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Polymerization of Pentachlorophenol and Ferulic Acid by Fungal Extracellular Lignin-Degrading Enzymes

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High-molecular-weight polymers were produced by a crude concentrated supernatant from ligninolytic *Phanerochaete chrysosporium* cultures in a reaction mixture containing pentachlorophenol and a humic acid precursor (ferulic acid) in the presence of a detergent and H_2O_2. Pure manganese peroxidase, lignin peroxidase, and laccase were also shown to catalyze the reaction.

When white-rot fungi are used for the remediation of soils contaminated with pentachlorophenol (PCP), a dramatic decrease of the extractable PCP occurs. From 88 to 90% of the PCP is depleted after 60 days, depending on the fungus used (20, 21, 22). A small part of it is converted to CO_2 and water (2 to 9%, depending on the fungus used) (22, 30), and another part is methylated by the fungus to yield pentachloroanisole (PCA). The percentage of PCA produced depends on the fungus and the soil type used (20, 21, 30). Evidence from a field study showed that once produced, PCA is also degraded (21). Finally, a large portion of the PCP is converted into soil-bound transformation products that are not extractable with organic solvents (30). Interaction of pesticides and other xenobiotic compounds with soil humic materials has been recognized for a long time (26).

Humic acid, a major component of soil organic matter, consists of complex polymers of hydroxyphenols, hydroxybenzoic acids, and other aromatic structures with linked peptides, amino sugar compounds, fatty acids, microbial cell wall and protoplasmic fractions, and possibly other constituents (14, 18, 27, 32). Phenolic compounds are primary precursors in the formation of humic acid polymers (14), undergoing either autooxidative or enzymatic polymerization, depending on their reactivity. Numerous xenobiotics are structurally aromatic. As with naturally occurring aromatic substrates, phenolic degradation products of aromatic xenobiotics (8) would be expected to become part of the pool of precursor molecules for humic acid synthesis and become constituents of soil humic polymers. Indeed, potentially toxic compounds have been linked into humic molecules since humus was first formed on earth (7). These compounds include phenols, naphthalenes, anthracene derivatives, aflatoxins, and many other toxic substances (25). Binding of xenobiotics to soil humic materials is mediated by a variety of biological (e.g., microbial and plant enzymes) and abiotic catalysts (e.g., metal oxides, clay minerals, and organomineral complexes) (5). Because of the complex and heterogeneous nature of soil, it has been difficult to resolve the relative contribution of the different catalysts to the oxidative polymerization of xenobiotics into humic acids. However, xenobiotic humification, the oxidative coupling of xenobiotics with soil humic materials, is thought to be mediated primarily by fungal extracellular monophenol monooxygenases, laccases, and peroxidases. The present work was undertaken to establish if lignin-degrading enzymes produced by white-rot basidiomycetes could catalyze the in vitro polymerization of PCP with a humic acid precursor, ferulic acid.

Polymerization of PCP and ferulic acid by the action of *Phanerochaete chrysosporium* enzymes. In vitro reactions containing PCP, ferulic acid, and concentrated supernatant fluid from ligninolytic *P. chrysosporium* BKM F-1767 cultures were conducted to determine if PCP and humic-acid precursors polymerize by the action of extracellular lignin-degrading enzymes produced by white-rot fungi. Full reactions contained (final concentrations) 20 mM sodium malonate (pH 4.5), 0.2 mM $MnSO_4$, 0.2 mM *n*-dodecyl-β-D-maltoside (Sigma), 173 μM ferulic acid, and 75 μM [^{14}C]PCP (300,000 dpm/reaction) in a final volume of 2.0 ml. H_2O_2 (0.2 mM final concentration) and 6 μl of concentrated supernatant containing 83.4 U ml^{-1} of manganese peroxidase (MnP) (assayed using 0.1 mM vanillylacetone as a substrate, 0.1 mM $MnSO_4$, and 100 mM sodium tartrate buffer [pH 4.5]) and 82 U ml^{-1} of lignin peroxidase (LiP) (assayed using 2 mM veratryl alcohol as a substrate, 25 mM sodium tartrate buffer [pH 2.5], and 0.4 mM H_2O_2) were added at the beginning of the reaction and after 24 h of incubation. Control reactions contained all of the above-mentioned components, except for concentrated supernatant, H_2O_2, or $MnSO_4$. The reactions were incubated at 30°C with shaking (200 rpm) for 48 h. They were stopped by placing them at 4°C. The enzyme preparation was obtained by growing *P. chrysosporium* in shaking liquid cultures under conditions optimal for the production of ligninolytic enzymes, which have been previously described (35). Cultures were incubated for 6 days, after which the supernatant fluid was concentrated 360 times, dialyzed against 10 mM sodium acetate (pH 5.5), and kept frozen. The molar ratio of ferulic acid to PCP (2.3/1) was chosen considering that the concentration of PCP in the soil would normally be lower than that of phenolic humus precursors. The presence of the detergent (*n*-dodecyl-β-D-maltoside) was found to be necessary for the reaction, probably because it aided the solubilization of the PCP. The products of the reaction were analyzed by gel permeation chromatography in a column (1.8 by 47 cm) of Sephadex G-75 (Pharmacia, Piscataway, N.J.) with 0.1 M potassium phosphate buffer (pH 7.5) containing 0.02% sodium azide as the mobile phase. The flow rate was approximately 60 ml h^{-1}, and 2.0-ml fractions were collected. Sodium sulfonate polystyrene molecular weight (MW) standards (Scientific Polymer Products Inc.,

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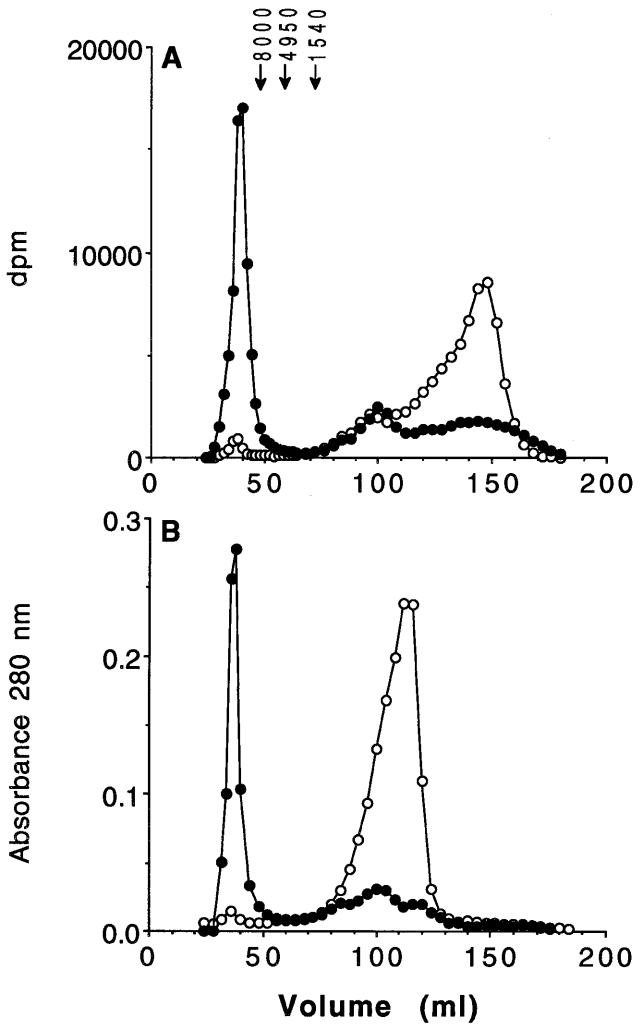

FIG. 1. Size exclusion chromatography of the products of the reaction between PCP and ferulic acid, catalyzed by concentrated supernatant fluid of *P. chrysosporium* cultures (●), and of a control reaction containing no enzyme (○). Radioactivity of the fractions (A) and A_{280} (B) are shown. Arrows show the elution volumes of sodium sulfonate polystyrene standards with the MWs indicated.

Ontario, N.Y.) were used to calibrate the column. The radioactivity of the fractions was counted in Polyfluor (Instrument Company, Meriden, Conn.) scintillation fluid.

In full reactions most of the radioactivity was eluted as a high-molecular-weight material (Fig. 1A). This material was excluded from the column (i.e., it was eluted with the void volume). In control reactions (i.e., those without enzyme preparation, H_2O_2, or $MnSO_4$) the PCP was eluted as a broad peak around 150 ml, which was beyond the total effective column volume (Vt). Aromatic hydroxyl and methoxyl substituents are known to increase adsorption onto Sephadex (11). Therefore, elution of PCP in fractions beyond the Vt was probably due to interactions of the phenolic group with the Sephadex gel matrix. A small peak corresponding to unreacted PCP was also observed in the full reaction. Production of the high-molecular-weight material in full reactions and failure of the material to form in reactions in which the supernatant or H_2O_2 was excluded indicated that either LiP and/or MnP was capable of polymerizing PCP into a higher-molecular-weight oligomer. No polymerization was observed when the reaction was conducted in the absence of $MnSO_4$ at pH 4.5 (not shown), which was an indication that, under these conditions, MnP catalyzed the reaction.

To demonstrate that ferulic acid was being incorporated into the oligomeric material, the A_{280} of the fractions from the Sephadex column was measured (potassium pentachlorophenate, the form of PCP present in the fractions, did not absorb at 280 nm). As expected, only one peak was observed for the control reaction, at the elution position of ferulic acid (110 ml). The elution profile of the full reaction showed a large peak that was excluded from the Sephadex G-75 column and only a small peak corresponding to free ferulic acid, indicating that the acid was indeed being polymerized into a higher-molecular-weight material (Fig. 1B). No other peaks of intermediate molecular weights were observed at A_{280} or in the radioactivity profiles. All the oligomeric material formed was excluded from the Sephadex column. Whether the MW of this material was really higher than 8,000 (the highest MW standard that eluted after the void volume) is not clear, since the structure of the MW standards used differed from the probable structure of the oligomer. The disappearance of PCP and ferulic acid from the reaction mixture was further confirmed by gas chromatography-mass spectrometry (GC-MS). Reactions were extracted three times with 2 ml of ethyl acetate at pH 2.0, and the extracts were dried by passing them through anhydrous sodium sulfate columns, concentrated to 4 ml, derivatized with *N*,*O*-bis(trimethylsilyl)acetamide under conditions previously described (12), and analyzed by GC-MS. Conditions for the GC were as described by Lamar et al. (21), and conditions for the MS were as described by Dietrich et al. (12). The extracts from control reactions showed peaks corresponding to the trimethylsilyl (TMS) derivatives of PCP (retention time, 13.67 min) and ferulic acid (retention time, 18.62 min). The peak corresponding to the TMS derivative of ferulic acid could not be detected in the extracts from the full reactions, and the peak corresponding to the TMS derivative of PCP was very small compared with the control reaction (not shown). Thus, both monomeric compounds were consumed during the reaction.

To determine if PCP polymerized into a higher-molecular-weight material in the absence of a humic acid precursor, the experiment was repeated excluding ferulic acid from the reaction. All other conditions were as before. As can be seen in Fig. 2, PCP was cross-linked to itself to form a higher-molecular-weight material that, as before, was excluded from the column. However, polymerization was enhanced by the presence of ferulic acid, as shown by a decrease in the area under the PCP peak and an increase in the height of the excluded peak (Fig. 2). The fact that polymer formation is enhanced in the presence of ferulic acid constitutes indirect evidence for copolymerization of PCP and ferulic acid.

Catalysis of the reaction by purified ligninolytic enzymes. Lignin-degrading fungi that have been shown to bind PCP to humic material in soil (30) produce different types of extracellular enzymes that could be involved in this process. Reactions using purified enzymes were conducted to determine which of them were able to catalyze the polymerization reaction of PCP and ferulic acid in vitro. All enzymes were used in amounts equivalent to 1 U ml^{-1} final concentration in the reaction medium. Pure MnP I and MnP II from *Phanerochaete sordida* (29) were used in reactions done in the same conditions as described above. Reactions containing laccase I of *Ceriporiopsis subvermispora* (15) and LiP2 of *P. chrysosporium* (13) were done at pH 3.0 and 4.5. The rest of the conditions were as before, except that laccase reactions did not contain $MnSO_4$ or H_2O_2 and LiP reactions did not contain $MnSO_4$.

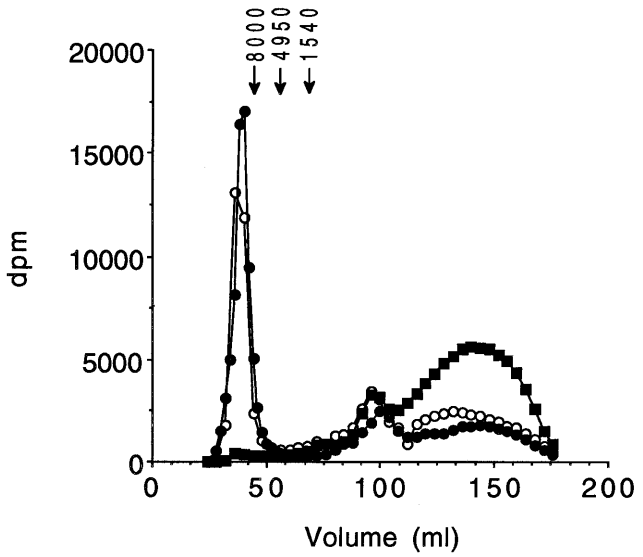

FIG. 2. Molecular size distribution of the polymerization products obtained from reactions containing only PCP (●) or PCP and ferulic acid (○) by the action of concentrated supernatant fluid of *P. chrysosporium* cultures and of a control reaction containing no enzyme (■). All other conditions were as described in the text. Arrows show the elution volumes of sodium sulfonate polystyrene standards with the MWs indicated.

Both MnPs were able to catalyze the reaction, producing a higher-molecular-weight oligomer (Fig. 3A). Although the optimal pHs of laccase and LiP have been described to be between 2.0 and 3.0 (15, 36), no polymerization reaction was observed with these two enzymes when the incubations were done at pH 3.0 (Fig. 3B). However, when the reactions were done at pH 4.5, both laccase and LiP produced the higher-molecular-weight material from PCP and ferulic acid (Fig. 3B). To reach an activity of 1.0 U ml^{-1} in the reaction mixture with laccase and LiP at pH 4.5, it was necessary to add 23 and 16 times more enzyme, respectively, than the amounts needed to reach this activity at pH 3.0. This was estimated on the basis of standard assay methods (laccase was assayed using 100 μM 2,6-dimethoxyphenol as a substrate and 50 mM sodium tartrate buffer, and LiP was assayed as described above) done at pHs 3.0 and 4.5. The lack of catalysis of the cross-linking reaction at pH 3.0 by LiP could be due to instability of this enzyme at that pH (36). The stability of laccase I of *C. subvermispora* at different pHs has not been described. As stated before, the oligomeric material was not produced when concentrated supernatant fluid of *P. chrysosporium* cultures was used in the absence of $MnSO_4$, even though this preparation contained LiP activity. The LiP activity was probably too low at pH 4.5 to produce a significant amount of product.

All the ligninolytic enzymes tested were able to catalyze the polymerization reaction. This result is not surprising in view of the mechanism of action of these enzymes. It has been shown that LiP catalyzes the 4-dechlorination of PCP to yield a *p*-benzoquinone (16). A similar reaction was catalyzed by MnP on 2,4,5-trichlorophenol (19). Laccase was also able to dechlorinate PCP and other chlorinated phenols (28). Quinones produced by these enzymes could easily polymerize into higher-molecular-weight material.

Ferulic acid was chosen for this study because it has been previously described to efficiently bind to other xenobiotic compounds like anilines, by the action of laccase, yielding hybrid trimers (1, 33). Its high reactivity is due to the acrilic group. Moreover, the reactivity of anilines to cross-coupling has been shown to be enhanced by the presence of highly reactive electron donors like ferulic acid (1). It has also been suggested that lignin-derived phenols that contain an acrilic group are preferentially utilized during peroxidase-mediated synthesis of humic material in the soil (1). Finally, it has been shown that the addition of ferulic acid and H_2O_2 to 3,3′-dichlorobenzidine-contaminated soil accelerates the binding of the latter compound to the soil (2).

Extensive work on the polymerization of phenolic compounds and on the binding of xenobiotic compounds to humic and fulvic acid has been done by Bollag and coworkers (for reviews, see references 3 and 4). Different chlorophenols have been shown to polymerize by the action of laccase, producing dimeric, trimeric, and tetrameric oligomers (6). The extent of polymerization of chlorinated phenols by laccase, horseradish peroxidase, and tyrosinase was shown to depend on the number of chlorines (9). The coupling reactions occur with deha-

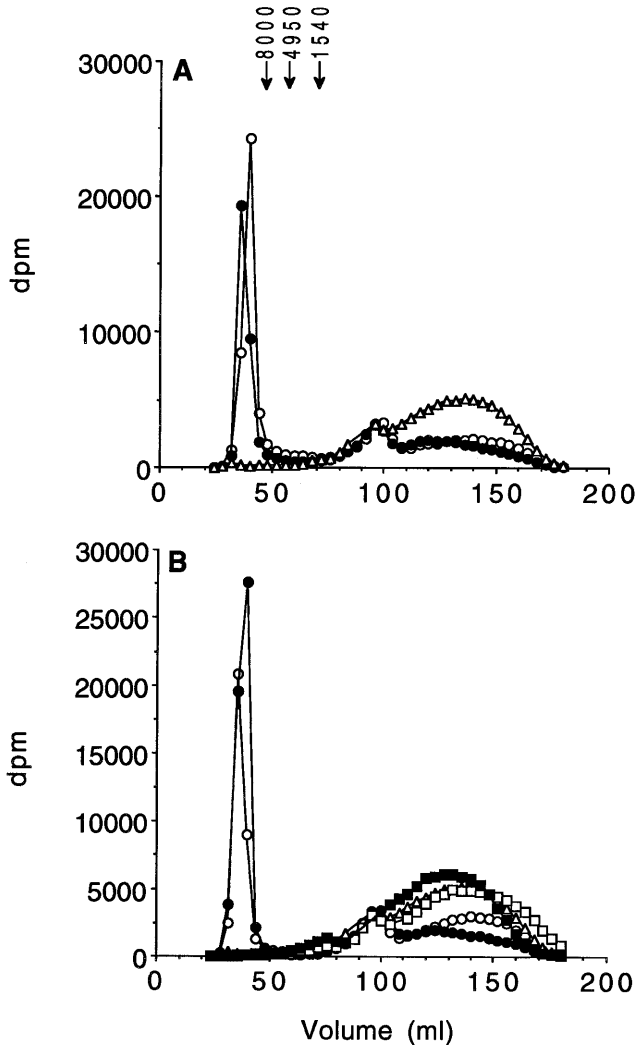

FIG. 3. Size exclusion chromatography of the products of the reaction between PCP and ferulic acid, catalyzed by pure MnP (A). MnP I (○) or MnP II (●) of *P. sordida* or no enzyme (△) was used. (B) Molecular size distribution of the products of the same reaction catalyzed by laccase I of *C. subvermispora* at pH 3.0 (■) and pH 4.5 (○), LiP2 of *P. chrysosporium* at pH 3.0 (□) and 4.5 (●), and a control reaction containing no enzyme (△). Arrows show the elution volumes of sodium sulfonate polystyrene standards with the MWs indicated.

logenation of the chlorinated phenols (10). Work done with chlorinated anilines showed that these compounds can be oxidized and cross-linked by laccase only in the presence of phenolic acids like guaiacol (34). Similarly, different chloroanilines have been shown to form cross-coupling products with ferulic acid by the action of laccase (33). Hybrid dimeric and trimeric products were identified in this reaction. Phenol and different chlorophenols and dichlorophenols have been shown to bind to humic or fulvic acids in the presence of lactoperoxidase, chloroperoxidase, horseradish peroxidase tyrosinase, or laccases (17, 23, 24, 31).

We believe that the process described in this work might mimic the irreversible binding of PCP to soil humic material. PCP and other pollutants may be copolymerized with low-molecular-weight aromatic compounds derived from the decay of lignin during the synthesis of humic material in the soil. Humification of these xenobiotics could greatly reduce their availability and thus their toxicity. White-rot fungi have potential for the remediation of soils contaminated with aromatic xenobiotics. However, the stability and toxicity of the oligomeric material need to be established.

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