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In Vitro Degradation of Insoluble Lignin in Aqueous Media by Lignin Peroxidase and Manganese Peroxidase

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ABSTRACT

The abilities of lignin peroxidase (LIP) and manganese peroxidase (MNP) from *Phanerochaete chrysosporium* to degrade an insoluble hardwood lignin in vitro in aqueous media were tested. Neither LIP nor MNP appreciably changed the mass or lignin content, although both produced small amounts of unique solubilized lignin fragments. Treatment with both LIP and MNP, however, decreased the mass by 11%, decreased the lignin content by 5.1% (4.2% as total weight), and solubilized unique lignin-derived molecules. These results suggest that LIP and MNP synergistically degrade high molecular weight insoluble lignin, but singly, neither enzyme is sufficient to effect lignin degradation.

Index Entries: Lignin peroxidase (LIP); manganese peroxidase (MNP); *Phanerochaete chrysosporium*.

INTRODUCTION

Billions of tons of lignocellulosic biomass are produced each year worldwide (*1*). Lignin biodegradation is a rate-limiting step in the mineralization of lignocellulosic biomass in the biosphere. Lignin removal in the pulp and paper industry is costly, energy-intensive, and toxic effluents are produced (*2*). Thus, microbial delignifying enzymes have important potential applications in the pulp and paper industry, and also in the bio-

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remediation of aromatic xenobiotics (3). Reactions catalyzed by the extracellular peroxidases of *Phanerochaete chrysosporium* have been well characterized on soluble lignin model compounds (4). In contrast, attempts to degrade the insoluble, high molecular weight polymer in completely aqueous media have been largely unsuccessful, casting considerable doubt on the putative roles of these peroxidases in the in vivo degradation of natural lignins (4).

The goal of this study was to determine whether it is possible to degrade a water-insoluble natural lignin polymer in aqueous media using the extracellular ligninolytic enzymes of *P. chrysosporium*. This was done using the two classes of peroxidases singly and in combination to treat an isolated water-insoluble lignin, which was shown by thioacidolysis and syringyl:guaiacyl ratio to be chemically and structurally similar to the initial poplar substrate. Fourier transform infrared spectroscopy (FTIR), gas chromatography/mass spectrometry (GC/MS), and mass balances were used to indicate lignin degradation. The results indicate that LIP and MNP act synergistically to degrade insoluble lignin in aqueous media, but singly, little or no degradation of the solid is observed.

MATERIALS AND METHODS

Lignin Preparation

The lignin substrate used in this study was isolated from hybrid poplar (*Poplus eugeneii*), as previously described (5,6), using a sequential treatment of dilute-acid hydrolysis, cellulolytic hydrolysis, and exhaustive extractions at 37°C with chloroform, ethyl acetate, and methanol. Carbohydrate and lignin compositions were measured by quantitative saccharification (7). In this technique, the sample was subjected first to hydrolysis with 72 wt% H_2SO_4 for 1 h at 30°C, and then with 4 wt% H_2SO_4 for 1 h at 121°C in an autoclave. The liquid fraction was quantitatively collected, diluted to a known volume, and analyzed for carbohydrates by HPLC, using a Bio-Rad (Hercules, CA) HPX-87P column with deashing system. Carbohydrate values were corrected for losses incurred during the high-temperature step through the use of a carbohydrate recovery experiment at 4 wt% H_2SO_4 and 121°C. The remaining insoluble portion was defined as Klason lignin with extractives and ash.

Enzymes

LIP and MNP enzymes were produced at 37°C from the extracellular fluid of agitated nitrogen-limited, acetate-buffered cultures of *P. chrysosporium*, strain BKM-F-1767 (ATCC 24725), as described by Tien and Kirk (8). Agitated cultures were inoculated with mycelia from stationary cultures containing 11.5 ppm of Mn(II) (8), as previously described (9). Basic media for these cultures were prepared as previously described (5,9), except that Mn(II) was added as $MnSO_4$ to production cultures at 100 ppm, to produce MNP for harvest on d 4, and at 0 ppm to produce LIP for

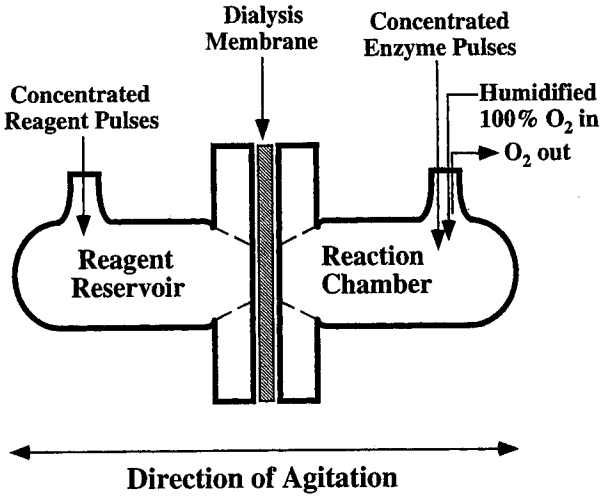

Fig. 1. Schematic of the dialysis reactor.

harvest on d 5. After concentration by ultrafiltration and dialysis *(5,6)*, aliquots of the dialyzed concentrates for each were stored at −20°C.

Activity Assays

LIP activity was determined spectrophotometrically at 310 nm *(8)*, defining 1 unit (U) of LIP activity as the oxidation of 1 μmol of veratryl alcohol to veratraldehyde per min, using a molar absorptivity of 9300 *M*/cm. MNP activity was measured spectrophotometrically at 610 nm *(2)*, with a reaction time of 4 min, defining 1 U of MNP activity as the oxidation of 1 μmol of phenol red per min, using a molar absorptivity of 4460 *M*/cm. Note that 1 U/L of LIP activity represents a much higher amount of heme protein than 1 U/L of MNP.

Lignin Treatment and In Vitro Reactor System

Lignin treatment with peroxidases was done in a small-scale dialysis reactor (Fig. 1) for 12 h at 37°C, 2% (w/v) solids, with approx 50 U LIP/g lignin and/or 2800 U MNP/g lignin in 20 m*M* tartrate buffer, pH 3.5, at 350 strokes/min, as previously described *(5,6)*. A 6000–8000 mol wt cutoff dialysis membrane, with a measured wet membrane thickness of 61 μm, was used to separate the chambers. Only silanized glassware, including the reactor halves and all sample handling equipment, were contacted with reaction mixtures, samples, or solvent extracts, to prevent compounds released from the lignin from binding to the glass. This reactor system was used to control the levels of H_2O_2 and veratryl alcohol (VA) in contact with the peroxidases over the course of the reaction. Control was achieved through use of a predictive control scheme *(5)*, which allowed adjustments of H_2O_2 and/or VA in the reagent reservoir (1 mL liquid), and LIP and/or

Table 1
Working Concentration Ranges in the Reaction Chamber
Over 12 h of Treatment

Enzyme(s) used	H_2O_2 Concentration range (μM) (Average in parentheses)	VA[a] Concentration range (μM) (Average in parentheses)
LIP	$10 < [H_2O_2]$ (15) < 25	$60 <$ [VA] (360) < 550
MNP	$15 < [H_2O_2]$ (40) < 80	–[b]
LIP + MNP	$5.0 < [H_2O_2]$ (8.0) < 15	$20 <$ [VA] (340) < 550

Brackets indicate concentration.
[a] VA, veratryl alcohol.
[b] VA was not present for this enzyme case.

MNP in the reaction chamber (1 mL slurry). Adjustments were necessary because of the small volume of the system. Concentration ranges maintained in contact with the enzymes over the course of the various runs are listed in Table 1. Initial conditions for each run, including controls, are presented in Table 2. Tartrate was used both as buffer and as the Mn(III) chelator *(10)*. After 12 h of reaction, the slurry in the reaction chamber was quantitatively collected (95% recovery of solids) by centrifugation. The supernatant was stored at −80°C for future GC/MS analyses. The resulting lignin pellet was sequentially washed free of protein by centrifugation with 1 *M* NaCl, followed by distilled H_2O. The pellet was then suspended in 1 mL of distilled H_2O and stored at −80°C.

Mass Balance and FTIR Analyses

Fourier transform infrared spectra were measured for all lignin samples using a Nicolet 5SXC FTIR spectrophotometer equipped with a deuterated triglycine sulfate detector. Transmission FTIR spectra of the pellets, averaged from 50 scans, were measured from 4000 to 650 cm^{-1} with 2 cm^{-1} resolution. A KBr background spectrum was subtracted from each spectrum. Measurements were performed on wafers containing approximately 0.5 wt% of solvent extracted (*see* extraction procedure below), quantitatively lyophilized lignin, pressed under vacuum, as previously described *(5,11)*. The mass balance was calculated from the lyophilized weight prior to pressing the wafer. Spectra were analyzed by partial least-squares (PLS) regression *(11)* for methoxyls/C_9, phenolic hydroxyls/C_9, carbohydrate, and lignin contents for each sample, using methods that were procedurally similar to that for the methoxyl content *(11)*. The regression methods were calibrated separately, as follows: The methoxyl method was calibrated with milled wood lignins *(11)*; the carbohydrate and lignin methods were calibrated with a combination of whole-wood samples and wood pulps in a manner analogous to that for methoxyl content; finally, the phenolic hydroxyl method was calibrated with organic solvent-isolated kraft lignins.

Table 2
Initial Conditions and Experimental Design for Reactor Runs and Controls

Enzyme(s)[a] used	Condition	Lignin (mg)	[LIP] (U/L)	[MNP] (U/L)	Reactant reservoir [H_2O_2] (m*M*)	Reactant reservoir [VA] (m*M*)	Reaction chamber [H_2O_2] (μM)[c]	Reaction chamber [VA] (μM)[c]	Reaction chamber [Mn(II)] (μM)[d]
LIP	Base case	22.8	1000	899	38.8	149	100	600	
LIP	No lignin control		1000	899	38.8	149	100	600	
LIP	No reagent control	23.1	1000	899					
LIP	No enzyme control	22.9			0.1	0.6	100	600	
LIP	Autoclaved enzyme control	23.1	nd[b]	nd	0.1	0.6	100	600	
LIP	No enzyme, no reagent control	22.9							
MNP	Base case	22.8	nd	56000	11.0		100		100
MNP	No lignin control		nd	56000	11.0		100		100
MNP	No reagent control	23.0	nd	56000					
MNP	No enzyme control	22.8			0.1		100		100
MNP	Autoclaved enzyme control	22.9	nd	nd	0.1		100		100
MNP	No enzyme, no reagent control	23.1							
LIP + MNP	Base case	22.8	1000	56000	44.4	120	100	600	100
LIP + MNP	No lignin control		1000	56000	44.4	120	100	600	100
LIP + MNP	No reagent control	22.8	1000	56000					
LIP + MNP	No enzyme control	23.1			0.1	0.6	100	600	100
LIP + MNP	Autoclaved enzyme control	23.0	nd	nd	0.1	0.6	100	600	100
LIP + MNP	No enzyme, no reagent control	23.0							

Brackets indicate concentration, while blanks indicate the absence of the species.

[a] The LIP concentrate consisted mainly of isoenzymes H2, H6, and H8, and also contained a very small amount of MNP, essentially all H4. The MNP concentrate consisted mainly of H3. Note that 1 U/L of LIP activity represents a much higher relative activity than 1 U/L of MNP activity; i.e., the LIP + MNP mixture contained 56% LIP and 44% MNP measured as heme protein.

[b] nd, None detected.

[c] Note that these values are higher than the maximum levels listed in Table 1 in which enzyme(s) are present. This is because conditions were set initially to quickly drive the H_2O_2 and VA levels down into the desired ranges (listed in Table 1) and maintain them there.

[d] Added as tartrate-chelated Mn(III) by first mixing it with the active MNP and stoichiometric H_2O_2.

Extraction of Liquid and Solid Samples for GC/MS

Recovered liquid fractions were thawed and extracted sequentially with three equal volumes of chloroform, recovering the chloroform layer each time. The process was repeated with ethyl acetate. Recovered solid fractions were thawed and pelleted by centrifugation, discarding the supernatant. The pellet was extracted twice at 37°C with 1 mL of chloroform, and then twice with 1 mL of ethyl acetate. The extracted solids were then washed free of solvents by centrifugation with distilled H_2O and stored at −80°C under 1 mL of distilled H_2O. To roughly quantify unknown peaks, internal standard, 2-chloro-5-(trifluoromethyl)-benzoic acid was added to each vial just prior to GC/MS analysis at 1020 ng/vial, using a clean glass microliter syringe. The extracts were then evaporated nearly to dryness under a light stream of nitrogen and derivatized for GC/MS (see below). Once samples were extracted, the extracts were stored at −20°C for no longer than 24 h before analysis, to guard against excessive volatilization.

GC/MS Analyses

Chloroform and ethyl acetate extracts of the liquid and solid samples were analyzed as trimethylsilane (TMS) ethers by GC/MS, as previously described *(5)*. Derivatization was done with *N,O-bis*-(trimethylsilyl)-trifluoroacetamide (BSTFA). Extracts were analyzed at 70 eV, using a JEOL AX 505 double-focusing magnetic sector mass spectrometer equipped with an HP5890 gas chromatograph. Separation of extracts was achieved with either a 30 m DB5-MS capillary GC column (J and W, Folsom, CA), or with a 30 m DB-1 capillary GC column (SPB-1, Supelco, Bellefonte, PA) *(5,6)*. Run conditions and the column used were taken into account when comparing retention times for comparison of controls and base case ion chromatograms.

Treatment of GC/MS Data

Unique peaks were identified in the base case chromatograms by comparison with the chromatograms of the controls. Peaks that were not unique to the base cases were excluded, unless the relative concentration was at least 3× higher in the base case. The selected peaks were integrated and their concentrations estimated from the internal standard. Commercially available databases were searched for matches to the mass spectra of the unknowns. Structures were suggested for each remaining unknown, using the mass spectrum, the derivatization pattern, and the following assumptions: It was assumed that the compounds were lignin-derived, and thus contained only carbon, hydrogen, and oxygen. Next, it was assumed that the products retained ring structures in aromatic or quinone forms, which limited the number of rings plus double bonds *(12)*.

RESULTS

Substrate Composition

Substrate compositions and physical characterization data were obtained in order to demonstrate the similarity of this lignin substrate to natural, insoluble lignin as it occurs in wood. The compositions of the poplar residue at each step of the lignin isolation procedure are presented in Table 3. The composition of the untreated poplar was typical for this type of hardwood *(13)*. Cellulose/hemicellulose removal yielded a substrate containing 74.5% lignin and 12.2% carbohydrates, after exhaustive solvent extraction. The final lignin residue was insoluble in dioxane, dimethylformamide, and several aqueous solutions of the two *(5)*. Acetylation *(14)* did not produce a soluble lignin, and thioacidolysis *(15,16)* produced yields of syringyl and guaiacyl monomer products of 78 and 87%, relative to those from the native poplar, respectively, indicating no significant condensation of β-O-4 intermonomer linkages. The syringyl: guaiacyl ratios for the isolated lignin and the untreated poplar were not significantly different *(16)*, at 1.02 and 1.15, respectively. These data indicate that the isolated lignin was similar to the untreated poplar lignin in terms of monomer contents, ratio of monomer types, and frequency of intact interunit linkages.

LIP and MNP Production

The LIP concentrate consisted mostly of isoenzymes H2, H6, and H8, and also contained a very small amount of MNP (*see* Table 2), essentially all H4. This probably occurred because of a small amount of Mn(II) carryover in the mycelial inoculum to the production cultures. The effect of this MNP in the LIP-alone lignin treatment runs was negated, however, by excluding Mn(II) from the reaction mixture, so that only LIP was active. The MNP produced consisted mainly of H3 and contained no detectable LIP activity.

Treatment of Lignin

Mass balances on the solid lignin before and after treatment with LIPs and/or MNPs were calculated as a simple measure of whether mass was lost because of enzyme activity. Results of the mass balances are presented in Fig. 2. The statistical significance of the base case measurements were tested using the null hypotheses that they were part of their control sets, since the controls have the same systematic errors associated with their measurement. The significance levels, using the Student's *t*-distribution with three degrees of freedom as the reference distribution, were 0.376, 0.130, and 0.018 for the LIP, MNP, and LIP + MNP base cases, respectively. Note that each control set was used separately, with its corresponding base cases (with which it was run). Thus, the null hypothesis was rejected

Table 3
Compositions of Untreated Poplar and Residue After Each Step of Lignin Isolation Procedure

Component	Untreated poplar (%)	Acid hydrolyzed (%)	Cellulase hydrolyzed (%)	Solvent extracted (%)
Glucan	42.9	62.9	8.1	10.1
Xylan	13.8	0.9	0.9	1.1
Galactan	0.1	nd[a]	0.3	1.0
Arabinan	0.6	nd	nd	nd
Mannan	1.0	nd	nd	nd
Lignin[b]	25.4	29.3	78.1	74.5
SUM[c]	83.8	93.1	87.4	86.7

Sequential isolation steps proceed from left to right in the table.
[a] nd, none detected.
[b] Includes Klason lignin, extractives, and ash.
[c] Not equal to 100% because of recovery errors and unknown uronic acid content.

for LIP + MNP, meaning that it was not part of its control set. The null hypothesis was not rejected for LIP or MNP alone. Comparatively, the significance level for a control data point showing a 7% loss, which was the maximum deviation seen among the control sets, was 0.208 in its control set, and 0.057 over all the control sets combined. Thus, the LIP + MNP base case data point, at 11% release of mass, was not part of its control set to 98.2% certainty. This indicates that neither LIP nor MNP solubilized the solid singly, but together they were effective in solubilizing the solid. The solid released may have included lignin and/or carbohydrates.

FTIR/PLS Analyses

FTIR/PLS measurements of lignin, carbohydrate, methoxyl, and phenolic hydroxyl contents were performed to provide an independent means of demonstrating lignin loss from the solid. The PLS software used to analyze the data indicated that the carbohydrate spectra for all samples varied significantly from the spectra used for the whole-wood and woodpulp calibration set (data not shown). This is probably because of the comparatively low carbohydrate content in the residual lignin used. Therefore, the carbohydrate predictions cannot be used with confidence, and are not included.

The FTIR spectra (Fig. 3) for all samples were typical for hardwood lignins *(11,17,18)*. The spectra were normalized so that the absorbance at 1504 cm^{-1} was proportional to the known lignin concentration in the pellets, as suggested by Faix *(19)*, since the aromatic skeletal vibrations at this band correlate well with lignin concentration. Results of the lignin content measurements are presented in Fig. 4. The statistical significance of the

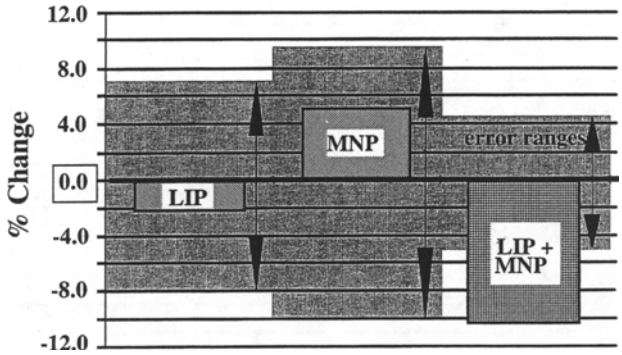

Fig. 2. Results of the mass balances for the lignin treatment runs. Controls are represented by the shaded error ranges, which are the averages ± 2 σ for the control mass balance data sets done with each base case. The LIP + MNP result is statistically different from the control set to 98.2% certainty.

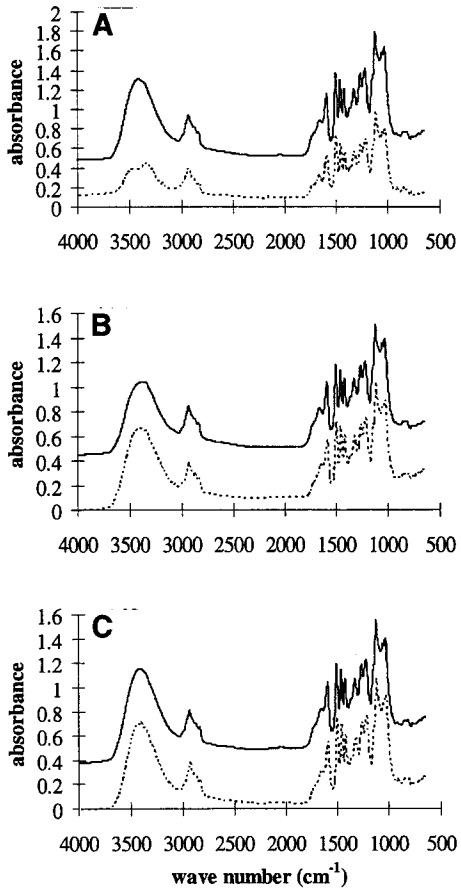

Fig. 3. Raw, unnormalized FTIR spectra of base cases (solid curves) and their ER⁻ controls (dashed curves). **(A)** LIP alone; **(B)** MNP alone; and **(C)** LIP + MNP. Note that 0.5 absorbance units have been added to each base case spectrum to proportionally separate it from its controls.

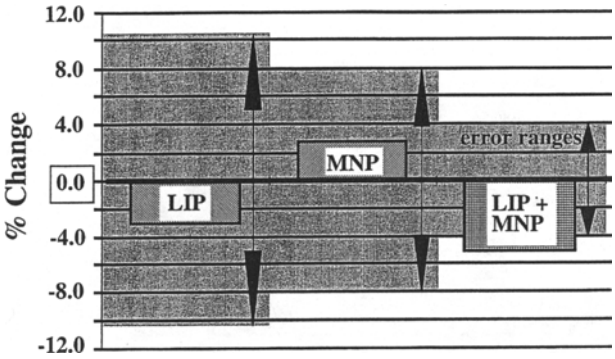

Fig. 4. Results of the FTIR/PLS determination of lignin content in the residue from the lignin treatment runs. Controls are represented by the shaded error ranges, which are the averages $\pm$ 2 σ for the control mass balance data sets done with each base case. The LIP and MNP results are clearly part of their control sets; the LIP + MNP result is statistically different from the control set to 98.8 or 92.3% certainty, using the normal distribution or the *t*-distribution, respectively.

base case measurements were tested as above, using the same null hypothesis, again using each control set separately with its base case. The *t*-distribution significance levels for LIP, MNP, and LIP + MNP base case mass balances were 0.324, 0.319, and 0.077, respectively, with seven degrees of freedom for each (duplicate measurements). Using the normal distribution, these were 0.301, 0.247, and 0.012, respectively. The null hypothesis was accepted for both LIP and MNP, using either distribution. It was rejected for LIP + MNP using the normal distribution, and borderline rejected using the *t*-distribution. It thus seems clear that the loss of mass shown above was at least in part a result of lignin release by LIP + MNP.

There was no change in the methoxyl content for any base case or control, with all measurements not statistically different from 1.34 $\pm$ 0.03 methoxyls/C_9 (average $\pm$ 2σ). This indicates that any lignin removal that may have occurred was not preferential for syringyl or guaiacyl units. The PLS analyses for phenolic hydroxyl contents predicted values well below the range of the Kraft lignin calibration set, for which the lower boundary was 0.70 OH groups per aromatic unit. All samples from this study were indicated at 0.00 $\pm$ 0.07 OH groups per C_9, which indicates that the lignin samples were highly polymeric, and also suggests that any lignin removal that may have occurred was at the free-phenolic ends of the lignin polymer.

GC/MS Analyses

The GC/MS analyses of solvent extracts of the aqueous and solid phases after enzymatic treatment were performed to present an additional independent verification of enzymatic lignin solubilization. Syringalde-

hyde, vanillic acid, and syringic acid were identified in all samples. 2,6-dimethoxyhydroquinone was also present in all samples, although much more was present in the base case extracts, while 2-methoxyhydroquinone was present in some, but not all, controls. Retention times, concentrations, and functional information derived from the mass spectra for unique and higher relative concentration peaks (*see* above for peak selection criteria), identified in the base case extracts, are presented in Table 4. Also included in Table 4 are the possible molecular formulas based on the available data and the procedure outline above. These are not meant to be confirmed identifications of the compounds, but are merely suggestions that fit the available information, since identification was not possible. Please refer to Thompson *(5)* and Thompson et al. *(6)* for the mass spectra and some possible structures.

DISCUSSION

The ideal substrate for lignin depolymerization studies would be an unmodified, carbohydrate-free lignin as it occurs in wood *(4)*. The methods chosen for the isolation of the lignin substrate used here represent an attempt to approach this ideal. Although the lignin used in this study has been modified to some extent during the isolation process and still contains at least 12% carbohydrates, thioacidolysis and other tests indicated that it was not significantly different from the original lignin *(16)*. Thus, the lignin used in this study was closer to the ideal substrate than many substrates used previously.

The mass balances for the base cases vs the controls provide some evidence that degradation and perhaps modification occurred for LIP + MNP, but not for either enzyme singly. The loss of mass was statistically different from its controls to 98.2% certainty. An interesting observation, noted during treatment runs containing MNP, was that the base case lignin color darkened over the course of the run from light to dark brown, and remained dark after treatment *(5)*. When LIP + MNP was used, the base case lignin first darkened, and then lightened back to its original color. With LIP singly, no color change was observed. Although this color change could be a result of precipitation of MnO_2, controls containing MNP and reagents, but devoid of lignin, did not form the characteristic MnO_2 precipitate, suggesting that the color changes may have been caused by modification of the lignin by MNP, and then release of the modified fragments by LIP.

The FTIR/PLS analyses indicate that neither LIP nor MNP singly altered the lignin content of the substrate, but together the two enzymes released 5.1% of the lignin (4.2% as total weight). This is an important verification that at least part of the 11% weight loss seen in the mass balance was caused by release of lignin, and not to carbohydrate solubilization. The carbohydrate contents were not well modeled by the calibration

Table 4
Summary of GC Data, Including Concentration and Functional Information, and Possible Molecular Formulas for Peaks of Interest Identified by GC/MS

RT (min:s)[a]	Derivatized mol wt (g/mol)	Base case	Unique? (y/n)	Extr.[f]	Est. conc. (ng/mL)	Highest control conc.[e] (ng/mL)	Structural analyses: Possible no. of TMS groups	Structural analyses: No. of rings + double bonds	Structural analyses: Possible formula
6:22	280	LIP + MNP	Yes	L-EA	0.0235	–	1	7	$C_{10}H_8O_5$
							1	6	$C_{11}H_{12}O_4$
6:32	318	MNP	Yes	S-EA	0.0189	–	1	?	Unknown[c]
8:08	182	LIP	Yes	L-EA	0.1656	–	0	5	$C_9H_{10}O_4$
							0	4	$C_{10}H_{14}O_3$
8:59	226	LIP	Yes	L-EA	0.8820	–	1	4	$C_8H_{10}O_3$[d]
9:17	308	LIP + MNP	No	S-C	0.0532	0.0065	1	6	$C_{13}H_{16}O_4$
10:12	298?	MNP	Yes	L-EA	0.0358	–	2?	?	Unknown[c]
11:10	282	LIP	Yes	L-EA	0.0925	–	2	5	$C_7H_6O_3$[b]
11:15	256	LIP	Yes	L-EA	0.9226	–	0	4	$C_9H_{12}O_4$
12:07	356	LIP + MNP	Yes	S-EA	0.0252	–	2	5	$C_{10}H_{12}O_5$
							2	4	$C_{11}H_{16}O_4$
							3	4	$C_7H_8O_3$
13:36	372	MNP	Yes	L-C	0.0249	–	2	4	$C_{11}H_{16}O_5$
							2	9	$C_{14}H_{12}O_5$
		LIP + MNP	No	S-C	0.0450	0.0112	3	4	$C_7H_8O_4$

15:25	342	MNP	Yes	L-C	0.0445	–	2	5	$C_9H_{10}O_5$
							2	4	$C_{10}H_{14}O_4$
15:54	400	MNP	No	L-EA	0.1499	0.0179	1	12	$C_{17}H_{12}O_7$
							1	11	$C_{18}H_{16}O_6$
			No	S-EA	0.0902	0.0179	1	10	$C_{19}H_{20}O_5$
							2	6	$C_{11}H_{12}O_7$
		LIP + MNP	No	L-EA	0.1933	0.0405	2	5	$C_{12}H_{16}O_6$
							2	4	$C_{13}H_{20}O_5$
							3	5	$C_8H_8O_5$
							3	4	$C_9H_{12}O_4$
16:00	370?	LIP	Yes	L-C	0.0256	–	2?	?	Unknown[c]
21:37	526	MNP	Yes	L-C	0.0498	–	2	14	$C_{20}H_{14}O_8$
							3	7	$C_{15}H_{18}O_7$
							3	6	$C_{16}H_{22}O_6$
22:26	552	MNP	Yes	S-C	0.0988	–	1	16	$C_{25}H_{20}O_{10}$
							1	15	$C_{26}H_{24}O_9$
							2	10	$C_{19}H_{20}O_{10}$
							2	9	$C_{20}H_{24}O_9$
							3	9	$C_{16}H_{16}O_8$
							3	8	$C_{17}H_{20}O_7$

Peaks are listed by increasing retention time, and by the extracts from which they were identified.

[a] Retention time with a DB-1 GC column and 3.75 min solvent hold for LIP and LIP + MNP runs, and retention time with a DB5-MS GC column and 2.00 min solvent hold for MNP runs.

[b] Identified as *p*-hydroxybenzoic acid by comparison with spectrum in NIST database.

[c] The noise level in the mass spectrum for this peak is too high to identify the molecular ion with a high degree of certainty.

[d] Identified as 3,4-dimethoxyphenol by comparison with spectrum of authentic standard.

[e] Highest concentration in any control extract for that enzyme case.

[f] L or S, Extract of liquid or solid phases, respectively; C, chloroform; EA, ethyl acetate.

method and so are not included here. The lack of differences in the methoxyl contents of all samples indicates that degradation took place in the LIP + MNP mixtures without preference for syringyl or guaiacyl units, and that no significant demethoxylation of the lignin occurred. Finally, the very low phenolic hydroxyl contents for all samples indicate that the lignin samples are highly polymeric, which suggests that intermonomer bond cleavage did not occur to any significant extent in the interior of the polymer, but must have occurred near the end units where the free-phenolic hydroxyl content is highest *(14)*.

The presence of unique products in the base case extracts for all three enzyme cases suggests that there was a release of lignin fragments catalyzed by both enzymes over the course of the 12 h reaction, alone and in combination. Reasonable structures that could be lignin-derived are possible for all unique products found in the extracts of all three enzyme cases *(5)*. It should be noted that no ring-opened structures were considered during the structural analysis procedure. It is possible that the unique products observed are enzyme oxidation products of soluble lignin extractives, such as syringaldehyde, not completely removed during the exhaustive solvent extractions of the dilute-acids/cellulase-treated poplar. The inclusion of a control to eliminate this type of product is, in retrospect, desirable. Although these data do not provide direct proof of release of lignin fragments by identification and quantification of the products, and elucidation of their formation pathways, the data are still useful, because they provide verification that products appear in the liquid phase that have mass spectra and functionalities characteristic of lignin-derived products. As such, they play an important supportive role to the FTIR and mass balance data.

This is the first in vitro evidence with an insoluble natural lignin in aqueous media, that these enzymes take part in lignin degradation. Each piece of evidence is insufficient to verify ligninolytic activity, but, when considered as a whole, it is clear that lignin degradation occurred with LIP and MNP present, but not to any significant extent with LIP or MNP singly. These results support those of Perez and Jeffries *(20)*, that MNP performs the initial stages of depolymerization and that LIP performs the bulk of the depolymerization, and of Tuor et al. *(21)*, that LIP and MNP act synergistically to degrade lignin.

CONCLUSIONS

The results of this study indicate that LIP and MNP from *P. chrysosporium* synergistically effect the degradation of lignin in aqueous media, with MNP perhaps catalyzing the initial steps to make the lignin a more suitable substrate for LIP. Because degradation was observed with control of H_2O_2 and VA levels, but not in previous studies without control, it is plausible that the previous lack of observable aqueous depolymerization of lignin

in vitro may have been caused by inactivation of the enzymes by H_2O_2, or to competitive inhibition by H_2O_2. It has also been demonstrated (22) that many peroxidases not generally capable of degrading lignin in aqueous media are able to depolymerize lignin in aqueous organic solvents. It is likely that the previous lack of observable aqueous lignin degradation was caused by a combination of these factors.

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