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PROGRESS REPORT

Identification of the Primary Mechanism for Fungal Lignin Degradation
DE-FG02-94ER20140

MAJOR ACCOMPLISHMENTS

Many lignin-degrading fungi appear to lack lignin peroxidase (LiP), an enzyme generally thought important for fungal ligninolysis. We are working with one of these fungi, *Ceriporiopsis subvermispora*, an aggressive white-rotter that selectively removes lignin from wood. During this project period, we have obtained the following principal results:

- New polymeric lignin model compounds were developed to assist in the elucidation of fungal ligninolytic mechanisms.
- Experiments with one of the polymeric lignin models showed that *C. subvermispora* cultures which express no detectable LiP activity are nevertheless able to degrade nonphenolic lignin structures. This result is significant because LiPs were previously considered essential for fungal attack on these recalcitrant structures, which constitute about 90% of lignin.
- Manganese peroxidases (MnPs), which *C. subvermispora* does produce, catalyze the peroxidation of unsaturated fatty acids to give fatty acid hydroperoxides. Fatty acid hydroperoxides are also used by MnP as oxidants (in place of H_2O_2) that support the MnP catalytic cycle. These results indicate that MnP turnover in the presence of unsaturated lipids generates reactive lipid oxyradicals that could act as oxidants of other molecules.
- MnP-mediated lipid peroxidation results in the co-oxidative cleavage of nonphenolic lignin structures. The MnP/lipid peroxidation system may therefore provide *C. subvermispora* and other LiP-negative fungi with a mechanism to degrade the principal structures of lignin.

The project has deviated only in one respect from the plan we gave in our 1994 Project Summary. We did not know at that time that MnP-mediated lipid peroxidation might account for ligninolysis by *C. subvermispora*, and experiments in this area were not planned. These experiments were added, and the ^{13}C-labeling experiments we projected are planned for the proposed new project period instead. The ^{14}C-labeling experiments we projected are nearly complete, as is the synthesis of several ^{13}C-labeled lignin models we will need for the upcoming work.

PUBLICATIONS

Srebotnik, E., Jensen, K. A., Jr., and Hammel, K. E. (1994) Fungal degradation of recalcitrant nonphenolic lignin structures without lignin peroxidase. ***Proc. Natl. Acad. Sci. U.S.A.*** **91:12794-12797.** This work was begun before the current project period, and was partly supported by a USDA grant during that time.

Lignin peroxidases (LiPs) are generally considered likely catalysts of ligninolysis by white-rot fungi, because they have the unusual ability to depolymerize the major, recalcitrant, nonphenolic structures of lignin [1]. Some white-rot fungi have been reported to lack LiP when grown on defined media, but it is not clear whether they exhibit full ligninolytic competence under these conditions. To address this problem, we compared the abilities of a known LiP producer, *Phanerochaete chrysosporium*, with those of a reported nonproducer, *Ceriporiopsis subvermispora*, to degrade a synthetic lignin with normal phenolic content, a lignin with all phenolic units blocked, and a dimer, 1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol, that represents the major nonphenolic structure in lignin. *P. chrysosporium* mineralized all three models rapidly in the standard defined medium that is used

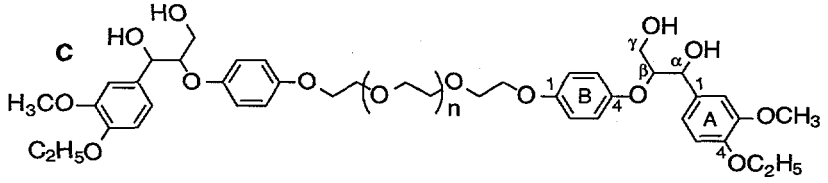
for most ligninolysis studies, but *C. subvermispora* showed much lower activity towards the nonphenolic models in defined medium. However, in wood, its natural environment, *C. subvermispora* mineralized all of the models as rapidly as *P. chrysosporium* did. The standard medium therefore fails to elicit a key component of the ligninolytic system in *C. subvermispora*. An isotope trapping experiment with the dimeric model showed that only negligible quantities of C_α-C_β cleavage product could be detected in the *C. subvermispora* cultures, and therefore we concluded that LiP did not play an important role in model compound degradation by this fungus. Our later work with a polymeric lignin model compound (see Jensen et al., below) has shown that this assessment was not entirely correct: C_α-C_β cleavage does in fact occur in *C. subvermispora* cultures. However, these later experiments have confirmed our original conclusion that LiP is not involved.

Bao, W., Fukushima, Y., Jensen, K. A., Jr., Moen, M. A., and Hammel, K. E. (1994) Oxidative degradation of nonphenolic lignin during lipid peroxidation by fungal manganese peroxidase. ***FEBS Lett.*** **354:297-300.** Funded entirely by this project.

In previous work, we showed that fungal manganese peroxidase (MnP) promotes the peroxidation of unsaturated fatty acids, and that a variety of polyaromatic hydrocarbons are co-oxidized during this reaction [2, 3]. *C. subvermispora* and many other LiP-negative fungi produce MnPs [4, 5], and therefore we were interested in the possibility that MnP-mediated lipid peroxidation might result in the co-oxidative cleavage of nonphenolic lignin structures. Results showed that a nonphenolic lignin model dimer, 1-(4-ethoxy-3-methoxyphenyl)-2-phenoxypropane-1,3-diol, was oxidized by a lipid peroxidation system that consisted of MnP, Mn(II), and unsaturated fatty acid esters. The reaction products included 1-(4-ethoxy-3-methoxyphenyl)-1-oxo-2-phenoxy-3-hydroxypropane and 1-(4-ethoxy-3-methoxyphenyl)-1-oxo-3-hydroxypropane, indicating that substrate oxidation occurred in part via benzylic hydrogen abstraction. The peroxidation system depolymerized both exhaustively methylated (nonphenolic) and unmethylated (phenolic) synthetic lignins efficiently. It may therefore enable white-rot fungi, especially those that lack LiP, to accomplish the initial delignification of wood.

Kawai, S., Jensen, K. A., Jr., Bao, W., and Hammel, K. E. (1995) New polymeric model substrates for the study of microbial ligninolysis. ***Appl. Environ. Microbiol.*** **61:3407-3414.** Funded by this project, except that one of the personnel (S. Kawai) did some of the work before the present project period, and was paid by a Consortium for Plant Biotechnology grant (T. K. Kirk, principal investigator).

Lignin model dimers are valuable tools for the elucidation of microbial ligninolytic mechanisms, but their low molecular weight (MW) makes them susceptible to nonligninolytic intracellular metabolism. This limitation was probably responsible for our inability to observe C_α-C_β cleavage of a low MW β-*O*-4-linked lignin model dimer when it was metabolized by *C. subvermispora* in wood (see Srebotnik et al. above). To address the problem, we prepared lignin models in which unlabeled and α-^{14}C-labeled β-*O*-4-linked dimers were covalently attached to 8000 MW polyethylene glycol (PEG) or to 45,000 MW polystyrene (PS). The water-soluble PEG-linked model **C**.



was mineralized extensively in liquid medium and in solid wood cultures of the white-rot fungus *Phanerochaete chrysosporium*, whereas the water-insoluble PS-linked model was not. Gel

permeation chromatography showed that *P. chrysosporium* degraded the PEG-linked model by cleaving its lignin dimer substructure rather than its PEG moiety. C_α-C_β cleavage was the major fate of the PEG-linked model after incubation with *P. chrysosporium* in vivo and also after oxidation with *P. chrysosporium* lignin peroxidase in vitro. The brown-rot fungus *Gloeophyllum trabeum*, which unlike *P. chrysosporium* lacks a vigorous extracellular ligninolytic system, was unable to degrade the PEG-linked model efficiently. These results show that PEG-linked lignin models are a marked improvement over the low MW models that have been used in the past.

Jensen, K. A., Kawai, S., Bao, W., Srebotnik, E., and Hammel, K. E. Manganese-dependent cleavage of nonphenolic lignin structures by *Ceriporiopsis subvermispora* in the absence of lignin peroxidase. Submitted to *Appl. Environ. Microbiol.* and favorably reviewed (see editor's letter attached to preprint). Funded entirely by this project.

Many ligninolytic fungi appear to lack lignin peroxidase (LiP), the enzyme generally thought to cleave the major, recalcitrant, nonphenolic structures in lignin. At least one such fungus, *Ceriporiopsis subvermispora*, is nevertheless able to degrade these nonphenolic structures. Experiments showed that wood specimen cultures and defined medium liquid cultures of *C. subvermispora* rapidly depolymerized and mineralized a ^{14}C-labeled, polyethylene glycol-linked, (PEG-linked) high MW β-*O*-4 lignin model compound (model **C**, see above) that represents the major nonphenolic structure of lignin. The fungus cleaved **C** between C_α and C_β to release benzylic fragments, which were shown in isotope trapping experiments to be major products of model **C** metabolism. The C_α-C_β cleavage of β-*O*-4 lignin structures to release benzylic fragments is characteristic of LiP catalysis, but assays of *C. subvermispora* liquid cultures that were metabolizing **C** confirmed that the fungus produced no detectable LiP activity. Three results pointed instead to the participation of a different enzyme, manganese peroxidase (MnP), in the degradation of nonphenolic lignin structures by *C. subvermispora*: (*a*) The degradation of **C** and of exhaustively methylated (nonphenolic) ^{14}C-labeled synthetic lignin by the fungus in liquid cultures was almost completely inhibited when the Mn concentration of the medium was decreased from 35 μM to approximately 5 μM. (*b*) The fungus degraded **C** and methylated lignin significantly faster in the presence of Tween 80, a source of unsaturated fatty acids, than it did in the presence of Tween 20, which contains only saturated fatty acids. Our previous work (see Bao et al., above) has shown that nonphenolic lignin structures are degraded during the MnP-mediated peroxidation of unsaturated lipids. (*c*) experiments with MnP, Mn^{2+}, and unsaturated lipid in vitro showed that this system mimicked intact *C. subvermispora* cultures in that it cleaved a nonphenolic β-*O*-4 lignin model compound between C_α and C_β to release a benzylic fragment.

Hammel, K. E., Mozuch, M. D., Jensen, K. A., Jr., and Kersten, P. J. (1994) H_2O_2 recycling during oxidation of the arylglycerol-β-aryl ether lignin structure by lignin peroxidase and glyoxal oxidase. *Biochemistry* 33:13349-13354. This work was begun before the current project period, and was partly supported by a USDA grant during that time.

These experiments were done at the beginning of the project period before we discovered that MnP-mediated lipid peroxidation results in the degradation of nonphenolic lignin structures. At that time, we were focusing on lignin peroxidase (LiP) and trying to determine what processes provide the extracellular H_2O_2 that this enzyme needs for turnover. We found that oxidative C_α-C_β cleavage of the arylglycerol-β-aryl ether lignin model 1-(3,4-dimethoxyphenyl)-2-phenoxypropane-1,3-diol by *Phanerochaete chrysosporium* LiP in the presence of limiting H_2O_2 was enhanced four- to fivefold by glyoxal oxidase from the same fungus. Further investigation showed that each C_α-C_β cleavage reaction released 0.8-0.9 equivalent of glycolaldehyde, a glyoxal oxidase substrate. The identification of glycolaldehyde was based on ^{13}C NMR spectrometry of reaction products obtained from β-, γ-, and β,γ-^{13}C-substituted model compound, and quantitation was based on an enzymatic NADH-linked assay. The oxidation of glycolaldehyde by glyoxal oxidase yielded 0.9 oxalate and 2.8 H_2O_2 per reaction, as shown by

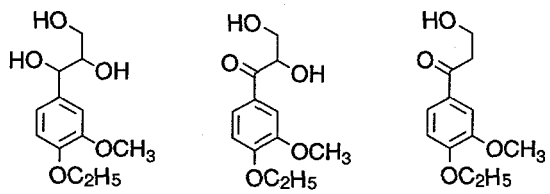
quantitation of oxalate as 2,3-dihydroxyquinoxaline after derivatization with 1,2-diaminobenzene, and by quantitation of H_2O_2 in coupled spectrophotometric assays with veratryl alcohol and LiP. These results suggest that the C_α-C_β cleavage of the model compound by LiP in the presence of glyoxal oxidase should regenerate as many as 3 H_2O_2. Calculations based on the observed enhancement of LiP-catalyzed C_α-C_β cleavage by glyoxal oxidase showed that approximately 2 H_2O_2 were actually regenerated per cleavage of model compound when both enzymes were present. The cleavage of arylglycerol-β-aryl ether structures by ligninolytic enzymes thus recycles H_2O_2 to support subsequent cleavage reactions.

UNPUBLISHED WORK IN PROGRESS.

In the final year of the current project , we intend to reach three goals:

- We will complete our analysis of the cleavage products that *C. subvermispora* forms from ^{14}C-labeled PEG-linked model **C**.
- We will finish preparing ^{13}C-labeled PEG-linked models that we anticipate will give an improved picture of the spectrum of degradative reactions that occur when *C. subvermispora* attacks nonphenolic lignin structures.
- We will begin to investigate the mechanism by which MnP reacts with lipid hydroperoxides and initiates the lipid peroxidation reactions that appear to be responsible for nonphenolic lignin degradation by *C. subvermispora.*

Experiments with ^{14}C-labeled model C. We have shown that nonphenolic β-*O*-4-linked lignin models undergo C_α-C_β and C_β-*O*-aryl cleavage during MnP-mediated lipid peroxidation in vitro, and we have also demonstrated in isotope trapping experiments that *C. subvermispora* cleaves the polymeric lignin model **C** to give products consistent with a C_α-C_β cleavage reaction. We do not yet know whether the intact fungus also yields C_β-*O*-aryl cleavage products, and therefore we plan isotope trapping experiments in which *C. subvermispora* cultures will be given radiolabeled model **C** and unlabeled isotope traps **K, L,** or **I**. All of the needed compounds are in hand, and the experimental procedures will be as we described in Jensen et al. (see above).



K **L** **I**

Experiments with ^{13}C-labeled PEG-linked lignin models. Experiments with ^{14}C-labeled model **A** are yielding valuable data about ligninolytic reactions in *C. subvermispora*, but because this fungus rapidly metabolizes the diagnostic low MW fragments that are produced, it is necessary to add isotope traps to the cultures. It is possible that these compounds perturb ligninolytic metabolism in unpredictable ways, and therefore it would be better to follow degradation by monitoring functional group changes that occur on the residual extracellular polymer rather than on the low MW products that are released. To address this problem, we will use ^{13}C NMR spectrometry to analyze functional group changes that white-rot fungi bring about in specifically ^{13}C-labeled polymeric lignin models. This approach has many advantages over NMR analysis of lignin itself: the principal β-*O*-4 structure of lignin is represented without interference from the numerous minor structures that are present in the natural polymer, and moreover only one carbon in this structure is detected. The resulting spectra are much simpler to interpret than those of natural lignin, and individual functional group changes are easily

pinpointed and characterized because an extensive database of diagnostic ^{13}C NMR chemical shifts has been accumulated by lignin NMR researchers.

We have completed the synthesis and characterization of one β-*O*-4 lignin model that is specifically ^{13}C-labeled at C_α of the propyl sidechain and which is attached to PEG via the phenylpropane ring (FIG. 1). The model exhibits a simple ^{13}C NMR spectrum with only two major signals. One of these is due to the enriched α-carbon (74.0 ppm) and the other to the natural abundance repeating internal methylene carbon of the PEG backbone (70.6 ppm). The other natural abundance signals due to the lignin model and to the nonrepeating methylenes at each end of the PEG are much weaker in relative intensity and therefore will not interfere with the analysis of functional group changes after fungal degradation. Other ^{13}C-labeled models will be prepared during the remainder of this project period and NMR experiments are planned to commence with the new project. These experiments are described in more detail in our proposal.

Experiments on the MnP/lipid peroxidation system. The results we have so far indicate that lipid oxyradicals are produced by MnP and may be the species that degrade nonphenolic lignin structures in *C. subvermispora*. To understand how these reactions occur, we need to identify the oxyradical species that are produced by the MnP/lipid peroxidation system. In our original work [3], we showed that the enzyme oxidizes linolenic acid (C18:3) to give products that give a color reaction in the standard thiobarbituric acid assay, and we showed that it oxidizes linoleic acid (C18:2) to give products that include the late-stage scission product hexanal. These reactions require Mn^{2+}. They proceed most rapidly in the presence of H_2O_2, but also occur after a brief lag in its absence.

We have now identified the initial products of linoleic acid peroxidation by MnP/Mn^{2+} as the *E,E* and *E,Z* isomers of 9- and 13-hydroperoxyoctadeca-9,11-dienoic acid (FIG. 2), using an HPLC procedure that resolves these isomers [6]. This product profile is virtually the same as the one we obtain by autooxidation of linoleic acid (data not shown), and therefore it can be concluded that MnP oxidizes linoleic acid with little or no regioselectivity. In this regard, MnP differs from myoglobin, which displays H_2O_2-dependent lipoxygenase activity, as well as from the true lipoxygenases [7]. The lack of regioselectivity in the MnP-catalyzed reaction is preliminary evidence that specific binding of the fatty acid near the MnP heme is not involved in lipid peroxidation by this enzyme.

If MnP does not act directly on unsaturated fatty acids as a lipoxygenase, another likely mechanism is that it might react with fatty acid hydroperoxides (LOOH), which are always present in unsaturated lipids when O_2 is present, to produce oxidants that promote lipid peroxidation away from the enzyme active site. The two principal mechanisms by which this could occur are outlined in the main proposal, but can be summarized briefly here as follows: (*a*) MnP might undergo catalytic turnover with fatty acid hydroperoxides in the same way that it does with H_2O_2, cleaving them heterolytically and yielding Mn^{3+}. Mn^{3+} would then oxidize other hydroperoxide molecules to give peroxyl radicals, which are propagators of the oxyradical chain reaction that leads to lipid peroxidation. (*b*) MnP might cleave lipid hydroperoxides homolytically to give alkoxyl radicals, which also propagate lipid peroxidation and are considerably more reactive than peroxyl radicals [8]. The two mechanisms are not mutually exclusive, i.e. the enzyme might be capable of both.

We have begun experiments to address these questions. When MnP (isozyme H4, 1 μM) was combined with 30 equivalents of 9-hydroperoxyoctadeca-9(*Z*),11(*E*)-dienoic acid at pH 4.5 and ambient temperature in sodium tartrate buffer, the visible absorption spectrum of the MnP heme showed a gradual decrease at its 407 nm Soret maximum after 4 min of incubation. After 10 min of incubation, the absorption maximum was red-shifted to 416 nm (FIG. 3). At this point excess (200 μM) Mn^{2+} was added, and the spectrum shifted back to that of the original resting state enzyme (not shown). The following control experiments were also done: (*a*) if 1 standard unit of

catalase was included in the incubation, the spectral changes occurred just as they did without catalase; (*b*) if lipid hydroperoxide was replaced with an equivalent amount of H_2O_2, the addition of 1 unit of catalase completely inhibited the spectral changes. These results show that linoleic acid hydroperoxide supported MnP turnover even in the presence of catalase at a level that would have scavenged any H_2O_2 that was present. Therefore, the results are not attributable to contamination of the sample with H_2O_2, and linoleate hydroperoxide must support MnP turnover.

The initial decrease in the Soret band at 407 nm is probably attributable to the formation of the two electron-oxidized form of the enzyme, Compound I:

$$MnP\text{–}Fe(III) + LOOH + 2H^+ \rightarrow MnP^{+\bullet}\text{–}Fe(IV) + LOH + H_2O,$$

and the subsequent red-shift in the spectrum is probably due to the oxidation by Compound I of a second molecule of lipid hydroperoxide to give the one electron-oxidized form of the enzyme, Compound II [9]:

$$MnP^{+\bullet}\text{–}Fe(IV) + LOOH \rightarrow MnP\text{–}Fe(IV) + LOO\bullet + H^+.$$

In this experiment, a heterogeneous mixture of MnP electronic states was clearly produced. Compound II formation did not proceed to completion, and it is additionally possible that subsequent oxidation of Compound II occurred to give MnP Compound III, which is also red-shifted, but the preliminary data do not permit a clear assignment of the structures. The results lead us to the tentative conclusion that lipid hydroperoxides are cleaved by MnP in a heterolytic reaction, and that peroxyl radicals will therefore be produced. During the remainder of the present project period, we will investigate the reaction of MnP with lipid hydroperoxides added in differing stoichiometries, which will allow us to determine whether MnP Compound I is in fact formed. The proposed new project period will include experiments to determine whether homolytic peroxide cleavage also occurs to give alkoxyl radicals, and these experiments are described in the main part of the proposal.

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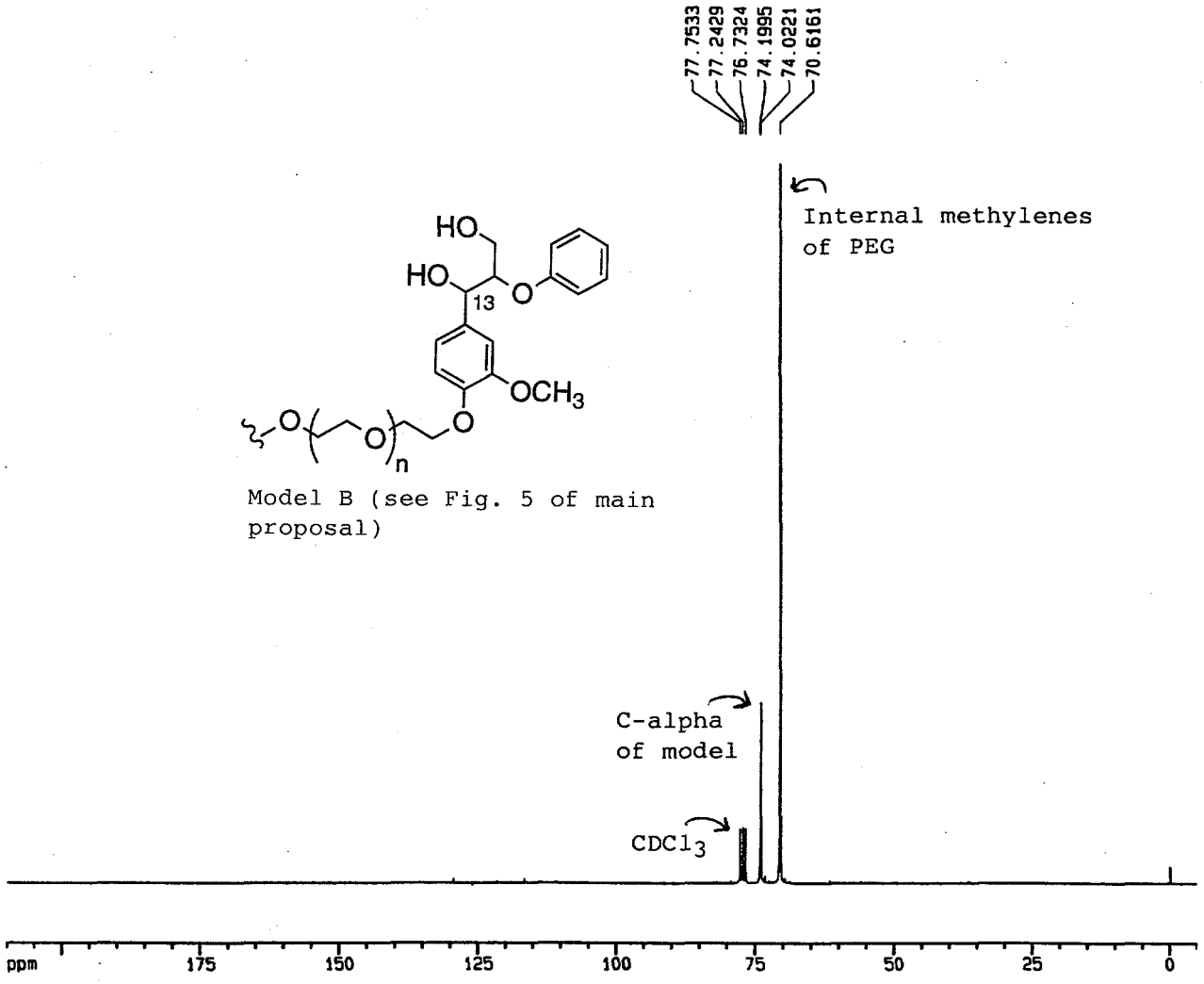

FIG. 1. ^{13}C NMR spectrum in deuterochloroform of a beta-O-4 lignin model compound that is 100% enriched with ^{13}C at C-alpha and linked to polyethylene glycol (PEG) at the 4-position of its phenylpropane moiety.

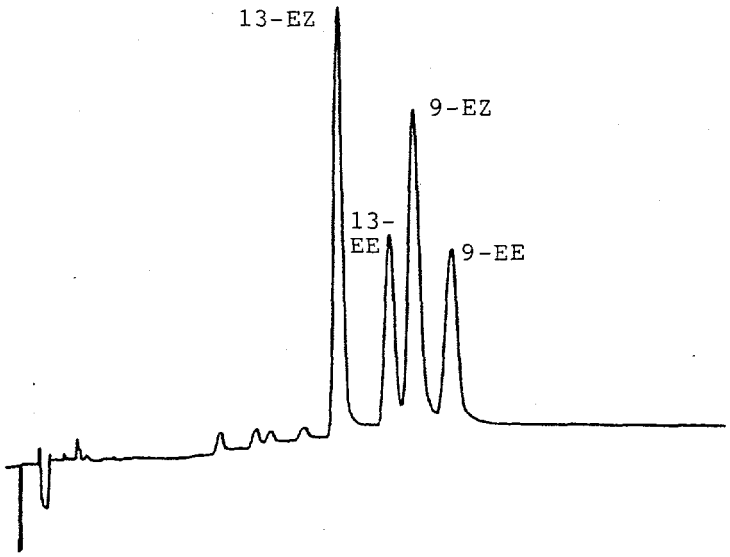

FIG. 2. Isomers of linoleic acid hydroperoxide formed by the action of P. chrysosporium MnP on linoleic acid. HPLC was performed as in ref. (6). Detection was at 234 nm. For reaction conditions see ref. (3).

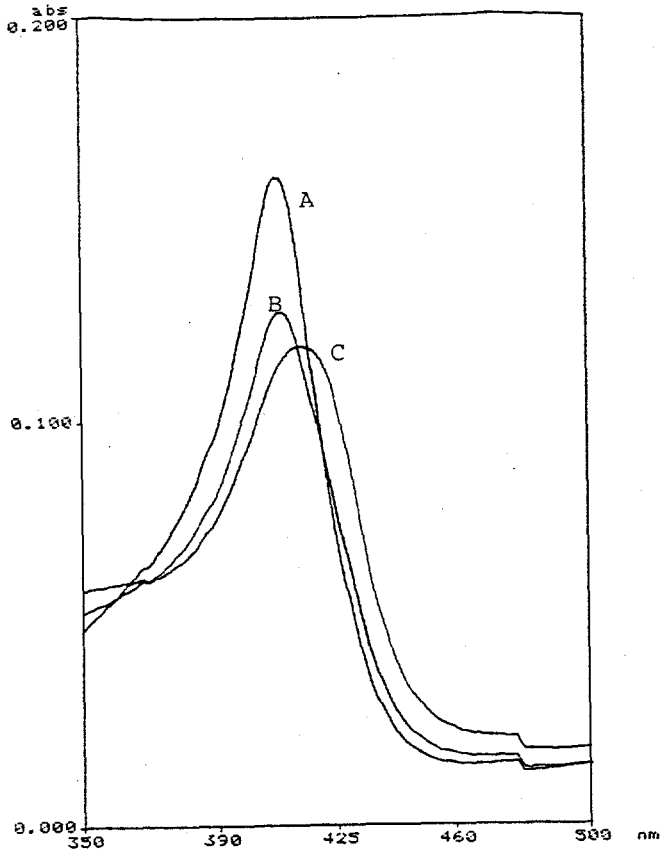

FIG. 3. UV absorption spectra of P. chrysosporium MnP in the resting state (A), 4 min after 30 equiv. of linoleate hydroperoxide was added (B), and 10 min after the hydroperoxide was added (C). Addition of $MnSO_4$ returned the spectrum to state (A). See text for details.