

## Conversion of Milled Pine Wood by Manganese Peroxidase from *Phlebia radiata*

MARTIN HOFRICHTER,\* TAINA LUNDELL, AND ANNELE HATAKKA

Division of Microbiology, Department of Applied Chemistry and Microbiology,  
University of Helsinki, FIN-00014 Helsinki, Finland

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Purified manganese peroxidase (MnP) from the white-rot basidiomycete *Phlebia radiata* was found to convert in vitro milled pine wood (MPW) suspended in an aqueous reaction solution containing Tween 20, Mn<sup>2+</sup>, Mn-chelating organic acid (malonate), and a hydrogen peroxide-generating system (glucose-glucose oxidase). The enzymatic attack resulted in the polymerization of lower-molecular-mass, soluble wood components and in the partial depolymerization of the insoluble bulk of pine wood, as demonstrated by high-performance size exclusion chromatography (HPSEC). The surfactant Tween 80 containing unsaturated fatty acid residues promoted the disintegration of bulk MPW. HPSEC showed that the depolymerization yielded preferentially lignocellulose fragments with a predominant molecular mass of ca. 0.5 kDa. MnP from *P. radiata* (MnP3) turned out to be a stable enzyme remaining active for 2 days even at 37°C with vigorous stirring, and 65 and 35% of the activity applied was retained in Tween 20 and Tween 80 reaction mixtures, respectively. In the course of reactions, major part of the Mn-chelator malonate was decomposed (85 to 87%), resulting in an increase of pH from 4.4 to >6.5. An aromatic nonphenolic lignin structure ( $\beta$ -O-4 dimer), which is normally not attacked by MnP, was oxidizable in the presence of pine wood meal. This finding indicates that certain wood components may promote the degradative activities of MnP in a way similar to that promoted by Tween 80, unsaturated fatty acids, or thiols.

Biodegradation of lignin by basidiomycetous white-rot fungi is, at least partly, brought about by extracellular lignin and manganese peroxidases (12, 26). MnP discovered in *Phanerochaete chrysosporium* in 1984 (28) is obviously produced exclusively by wood and soil-litter-decomposing basidiomycetes (14). Evidence has been provided that the enzyme is involved in the biodegradation of lignin (14, 36, 38, 40) and other recalcitrant substances such as humic acids (25) or organopollutants (1, 5, 15). There are a number of efficient delignifying fungi that secrete MnP as the only extracellular peroxidase, including *Lentinula (Lentinus) edodes* (30), *Panus tigrinus* (35), *Ceriporiopsis subvermisporea* (32), *Phanerochaete sordida* (41), *Dichomitus squalens* (39), and *Pleurotus ostreatus* (7); some other ligninolytic fungi produce MnP, along with lignin peroxidase (e.g., *Phanerochaete chrysosporium*, *Phlebia radiata*, *Trametes versicolor*, and *Nematoloma frowardii*) (12, 14, 19, 26). MnP works via chelated Mn<sup>3+</sup>, which acts as a small diffusible oxidant (redox mediator) attacking preferentially phenolic lignin structures (8, 9). The oxidative strength of the MnP-Mn<sup>2+/3+</sup> system is enhanced in the presence of certain cooxidants as glutathione or unsaturated lipids, which enable it to oxidize also recalcitrant nonphenolic lignin moieties with higher redox potential (4, 22, 23).

MnP was found to depolymerize and even mineralize part of polymeric lignin in cell-free systems (20, 29, 44). Most of these investigations, however, were carried out by using isolated dispersed, natural, or synthetic lignins (DHP) as substrates, and so far only a few studies have been published in which solid

lignocelluloses (e.g., pulp or straw) were treated with MnP (18, 27, 40). The present study therefore focuses on the action of purified MnP enzyme from the white-rot fungus *P. radiata* on solid pine wood that was merely milled prior to use. In this context, the effect of lipid surfactants such as Tween 20 and Tween 80 was examined.

### MATERIALS AND METHODS

**Organism and enzyme preparation.** The corticoid white-rot fungus *P. radiata* 79 (ATCC 64658) used for the production of MnP was isolated and characterized as described earlier (10, 37). The fungus was cultured in a nitrogen-limited succinate-buffered medium (pH 4.5) containing 200  $\mu$ M Mn<sup>2+</sup> in a 20-liter bioreactor. MnP from *P. radiata* (MnP3; pI 3.6) was purified from the culture liquid of 8- to 10-day-old cultures as described previously (37).

**MPW.** Milled pine wood (MPW) applied as substrate for MnP was prepared from a mixture of air-dried sap and heart wood of Scots pine (*Pinus sylvestris*) by using a rotating jar ball mill according to the method of Lundquist (34) except that no organic solvent or water was added during the milling process. Thus, the obtained powder (MPW1) contained all wood components including the extractives. Part of the MPW1 was further extracted in 5-mg portions with 1 ml of 0.5% Tween 20 or Tween 80 in 50 mM sodium malonate (also containing 2 mM MnCl<sub>2</sub>) with vigorous shaking for 2 days. After centrifugation, the supernatant containing the extractable wood constituents (MPWX, mostly aromatic compounds) was separated from the insoluble wood pellet (MPW2). The latter was washed twice with ethyl acetate and water prior to use. Dry weight measurements showed that about 20% (19.9%  $\pm$  2.3% = 99.5  $\pm$  11.5  $\mu$ g ml<sup>-1</sup>,  $n$  = 5) of the MPW could be dissolved in both Tween 20 and Tween 80 under these conditions. Most experiments were carried out with MPW1; MPW2 and MPWX were used as additional substrates to distinguish between depolymerizing and polymerizing MnP activities.

**HPSEC.** High-performance size exclusion chromatography (HPSEC) was used for the determination of the molecular mass distribution of lignocellulose fragments formed by MnP (18, 20). The high-performance liquid chromatography (HPLC) system (HP 1090 Liquid Chromatograph; Hewlett-Packard, Waldbronn, Germany) equipped with a diode array detector was fitted with a HEMA-Bio linear column (8 by 300 mm, 10  $\mu$ m; Polymer Standard Service, Mainz, Germany). The mobile phase consisted of 20% acetonitrile and 80% of an aqueous solution of 0.5% NaNO<sub>3</sub> and 0.2% K<sub>2</sub>HPO<sub>4</sub>; the pH was adjusted to 10.0 by the

\* Corresponding author. Mailing address: Department of Applied Chemistry and Microbiology, P.O. Box 56, Biocenter 1, Viikinkaari 9, FIN-00014 Helsinki, Finland. Phone: 358-9-191-59321. Fax: 358-9-191-59322. E-mail: martin.hofrichter@helsinki.fi.

addition of NaOH. Sodium polystyrene sulfonates (1.3 to 168 kDa, Polymer Standard Service) and biphenyl dicarboxylic acid (0.246 kDa) served as molecular mass standards (16).

**Determination of organic acids.** The concentrations of malonate and other organic acids (oxalate, tartrate, and malate) acting as Mn chelator and buffer substances in the reaction system were determined after 48 h by using the HPLC system mentioned above but fitted with a Aqua-C<sub>18</sub> column (4.6 by 250 mm, 5  $\mu$ m; Phenomex, St. Torrance, Calif.). Phosphoric acid (10 mM) served as eluent under isocratic conditions, and authentic standards of the organic acids served as references (detection wavelength, 210 nm) (17).

**Enzyme assay.** MnP activity and Mn<sup>3+</sup> concentration were measured at 420 nm by monitoring the oxidation of ABTS [2,2'-azinobis(3-ethylbenzthiazoline-sulfonic acid);  $\epsilon_{420} = 36 \text{ mM}^{-1} \text{ cm}^{-1}$ ] as described previously (17, 20).

**Enzymatic reactions.** The basic reaction mixture contained in a total of 1 ml of 50 mM sodium malonate (pH 4.5), 2 mM MnCl<sub>2</sub>, 0.5% Tween 20 or 80, 10 mM glucose, 0.1 U of glucose oxidase (from *Aspergillus niger*; Sigma-Aldrich) ml<sup>-1</sup>, and 2 U of MnP3 from *P. radiata* (40 nM) ml<sup>-1</sup>. MPW1 (5 mg ml<sup>-1</sup>) or MPW2 (ca. 4 mg ml<sup>-1</sup>) served as solid target substrates. The soluble MPWX fraction (1 ml) was supplemented with the respective reactants and was, like MPW1 and MPW2, incubated in 10-ml reaction tubes closed with parafilm with vigorous stirring at 37°C for 48 h. Samples (10  $\mu$ l) for the measurement of MnP activity and Mn<sup>3+</sup> concentration were collected immediately after the reaction was started, as well as in 0.5- to 10- h intervals; larger samples (50  $\mu$ l) for HPSEC analyses were obtained after 24 and 48 h. Considering the amount of extractable wood components (~1 mg of lignin plus other aromatics ml<sup>-1</sup>) and the change in the extinction coefficient due to oxidation of this material, we calculated roughly how much aromatic material was released by MnP from solid wood (MPW1, MPW2) by comparing the area below the respective HPSEC elution profiles.

In order to check the stability of alternative chelators for the MnP system, MPW1 was incubated in the reaction mixture mentioned above (with Tween 20) but replacing malonate by equivalent amounts of oxalate, malate, or tartrate. After a reaction of 48 h, the samples were analyzed by HPSEC, as well as for their organic acid concentration, pH, and remaining MnP activity.

**Oxidation of a nonphenolic LMC.** The aromatic dimer 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol (11) was used to examine the influence of MPW1 on the oxidation of recalcitrant nonphenolic  $\beta$ -O-4 lignin structures by MnP. The conditions were identical to those described above except that the Tween 20-containing reaction solution (total of 1 ml) was additionally supplemented with 100  $\mu$ M lignin model compound (LMC). The keto-form of LMC [keto-LMC = 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)propane-1-one (11)], veratryl alcohol, veratryl aldehyde, veratric acid, and guaiacol (2-methoxyphenol) served as authentic references of possible oxidation products. HPLC was used for quantitative analysis after 24 h by using the equipment described above, including the Aqua-C<sub>18</sub> column (Phenomex). Separations were run at constant 40°C by using a stepwise gradient of 0 to 45% acetonitrile (0% [0 min], 0% [10 min], 40% [20 min], 45% [25 min], 0% [30 min], and 0% [35 min]) in 0.05% phosphoric acid for 35 min with a constant flow rate of 0.75 ml min<sup>-1</sup>. Eluted substances were detected at 275 and 310 nm.

## RESULTS

**MnP activity during MPW treatment.** Time courses of MnP activity during the treatment of MPW1 are shown in Fig. 1 and were very similar regardless of whether the reaction mixture contained Tween 20 or Tween 80. The activity of MnP increased in the very beginning (from 2 to 2.4 U ml<sup>-1</sup>), which was due to the presence of Tween 20 or Tween 80 that somehow enhanced the enzyme activity (probably by improving the accessibility of ABTS). After 1 h, MnP activity started to decline, reaching temporary minima of 0.55 and 0.43 U ml<sup>-1</sup> after 18 h for Tween 20 and Tween 80 containing samples, respectively. Simultaneous with the decrease of MnP activity, Mn<sup>3+</sup> appeared in the reaction solution and reached its maximum concentration of 200  $\mu$ M after 18 h. Within the next 6 h, the Mn<sup>3+</sup> level dropped noticeably (to 70  $\mu$ M), which was accompanied by a partial recovery of MnP activity, particularly in the Tween 20-containing samples (from 0.55 to 1.3 U ml<sup>-1</sup>; and from 0.43 to 0.7 U ml<sup>-1</sup> in Tween 80 samples). Afterward,

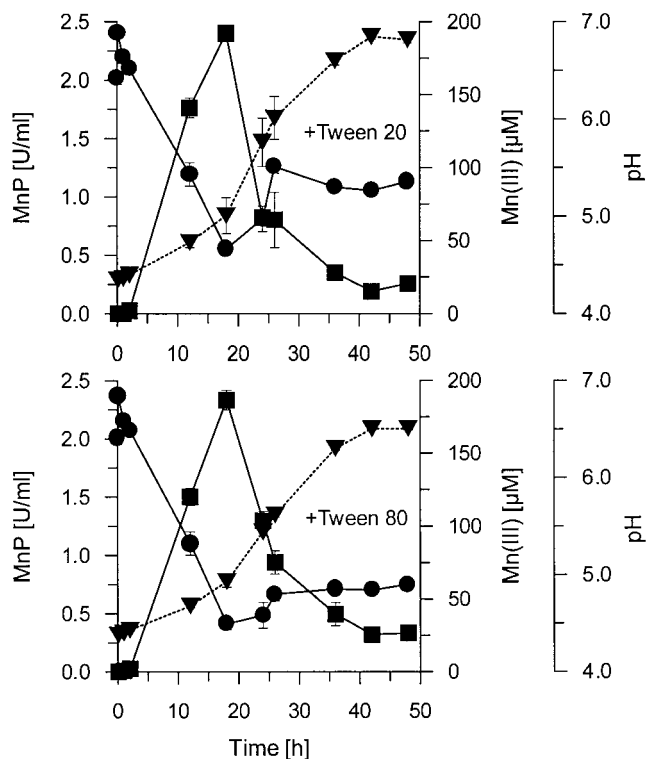


FIG. 1. Activity of MnP, concentration of Mn<sup>3+</sup>, and pH during the in vitro treatment of MPW1 with MnP. The upper graph refers to Tween 20-containing samples; the lower one refers to Tween 80-containing samples. Symbols: ●, actual MnP activity; ■, Mn<sup>3+</sup> concentration; ▼, pH.

the MnP activity remained nearly constant until the end of the experiment.

In the course of the experiment, the pH of the reaction solutions increased from ca. 4.5 to 6.5 to 6.8 (Fig. 2). HPLC analyses revealed that the pH increase was caused by a substantial decomposition of malonate during MnP catalysis (Table 1). More than 80% of malonate disappeared and was very probably converted into carbon dioxide and only to a small extent into oxalate. Alternative organic acids acting as buffer substances and chelators for manganese were also decomposed by the MnP system, although to a noticeably lesser extent than was the malonate (Table 2). Only in the case of oxalate was a similar drastic increase in pH as with malonate observed. However, ca. 60% of oxalate remained intact after 48 h. On the other hand, the organic acids tested were not as efficient as malonate in promoting the attack on MPW1 by MnP (see below).

**HPSEC analyses.** Figure 2 summarizes the HPSEC elution profiles of the water-soluble products formed as the result of the MPW treatment with MnP. Although we do not know anything about the molecular weight of native lignin in the untreated bulk of wood, it is obvious that both polymerizing and depolymerizing reactions took place and that these reactions were dependent on the type of Tween used.

MPW2 (free of soluble wood components) was only slightly attacked by MnP in the presence of Tween 20, resulting in the formation of moderate amounts (ca. 300  $\mu$ g ml<sup>-1</sup>) of low-

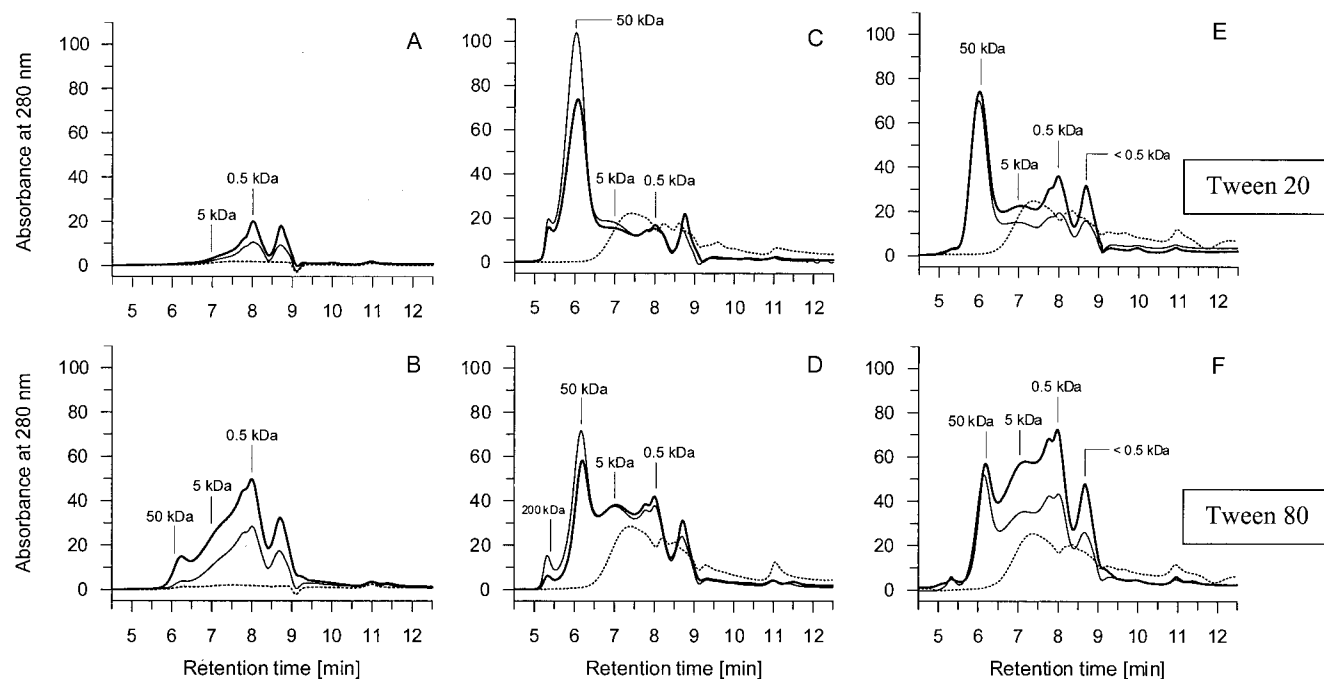


FIG. 2. HPSEC elution profiles of soluble products from different MPW preparations treated with MnP3 in the presence of malonate for 48 h. The upper row refers to Tween 20-containing samples; the lower row to Tween 80-containing samples. Key: dotted lines, controls without MnP after 48 h; thin lines, MnP-containing samples after 24 h; bold lines, MnP-containing samples after 48 h. (A) MPW2 plus Tween 20. (B) MPW2 plus Tween 80. (C) MPWX plus Tween 20. (D) MPWX plus Tween 80. (E) MPW1 plus Tween 20. (F) MPW1 plus Tween 80.

molecular-mass fragments ( $\sim 0.5$  kDa and smaller; Fig. 2A). Tween 80 stimulated noticeably the depolymerization process, resulting in an increase of water-soluble fragments, among which were also higher-molecular-mass products ( $>5$  kDa; Fig. 2B), and we estimated that ca.  $800 \mu\text{g}$  of lignin fragments and other aromatics  $\text{ml}^{-1}$  was released.

The conversion of MPWX obviously resulted in the polymerization of soluble wood constituents to high-molecular-mass products ( $\sim 50$  kDa), although individual differences were ascertained for Tween 20- and Tween 80-containing samples (Fig. 2C and D). High-molecular-mass products ( $\sim 50$  kDa) were predominantly formed in the presence of Tween 20 (Fig. 2C), whereas the elution profiles of Tween 80-containing samples also included more of the lower-molecular-mass fractions ( $\sim 0.5$  and  $5$  kDa; Fig. 2D). Elution profiles of the MPW1 products show characteristics of both MPW2 and MPWX (Fig. 2E and F). Depolymerization was particularly pronounced in

the presence of Tween 80, where the low-molecular-mass fractions dominated in the end over the high-molecular-mass ones. On the other hand, estimation of total aromatic fragments indicates that in the presence of both Tween 20 and Tween 80, similar amounts were released ( $600$  and  $700 \mu\text{g ml}^{-1}$ , respectively).

The replacement of malonate as a chelator for  $\text{Mn}^{3+}$  did not lead to an increased depolymerization of MPW1. On the contrary, the respective HPSEC profiles for tartrate-, malate-, and oxalate-containing samples demonstrated that malonate was the most efficient chelator of the  $\text{MnP-Mn}^{2+/3+}$  system (data not shown). Whereas the formation of high-molecular-mass fragments was not influenced or even enhanced, the resulting amounts of low-molecular-mass substances were in all three cases lower. Furthermore, the remaining MnP activities after

TABLE 1. Decomposition of malonate as the result of MnP catalysis during the treatment of MPW1<sup>a</sup>

Sample	pH	Malonate (mM)	Oxalate (mM)
Control without MnP plus Tween 20	4.4	51.3	$<0.3$
MnP plus Tween 20	6.8	6.8	3.5
Control without MnP plus Tween 80	4.4	52.5	$<0.3$
MnP plus Tween 80	6.6	8.1	5.8

<sup>a</sup> All reaction mixtures contained  $2 \text{ mM Mn}^{2+}$  in  $50 \text{ mM Na malonate}$  buffer. The data represent the average values of three reaction tubes after 48 h of incubation (the standard deviation was  $<10\%$ ). The amounts of malonate and oxalate were determined by HPLC analysis.

TABLE 2. Decomposition of alternative dicarboxylic acids in the course of MPW1 treatment with MnP<sup>a</sup>

Sample	pH	Organic acid (mM)	Remaining MnP activity (%) <sup>b</sup>
Oxalate (control without MnP)	3.8	52.1	
Oxalate (plus MnP)	6.6	34.9	40
Malate (control without MnP)	3.8	50.6	
Malate (plus MnP)	4.1	41.5	0
Tartrate (control without MnP)	4.1	51.6	
Tartrate (plus MnP)	4.2	39.1	3

<sup>a</sup> Reactions were performed in the presence of  $2 \text{ mM Mn}^{2+}$  and Tween 20 ( $0.5\%$ ). The organic acid concentration and pH were determined after 48 h as in Table 1 (the standard deviation was  $<10\%$ ).

<sup>b</sup> For comparison, the remaining MnP activity of malonate containing samples was  $65\%$ .

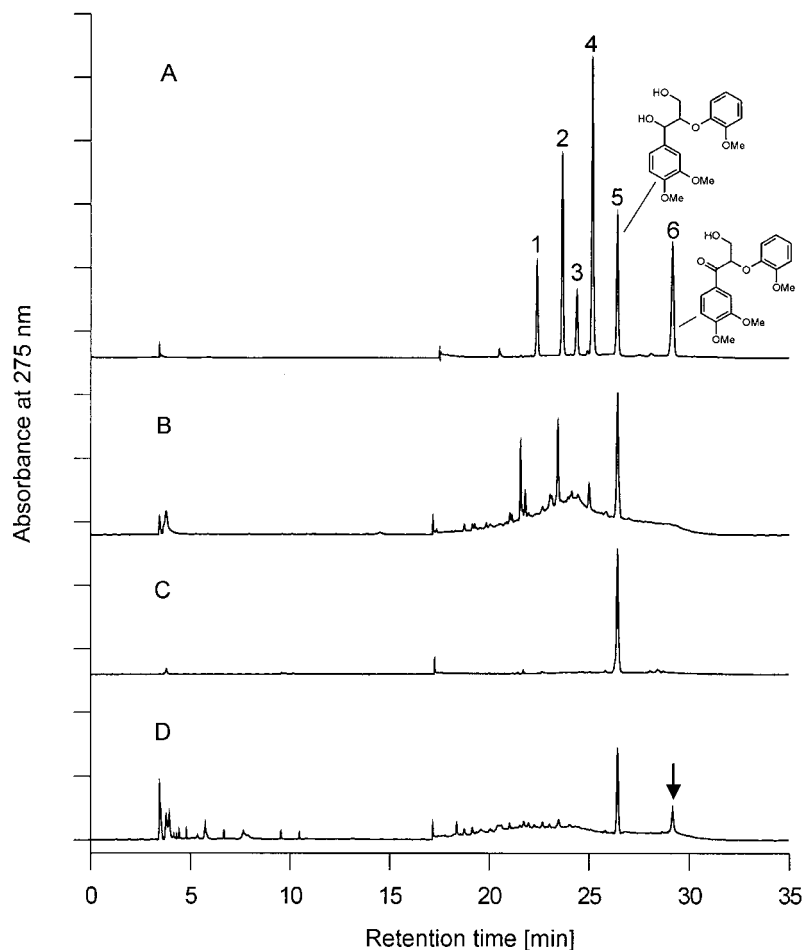


FIG. 3. Oxidation of a nonphenolic LMC by MnP in the presence of MPW (MPW1) for 24 h. Reversed-phase HPLC analysis was performed at 275 nm by using a water-acetonitrile gradient. (A) Standards (100  $\mu\text{M}$ ) of veratryl alcohol (peak 1), veratric acid (peak 2), guaiacol (peak 3), veratryl aldehyde (peak 4), LMC (peak 5), and keto-LMC (peak 6). (B) Complete reaction mixture plus LMC plus MPW1, but without MnP. (C) Complete reaction mixture plus LMC plus MnP, but without MPW1. (D) Complete reaction mixture plus LMC plus MnP plus MPW1.

MPW1 treatment show that oxalate, malate, and tartrate were not as efficient as malonate in maintaining MnP activity (Table 2).

**Oxidation of LMC.** MnP3 from *P. radiata* was not able to attack a nonphenolic dimer (LMC) in the presence of Tween 20. Within 48 h of incubation, only a negligible part of LMC (<3%) was converted, and no indication for the formation of the keto-form of LMC was found (Fig. 3C). However, in the presence of MPW1 (5 mg ml<sup>-1</sup>), a significant amount of LMC (ca. 35% = 35  $\mu\text{M}$ ) disappeared in the reaction solution accompanied by the nearly equivalent formation of the corresponding keto-form of LMC (31.9  $\mu\text{M}$ , arrow in Fig. 3D); other oxidation products, such as monomeric fragments (e.g., veratric acid or veratryl aldehyde), were not observed (Fig. 3A and D). The keto-form of LMC was also detected when MPW1 was used instead of MPW1, although oxidation was less pronounced (data not shown). In addition, Fig. 3 shows that MnP treatment reduced the amount of soluble lower-molecular-mass constituents of MPW1, which formed a broad peak absorbing at 275 nm in the HPLC elution profile of MnP-free controls (Fig. 3B). A number of sharp, distinct peaks

sitting up on this broad peak and with characteristic aromatic UV spectra disappeared completely from the reaction solution as a result of MnP treatment (Fig. 3D). It can be concluded that these soluble aromatic monomers and oligomers were preferentially polymerized by the MnP system, which matches well the results of the HPSEC analyses. Last, but not least, there was also an indication for the depolymerizing activity of MnP. Thus, the elution profile of treated MPW1 was altered toward more-hydrophilic products eluting very early from the reversed-phase column (Fig. 3B and D).

## DISCUSSION

MnP3 from *P. radiata* is able to convert MPW suspended in an appropriate reaction solution in vitro. The enzymatic attack results in the polymerization of lower-molecular-mass, soluble wood components and in the partial depolymerization of the insoluble bulk of pinewood. Tween 80, acting as both a surfactant and cooxidant for the MnP system (23), promotes the disintegration of insoluble MPW into smaller, soluble lignocellulose fragments. MnP3 was found to be a rather stable enzyme

that remained active for at least 2 days under the reaction conditions applied. An aromatic nonphenolic lignin structure ( $\beta$ -O-4), which is normally not attacked by MnP but is cleaved by LiP (33), can be oxidized in the presence of pinewood meal, indicating the support of MnP activities by certain wood components.

The ability of MnP from different fungi to depolymerize isolated lignins partly has been demonstrated in vitro within some studies. Among the lignins tested were synthetic preparations (19, 20, 44), chlorolignin (29) and natural lignins from spruce or pine (4, 13), *Hevea* spp. (6), and wheat straw (18). Our results also show that MnP not only attacks isolated lignins in aqueous reaction systems but also attacks more-complex solid lignocellulose such as MPW.

There are only a few reports on the conversion of entire lignocelluloses (wood, straw, or pulp) by oxidative fungal enzymes. Softwood kraft pulp was treated with MnP from *Trametes versicolor* to oxidize residual lignin (38, 40). In contrast to our present results, MnP was not able to "solubilize" lignin directly in previous studies, i.e., no water-soluble lignin fragments were found in the reaction mixtures after MnP treatment (38, 40). The reason for this might be the use of a low malonate concentration (5 mM) and the lack of a surfactant such as Tween. On the other hand, it has been shown that the MnP treatment lowered the kappa number of kraft pulp and increased the alkali extractability of residual lignin (38), indicating that MnP partially oxidized the lignin in pulp under the conditions applied. Similar results were obtained with hardwood kraft pulp and wheat straw meal when MnP from *Phanerochaete sordida* (27) and the abiotic model system  $\text{MnO}_2$ -oxalate, respectively, were used (31). The authors of the former study noticed, similar to our observations, a positive effect of Tween 80 toward the oxidation of lignin by MnP and the bleaching of pulp.

Unlike isolated dispersed lignins such as DHP, the disintegration of nonextracted solid lignocellulose (MPW1) was accompanied in our present study (at least in the early stages of attack) by substantial formation of high-molecular-mass, water-soluble fragments resulting from the obvious polymerization of low-molecular-mass wood components. The substantial polymerization of aromatic monomers such as guaiacol (2-methoxyphenol) and 2,6-dimethoxyphenol to insoluble macromolecules has recently been described for an MnP from *Bjerkandera adusta* (21). The fact that the polymers formed by MnP in our experiments did not precipitate suggests that their highly hydrophilic nature is based on a high grade of initial oxidation.

It is interesting that the  $\text{Mn}^{3+}$  concentration seems to regulate the MnP activity; the higher the  $\text{Mn}^{3+}$  concentration was during the course of MPW conversion, the lower was the actual activity of MnP, and vice versa. It is still unclear, however, just what is responsible for this endproduct regulation, but some kind of feedback control on the enzyme level cannot be ruled out. Some indication for the regulation of MnP activity by  $\text{Mn}^{3+}$  was already found in a previous study (45). The activity of crude MnP from *Clitocybula dusenii* declined considerably when  $\text{Mn}^{3+}$  pyrophosphate was added to the assay mixture.

The decomposition of malonate in the course of MnP catalysis is a phenomenon that we already observed in an earlier study in the absence of any secondary substrate (17). This

radical process, which also applies to oxalate and other organic acids, limits the efficiency of MnP catalysis in cell-free batch systems, since the concomitant degradation of the required chelator and buffer agent leads to disproportionation of  $\text{Mn}^{3+}$  and to an unfavorable increase of pH. To overcome this problem in future experiments, malonate could be re-added continuously, holding the pH constant.

The MnP system obviously has an effect on certain lignin structures in pine wood, but only the "cooperation" with cooxidants as the unsaturated fatty acid residues in Tween 80 may enable the substantial disintegration of intact lignin. Reactive peroxy radicals derived from unsaturated lipids (24) might be responsible for the enhanced pine wood conversion. Whether the lipid substances in question are produced by the fungus (3), are present in the wood, or originate from both sources has to be proven in each particular case. In this context, our finding that the presence of MPW1 enables MnP to oxidize a nonphenolic lignin model dimer is remarkable. It can be concluded that certain substances either contained in MPW or formed from MPW by MnP somehow promote the oxidation of more-recalcitrant lignin structures. The nature of these wood constituents is still unknown. As already mentioned, lipids comprising unsaturated fatty acids (22, 23, 24), but also thiols (4, 43) or aromatic mediators, which have been shown to expand laccase activities in vitro (2, 42), should be taken into consideration.

Our future studies will focus on the action of MnP on other types of wood (e.g., other softwoods such as spruce or larch and also hardwood), on wood constituents acting as natural cooxidants (redox mediators), and on synergistic effects of other lignin-modifying enzymes (lignin peroxidase and laccase) on the MnP-catalyzed conversion of wood.

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