FEBS Letters 354 (1994) 297–300

FEBS 14759

Oxidative degradation of non-phenolic lignin during lipid peroxidation by fungal manganese peroxidase

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Received 3 October 1994

Abstract A non-phenolic lignin model dimer, 1-(4-ethoxy-3-methoxyphenyl)-2-phenoxypropane-1,3-diol, was oxidized by a lipid peroxidation system that consisted of a fungal manganese peroxidase, 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 via benzylic hydrogen abstraction. The peroxidation system depolymerized both exhaustively methylated (non-phenolic) and unmethylated (phenolic) synthetic lignins efficiently. It may therefore enable white-rot fungi to accomplish the initial delignification of wood.

Key words: Manganese peroxidase; Lipid peroxidation; Ligninolysis; Non-phenolic lignin; White-rot fungus

1. Introduction

White-rot fungi have evolved unique ligninolytic mechanisms, which enable them to play an essential role in terrestrial ecosystems by degrading and recycling lignified biomass. The chemical recalcitrance, random structure, and large size of lignin require that these mechanisms be oxidative, non-specific, and extracellular [1]. The non-phenolic structures that comprise approximately 90% of lignin present a particular problem, because they resist oxidation by most known biochemical agents.

Many white-rot fungi produce highly oxidizing extracellular lignin peroxidases that cleave the non-phenolic linkages of lignin directly [1–3], and it is likely that these enzymes provide one important route for fungal ligninolysis. However, other ligninolytic fungi appear to lack lignin peroxidase [4–6], and it has recently been shown that one such basidiomycete, *Ceriporiopsis subvermispora*, degrades non-phenolic lignin structures in wood without significant involvement of this enzyme [7]. Other, as yet undiscovered, mechanisms of fungal ligninolysis must exist.

Manganese peroxidases (MnPs) are a second group of extracellular enzymes secreted by white-rot fungi. MnPs operate by oxidizing Mn(II) to chelated Mn(III), which acts as a diffusible oxidant at locations remote from the enzyme active site [8,9]. Although MnPs are widely distributed in white-rot fungi [5], they have not been considered efficient ligninolytic agents because Mn(III) chelates are weak oxidants under physiological conditions. The MnP/Mn couple oxidizes phenolic lignin structures, which are minor components of lignin [10], but fails to oxidize or depolymerize non-phenolic lignin [3].

Recently, we reported that MnP promotes the peroxidation of unsaturated fatty acids, and that this system accomplishes the oxidative cleavage of phenanthrene, a polycyclic aromatic hydrocarbon that otherwise resists oxidation by fungal peroxidases [11]. Here we show that the MnP/lipid peroxidation system cleaves a non-phenolic lignin model compound oxidatively, and that it depolymerizes both non-phenolic and phenolic lignin.

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2. Materials and methods

2.1. Reagents

1-(4-Ethoxy-3-methoxyphenyl)-2-phenoxypropane-1,3-diol (**I**) [12], 1-(4-ethoxy-3-methoxyphenyl)-1-oxo-2-phenoxy-3-hydroxypropane (**II**) [13], and 1-(4-ethoxy-3-methoxyphenyl)-1-oxo-3-hydroxypropane (**III**) [14] were prepared by the indicated methods. I labeled with ^{14}C at C1 (0.066 mCi·mmol^{-1}) was prepared in the same manner as the unlabeled compound, using [1-^{14}C]acetic acid as the labeled precursor.

Synthetic guaiacyl lignin labeled with ^{14}C at C2 of the propyl sidechain (0.01 mCi·mmol^{-1} of phenylpropane substructures) and an exhaustively methylated preparation of the same lignin were prepared as described previously [3].

MnP isozyme H4 was purified from N-limited cultures of *Phanerochaete chrysosporium* by ion-exchange chromatography, followed by chromatography on Cibacron blue agarose [3]. The preparation was free of detectable lignin peroxidase activity.

Tween 20, a mixture of polyoxyethylene sorbitan esters of saturated fatty acids (predominantly 12:0, 14:0, and 16:0), and Tween 80, a mixture of polyoxyethylene sorbitan esters of unsaturated and saturated fatty acids (predominantly 18:1, 18:2, 16:0, and 18:0), were the purified low peroxide/low carbonyl grade (Surfact-Amps) from Pierce Chemical Co. Monolinolein (monolinoleoyl-rac-glycerol) was obtained from Nu Chek Prep. Lipids and surfactants were stored under argon at −20 °C. All other chemicals were reagent grade or better.

2.2. Oxidation of I

I was oxidized with MnP in the presence of unsaturated lipids by a modification of the procedure used earlier to oxidize phenanthrene [11]. The reactions (2.0 ml, rotary-shaken at ambient temperature) contained ^{14}C-labeled I (340 μM, 1.0×10^5 dpm, added as a stock solution in 25 μl methanol), Tween 80 or 20 (1.0%), and $MnSO_4$ (0.4 mM), in Na-tartrate (20 mM, pH 4.5). Reactions done with monolinolein (10 mM) as the source of unsaturated lipid, as well as controls without monolinolein, were done with 3.0% Tween 20 as the surfactant. Reactions were initiated with 0.04 μM MnP, and an additional 0.04 μM enzyme was added at 24 h intervals for a total reaction time of 112 h.

A portion (0.50 ml) of each reaction mixture was then subjected to reverse-phase HPLC on a styrene divinylbenzene column (PRP-1; Hamilton). The column was eluted at 1.0 ml·min^{-1} and ambient temperature with acetonitrile/H_2O (15:85) for 15 min, followed by a 35 min linear gradient to acetonitrile/H_2O (65:35). Fractions (1.0 ml) were collected and analyzed for ^{14}C by scintillation counting. Preliminary product identifications were obtained by comparing retention times with those of authentic standards, and were then confirmed by gas chromatography/electron impact mass spectrometry (GC/MS) [15] of pooled and extracted HPLC fractions from a scaled-up reaction.

2.3. Oxidation of synthetic lignins

Synthetic lignins were oxidized in reactions (40.0 ml, rotary-shaken at ambient temperature) that contained ^{14}C-labeled lignin (180 μg,

SSDI 0014-5793(94)01146-X

2.0×10^4 dpm, added as a stock solution in 25 μl *N,N*-dimethylformamide), Tween 80 or 20 (0.25%), and $MnSO_4$ (0.4 mM), in Na-tartrate (10 mM, pH 4.5). Reactions were initiated with 0.04 μM MnP, and an additional 0.04 μM enzyme was added at 24 h intervals for a total reaction time of 72 h.

The completed reactions were concentrated by rotary vacuum evaporation, clarified by centrifugation, and subjected to gel-permeation chromatography (GPC) on a 1.8×33 cm column of Sephadex LH60 in *N,N*-dimethylformamide that contained 0.1 M LiCl, all steps being carried out as described previously [3]. Post-reaction mass balances for experiments with methylated lignin showed that both control and complete reactions gave 70–76% soluble ^{14}C, 13–19% insoluble ^{14}C, and 2–5% ^{14}C on the glassware. Control and complete reactions with unmethylated lignin both gave ^{14}C that was 60–70% soluble and 17–28% insoluble, with 2–9% on the glassware.

The molecular weight distributions of soluble treated and control ^{14}C-labeled lignins were determined by scintillation counting of the collected (1.5 ml) GPC fractions. Nominal weight-average molecular weights (M_w) and number-average molecular weights (M_n) of the chromatographed lignins were calculated using the standard formulas [16], based on calibrations of the GPC column with polystyrene standards.

3. Results and discussions

3.1. Oxidation of I

Model dimer **I**, which represents the major arylglycerol-2-aryl ether substructure of lignin, was oxidized in the presence of MnP, Mn(II), Tween 80, and oxygen. Exogenous H_2O_2 was found to be unnecessary for the reaction, as observed previously for the oxidation of phenanthrene by this system [11]. HPLC showed that the complete system converted **I** to **II** and a complex mixture of products more polar than **I**. The yields of **II** and of polar oxidation products in 112 h were 26% and 20%, respectively.

GC/MS analysis of the major oxidation product confirmed that it was **II**. MS: (TMS ether) (*m/z*, relative intensity) 388 (M^+, 9), 373 (1), 268 (31), 179 (100), 151 (25). GC/MS also showed that the HPLC fractions eluting at 27–29 min contained **III**, a monomeric product derived from the cleavage of **I**. MS: (TMS ether) (*m/z*, relative intensity) 296 (M^+, 47), 281 (63), 253 (15), 223 (14), 206 (61), 179 (100), 151 (65). The chromatographic

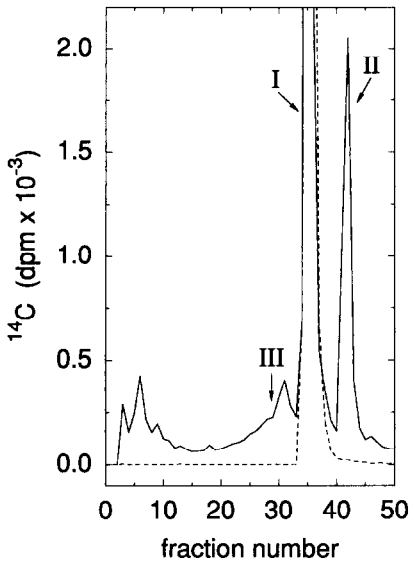

Fig. 1. HPLC analysis of products from the oxidation of **I** with the MnP/lipid peroxidation system, in reactions with (——) and without (- - -) monolinolein. The total ^{14}C per chromatogram was 2.5×10^4 dpm.

retention times and mass spectra obtained with authentic standards of **II** and **III** were the same as those found for the reaction products.

I was not oxidized when MnP or Mn(II) was omitted, when the free radical scavenger butylated hydroxytoluene (300 μM) was added, or when the reaction was conducted under argon. There was also no reaction when Tween 80, the source of unsaturated fatty acid esters, was replaced with Tween 20, which contains saturated fatty acid esters. However, reaction mixtures containing Tween 20 did oxidize **I** when they were supplemented with the unsaturated fatty acid ester monolinolein. The yields of **II** and of polar oxidation products in 112 h under these conditions were 16% and 22%, respectively (Fig. 1).

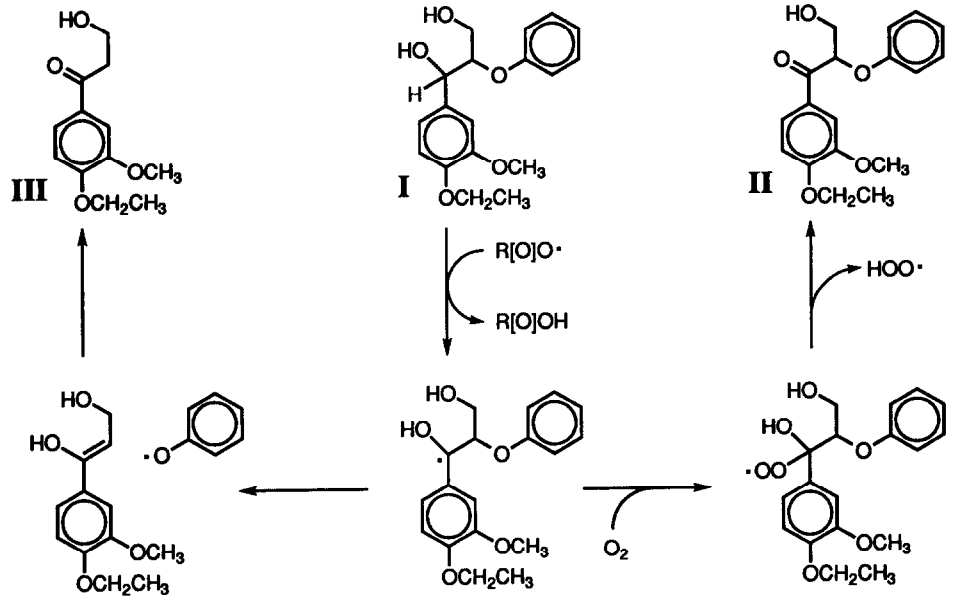

Fig. 2. Proposed scheme for the oxidation of **I** to **II** and **III**. R represents a moiety derived from unsaturated lipid.

This result shows that the difference between Tween 80 and Tween 20 in their ability to support I oxidation was not due merely to differences in their surfactant properties, but rather to the fact that one contains unsaturated lipids whereas the other does not.

The data show that the oxidation of I was associated with MnP-mediated lipid peroxidation. The formation of products II and III is most easily explained by a reaction mechanism in which the benzylic hydrogen at C1 of the substrate is abstracted, breaking the relatively weak C–H bond at that position [17] and yielding a resonance-stabilized benzylic C-centered radical. Addition of O_2 at C1 followed by loss of ·OOH [15] then yields II, whereas homolytic C–O fission at C2 expels a phenoxy radical and yields the enol form of III (Fig. 2). This mechanism is the same as that demonstrated earlier for the thiyl radical-mediated oxidation of a non-phenolic lignin model dimer by MnP in the presence of thiols [14]. However, white-rot fungi do not produce extracellular thiols [14], whereas they do contain extracellular membrane lipids. The proximal oxidant of I in the system we describe here is unknown, but lipid-derived peroxy or alkoxy radicals are likely candidates.

3.2. Ligninolysis

The results obtained with I show that MnP-mediated lipid peroxidation co-oxidizes the major non-phenolic subunit of lignin, but they do not indicate whether a significant proportion of the diverse oxidation products formed result from cleavage reactions that could lead to ligninolysis. To address this problem, we investigated the ability of the system to depolymerize non-phenolic and phenolic lignins.

Exhaustively methylated synthetic lignin was efficiently depolymerized by the MnP/lipid peroxidation system in 72 h, giving products with M_W = 7000 and M_n = 1700 (Fig. 3). By contrast, a control reaction done with Tween 20 instead of Tween 80 failed to depolymerize the lignin, giving products with M_W = 11,300 and M_n = 6100 that resembled the starting material. Control reactions lacking MnP or Mn(II) gave similar negative results. We conclude that the MnP/lipid peroxidation system, unlike MnP alone, depolymerizes the non-phenolic units of lignin.

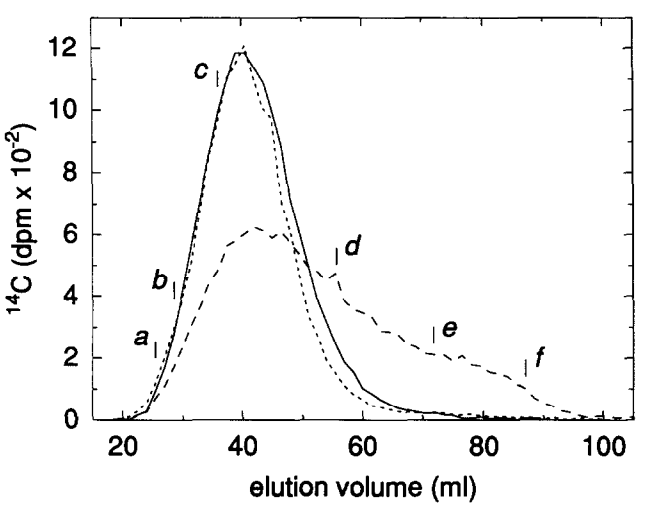

Fig. 3. GPC molecular weight distributions of exhaustively methylated lignin treated for 72 h with the MnP/lipid peroxidation system, using Tween 80 as the lipid source (– – –), using Tween 20 as the lipid source (- - -), and using Tween 80 but without Mn(II) (——). The designations a–e correspond to the elution positions of polystyrene standards with the following molecular weight values: a, 34,500 (excluded from gel); b, 22,000; c, 10,000; d, 3,100; e, 1,050. Standard f was veratraldehyde (166).

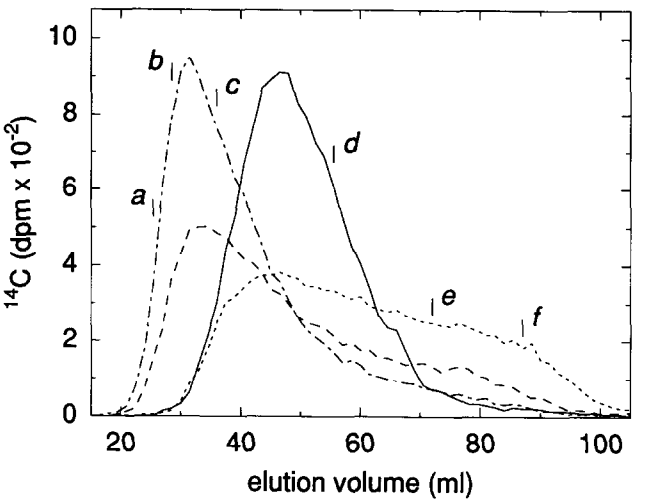

Fig. 4. GPC molecular weight distributions of phenolic lignin treated with the MnP/lipid peroxidation system in the presence of Tween 80 for 24 h (–·–·–), 48 h (– – –), and 72 h (- - -), and of a 72 h reaction done without Mn(II) (——). Molecular weight standards are as described in Fig. 3.

The system also degraded synthetic lignin with a normal (approx. 10%) phenolic content. Oxidation for 24 h caused polymerization of the lignin, presumably via coupling reactions between MnP-generated lignin phenoxy radicals, but net depolymerization resulted after 72 h, giving products with M_W = 4200 and M_n = 800 (Fig. 4). By contrast, a control reaction without Mn(II) failed to depolymerize the lignin, giving products with M_W = 6000 and M_n = 2800 that resembled the starting material. Therefore, the MnP/lipid peroxidation system is ligninolytic even in the presence of polymerizable phenols.

Past work has shown that the extracellular peroxidases of white-rot fungi are associated with the cell membrane and with extracellular membranous structures [18,19]. MnP-mediated lipid peroxidation is likely in this environment, and would generate lipid peroxy radicals. These radicals are relatively stable, with half-lives measured in seconds [20]. They are also effective hydrogen- abstracting agents [21], and are small enough to enter pores in wood where enzymes such as lignin peroxidase cannot penetrate [22]. We propose that white-rot fungi use MnP-mediated lipid peroxidation to accomplish the initial delignification of wood.

Acknowledgements: We thank R. Pettersen for mass spectral analyses. Supported by US Department of Energy Grant DE-FG02–94ER20140 (to K.E.H.).

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