Vol. 176, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1991 Pages 269-275

IN VITRO DEPOLYMERIZATION OF LIGNIN BY MANGANESE PEROXIDASE OF Phanerochaete chrysosporium

Hiroyuki Wariishi, Khadar Valli, and Michael H. Gold+

Dept. of Chemical & Biological Sciences, Oregon Graduate Institute of Science & Technology, 19600 N.W. Von Neumann Drive, Beaverton, OR 97006-1999

Received February 19, 1991

Summary: Homogeneous manganese peroxidase catalyzed the in vitro partial depolymerization of four different ^{14}C-labeled synthetic lignin preparations. Gel permeation profiles demonstrated significant depolymerization of ^{14}C-sidechain-labeled syringyl lignin, a ^{14}C-sidechain-labeled syringyl-guaiacyl copolymer (angiosperm lignin), and depolymerization of ^{14}C-sidechain- and ^{14}C-ring-labeled guaiacyl lignins (gymnosperm lignin). 3,5-Dimethoxy-1,4-benzoquinone, 3,5-dimethoxy-1,4-hydroquinone, and syringylaldehyde were identified as degradation products of the syringyl and syringyl-guaiacyl lignins. These results suggest that manganese peroxidase plays a significant role in the depolymerization of lignin by Phanerochaete chrysosporium.

The white rot basidiomycetous fungus Phanerochaete chrysosporium secretes two extracellular heme peroxidases which are thought to constitute the major components of its lignin degradative system (1,2). Lignin peroxidase catalyzes the oxidative cleavage of nonphenolic lignin model compounds by a mechanism involving the formation of a substrate aryl cation radical (1-4). A recent report demonstrated that crude lignin peroxidase preparations catalyzed at least the partial depolymerization of synthetic syringyl/guaiacyl lignin (5).

Manganese peroxidase catalyzes the oxidation of Mn^{II} to Mn^{III} and the latter, complexed with an organic acid, readily oxidizes phenolic lignin model dimers (6-10). It has been proposed that the freely diffusible, enzyme-generated Mn^{III}-organic acid complex would be an excellent catalyst for the oxidative depolymerization of lignin, an insoluble phenolic polymer which may be partially inaccessible to lignin peroxidase (2,6-10). Herein, we report for the first time that in the presence of Mn^{II} and H_2O_2, homogeneous manganese peroxidase catalyzes the effective depolymerization of synthetic syringyl, syringyl/guaiacyl, and guaiacyl lignins in vitro.

+ To whom correspondence should be addressed.

Abbreviations used: DHP, dehydropolymerizate; GCMS, gas chromatography/mass spectrometry; HMW, high molecular weight fraction; LMW, low molecular weight fraction; R-G*-DHP, ring-labled guaiacyl DHP; SC-G*-DHP, Cβ sidechain-labeled guaiacyl DHP; SC-S*-DHP, Cβ sidechain-labeled syringyl DHP; SC-SG*-DHP, syringyl-guaiacyl copolymer, Cβ sidechain-labeled in the guaiacyl moiety.

0006-291X/91 $1.50

MATERIALS AND METHODS

Enzymes: Manganese peroxidase was purified from cultures of P. chrysosporium and enzyme concentrations were determined as described (6,9). Glucose oxidase was purchased from Boehringer Mannheim.

Preparation of radiolabeled lignins: 2-[^{14}C]-sidechain-labeled guaiacyl-DHP (SC-G*-DHP) and [^{14}C]-U-ring-labeled guaiacyl-DHP (R-G*-DHP) were prepared from 2-[^{14}C]-1-(4-hydroxy-3-methoxyphenyl)-3-hydroxy-1-propene and [^{14}C]-U-ring-1-(4-hydroxy-3-methoxyphenyl)-3-hydroxy-1-propene as described (11). 2-[^{14}C]-sidechain-labeled syringyl/guaiacyl-DHP (2-[^{14}C] in guaiacyl unit, SC-SG*-DHP) was prepared by the copolymerization of 2-[^{14}C]-1-(4-hydroxy-3-methoxyphenyl)-3-hydroxy-1-propene and unlabeled 1-(3,5-dimethoxy-4-hydroxyphenyl)-3-hydroxy-1-propene as previously described (12). 2-[^{14}C]-sidechain-labeled syringyl-DHP (SC-S*-DHP) was prepared as previously described (13). Each synthetic lignin was fractionated by gel permeation chromatography through a Sephadex LH-20 column (1.8 x 50 cm, eluted with dioxane:H_2O 9:1) to remove low-molecular-weight material prior to the depolymerization experiments. All 4 DHPs eluted with the void volume, indicating a molecular weight >2000. SC-G*-, R-G*-, SC-SG*-, and SC-S*-DHP had specific radioactivities of 3.3×10^5, 3.2×10^5, 5.1×10^5, and 6.3×10^5 cpm/mg, respectively.

Enzyme reactions: In vitro depolymerizations were carried out at 37°C for the indicated times under 100% O_2 in 50 mM Na-malonate, pH 4.5 (1.0 ml), containing manganese peroxidase (5 μg), DHP (~5 x 10^4 cpm), $MnSO_4$ (0.2 mM), glucose (2.5 mM), and glucose oxidase (0.025 U). DHPs were added as DMF solutions (30 μl/ml). H_2O_2 was continuously generated at the rate of 6.3 nmol/min during the course of the reactions.

Analysis of lignin degradation: After completion of the reactions, 1.0 ml of dioxane was added and aliquots were removed and radioactivity was counted in EcoLite (ICN Biochemicals) before and after centrifugation (3000 rpm, 2 min) to determine the amount of reaction-generated insoluble material. Subsequently, 0.5 ml of supernatant was mixed with 1.5 ml of dioxane and applied to a Sephadex LH-20 column (1.2 x 40 cm) equilibrated in degassed dioxane-H_2O (9:1) (14). The column was eluted with dioxane-H_2O (9:1), and 1-ml fractions were collected and counted. The column was calibrated using standard model compounds (10,11). The HMW fraction (tubes 11-20) and LMW fraction (tubes 21-35) were pooled and counted.

Analysis of monomeric degradation products: LH-20 column fractions 28-32 from the oxidation of SC-SG*- and SC-S*-DHPs, corresponding to the monomer elution peak, were pooled. After acidification and extraction with chloroform, the products were derivatized (BSTFA:pyridine 2:1) and analyzed by GCMS (10).

RESULTS

Time dependence of DHP degradation by manganese peroxidase: Table I shows the recovery of ^{14}C-labeled products in the HMW, LMW, and insoluble fractions for reactions with each labeled DHP. For each reaction the total radioactivity recovered approached 100%. Oxidation of SC-G*-DHP for 30 min resulted in significant depolymerization (19% of the radioactivity in the LMW fraction) with an equal amount of insoluble polymer formed. Further reaction resulted in an increase in the insoluble polymeric material. The profile for R-G*-DHP was similar; however, after 30 min, 25% of the material was recovered in the LMW and only 15% was recovered as insoluble polymer. The syringyl-guaiacyl polymer

Table I

Recovery of fractions from the manganese peroxidase-catalyzed oxidation of 14C-labeled DHPs [a]

Substrate	Reaction time (hrs)	% Recovery (fractions)[b]			
		HMW	LMW	Insoluble	Total
SC-G*-DHP	0	92	3	<1	95
	0.5	61	19	18	98
	1.0	40	14	39	93
R-G*-DHP	0	96	3	<1	99
	0.5	53	25	15	93
	1.0	38	16	38	92
SC-SG*-DHP	0	91	6	1	98
	0.5	40	28	27	95
	1.0	18	21	52	91
SC-S*-DHP	0	91	4	1	96
	0.5	81	10	3	94
	1.0	74	12	8	94
	7.0	37	42	13	92

[a] Reactions were carried out for the indicated time periods, after which the insoluble material was removed by centrifugation and the soluble products were separated by gel permeation chromatography on Sephadex LH-20 as described in the text.

[b] % Recovery was calculated from the total cpm in each fraction.

was oxidized more rapidly than the guaiacyl polymers. After 30 min only 40% of the material remained in the HMW fraction, while 28% of the radioactivity was found in the LMW fraction. For both the guaiacyl and guaiacyl-syringyl DHPs, maximal depolymerization as measured by recovery of radioactivity in the LMW fraction occurred within 30 min, after which repolymerization predominated. In contrast, with SC-S*-DHP depolymerization continued throughout the course of the 7-hr reaction.

Negligible amounts of $^{14}CO_2$ determined as described (11) were released during the reactions. No oxidation occurred if either enzyme, Mn^{II}, or glucose/glucose oxidase was omitted from the reaction mixture.

Gel permeation profiles of enzymatically degraded DHPs: The gel permeation profiles for the enzymatically treated DHPs are shown in Figure 1. For each profile the peak of the LMW fraction corresponded to dimeric and monomeric standard compounds. The extensive depolymerization of SC-S*-DHP seen in Figure 1D confirms that minimal polymerization occurred with this substrate. The profiles confirm that during the initial 30 min, significant depolymerization also occurred with R-G*-DHP, SC-G*-DHP and SC-SG*-DHP (Fig. 1A-C).

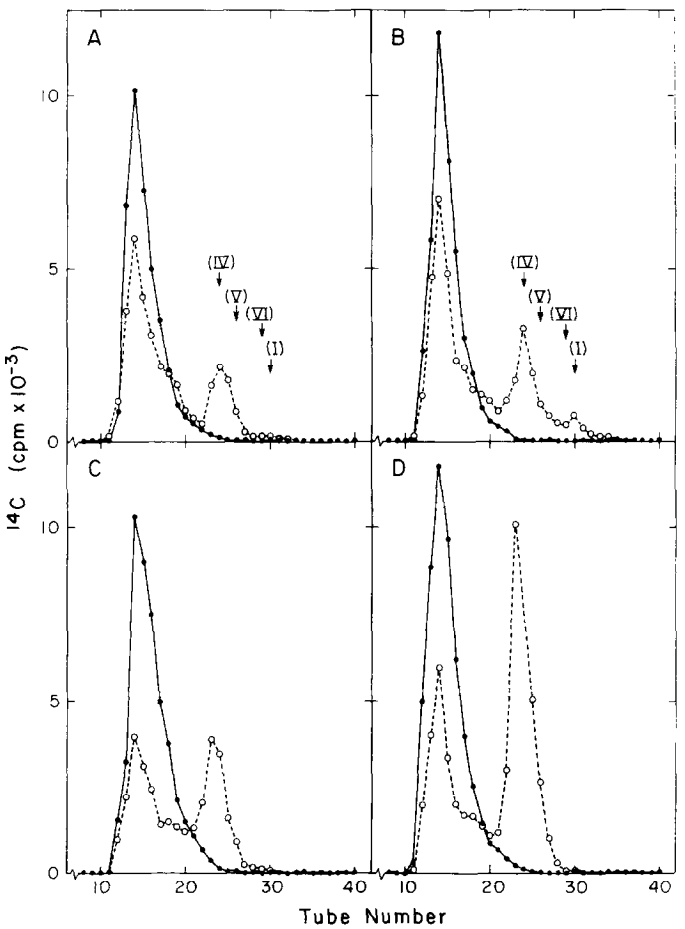

Figure 1. Gel permeation profiles of products of the manganese peroxidase catalyzed oxidation of ^{14}C-DHPs. ^{14}C-labeled DHPs were filtered on Sephadex LH-20 before (solid circle) or after (open circle) enzymatic oxidation. A. SC-G*-DHP treated for 30 min. B. R-G*-DHP treated for 30 min. C. SC-SG*-DHP treated for 30 min. D. SC-S*-DHP treated for 7 hr. Chromatographic standards included ^{14}C-guaiacylglycerol-β-guaiacyl ether (IV), ^{14}C-ferulic acid (V), ^{14}C-vanillic acid (VI), and 3,5-dimethoxy-1,4-benzoquinone (I), and were prepared as described (10,11).

Identification of monomeric cleavage products: Sidechain cleavage of 2-[^{14}C]-sidechain-labeled DHP would be expected to yield unlabeled C_6C_1 and benzoquinone monomeric products. Therefore, the nonradioactive fractions which coeluted with vanillic acid and benzoquinone standards (fractions 28-32, Figure 1) were collected from 1-hr degradation samples of SC-S*- and SC-SG*-DHP. The major product in these fractions was identified as 3,5-dimethoxy-1,4-benzoquinone (I). MS (m/z) 168 (M^+, 24%), 153 (2), 138 (10), 125 (8), 80 (26), 69 (100). Two other products were identified as 3,5-dimethoxy-1,4-dihydroxybenzene (II) and syringylaldehyde (III). II: MS (m/z) (diTMS ether) 314 (M^+, 100%), 299 (12), 284 (85), 73 (6). III: MS (m/z) (TMS ether) 254 (M^+, 42%), 239 (37), 224 (100), 153 (13), 73 (24).

DISCUSSION

P. chrysosporium and other white rot fungi degrade ^{14}C-labeled DHPs extensively to $^{14}CO_2$ and H_2O (1,2,11,12,15). Since lignin and manganese peroxidases from P. chrysosporium can degrade a variety of lignin model compounds, and since the mechanisms of these enzymes (1-4,10) can account for the products derived from the fungal degradation of lignin, both of these peroxidases have been implicated in lignin degradation. However, until recently little direct evidence for the in vitro depolymerization of lignins by pure preparations of these enzymes has been reported. Although an early report showed that a methylated derivative of lignin was partially depolymerized by a lignin peroxidase preparation (16), in subsequent experiments using unmodified lignins only polymerization was observed (17,18). Recently Hammel and Moen (5) reported the effective depolymerization of syringyl-guaiacyl DHP by a crude lignin peroxidase preparation using veratryl alcohol, 10% dimethylformamide, and low concentrations of H_2O_2 in the reaction mixtures. Several monomeric compounds have also been isolated from reactions of lignin peroxidase with DHP (19). Extensive degradation of unmodified guaiacyl lignin by purified lignin peroxidase has yet to be accomplished.

Several studies have indicated that manganese peroxidase activity appears prior to lignin peroxidase activity in culture (6,9,20) suggesting that manganese peroxidase may be responsible for initial depolymerization reactions. Furthermore, it has been suggested that the diffusible manganese peroxidase-generated Mn^{III} may be an effective catalyst for the oxidation of this heterogeneous, insoluble polymer (1,6-9). However, the in vitro oxidation of lignin by manganese peroxidase has not been reported previously. Guaiacyl- and syringyl-guaiacyl-DHPs represent gymnosperm and angiosperm lignins, respectively (21). Although syringyl lignin is not naturally prevalent, syringyl-rich lignins have been detected in some hardwood tissues (22). In this study, syringyl-DHP was utilized to compare the degradability of the syringyl and guaiacyl moieties in lignin.

Syringyl DHP was extensively depolymerized by the manganese peroxidase system (Table I and Fig. 1D). Since relatively little polymerization occurred with this substrate, the reaction was allowed to proceed for 7 hr, after which 42% of the starting material was depolymerized. Syringyl lignin contains relatively few condensed substructures (13,23); thus, it is probably more susceptible to depolymerization (1,2). Furthermore, guaiacyl lignins contain a greater proportion of unsubstituted C_5 aromatic carbons and consequently would be more susceptible to further polymerization or repolymerization (1,2,5,11).

3,5-Dimethoxy-p-quinone (I), 3,5-dimethoxy-p-hydroquinone (II), and syringylaldehyde (III) were identified as fragmentation products from both SC-S*- and SC-SG*-DHPs, indicating that both alkyl-phenyl and C_α-C_β cleavage of the lignin sidechain occurred. Depolymerization probably is initiated by the

oxidation of the free phenolic group to form a phenoxy radical as previously shown for the degradation of a phenolic diarylpropane model compound by manganese peroxidase where products I-III were also recovered (10). Both the hydroquinone (II) and syringylaldehyde (III) are oxidized to the p-quinone (I) by manganese peroxidase (10). The metabolism of p-quinones to CO_2 by P. chrysosporium has recently been demonstrated (24), suggesting that p-quinones are probably key intermediates in the degradation of lignin.

SC-SG*-DHP is also degraded significantly (Fig. 1C) and the syringyl moiety is released via alkyl-phenyl and C_α-C_β cleavage, yielding products I-III. Since this syringyl-guaiacyl copolymer is labeled in the guaiacyl moiety, the gel permeation profile (Fig. 1C) reflects depolymerization of the entire polymer.

As shown in Figs. 1A and B, purified manganese peroxidase also is able to depolymerize significantly both SC-G*- and R-G*-DHPs. As shown in Table 1, guaiacyl-containing DHPs are simultaneously depolymerized and polymerized or repolymerized by manganese peroxidase with polymerization reactions becoming dominant after the initial 30 minutes. It is likely that under in vivo conditions, LMW fragments are either taken up by the fungus and metabolized intracellularly (3,24), or are further degraded by lignin peroxidase (1-4), thus preventing repolymerization. Thus the depolymerization of 19% and 25% of the guaiacyl-labeled DHPs within 30 min is highly significant.

This is the first report of lignin depolymerization by manganese peroxidase, demonstrating that this enzyme probably plays an important role in the fungal degradation of lignin.

Acknowledgments: Supported by NSF grant DMB-8904358 and DOE grant DE-FG06-87-ER13715.

REFERENCES

1. Gold, M. H., Wariishi, H., and Valli, K. (1989) In Biocatalysis in Agricultural Biotechnology (J. R. Whitaker and P. E. Sonnet, Eds.), ACS Symposium Series 389, pp. 127-140, American Chemical Society, Washington DC.
2. Kirk, T. K., and Farrell, R.L. (1987) Annu. Rev. Microbiol. 41, 465-505.
3. Schoemaker, H. E. (1990) Recl. Trav. Chim. Pays-Bas 109, 255-272.
4. Higuchi, T. (1990) Wood Sci. Technol. 24, 23-63.
5. Glenn, J. K., and Gold, M. H. (1985) Arch. Biochem. Biophys. 242, 329-341.
6. Hammel, K. E. and Moen, M. A. (1991) Enz. Microb. Technol. 13, 15-18.
7. Glenn, J. K., Akileswaran, L., and Gold, M. H. (1986) Arch. Biochem. Biophys. 251, 688-696.
8. Wariishi, H., Akileswaran, L., and Gold, M. H. (1988) Biochemistry 27, 5365-5370.
9. Wariishi, H., Dunford, H. B., MacDonald, I. D., and Gold, M. H. (1989) J. Biol. Chem. 264, 3335-3340.
10. Wariishi, H., Valli, K., and Gold, M. H. (1989) Biochemistry 28, 6017-6023.
11. Gold, M. H., Mayfield, M. B., Cheng, T. M., Krisnangkura, K., Shimada, M., Enoki, A., and Glenn, J. K. (1982) Arch. Microbiol. 132, 115-122.
12. Faix, O., Mozuch, M. D., and Kirk, T. K. (1985) Holzforschung 39, 203-208.

13. Yamasaki, T., Hata, K., and Higuchi, T. (1973) Mokuzai Gakkaishi 19, 299-300.
14. Gold, M. H., Kutsuki, H., and Morgan, M. A. (1983) Photochem. Photobiol. 38, 647-651.
15. Kirk, T. K., Schultz, E., Connors, W. J., Lorenz, L. F., and Zeikus, J. G. (1978) Arch. Microbiol. 117, 227-285.
16. Tien, M., and Kirk, T. K. (1983) Science 221, 661-663.
17. Haemmerli, S. D., Leisola, M. S. A., and Fiechter, A. (1986) FEMS Microbiol. Lett. 35, 33-36.
18. Kern, H. W., Haider, K., Wim Pool, J. W. deL., and Ernst, L. (1989) Holzforschung 43, 357-384.
19. Umezawa, T., and Higuchi, T. (1989) Mokuzai Gakkaishi 35, 1014-1020.
20. Pease, E. A., Andrawis, A., and Tien, M. (1989) J. Biol. Chem. 264, 13531-13535.
21. Sarkanen, K. V., and Ludwig, C. H. (1971) Lignins: Occurrence, Formation, Structure and Reactions, Wiley-Interscience, New York.
22. Fergus, B. J., and Goring, D. A. I. (1970) Holzforschung 24, 118-124.
23. Yamasaki, T., Hata, K., and Higuchi, T. (1976) Mokuzai Gakkaishi 22, 582-588.
24. Valli, K., and Gold, M. H. (1990) J. Bacteriol. 173, 345-352.