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Penetrability of White Rot-Degraded Pine Wood by the Lignin Peroxidase of *Phanerochaete chrysosporium*

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The penetration of enzymes into wood cell walls during white rot decay is an open question. A postembedding immunoelectron microscopic technique was the method of choice to answer that question. Infiltration of pine wood specimens with a concentrated culture filtrate greatly improved the labeling density and, thereby, reproducibility. Characterization of the concentrated culture filtrate by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting (immunoblotting) revealed three closely spaced proteins of molecular weights about 42,000 showing immunoreactivity against anti-lignin peroxidase serum. It was shown by immunogold labeling that lignin peroxidase of *Phanerochaete chrysosporium* is located on the surface of the wood cell wall or within areas of heavy attack. It did not diffuse into undecayed parts of the cell wall. The reasons for preventing lignin peroxidase from penetrating wood cell walls during white rot decay are discussed.

Great importance is attached to the microbial degradation of lignin in connection with a future biotechnological use in the pulp and paper industry (5, 6) and for the treatment of bleach plant effluents (3). For this reason, intensive research has been carried out in the past few years to clarify the mechanisms of lignin degradation by basidiomycetes, above all *Phanerochaete chrysosporium*. The enzyme lignin peroxidase (8, 24), isolated from the culture filtrate of *P. chrysosporium*, is said to play one of the leading roles in lignin degradation (13). Lignin peroxidase catalyzes in vitro the oxidation of a great variety of simple lignin model substances. Little is known, however, about the action of lignin peroxidase on the lignin polymer. Cleavage of the lignin polymer by radicals, which is not catalyzed directly by enzymes, might play a decisive role (9, 11).

Biochemical studies were carried out mostly with soluble, low-molecular-weight substances in liquid systems in which the enzymes gain easy access to their substrate. However, in wood degradation by enzymes, not only biochemical, but also morphological and spatial aspects play important roles because the degrading agents have to be capable of reaching their substrate. Of course, this is also true for a biotechnological use of enzymes in biological bleaching and in biological pulping in which only a rapid penetration of the lignin-olytic system into the wood cell wall would guarantee an acceptable duration of the process. The pore size of the wood cell wall, resulting from its ultrastructural architecture, is relatively small compared with the size of enzymes; thus, the diffusion of enzymes within the wood cell wall has always been a matter for discussion (4, 23).

It may be concluded from light and electron microscopic studies of the morphology of fungal wood degradation carried out so far that white rot is characterized by a continuous degradation of the wood cell wall from the lumen to the middle lamella (10, 16, 26) and that the degrading agents therefore probably act only on the surface. Even if lignin is degraded preferably, it does not seem to be degraded via diffusion of enzymes into the entire cell wall, but also continuously from the lumen to the middle lamella (1, 2, 17, 18).

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A postembedding immunoelectron microscopic technique was the method of choice to clarify the problems of the diffusibility of enzymes into the wood cell wall. This method was successfully used in previous studies (7, 22) to localize the intracellular presence of lignin peroxidase. The object of the present study was the immunoelectron microscopic localization of the place of action of lignin peroxidase in white rot-degraded pine wood.

MATERIALS AND METHODS

Organisms and growth conditions. *P. chrysosporium* BKM-F-1767 (ATCC 24725) and *Trametes hirsuta* D37 (strains isolated at our institute) were grown on pine wood at 28°C (15). For production of lignin peroxidase, *P. chrysosporium* was cultivated in agitated liquid cultures at 39°C under conditions modified from the methods of Kirk et al. (12): basal medium B-III was supplemented with sevenfold the basal trace element solution–0.4 mM veratryl alcohol–0.05% Tween 20–1% glucose and buffered with 10 mM sodium hydrogen phthalate, pH 4.5. After inoculation with spore suspension (A_{650} of 0.5; 100 ml/1,000 ml of medium), the cultures were grown in 1-liter Erlenmeyer flasks containing 500 ml of medium on a rotary shaker at 125 rpm. The cultures were flushed with oxygen at the time of inoculation and after 3, 5, and 6 days of cultivation. Under those conditions, maximal lignin peroxidase activity was reached after 7 days of cultivation. Lignin peroxidase activity (oxidation of veratryl alcohol to veratryl aldehyde) was measured by the method of Kirk et al. (12) at 25°C.

Concentration of culture filtrate. The culture filtrate was concentrated 100-fold by ultrafiltration (Amicon YM10) and filtered through a membrane filter (0.45 μm). Further concentration to 1:300 and membrane filtration were followed by dialysis against 10 mM sodium acetate, pH 6.0. The concentrated culture filtrate had a lignin peroxidase activity of approximately 20 U/ml.

Characterization of culture filtrate. Gel permeation chromatography was performed on Bio-Gel P200 (Bio-Rad Laboratories) in 10 mM sodium acetate, pH 6.0. The column was calibrated with dextran blue, aldolase, ovalbumin, and cytochrome *c* (all from Pharmacia).

One-dimensional electrophoretic analysis of proteins was

performed on sodium dodecyl sulfate–10% polyacrylamide gels, using the discontinuous buffer system of Laemmli (14). Protein bands were stained with Coomassie brilliant blue.

For immunoblotting, slab gels containing separated proteins were soaked in 50 mM sodium borate (pH 9.0)–1 mM EDTA–5 mM 2-mercaptoethanol–10% (vol/vol) methanol for 30 min. The proteins were then electrophoretically transferred to 0.2-μm nitrocellulose sheets (25), using the same buffer. For immunoreaction, nitrocellulose sheets were incubated with 10 mM Tris hydrochloride (pH 8.0)–150 mM NaCl (buffer A) containing 1% bovine serum albumin and 3% milk powder for 3 h at 37°C. They were then overlaid with rabbit anti-lignin peroxidase serum or preimmune serum, both diluted 1:10,000 for 1 h at 37°C. After several washes in buffer A containing 0.05% (vol/vol) Tween 20 (six 10-min periods), blots were incubated in a 1:7,500 dilution of anti-rabbit immunoglobulin G coupled to alkaline phosphatase (Promega Biotec) in the same buffer for 1 h at 37°C. After extensive washing, color was developed as described by the suppliers.

Infiltration of pine wood samples and preparation for electron microscopy. Specimens of noninfected pine wood and pine wood degraded by wood-rotting fungi were infiltrated with concentrated culture filtrate of *P. chrysosporium* for 20 h at 4°C and fixed in 70% ethanol for 1 h at 4°C. After dehydration, up to 80 to 90% ethanol specimens were embedded in LR White resin (Polaron Equipment Ltd.) and polymerized for 24 h at 50°C (22).

Postembedding immunogold labeling. Ultrathin sections of LR White-embedded specimens were collected on Formvar-coated or uncoated hexagonal mesh nickel grids and labeled with rabbit antiserum against lignin peroxidase H8 (12) (primary antibody) and affinity-purified goat anti-rabbit immunoglobulin G (Janssen Life Sciences products), conjugated to 5-nm colloidal gold particles (secondary antibody). The complete labeling procedure was described previously (22). Some of the labeled sections were re-embedded in LR White and cut perpendicularly to the labeled surface. Ultrathin sections were examined in a JEOL 100C transmission electron microscope at 100 kV.

Labeling specificity controls. Several control experiments were performed to determine labeling specificity, i.e., nonspecific adsorption of immunoglobulins to thin sections and cross-reactivity of the antibodies to proteins of other fungi: (i) replacement of the anti-lignin peroxidase serum by normal rabbit serum (Sera-Lab), preimmune serum, or other immune sera raised in rabbits, but not specific for lignin peroxidase; (ii) labeling of uninfected wood specimens; (iii) labeling of ultrathin sections of *T. hirsuta*.

RESULTS

Labeling of lignin peroxidase excreted by *P. chrysosporium* in vivo. Without preserving the ultrastructure of the hyphae, it was possible by a mild ethanol fixation to localize the lignin peroxidase excreted by *P. chrysosporium* in vivo on the surface of the wood cell wall. This labeling was independent of a direct contact between the wood cell wall and the hyphae. Figure 1 (top to bottom) shows a pine wood cell wall which was degraded almost completely. Lignin peroxidase is localized only on the surface of the wood cell wall. In general, the intensity of labeling of lignin peroxidase produced by *P. chrysosporium* in vivo differed too much in the individual specimens investigated to make a clear statement on the possible penetration of wood fibers by lignin peroxidase of *P. chrysosporium* during decay.

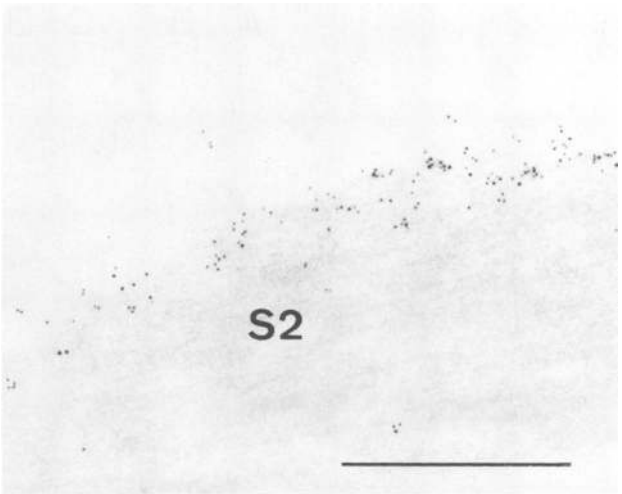
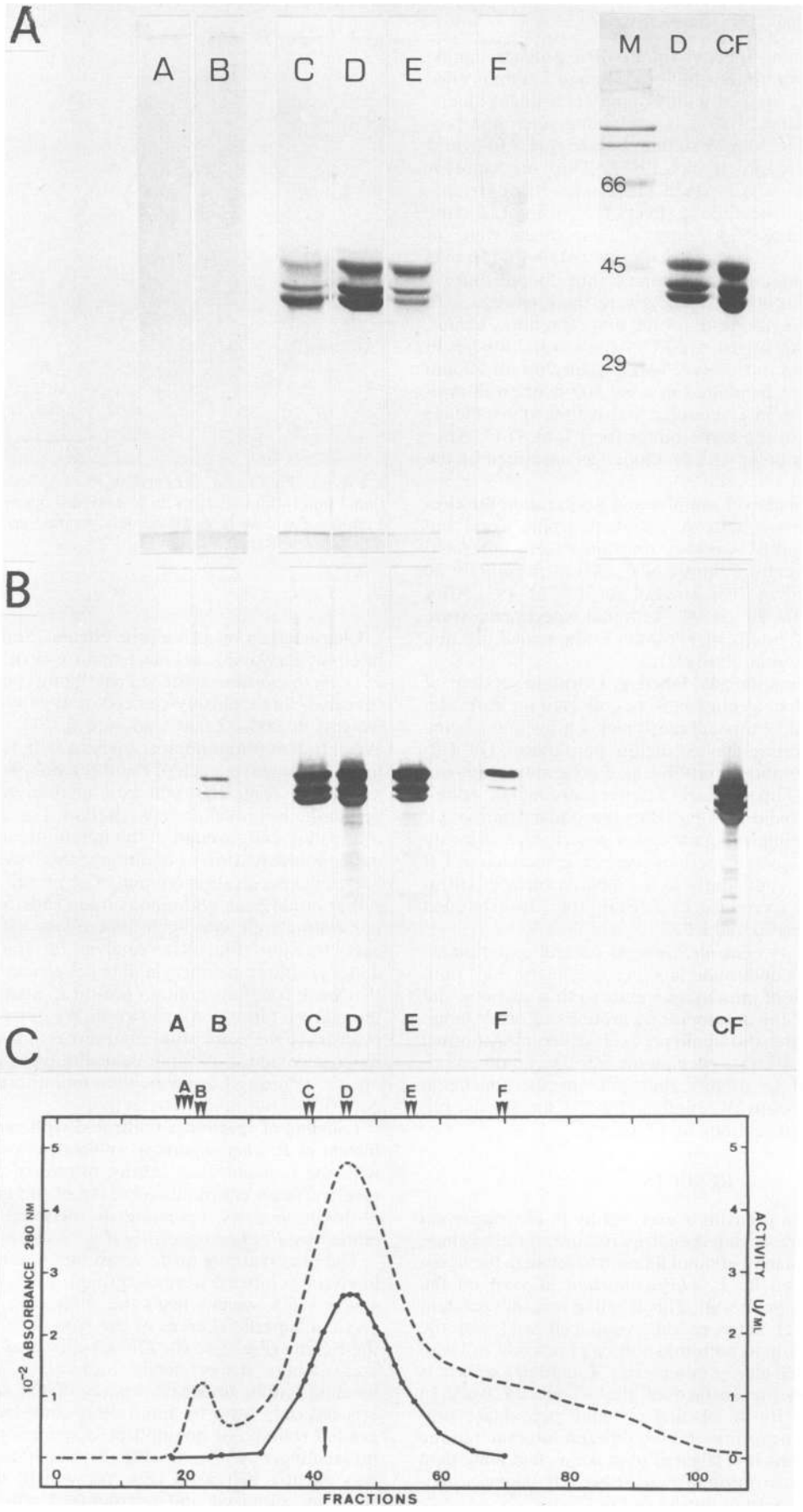


FIG. 1. Pine wood, decayed by *P. chrysosporium* (without additional infiltration). Part of an intensively degraded wood cell wall. Lignin peroxidase is localized only on the surface of the cell wall. S2, S_2 layer. Bar, 0.5 μm.

Characterization of culture filtrate. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of culture filtrates of *P. chrysosporium* enriched in lignin peroxidase activity revealed three closely spaced polypeptides of molecular weights 40,000, 42,000, and 46,000 (Fig. 2A). As shown in Western blot (immunoblot) analysis with rabbit antibodies to lignin peroxidase, each of the three polypeptides was immunoreactive (Fig. 2B), whereas incubation with preimmune serum did not result in any reaction. For a rough estimation of the molecular weight of the immunoreactive polypeptides under nondenaturing conditions, they were run on a calibrated gel permeation column. The proteins were eluted in a rather broad peak with a maximum shortly after ovalbumin, correlating well with lignin peroxidase activity measured in each fraction (Fig. 2C). Analysis of fractions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that peak fractions contain the three main protein bands of the culture filtrate. As shown in Western blot analysis, the peak fractions were still immunoreactive with antibodies to lignin peroxidase. A minor peak eluting near the void volume of the column showed neither immunoreactivity to lignin peroxidase nor enzymatic activity.

Labeling of specimens infiltrated with concentrated culture filtrate of *P. chrysosporium*. Infiltration of wood specimens with the concentrated culture filtrate of *P. chrysosporium* resulted in an enormous increase of the labeling density on ultrathin sections, revealing the penetrability of wood degraded by *P. chrysosporium* (Fig. 3 to 6).

The observations made when lignin peroxidase produced in vivo was labeled were confirmed. Also, in advanced decay stages (25% weight loss) the infiltrated lignin peroxidase stays in superficial areas of the wood cell walls attacked by the fungus (Fig. 3 to 6). The labeling was most intensive in areas where heavy decay took place. The intensity of labeling within undecayed parts of the wood cell wall increased only little by infiltration compared with the background staining of noninfiltrated specimens (blanks): partly no labeling was present (Fig. 3); partly the labeling density was slightly increased (Fig. 6), which, however, was the same in colonized and uncolonized pine wood specimens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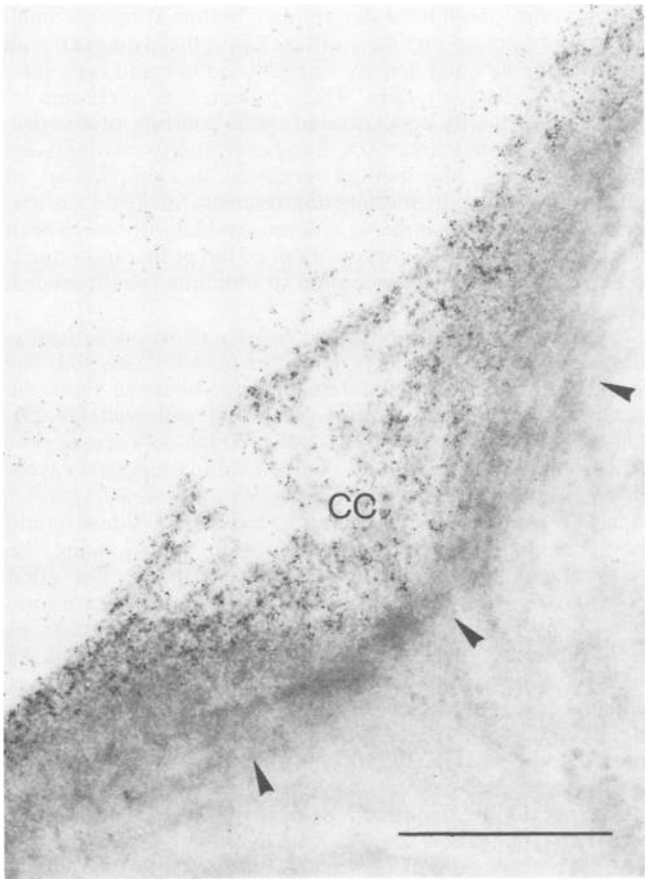

FIG. 3. White rot-degraded (*P. chrysosporium*) pine wood, infiltrated with concentrated culture fluid of *P. chrysosporium*. Cell corner (CC) is partly disintegrated into granular pieces on the surface areas. There is higher electron density in deeper, unlabeled areas (arrows). Bar, 1 μm.

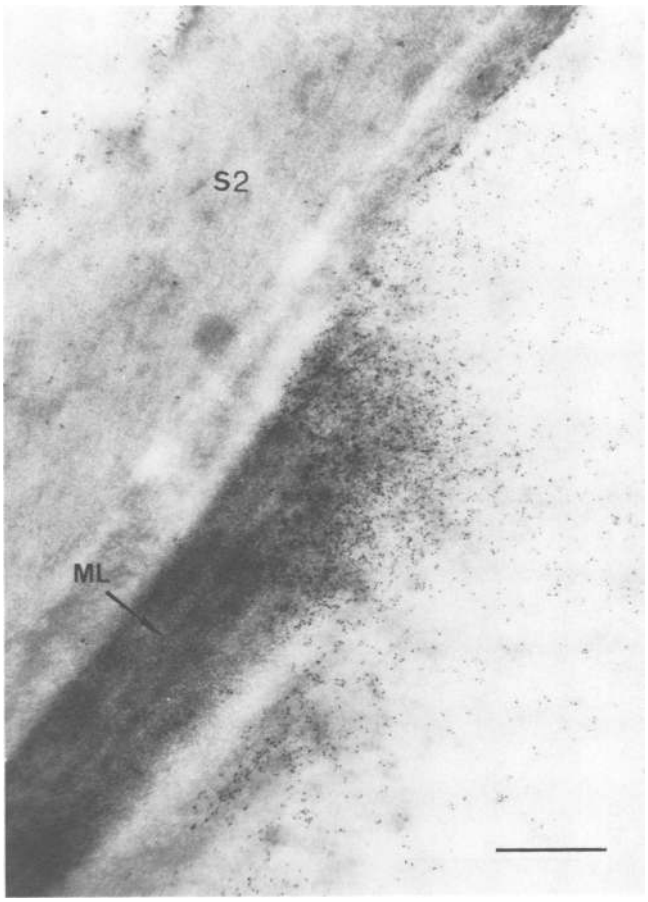

FIG. 4. White rot-degraded (*P. chrysosporium*) pine wood, infiltrated with concentrated culture fluid of *P. chrysosporium*. Heavily attacked wood cell wall is shown. There is high density of labeling where decay of the middle lamella (ML) took place. S2, S_2 layer. Bar, 1 μm.

and did not change in the course of decay. Figure 4 shows a heavily attacked wood cell wall partly degraded down to the middle lamella. It is noticeable that the labeling of lignin peroxidase is substantially denser in areas of heavy attack (Fig. 3 to 6) than in morphologically unchanged areas or cell walls of uncolonized wood, where labeling occurred mostly only in a very narrow area of the surface of the wood cell wall (Fig. 7), probably as a result of fibrillar remainders of degraded wood cell wall structures on the surface. The area of heavy attack shown in Fig. 6, where high labeling density is observed, has a thickness of 0.3 to 0.6 μm. Furthermore, it is noticeable (Fig. 3 and 6) that labeled and unlabeled areas of the wood cell wall are rather sharply separated from one another: there is no concentration gradient of lignin peroxidase in the direction of the middle lamella.

Sometimes it was apparently easier for lignin peroxidase to penetrate into deeper areas of the middle lamella than into other cell wall layers (Fig. 5). Figure 3 shows a heavily attacked cell corner. It can be seen electron microscopically that it has partly been disintegrated into granular pieces on the surface; lignin peroxidase is present only there. Nevertheless, it was often noticed that unlabeled deeper layers have a higher electron density up to a depth of a few 1/10 μm.

Re-embedding of labeled sections. In postembedding immunolabeling of lignin peroxidase, the antibodies themselves need not diffuse into the wood cell wall. Only antigens exposed on the surface of the ultrathin sections are accessible to the antibodies (Fig. 8).

Labeling specificity. Nonspecific adsorption of immunoglobulins to ultrathin sections was negligible; for detailed information, see Srebotnik et al. (22). Labeling ultrathin sections of *T. hirsuta* demonstrated the specificity of the antiserum for lignin peroxidase of *P. chrysosporium*: no cross-reactivity to extra- and intracellular proteins of this fungus was observed, although it can be assumed that the white rot fungus *T. hirsuta* produces ligninolytic enzymes similar to those produced by *P. chrysosporium*.

FIG. 2. Characterization of the concentrated culture filtrate of *P. chrysosporium*. (A) Sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis of the concentrated culture filtrate (CF) and gel permeation fractions A to F. M, Molecular weight markers. (B) Western blot analysis of the separated proteins, using anti-lignin peroxidase antiserum against lignin peroxidase H8. (C) Gel permeation profile of the concentrated culture filtrate with the active proteins eluting shortly after ovalbumin (arrow). Symbols: ---, A_{280}; •••, corresponding lignin peroxidase activity. A to F and arrowheads are fractions used for electrophoresis and Western blotting.

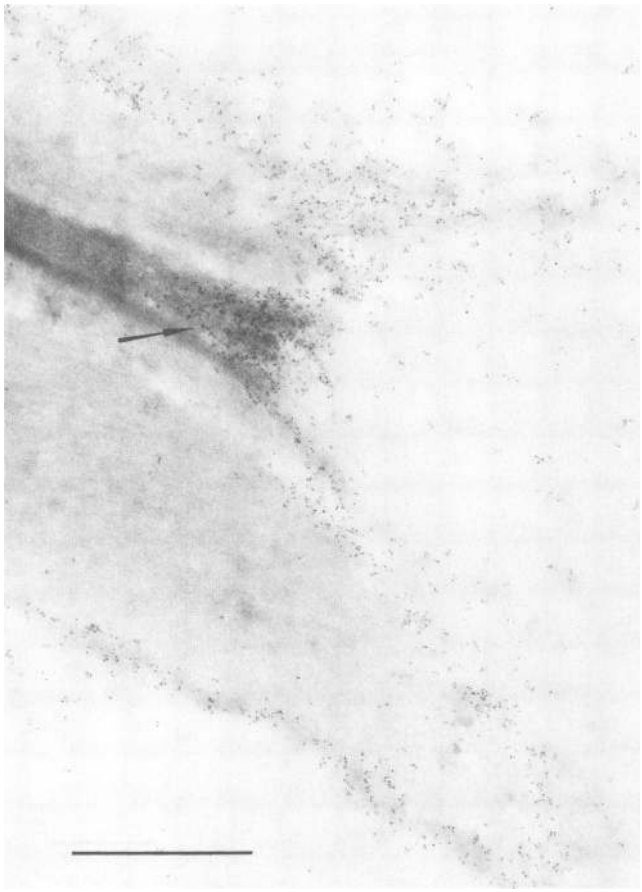

FIG. 5. White rot-degraded (*P. chrysosporium*) pine wood, infiltrated with concentrated culture fluid of *P. chrysosporium*. Heavily decayed wood cell wall is shown. Lignin peroxidase penetrates into the middle lamella for a short distance (arrow). Bar, 1 μm.

DISCUSSION

In a preceding study, by using different fixation methods and comparing the results, the problem with the postembedding technique, i.e., optimal fixation of the ultrastructure and preservation of the antigenicity of proteins at the same time, was overcome. Nevertheless, clear statements could only be made on the intracellular lignin peroxidase or its precursors, respectively (22). The question concerning the penetration of lignin peroxidase into the wood cell wall remained unanswered. Garcia et al. (7) concluded that, in naturally decayed wood, lignin peroxidase may not be released extracellularly, not least because they were unable to detect lignin peroxidase in unfixed samples by immunofluorescence. However, since in this method the unfixed samples have to be washed extensively after labeling, the lignin peroxidase-antibody complexes may simply have been washed out. We were able to show now that the very low concentration of the extracellular lignin peroxidase excreted by *P. chrysosporium* (12) is the reason for the weak labeling. Mild fixation with ethanol permitted a localization of the enzyme on the surface of the cell walls of colonized wood. It was the first indication that the extracellular lignin peroxidase of *P. chrysosporium* acts independently of a direct contact between hypha and wood cell wall, preferably on the surface thereof.

It was not possible to determine whether at least a small portion of lignin peroxidase diffused into the wood cell wall because the labeling density was still too low and reproducibility was relatively poor. This problem was overcome by the use of a highly concentrated culture filtrate of *P. chrysosporium* as a marker for the penetrability of degraded wood cell walls. This method permitted, in a simple way, an effective increase in the labeling intensity of lignin peroxidase on wood, which in our opinion could hardly have been achieved by a further improvement of the preparation methods, with the possible exception of immunocryoultramicrotomy.

Stone et al. (23) studied the porosity of wood with dextrans and discussed the penetration of cellulases into the wood cell wall. No clear statement could be made about the penetration of enzymes into the wood cell wall (4, 23). Differences between dextrans and proteins concerning possible interactions with the wood cell wall components were disregarded.

These unclear points regarding lignin peroxidase could now be eliminated by direct proof in wood, using the postembedding immunogold labeling technique. The great advantage of postembedding immunoelectron microscopy over many other labeling methods is its high specificity as well as the fact that only protein molecules exposed on the surface of the section are labeled. Therefore, any absence of labeling is not because the relatively big marker molecules (IgG coated with colloidal gold) do not have access to the protein to be detected (lignin peroxidase inside the wood cell wall).

The results obtained for *P. chrysosporium* suggest that a direct enzymatic conversion of the lignin polymer in the cell wall of pine wood can take place only on a surface which may lay somewhat deeper in the cell wall if lignin is degraded faster than cellulose (Fig. 6). The slightly higher labeling density inside the wood cell wall compared with uninfiltrated control specimens may be explained by the fact that the pore sizes in the wood cell wall differ greatly (4) and therefore a few pores may be sufficiently large for lignin peroxidase to diffuse. But the labeling density inside the cell wall is very low and not in proportion to the density on the lumen surface. Moreover, the labeling density inside the wood cell wall of both uncolonized and colonized infiltrated samples is the same, independent of the decay stage. Therefore, lignin peroxidase inside the wood cell wall does not seem to have a decisive share in lignin degradation.

The observed increase in electron density at attacked middle lamellae and cell corners suggests certain changes in deeper layers. If these are decay processes, they have to be attributed to nonenzymatic reactions, possibly nonenzymatic side chain cleavages by a cation-radical mechanism (9, 11).

Even if the strain of *P. chrysosporium* used by Ruel et al. (20) caused bore holes atypical of the strain used in our work, the morphological changes caused in the cell wall were rather restricted to the hyphal vicinity.

The incapability of lignin peroxidase to freely diffuse into the entire wood cell wall explains the long reaction time observed in tests for the biological delignification by *P. chrysosporium* (5).

A rapid degradation of lignin could be effected only by pretreatment of the wood, resulting in a widening of the pores, or by the use of lignin-degrading biomimetic catalysts which are substantially smaller than lignin peroxidase, recently achieved with porphyrins (19).

The question arises as to why lignin peroxidase cannot

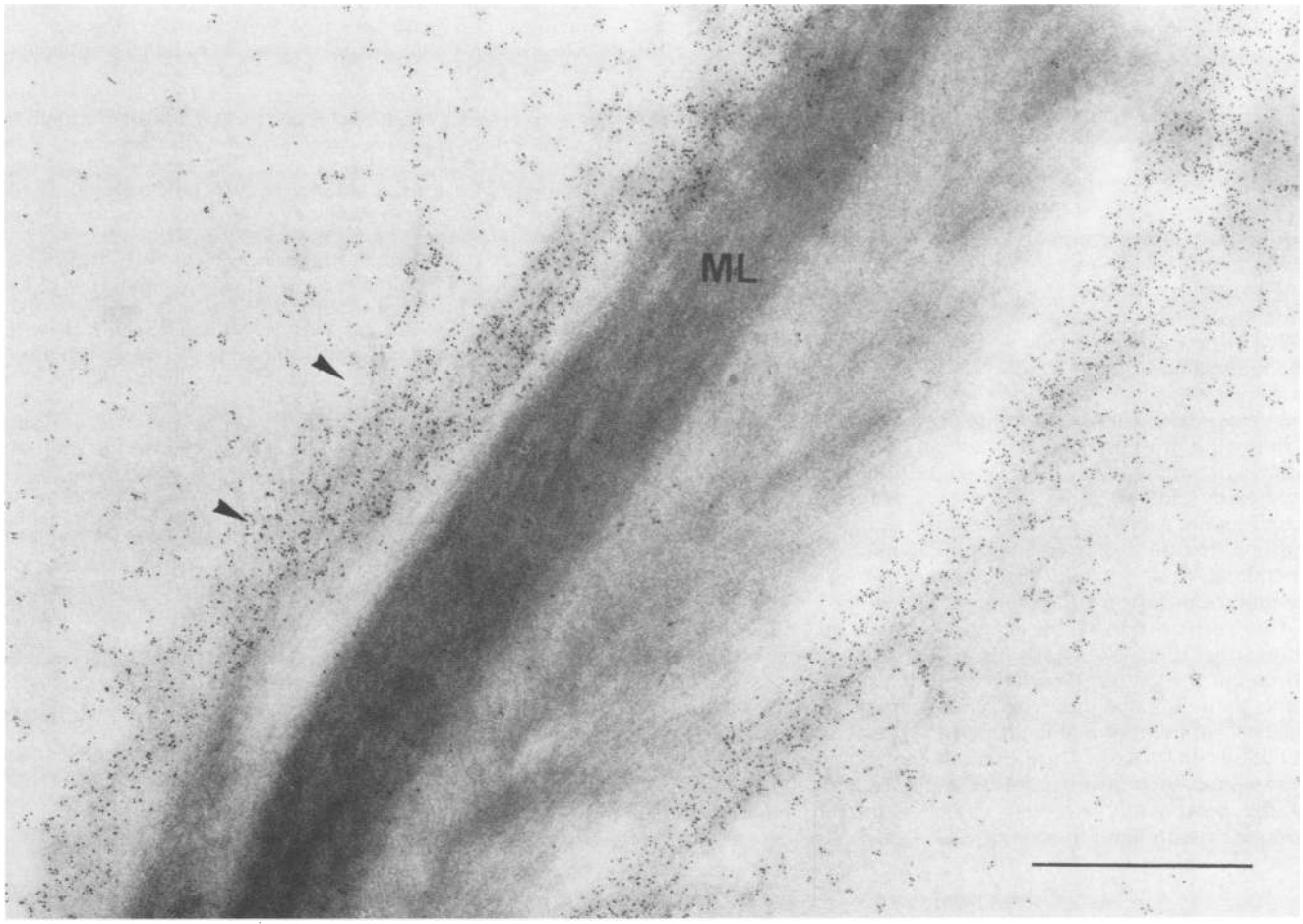

FIG. 6. White rod-degraded (*P. chrysosporium*) pine wood, infiltrated with concentrated culture fluid of *P. chrysosporium*. Shown is wood cell wall, decayed down to the middle lamella (ML) on the left side. There is fibrillar orientation of cell wall remedies in areas of cell wall attack (arrows). Bar, 1 μm.

diffuse into the wood cell wall. Since one of the requirements for the study of penetrability in general is a uniform size of the marker molecules, we characterized the culture filtrate by gel chromatography, electrophoresis, and Western blotting. The results prove that the major enzymatically, and above all immunologically, active protein species in the culture filtrate represent monomeric molecules in solution with molecular weights of around 42,000. Furthermore, it can be concluded that these active proteins, most likely multiple lignin peroxidases with high immunological homology (12), do not interact with each other to form high-

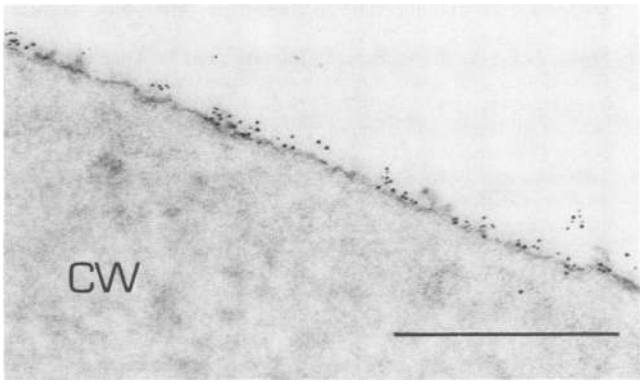

FIG. 7. Uncolonized pine wood cell wall (CW) infiltrated with concentrated culture fluid. Only narrow area on the surface of the wood cell wall is labeled. Bar, 0.5 μm.

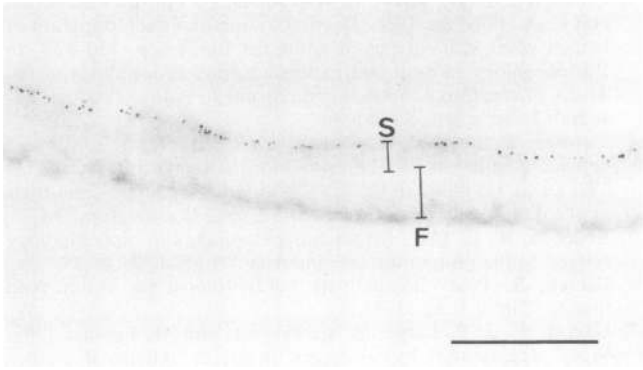

FIG. 8. Ultrathin section of wood degraded by *P. chrysosporium* which was re-embedded in LR White and cut perpendicularly to the labeled surface. S, Re-embedded ultrathin section; F, Formvar film. Bar, 0.5 μm.

molecular-weight complexes. Therefore, the following may prevent lignin peroxidase from penetrating wood cell walls. (i) The pores in the wood are so small that, as a result of steric hindrance alone, lignin peroxidase molecules cannot diffuse into the wood cell wall. (ii) The wood pore sizes are slightly greater than the size of the enzyme, in which case a sufficiently strong lignin peroxidase-lignin interaction might obstruct the pores.

The present state of knowledge of the properties of lignin peroxidase greatly supports the first possibility, namely, steric hindrance as a decisive factor: Garcia et al. (7) could not prove any lignin peroxidase-lignin interaction by an immunocytochemical method under various test conditions. If lignin peroxidase formed a strong bond with native lignin or lignin modified by *P. chrysosporium*, this would have been proved, as has been done with polysaccharide-cleaving enzymes; e.g., Ruel and Joseleau (21) succeeded in demonstrating the presence of glucomannan in spruce with mannanase with almost the same method. This as well as the studies by Harvey et al. (9) on the indirect conversion of lignin model substances with veratryl alcohol as a mediator suggest that the formation of stable lignin peroxidase-lignin complexes which would be necessary for an effective prevention of diffusion is unlikely.

Comparison of available gel filtration data on cellulases (4) shows that their size is similar to that of lignin peroxidase. Although a direct comparison of different enzymes as regards their diffusion into wood cell walls is uncertain only on the basis of hydrodynamic properties, it may be assumed for cellulases that, if they are at least the same size as lignin peroxidase, they also cannot diffuse into the wood because of the presumably stronger enzyme-substrate interaction compared with lignin peroxidase.

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LITERATURE CITED

1. **Blanchette, R. A.** 1984. Screening wood decayed by white rot fungi for preferential lignin degradation. Appl. Environ. Microbiol. **48**:647–653.
2. **Blanchette, R. A., and I. D. Reid.** 1986. Ultrastructural aspects of wood delignification by *Phlebia* (*Merulius*) *tremellosus*. Appl. Environ. Microbiol. **52**:239–245.
3. **Chang, H., T. W. Joyce, Y. Matsumoto, C. Yin, B. Vasudevan, and C.-A. Boechat.** 1986. Decolorization and dechlorination of bleach plant effluents by a white-rot fungus, p. 120–123. *In* Biotechnology in pulp and paper industry. Proceedings of the Third International Conference. Swedish Forest Product Research Laboratory, Stockholm.
4. **Cowling, E. B., and W. Brown.** 1969. Structural features of cellulosic materials in relation to enzymatic hydrolysis, p. 152–187. *In* G. J. Hajny and E. T. Reese (ed.), Cellulases and their applications. American Chemical Society, Washington, D.C.
5. **Eriksson, K. E.** 1985. Swedish developments in biotechnology related to the pulp and paper industry. Tappi J. **68**:46–55.
6. **Farrell, R.** 1984. Biocatalysts hold promise for better pulp quality. Tappi J. **67**:31–33.
7. **Garcia, S., J. P. Latge, M. C. Prevost, and M. Leisola.** 1987. Wood degradation by white rot fungi: cytochemical studies using lignin peroxidase-immunoglobulin-gold complexes. Appl. Environ. Microbiol. **53**:2384–2387.
8. **Gold, M. H., M. Kuwahara, A. A. Chiu, and J. K. Glenn.** 1984. Purification and characterization of an extracellular H_2O_2-requiring diarylpropane oxygenase from the white rot basidiomycete, *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **234**:353–362.
9. **Harvey, P. J., H. E. Schoemaker, and J. M. Palmer.** 1986. Veratryl alcohol as a mediator and the role of radical cations in lignin biodegradation by *Phanerochaete chrysosporium*. FEBS Lett. **195**:242–246.
10. **Highley, T. L., and L. L. Murmanis.** 1987. Micromorphology of degradation in western hemlock and sweetgum by the white-rot fungus *Coriolus versicolor*. Holzforschung **41**:67–71.
11. **Kersten, P. J., M. Tien, B. Kalyanaraman, and T. K. Kirk.** 1985. The ligninase of *Phanerochaete chrysosporium* generates cation radicals from methoxybenzenes. J. Biol. Chem. **260**:2609–2612.
12. **Kirk, T. K., S. Croan, M. Tien, K. Murtaugh, and R. Farrell.** 1986. Production of multiple ligninases by *Phanerochaete chrysosporium*: effect of selected growth conditions and use of a mutant strain. Enzyme Microb. Technol. **8**:27–32.
13. **Kirk, T. K., and R. L. Farrell.** 1987. Enzymatic "combustion": the microbial degradation of lignin. Annu. Rev. Microbiol. **41**: 465–505.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
15. **Messner, K., and H. Stachelberger.** 1984. Transmission electron microscope observations of brown rot caused by *Fomitopsis pinicola* with respect to osmiophilic particles. Trans. Br. Mycol. Soc. **83**:113–130.
16. **Messner, K., and H. Stachelberger.** 1984. Transmission electron microscope observations of white rot caused by *Trametes hirsuta* with respect to osmiophilic particles. Trans. Br. Mycol. Soc. **83**:209–216.
17. **Murmanis, L. L., T. L. Highley, and J. G. Palmer.** 1984. An electron microscopy study of western hemlock degradation by the white-rot fungus *Ganoderma applanatum*. Holzforschung **38**:11–18.
18. **Nyhlen, L., and T. Nilsson.** 1987. Combined T.E.M. and UV-microscopy on delignification of pine wood by *Phlebia radiata* and four other white rotters, p. 227–282. *In* E. Odier (ed.), Lignin enzymic and microbial degradation. Institut National de la Recherche Agronomique, Paris.
19. **Paszczynski, A., R. L. Crawford, and R. A. Blanchette.** 1988. Delignification of wood chips and pulps by using natural and synthetic porphyrins: models of fungal decay. Appl. Environ. Microbiol. **54**:62–68.
20. **Ruel, K., F. Barnoud, and K. E. Eriksson.** 1981. Micromorphological and ultrastructural aspects of spruce wood degradation by wild-type *Sporotrichum pulverulentum* and its cellulaseless mutant cel 44. Holzforschung **35**:157–171.
21. **Ruel, K., and J.-P. Joseleau.** 1984. Use of enzyme-gold complexes for the ultrastructural localization of hemicelluloses in the plant cell wall. Histochemistry **81**:573–580.
22. **Srebotnik, E., K. Messner, R. Foisner, and B. Pettersson.** 1988. Ultrastructural localization of ligninase of *Phanerochaete chrysosporium* by immunogold labeling. Curr. Microbiol. **16**:221–227.
23. **Stone, J. E., A. M. Scallan, E. Donefer, and E. Ahlgren.** 1969. Digestibility as a simple function of a molecule of similar size to a cellulase enzyme, p. 219–241. *In* G. J. Hajny and E. T. Reese (ed.), Cellulases and their applications. American Chemical Society, Washington, D.C.
24. **Tien, M., and T. K. Kirk.** 1984. Lignin-degrading enzyme from *Phanerochaete chrysosporium*: purification, characterization, and catalytic properties of a unique H_2O_2-requiring oxygenase. Proc. Natl. Acad. Sci. USA **81**:2280–2284.
25. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4353.
26. **Wilcox, W. W.** 1968. Changes in the wood microstructure through progressive stages of decay. U.S. For. Serv. Res. Pap. FPL **70**:1–46.