage to generate fragments terminated by new alcohol (**I**) and aldehyde (**II**) end groups (Fig. 2). This is the mechanism thought to operate in intracellular monooxygenase-catalyzed oxidations of certain low-M_r ethers (8).

Hydrogen abstraction from a PEO methylene group would yield a carbon-centered radical that would rapidly add O_2 to give a peroxyl radical. PEO peroxyl radicals have been proposed to fragment unimolecularly via hexagonal intermediates (22–24), but it appears equally possible that they would decompose via alkoxyl radical intermediates in reactions analogous to those that occur during fatty acid peroxidation (32) (Fig. 2). In any event, the unimolecular pathway proposed earlier and the alkoxyl radical pathway shown here lead to PEO fragments that carry the same end groups: alcohols (**I**) and aldehydes (**II**) via hemiacetal cleavage and other reactions and formate esters (**III**) via β-scission of a C—C bond. Products **I–III** are formed when PEO is cleaved by radiolytically generated ·OH (22–24).

Identification of New End Groups in Degraded PEO. To distinguish between oxygen insertion and hydrogen abstraction, we obtained ^{13}C NMR spectra of the products generated by *G. trabeum* from a 2×10^5 M_r unlabeled PEO. Unchanged PEO was recovered from uninoculated cultures after 13 days of incubation. The only significant NMR signal observed was caused by methylenes in internal repeating ethoxyl units (70.5 ppm). The two carbons in terminal hydroxyethyl groups (**Ia**, 61.4 ppm and **Ib**, 72.6 ppm) were undetectable because of their low frequency in this high-M_r polymer (Fig. 3*A*).

Prominent new end group signals appeared in PEO that had been degraded by *G. trabeum* for 13 days (Fig. 3*B*). The major terminal structures were alcohols (**Ia**, 61.4 ppm, ≈60% of total), formate esters (**IIIa**, 161.0 ppm, ≈30% of total), and aldehydes (**IIa**, 200.8 ppm, ≈10% of total). The other major NMR signals in the spectrum came from methylenes adjacent to the terminal alcohols (**Ib**, 72.6 ppm), aldehydes (**IIb**, 76.8 ppm), and formate esters (**IIIb**, 62.9 ppm and **IIIc**, 68.8 ppm). Integration of the end group and internal ^{13}C signals indicated that the material subjected to analysis had been depolymerized to a M_r of $\approx 2 \times 10^3$. Similar results were obtained when PEO was oxidized *in vitro* with Fenton reagent (Fig. 3*C*).

The identity of the formate ester was confirmed in several ways: (*i*) An authentic standard of PEO formate also exhibited signals **III a–c.** (*ii*) Distortionless enhancement with polarization transfer spectra of the degraded sample and of PEO formate showed that the carbon responsible for the 161.0-ppm signal (**IIIa**) carried a single proton. (*iii*) The degraded sample and PEO formate both exhibited a ^1H NMR signal characteristic of a formate ester proton at 8.1 ppm. A heteronuclear correlation experiment confirmed this assignment by giving a cross-peak between the 8.1-ppm proton signal and the 161.0-ppm carbon signal. (*iv*) When the degraded sample was hydrolyzed with aqueous NaOH, the 161.0-ppm ester carbonyl signal disappeared, the amplitude of the 61.4 ppm alcohol signal increased, and a new signal attributable to formic acid appeared at 162.3 ppm.

These data show that terminal formate esters were major products of PEO degradation by *G. trabeum*, but they do not exclude the possibility that these groups were derived from an esterification reaction between exogenous formate and new —CH_2OH termini on the cleaved polymer. To address this question, we repeated the biodegradation experiment with a 1.4×10^4 M_r perdeuterated PEO. ^{13}C NMR analysis of this sample showed a three-line signal centered at 160.8 ppm (coupling constant = 34 Hz), as expected for a deuterated formate ester carbonyl when the spectrum is not decoupled for deuterium (Fig. 3*B Inset*). This result proves that the formate ester was derived from the original PEO polymer and consequently that oxidative C—C bond cleavage is a major reaction in PEO degradation by *G. trabeum*.

Conclusions. These experiments show that *G. trabeum* produces a strong extracellular oxidant that leads to extensive PEO depolymerization by abstracting hydrogens from the polymer's internal methylene groups. One possibility is that this oxidant is Fenton reagent, which we found to cleave PEO by the same route *in vitro*. Previous work has shown that some brown-rot fungi produce extracellular Fe oxidoreductases (17) and Fe chelators (13, 15, 16, 18) that might reduce Fe^{3+} to Fe^{2+} as required for a Fenton mechanism, and it has been suggested that the necessary H_2O_2 might be produced via Fe^{2+} autooxidation (17).

Alternatively, the *G. trabeum* oxidant may be a less reactive free radical. The C—H bond dissociation energy for ether methylenes such as those in PEO is ≈90 kcal·mol^{-1} (33), which is approximately the same as the O—H bond dissociation energy for alkyl hydroperoxides (34). Therefore, the oxidant that initiates PEO cleavage could be a peroxyl radical or a similarly reactive species, and PEO peroxyl radicals could propagate the reaction. It is accordingly possible that the amount of initiating oxidant

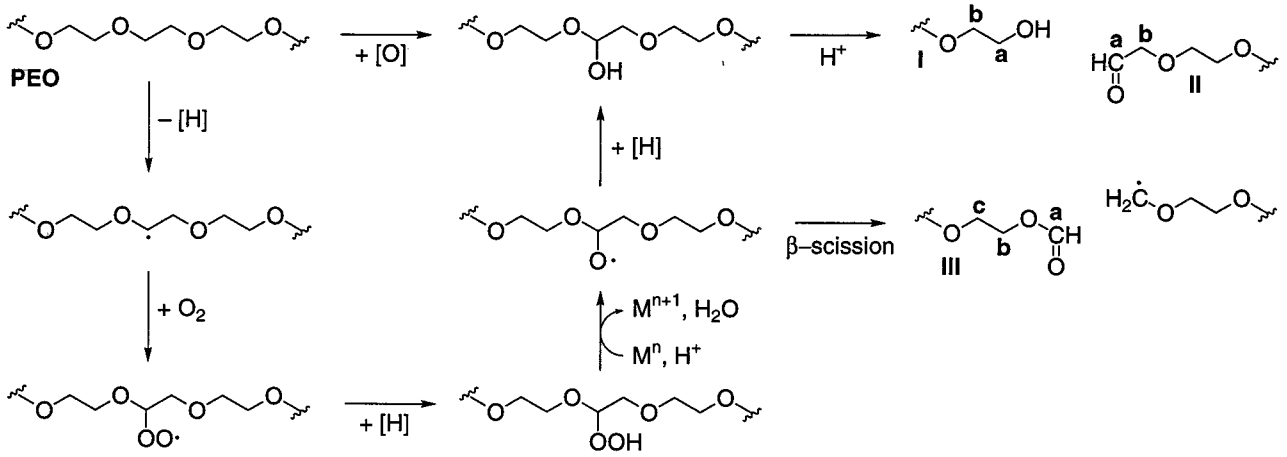

FIG. 2. Predicted pathways of PEO scission after oxygen insertion or hydrogen abstraction. Labeled structures are those identified in ^{13}C NMR experiments (see Fig. 3). M^n indicates a transition metal ion.

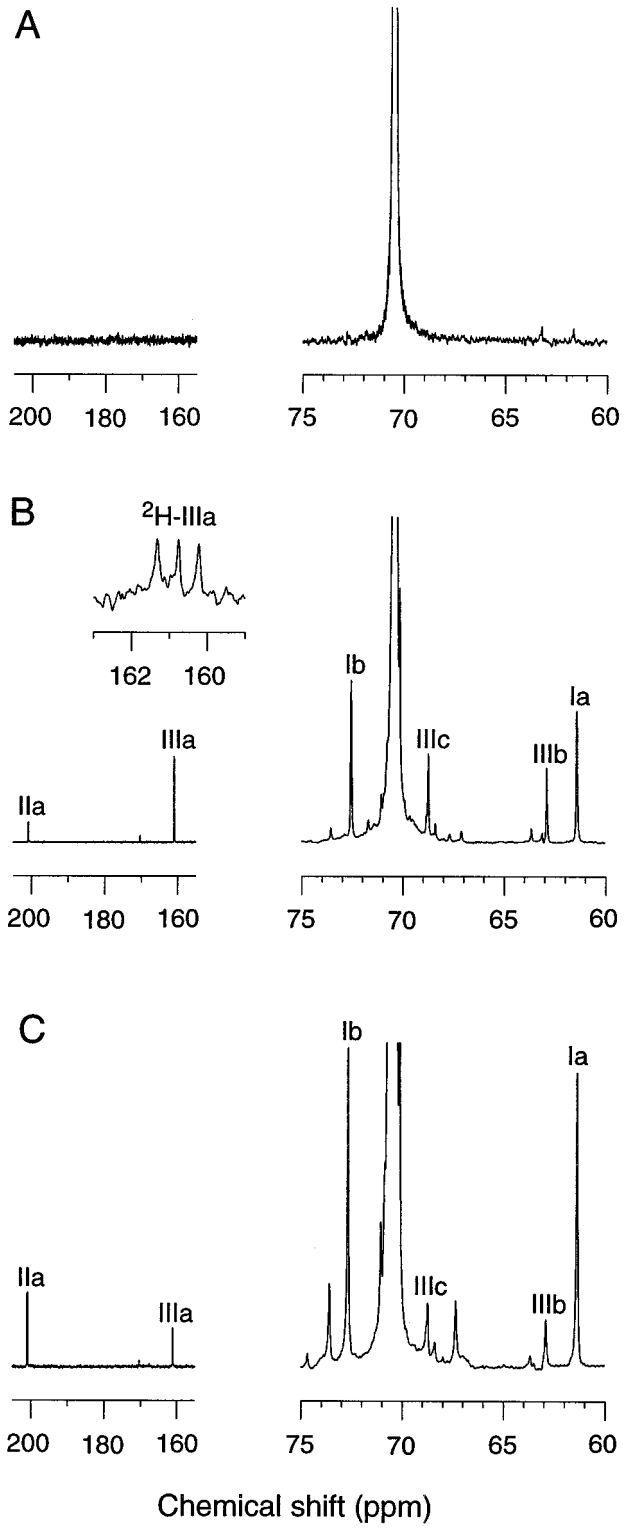

FIG. 3. ^{13}C NMR analyses of PEO. (*A*) PEO recovered from an uninoculated culture. (*B*) PEO recovered from a *G. trabeum* culture. (*Inset*) The spectrum obtained in the formate ester carbonyl region when perdeuterated PEO rather than natural abundance PEO was supplied. (*C*) PEO recovered after Fenton oxidation. Structures corresponding to the labeled NMR signals are shown in Fig. 2. The spectra have been normalized by setting the amplitude of the 70.5-ppm signals (which are truncated here) to a constant height. The signal for structure **IIb** was superimposed on the C^2HCl_3 signals and is not shown.

produced by the fungus is small relative to the amount of PEO cleaved, but, at present, we cannot distinguish between initiation and propagation reactions in this system.

We consider it likely that the *G. trabeum* oxidant's natural function is to participate in the brown rot of wood because the reactivity of C—H bonds in cellulose toward radical oxidants is expected to be similar to that of C—H bonds in PEO (33). However, a major difference between hydrogen abstraction from PEO and hydrogen abstraction from cellulose is that the latter process cannot yield chain-propagating peroxyl radicals on every carbon. Although α-alkoxyalkylperoxyl radicals can be formed at C1, C4, and C5 of each glucosyl unit in cellulose, α-hydroxyalkylperoxyl radicals will be produced instead at C2, C3, and C6, and will eliminate perhydroxyl radicals (·OOH) (21). However, at the high extracellular acidities (pH 2–4) generated by brown-rot fungi (17, 35), this ·OOH either could initiate new H-abstraction reactions from cellulose or could dismutate to provide H_2O_2 for Fenton oxidation of the polymer (32).

G. trabeum is unusual in its ability to degrade an aliphatic polyether via extracellular one-electron oxidation. Even white-rot basidiomycetes, which use radical chemistry to oxidize lignin and many other organic chemicals (4, 7, 36), are unable to depolymerize PEO significantly (28, 37). Our results and recent data from Wetzstein *et al.* (38) suggest that brown-rot fungi may be useful for organopollutant bioremediation as a consequence of the one-electron oxidants they produce.

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