APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1989, p. 871–881
0099-2240/89/040871-11$02.00/0

Vol. 55, No. 4

Intra- and Extracellular Localization of Lignin Peroxidase during the Degradation of Solid Wood and Wood Fragments by *Phanerochaete chrysosporium* by Using Transmission Electron Microscopy and Immuno-Gold Labeling

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Received 11 October 1988/Accepted 5 January 1989

The distribution of lignin peroxidase during degradation of both wood and woody fragments by the white rot fungus *Phanerochaete chrysosporium* was investigated by using anti-lignin peroxidase in conjunction with postembedding transmission electron microscopy and immuno-gold labeling techniques. The enzyme was localized in the peripheral regions of the fungal cell cytoplasm in association with the cell membrane, fungal cell wall, and extracellular slime materials. In solid wood, lignin peroxidase was detected in low concentrations associated with both superficial and degradation zones within secondary cell walls undergoing fungal attack. A similar but much greater level of extracellular peroxidase activity was associated with wood fragments degraded by the fungus grown under liquid culture conditions optimal for production of the enzyme. Efforts to infiltrate degraded wood pieces with high levels of lignin peroxidase showed the enzyme to be restricted to superficial regions of wood decay and to penetrate wood cell walls only where the wall structure had been modified. In this respect the enzyme was able to penetrate characteristic zones of degradation within the secondary walls of fibers to sites of lignin attack. This suggests a possibility for a close substrate-enzyme association during wood cell wall degradation.

Phanerochaete chrysosporium (wild type) is a white rot basidiomycete which can cause the simultaneous decay of wood components (cellulose, hemicellulose, and lignin) during wood tissue degradation. Morphologically, decay in wood is normally reflected by a pronounced thinning of fiber walls which is initiated through the activity of hyphae in lumina. The fungus and its mutants have been studied intensively over the past decade and are now known to produce a number of extracellular enzymes capable of cellulolytic, hemicellulolytic, or ligninolytic activity; several of these enzymes may act synergistically. Of the isolated ligninolytic enzymes, the H_2O_2-dependent lignin peroxidase (ligninase) has achieved considerable notoriety since its first isolation and purification (12, 30), as it is considered to play a major role in the oxidation of lignin (30, 31). The enzyme, a heme-containing glycoprotein with broad specificity, has been shown to be capable of the oxidative conversion of a range of lignin-related model compounds in vitro (19, 29, 31). Its role and localization with respect to the fungus during wood degradation are, however, poorly understood, and the few studies performed so far (10, 11, 27) have not shown conclusively its natural association with lignocellulosic substrates undergoing decay.

Until recently, the in situ localization and demonstration of enzymes involved in wood decay has been carried out by complexing heavy metals with the products produced by enzymatic action. Such reactions can be both unspecific and difficult to interpret. Recently, more-effective and more-specific techniques based on the use of antibodies raised against appropriately inoculated antigens (e.g., purified enzymes) have been developed. Such antibodies have the advantage of having a high specificity for their antigens and can be demonstrated directly or indirectly in situ by using a variety of immunological detection techniques at both light and electron microscopy levels.

In the present study, an indirect immunological technique involving anti-lignin peroxidase was used to study the intra- and extracellular distribution of lignin peroxidase during degradation of both birch (*Betula verrucosa*) and pine (*Pinus sylvestris*) wood and woody fragments by *P. chrysosporium*.

MATERIALS AND METHODS

Fungal strains. The white rot basidiomycete used in the experiments was *P. chrysosporium* P-127 (wild type) obtained from the collection maintained at the Department of Forest Products, Swedish University of Agricultural Sciences. The fungus, previously isolated from chip piles, has been shown to be very active on lignocellulose substrates. The fungus is routinely maintained on 2.5% malt agar plates.

Wood samples and decay tests. Small wood blocks (3.0 by

TABLE 1. Weight losses obtained with *P. chrysosporium* on pine and birchwood blocks

Time (wk)	% Wt loss [a]	
	Betula verrucosa	*Pinus sylvestris*
1	5.3	2.4
2	23.5	11.7
3	51.0	24.1
4	54.3	30.0
5	78.1	29.3
6	71.2	44.7
8	89.5	49.6
12	92.1	47.7

[a] Based on six replicates.

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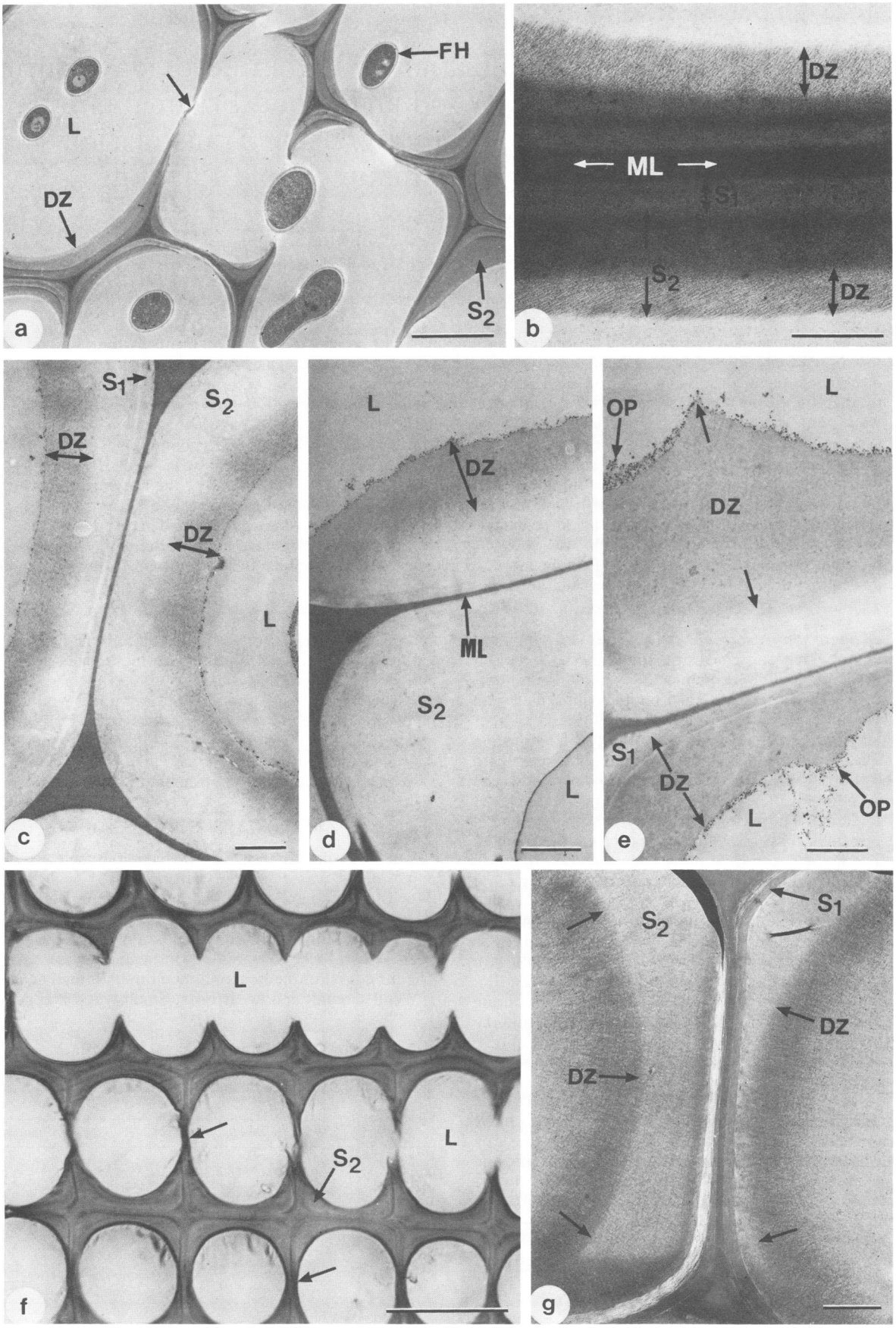
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1.5 by 0.5 cm) of birch (*B. verrucosa*) and pine (*P. sylvestris*) were placed in 125-ml Erlenmeyer flasks containing moist vermiculite. After being autoclaved, the flasks were inoculated with a buffered fungal spore-mycelium suspension of *P. chrysosporium* P-127-1 previously grown on 2.5% malt agar plates. To enhance growth, the flasks were also supplemented with 2 ml of a sterile 2% malt extract solution. Flasks were then incubated at 38 to 40°C. Weight losses under these conditions are shown in Table 1.

Small matchstick samples were dissected from the wood blocks for transmission electron microscopy (TEM) cytochemical and immunological studies. Matchsticks were taken from parallel samples after 3 weeks (*B. verrucosa*) and 5 weeks (*P. sylvestris*), when it was evident from light microscopy and Klason lignin (insoluble-lignin) analyses (data not shown) that lignin degradation was occurring.

Anti-lignin peroxidase and lignin peroxidase. Polyclonal anti-lignin peroxidase was raised in New Zealand White rabbits previously inoculated with a purified ligninase enzyme (31). The serum, which has been characterized previously (18), was a generous gift to B.P. from T. K. Kirk. Western blot (immunoblot) analyses have revealed cross-reactivity of the antiserum with at least three purified ligninase proteins produced in culture media by *P. chrysosporium* (18).

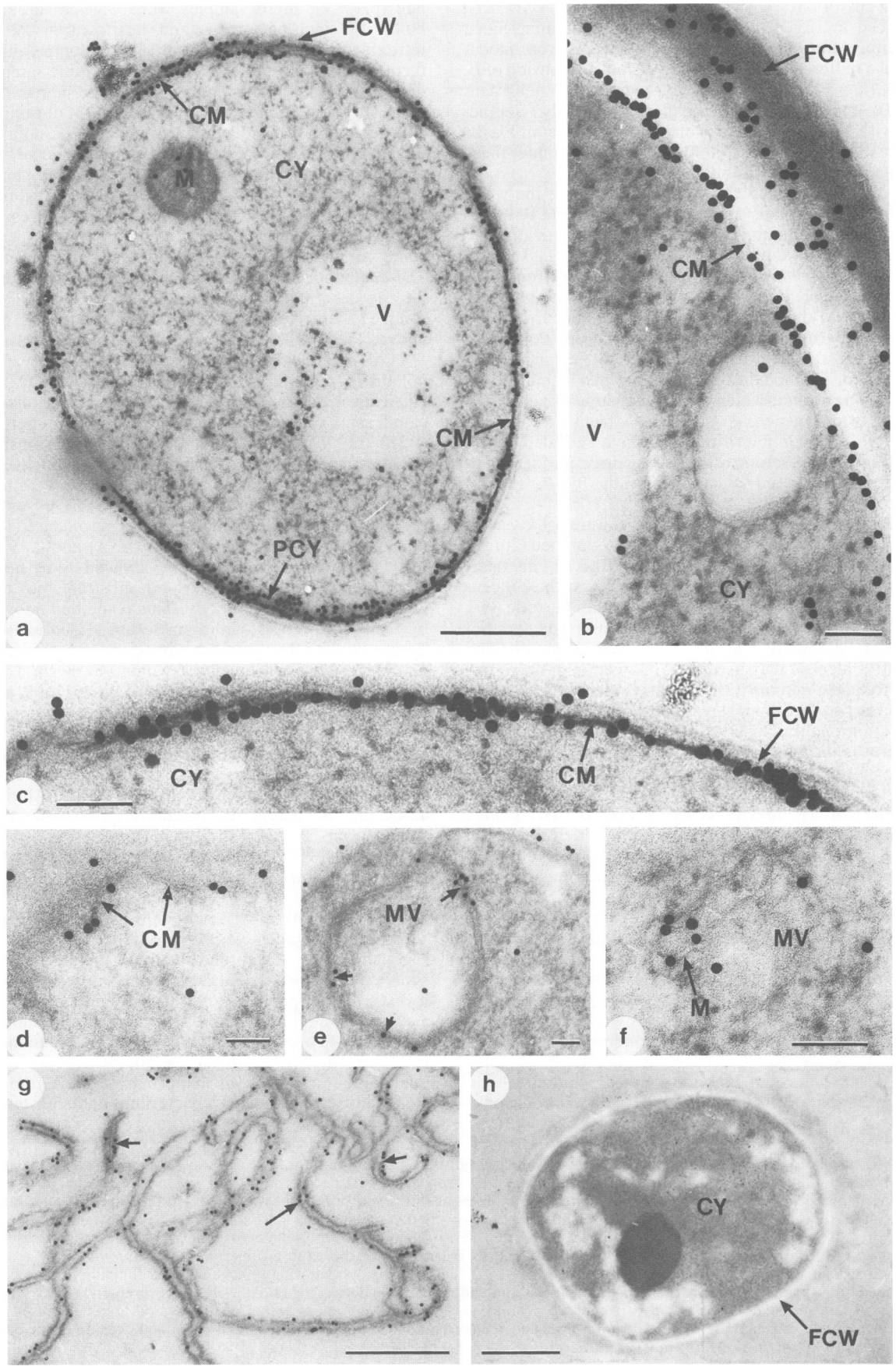
Liquid culture conditions. Nonagitated cultures of *P. chrysosporium* P-127-1 were set up as previously outlined (20) (10 ml of medium per 125-ml Erlenmeyer flask). The basal medium contained mineral salts and vitamins at the concentrations previously reported plus ammonium tartrate (5.1 mM) as the nitrogen source (21). The medium was buffered with 20 mM 2,2-dimethyl succinate (7), and vacuum-distilled (32) veratryl alcohol was used as an activator to maximize lignin peroxidase activity (21). Veratryl alcohol (final concentration per flask, 1.5 mM) was added as a sterile solution at inoculation of the fungal suspension. Ground birchwood (40 mesh) was used as the carbon source. After inoculation with spores, the cultures were grown for 40 h before being exposed to a 100% O_2 atmosphere (21). As an indicator of ligninolytic activity, loss of color of Poly-B (0.01 g/liter; Sigma Chemical Co., St. Louis, Mo.) was determined in parallel flasks. Under these conditions, loss of color of Poly-B occurred after 3 to 4 days of growth. Small samples of the fungal mycelium-wood sawdust mat were taken for immunolabeling studies after a 6-day growth period.

TEM. Samples (decay test and liquid culture material) for immunological studies were fixed for 3 h at 4°C in 4% (vol/vol) paraformaldehyde containing 1% glutaraldehyde or 8% paraformaldehyde in 0.1 M phosphate or sodium cacodylate buffer (pH 7.2). Thereafter, samples were dehydrated in an ethanol series and embedded in London resin (London Resin Co., Basingstoke, United Kingdom). Other samples were fixed for 3 h at room temperature in 3% glutaraldehyde containing 2% paraformaldehyde in 0.1 M sodium cacodylate (pH 7.2); these samples were washed three times in buffer (30 min per wash) and postfixed in 1% osmium tetroxide in 0.1 M buffer for 2 h at room temperature. After further washes in buffer and distilled water, samples were dehydrated and embedded as described above. Selected material was sectioned with a Reichert FC4 ultramicrotome, and sections were collected with nickel or copper grids. Poststaining, when performed, was conducted with 4% aqueous uranyl acetate (5 min) and Reynolds (25) lead citrate (15 min). Observations were made with a Philips 201 or JEOL 100B TEM at various accelerating voltages.

TEM cytochemistry. For detecting general peroxidase, some samples, fixed as described above, were treated with 3,3'-diaminobenzidine and H_2O_2 followed by osmium tetroxide, as described by Graham and Karnovsky (13). Such samples were resin infiltrated and sectioned as described above. TEM observations were made without poststaining.

TEM immunolabeling studies. Ultrathin sections for immunolabeling were pretreated with either 10% H_2O_2 (unosmicated material) or saturated sodium metaperiodate (osmicated samples) and washed in phosphate-buffered saline (PBS) (pH 7.2). Unosmicated samples were further incubated with 0.1 M glycine (35 min) to quench aldehyde groups induced during fixation (17). Next, sections were incubated in drops of normal goat serum in PBS (1:30) and then in previously cross-absorbed anti-lignin peroxidase (1:50) containing 1% bovine serum albumin (BSA) and 0.05% Tween 20. (Preliminary labeling studies showed some nonspecific background labeling of the wood substrate [not the background resin] by the antibody. This could be removed either by using a low titer of the antiserum [1:300 in PBS-BSA-Tween 20] or by cross-absorbing a higher titer of the antibody with birch or pine sawdust [40 mesh; 1 g/ml of a 1:50 ratio of antibody/PBS-BSA-Tween 20] for 2 h at room temperature.) Various antiserum dilutions and periods of incubation were tried to determine optimal labeling. Time differences were evident in optimal labeling of the fungus and degraded wood samples. In the present studies, labeling was performed overnight (12 to 14 h) at 4°C. After primary antiserum incubation, the sections were washed in PBS-BSA-Tween 20 (pH 7.4) and in Tris hydrochloride-BSA-Tween 20 (pH 8.2) and subsequently incubated with a gold-labeled anti-rabbit immunoglobulin G conjugate (goat anti-rabbit G15) (B-2340; Janssen Life Science Products, Beerse, Belgium) in Tris hydrochloride-BSA-Tween 20 (pH 8.4) for 1 h. Samples were then washed thoroughly in Tris hydrochloride (pH 8.4) and finally in distilled water. Poststaining, when performed, was done as described above. To investigate the specificity of the lignin peroxidase antiserum, a number of method and antiserum control experiments were performed. To provide method controls, immunolabeling was performed after the antiserum was replaced with a nonimmune serum or an inappropriate antiserum (a polyclonal antiserum made to a bacterium) and after omission of

FIG. 1. Transmission electron and light micrographs showing various stages of birch (a to e) and pine (f and g) fiber degradation by *P. chrysosporium*. (a to e) Early and late stages of fiber wall attack showing prominent decay zones (DZ) within the S_2 layer. (a) Transmission electron micrograph showing typical cell wall thinning of birch fibers and presence of fungal hyphae (FH) in the lumina (L). A thin decayed zone is apparent on the edge of the fibers. (b) Transmission electron micrograph showing selective removal of substances from the decay zones in more advanced stages. ML, Middle lamella. (c to e) Phases in decay zone formation across the S_2 layer. Note the absence of the decay zone in the lower unattacked fiber wall (d). Decay causes indentation of the exposed fiber wall regions, which are covered with osmiophilic particles (OP) produced during fixation with OsO_4. (f) Light micrograph showing typical cell wall thinning of pine fibers. As a consequence of the slightly thinner nature of the radial walls and uniform erosion decay, the radial walls are severed first (arrows), producing tangential wall remnants (see the upper part of the photograph). (g) Typical decay zones within pine latewood fibers after treatment with diaminobenzidine-H_2O_2-osmium tetroxide. A more pronounced electron-opaque reaction is apparent in the outer region of the decay zone. Bars: 5.0 μm (a), 1.0 μm (b to e), 10 μm (f), 2.0 μm (g).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the primary antiserum stage entirely. To provide an antiserum control, the anti-lignin peroxidase was preadsorbed with lignin peroxidase. Additional electron microscopy labeling studies were also performed on undegraded wood samples to provide a substrate control.

Penetration of lignin peroxidase into *P. chrysosporium*-degraded birchwood samples. To determine whether lignin peroxidase has a capacity to penetrate birchwood, small pieces were taken from decayed blocks (6 weeks old; see above) and incubated for 1 h under slight vacuum with lignin peroxidase (8 mg/ml) in 0.33 M sodium tartrate buffer (pH 3.0) (previously isolated and purified from liquid cultures of *P. chrysosporium*). Then the samples were washed three times (5 min per wash) in the same buffer, fixed, and processed as described for TEM. Such degraded wood samples were selected deliberately, since the nonhomogeneous nature of the decay process by white rot fungi in solid wood has the advantage of providing both the entire spectrum of stages of wood cell wall breakdown and examples of unattacked wood cells.

RESULTS

Micromorphology of *P. chrysosporium* degradation (general). Morphologically, decay in both birch and pine normally takes place by a progressive thinning of the fiber walls by the degradative action of hyphae in the cell lumina (Fig. 1a to f). Since the fiber tangential walls in latewood of pine are often slightly thicker or possibly are more resistant to decay (Fig. 1f, arrows), progressive thinning results in a rupture of the radial cell walls at an early stage, leaving a characteristic wall pattern (Fig. 1f). Staining of the wood samples shows that during degradation a prominent decay zone may be produced within the secondary S_2 cell wall region of attacked fibers (Fig. 1b to e and g). With birch fibers and with earlywood fibers of pine, this zone is often restricted to a thin layer immediately adjacent to the lumina of the degrading fibers (Fig. 1a to e). The zone may not always form a complete ring around the lumen wall and in earlywood pine fibers may be more restricted to the neighboring region of fungal hyphae in wood cell wall contact. The decay zones are best observed in latewood fibers of pine and birch, in which they may form a layer several micrometers thick in advance of cell wall thinning (Fig. 1g). Treatment of degraded fibers of pine with the diaminobenzidine reagent for general peroxidases produced a strong reaction localized in a narrow band in the outermost region of the decay zone (Fig. 1g). The zones are therefore a characteristic feature of decay of the wood cell walls and suggest that wood substance dissolution and agents of decay (e.g., peroxidases) are concentrated within a defined region. One goal of the present study was to determine whether lignin peroxidase was associated with these degradation zones.

The location of fungal hyphae may vary during the degradation process. While all hyphae are located in the lumina of tissue elements within the true wood structure, degradation may occur via the action of hyphae in direct (i.e., adpressed) wood cell wall contact or via the action of those hyphae apparently located more centrally in the cell lumen. However, no differences in the overall degradative pattern were noted among such hyphae, apart from the initial more-concentrated decay around hyphae adpressed to the wood cell wall. In addition, the latter hyphae along their lengths were often noted to be both adpressed and lying more centrally within the fiber lumen.

Detection of lignin peroxidase. (i) Degraded wood samples. Thin sections of degraded wood blocks treated with the lignin peroxidase antiserum generally showed intense labeling of the peripheral fungal cell cytoplasm, cell membrane, and cell wall regions (Fig. 2a to c). Labeling was frequently seen to occur preferentially on the fungal cell membrane and associated inner membrane(s) (multivesicular vesicles), although ultrastructural preservation of these structures was not optimal (Fig. 2d to f). Labeling was often prominent on the inner region (periplasmic space) of the fungal cell wall (Fig. 2b and c). The presence of extracellular lignin peroxidase was also frequently suggested by gold labeling of slime materials and membranous materials associated with the fungi and wood cell walls (Fig. 2g). Little nonspecific background labeling was observed in the preparations, and the results for all controls, including the preparation of cross-absorbed ligninase plus anti-lignin peroxidase (see Materials and Methods), were negative (Fig. 2h).

Immunolabeling of wood cell walls proved more difficult than immunolabeling of fungal hyphae, but longer antiserum incubation times provided increased labeling. Observation of degraded cell walls of birchwood in various stages of decay showed labeling of exposed cell wall regions, suggesting the presence of lignin peroxidase (Fig. 3a to f). Labeling was often associated with fibrillar elements protruding from the wall and occurred on all wood cell wall regions (i.e., tertiary wall, S_2, S_1, and middle-lamella regions) subjected to decay (Fig. 3a to e). A similar result was obtained for degrading pine fibers, in which labeling was also concentrated within exposed cell wall regions (Fig. 3g).

Evidence for increased lignin peroxidase levels was recognized in the confines of erosion-type troughs produced by the fungal hyphae within the secondary fiber cell walls during decay (Fig. 3c and h). Such troughs were generally associated with hyphae, although they were also produced in the apparent absence of hyphae (Fig. 3h).

For the degraded-wood preparations studied, some labeling within the wood cell wall in association with S_2 degradation zones was recognized, suggesting the presence of at least low levels of lignin peroxidase (Fig. 3f and g). No specific labeling was, however, recognized for undegraded or structurally unmodified cell wall regions not associated with the decay zones or exposed regions of fungal attack.

The results of control experiments, including attempts to label sections of undegraded birch and pinewood, were negative suggesting the validity of the labeling reactions. By

FIG. 2. Transmission electron micrographs showing fungal intracellular and extracellular distribution of lignin peroxidase in *P. chrysosporium* from degraded wood samples. (a) Transverse section of a hypha, giving an overview of the spatial distribution of lignin peroxidase in the peripheral cell cytoplasm (PCY), cell membrane (CM), and fungal cell wall (FCW) regions. M, Mitochondrion; V, vacuole. (b and c) Higher-magnification photos showing intense gold labeling and lignin peroxidase along the cell membrane and hyphal cell wall regions. CY, Cell cytoplasm. (d to f) Lignin peroxidase in association with the fungal cell membrane and associated cytoplasmic membranous vesicular (MV) structures. (g) Lignin peroxidase labeling (arrows) of fungally produced extracellular slime materials and membranous structures within the lumina of degraded wood fibers. (h) Control preparation showing absence of labeling of fungal hyphae when anti-lignin peroxidase was first cross-absorbed with lignin peroxidase followed by gold-labeled goat anti-rabbit serum and then used to label sections in the normal way followed by gold-labeled goat anti-rabbit serum. Bars: 0.5 μm (a, g, and h) and 0.1 μm (b to f).

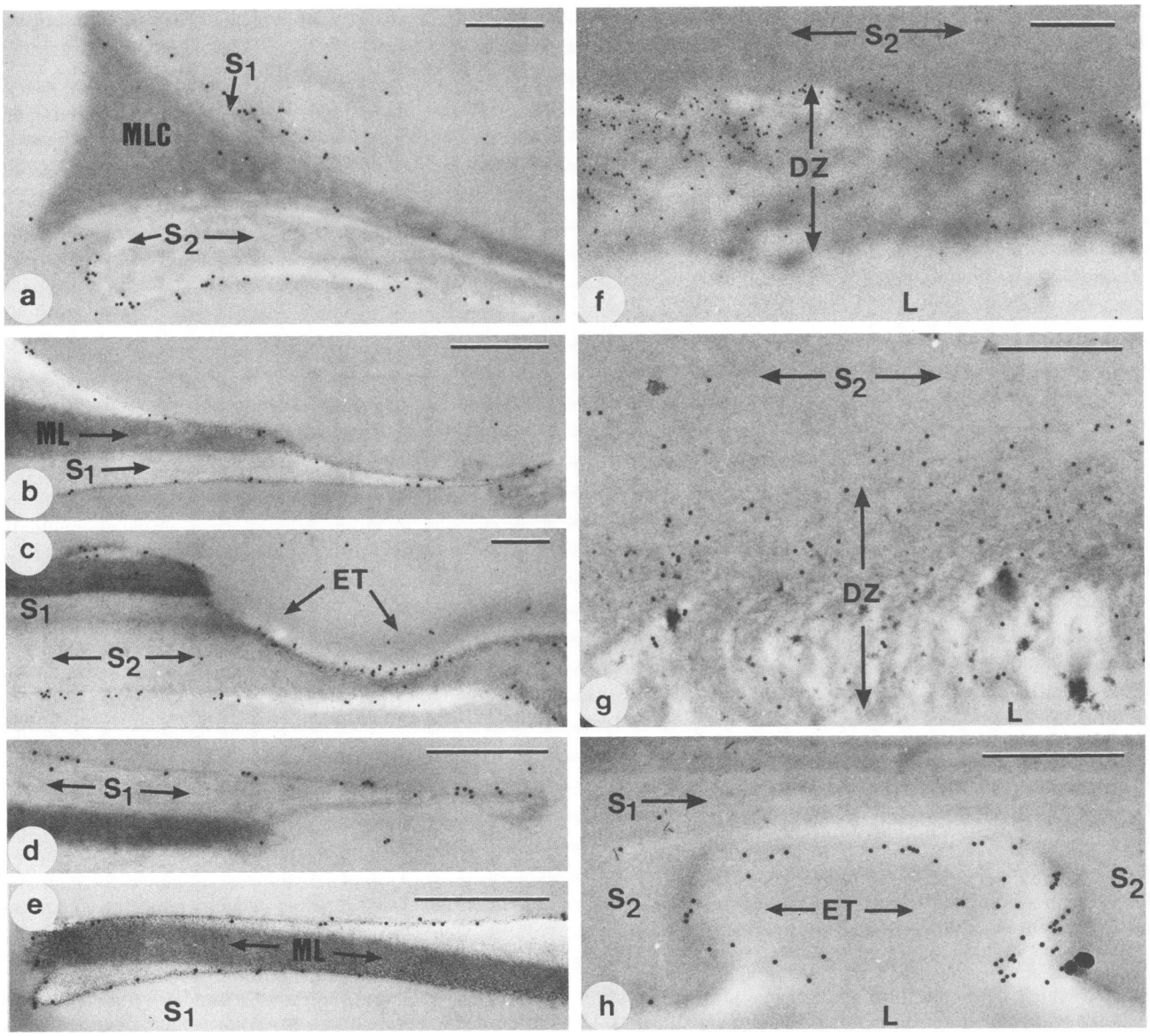
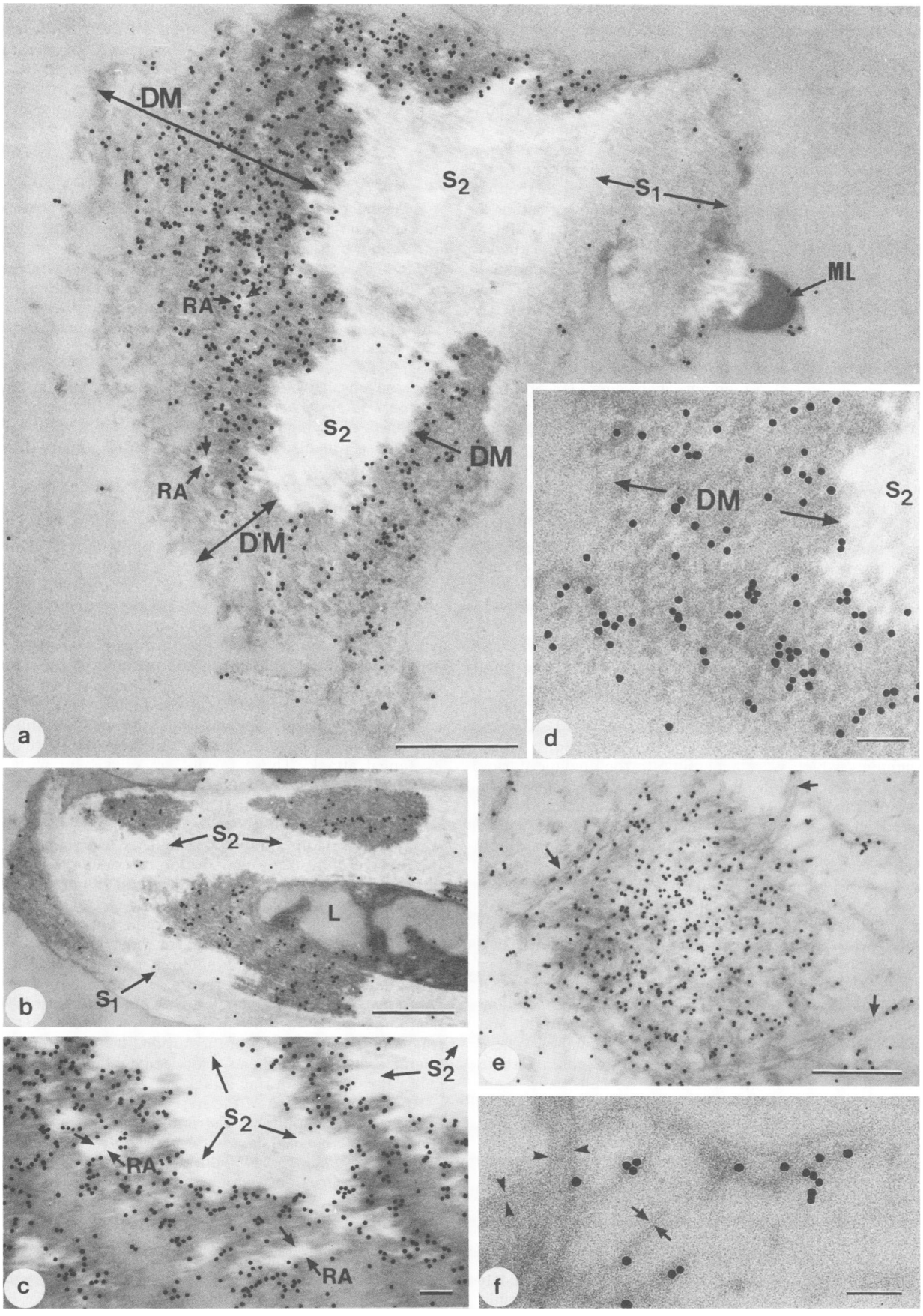


FIG. 3. Transmission electron micrographs showing the extracellular localization of lignin peroxidase in degraded wood samples of birch (a to f and h) and pine (g). (a to e) Micrographs showing various stages in birch cell wall degradation, with lignin peroxidase labeling of superficial cell wall regions subject to surface erosion decay. Labeling is visible on all cell wall layers, including exposed middle-lamella (ML) regions. MLC, Middle-lamella cell corner; ET, erosion trough. (f and g) Birch and pine secondary cell wall degradation with lignin peroxidase located in attacked peripheral cell wall regions and within the characteristic decay zones (DZ). L, Lumen. (h) Concentration of lignin peroxidase in surface regions of an erosion trough produced in a birch S_2 cell wall layer. Little nonspecific labeling is visible on the background resin and undegraded wood fiber regions. Bars, 0.5 μm.

the same indirect methods, labeling of milled-birchwood lignin was attempted to determine whether the lignin peroxidase antiserum had any affinity for lignin. No specific labeling was observed, although prior treatment of sections (resin embedded) with the lignin peroxidase enzyme preparation followed by the lignin peroxidase antiserum and then goat anti-rabbit immunoglobulin G15 produced intense labeling (results not shown).

FIG. 4. Transmission electron micrographs showing the extracellular localization of lignin peroxidase in degraded birchwood fragments from liquid cultures. (a to c) Lignin peroxidase concentrated in and penetrating attacked and exposed cell wall regions within the S_2 cell wall of birch undergoing decay. The enzyme is also concentrated at the interface between attacked and undegraded regions, and residual undegraded areas (RA) are present within the highly attacked regions (arrows). L, Lumen; DM, residual degraded cell wall matrix; ML, middle lamella. (d) Higher-magnification view of the micrograph in panel a, showing the characteristic nature of the residual degraded cell wall matrix and that of undegraded regions. (e) Highly degraded birch fragment with lignin peroxidase still associated (arrows). (f) Higher-magnification view of the micrograph in panel e, showing the individual elements forming the degraded matrix. Note indications of the presence of microfibril remnants (arrows and arrowheads) within the elements of the matrix. Bars: 0.5 μm (a, b, and e) and 0.1 μm (c, d, and f).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(ii) Degraded wood fragments. Immunolabeling studies of *P. chrysosporium* hyphae grown for 6 days under liquid culture conditions optimal for lignin peroxidase production gave results similar to those observed for degraded solid wood (pine and birch), with lignin peroxidase localized primarily in the peripheral cell cytoplasm, cell membrane, and fungal cell wall regions. More intense labeling was, however, generally recorded for the fungal cell walls, suggesting passage of the enzyme through the walls. In contrast, with solid wood, birch fragments exhibited intense labeling of regions where degradation was occurring, indicating a high concentration of and association with extracellular lignin peroxidase (Fig. 4a to d). At early stages of attack, lignin peroxidase was restricted to surrounding superficial regions of the fragments, but as decay progressed across the fragments, lignin peroxidase was localized within the matrix of the attacked fragments themselves (Fig. 4a to d). Frequently, the enzyme appeared to be more concentrated at the interface between undegraded and obviously attacked regions (Fig. 4a and c). Fragments were degraded progressively from the outer regions, with the characteristic fibrillar electron-lucent appearance of the fragments changing gradually to a looser but electron-dense matrix-type material (Fig. 4a to e). Ultimately, entire fragments were reduced to the matrix-type material, which appeared more loosely associated but still had lignin peroxidase associated with its individual components (Fig. 4d to f). The matrix had a characteristic appearance (Fig. 4b to d) of individual elements often running in a direction similar to that of the original microfibrillar elements (Fig. 4b). Detailed observations showed indications of microfibril remnants (Fig. 4f, arrows) within the highly degraded matrix.

Overall, extracellular lignin peroxidase labeling was restricted either to peripherally attacked regions of the fragments or to regions where their original structure was made porous by progressive degradation or the initial milling procedure for fragment production (Fig. 4a to d). Consequently, labeling also occurred in degraded regions within the fragments which were accessible to the enzyme during the 6-day culture period (Fig. 4b). Frequently, isolated or residual regions not fully degraded also remained conspicuous within the attacked matrix (Fig. 4a to c, RA). Very little nonspecific labeling of undegraded regions within the birch fragments occurred (Fig. 4a to d), and the results for all controls (see Materials and Methods) were negative.

***P. chrysosporium*-degraded birchwood impregnated with lignin peroxidase.** Degraded birch samples (6 weeks old) incubated with purified lignin peroxidase reacted intensely with the lignin peroxidase antiserum (Fig. 5a to e). Lignin peroxidase was present in high levels associated with all degraded and exposed wood cell wall regions, including secondary cell wall layers (i.e., S_2 and S_1 [Fig. 5a to d]) and the middle-lamella cell corner regions (Fig. 5d and e). Where prior fungal degradation had produced the characteristic zones of decay within the secondary S_2 cell wall, lignin peroxidase was present at high levels, with the greatest concentration occurring at the interfacial regions between undegraded and degraded or otherwise structurally modified regions (Fig. 5a to c). No specific labeling of the corresponding but adjacent undegraded S_2 cell wall regions was noted (Fig. 5a to c). With whole, undegraded fiber cell wall regions, lignin peroxidase labeling was restricted to the lumen cell wall, indicating a limited capacity of the enzyme to penetrate the undegraded wood substrate itself (Fig. 5a). In wood cell wall regions where advanced fungal degradation had left only S_1 and middle lamella cell wall corners remaining, lignin peroxidase was again concentrated in peripheral regions (Fig. 5d and e). Some penetration into remaining middle-lamella cell wall corners was also recorded, particularly where these had been made more porous by fungal attack. In such cases, the enzyme was localized either in peripheral regions or within cell corners in more electron-lucent areas from which lignin had presumably been modified (Fig. 5e).

DISCUSSION

By using a TEM approach in conjunction with postembedding immuno-gold labeling, it has been possible to study the intra- and extracellular distribution of lignin peroxidase during the degradation of lignocellulose in fragment form or as solid wood. Lignin peroxidase was found to be associated with both fungal hyphae and degrading wood fiber elements. The enzyme was localized along the inner wall lumina or exposed surfaces of degrading birch and pine fibers in which progressive decay was occurring. Lignin peroxidase was detected on all wood layers, including the middle lamella, but only after its exposure in advanced stages of decay. Evidence of a high concentration of extracellular lignin peroxidase was also obtained for birch fragments degraded by *P. chrysosporium* grown under liquid culture conditions optimal for secretion and production of the enzyme.

Under these conditions the fragments showed intense labeling for lignin peroxidase at all stages of degradation, from initial superficial attack to complete disorganization of the wood substance. The enzyme was not restricted to the outer regions of the fragments but penetrated the wood substrate as the degradation process continued.

Labeling studies with *P. chrysosporium*-degraded birchwood samples impregnated with purified lignin peroxidase provided additional information on the ability of the enzyme to penetrate degraded but not undegraded wood cell wall regions. The results highlight the ability of the purified enzyme to penetrate regions (decay zones) of the wood substrate which have been structurally modified (exposed) by fungal attack. These results are consistent with those obtained for solid wood and woody fragments degraded in situ, which also showed that the enzyme was unable to penetrate undegraded or structurally unmodified wood cell walls. In a previous study (27) (published after submission of the present study), the penetration of concentrated culture filtrates containing lignin peroxidase into *P. chrysosporium*-degraded pinewood was also studied, since adequate and consistent levels of the enzyme could not be detected in vivo. Those investigators found for pine, as we report here for birch, the inability of lignin peroxidase to penetrate undegraded wood cell wall layers. But unlike those investigators, we found an association of lignin peroxidase with the degrading wood in vivo in both wood blocks and wood fragments and an ability of the purified enzyme to penetrate characteristic decay zones produced within the secondary cell walls of fibers during attack.

Apart from labeling of the hyphal cell wall, the cytoplasmic and cell membrane distribution of lignin peroxidase reported here is consistent with that reported elsewhere (10, 11) for *P. chrysosporium* during degradation of *Populus tremuloides*. Previous cytochemical studies (8) have shown evidence of sites of peroxidatic activity within the periplasmic space of this fungus (i.e., the fungal cell membrane-cell wall interface), which is also consistent with the present results. In other studies (22, 28), however, it was concluded that lignin peroxidase is more closely associated with the cell membrane in *P. chrysosporium*. Slight differences in the

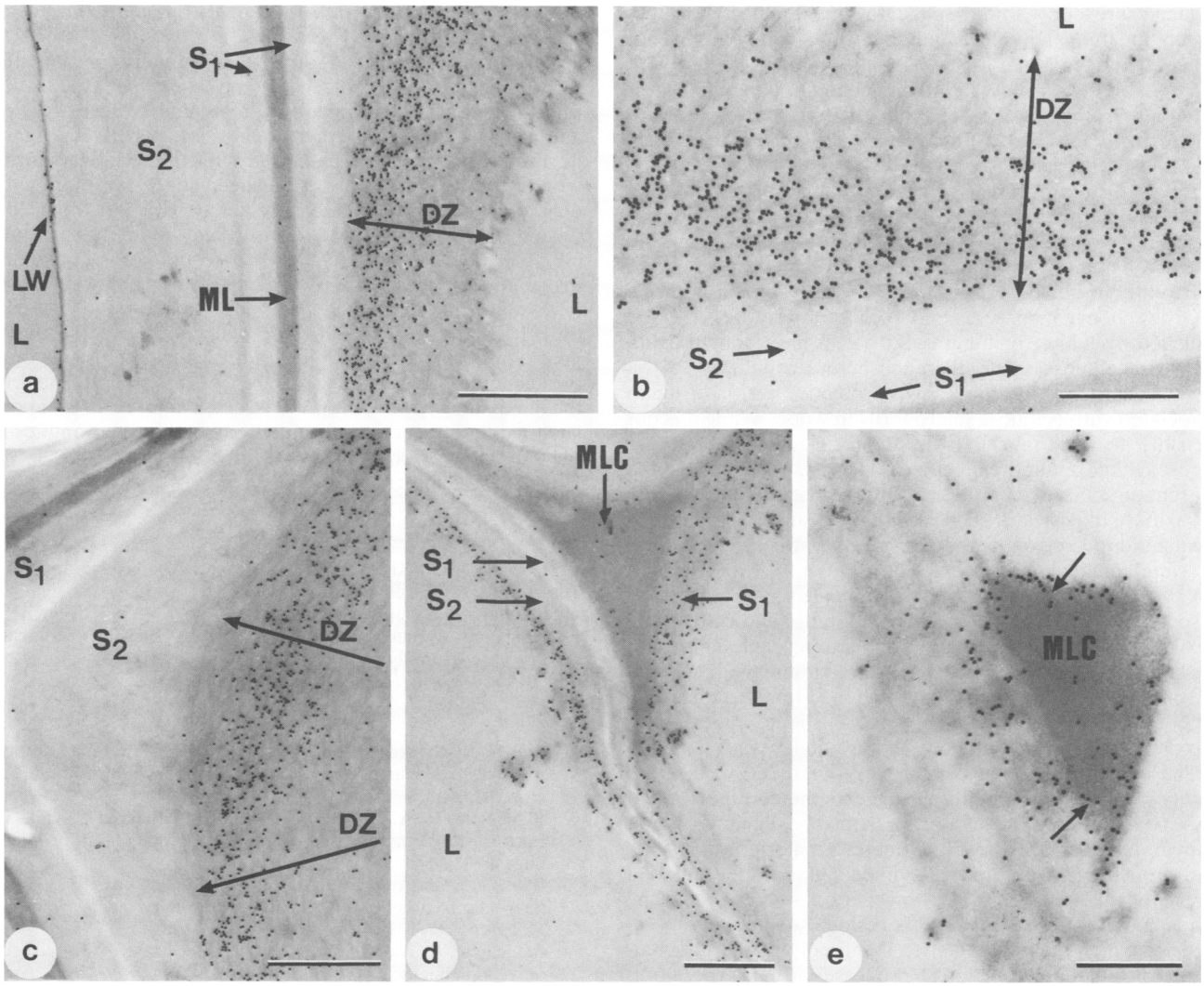

FIG. 5. Transmission electron micrographs showing anti-lignin peroxidase immunolabeling of lignin peroxidase-impregnated *P. chrysosporium*-degraded birchwood. (a to c) Localization of lignin peroxidase in degradation zones (DZ) produced within the S_2 cell wall. The enzyme is restricted to exposed cell wall areas and appears to be concentrated in the interfacial region between undegraded and degraded or otherwise structurally modified S_2 cell wall regions. Note the inability of the enzyme to penetrate the undegraded birch fiber secondary cell wall regions, with localization occurring only on the lumen wall (LW). ML, Middle lamella; L, Lumen. (d) Advanced stage of decayed wood, with lignin peroxidase concentrated in peripheral cell wall regions including exposed S_1, S_2, and middle-lamella cell corner (MLC) regions. (e) Labeling of residual middle-lamella cell wall corner, with lignin peroxidase concentrated in exposed outer regions and also penetrating (arrows) partially degraded regions. Bars: 1.0 μm (a, c, and d) and 0.5 μm (b and e).

specificities of the polyclonal antisera or more possibly in the movement of the enzyme during fixation (dependent on fixative) and electron microscopy processing may be responsible for the variations over the limited area involved.

A characteristic feature of the decay induced by *P. chrysosporium* in birch and pine was the occurrence of prominent decay zones within the S_2 cell wall regions, particularly in pine latewood fibers. Similar zones have been observed with other white rot fungi (1, 23, 33) and are thought to reflect stages in progressive lignin attack and possibly depolymerization across the S_2 cell wall. Previously it was postulated that lignin degradation across wood cell walls occur by a nonenzymatic means (e.g., free radicals) (26). This would agree with current knowledge on the known limited porosity of wood cell walls to large molecules (5, 14) and with recent findings obtained by using biomimetric catalysts to delignify wood (24).

In the present studies with degraded wood samples, lignin peroxidase was detected, albeit at reduced levels, in both superficially degraded cell wall regions and within the characteristic S_2 decay zones in both birch and pine. Similarly, the wood fragment samples showed lignin peroxidase to both penetrate and be concentrated at the interface between degraded and nondegraded wood cell wall regions. Penetration of lignin peroxidase into decay zones of partially degraded fibers was also recognized in wood samples infiltrated with the enzyme. This indicates, therefore, a capacity of the enzyme (molecular weight, 42,000) to penetrate attacked wood cell walls and to be located at the sites where lignin degradation and depolymerization are occurring. In addition, the inability of the enzyme to penetrate unattacked wood cell walls in any of the three experiments is consistent with a limited porosity of wood cell walls to molecules of this dimension (5).

The microscopy studies indicate that direct contact between the fungal hyphae and the wood cell walls is not necessary for degradation. A similar situation existed with the liquid cultures, in which the fragments, although aggregated into a closely adhering mat by the fungal hyphae, were not normally in direct contact with the fungus, judging from TEM observations. The present results are therefore consistent with the extracellular secretion of lignin peroxidase and its subsequent association and presumed activity with the degrading wood substrate and lignin at a distance from the fungal hyphae, direct contact between fungus and substrate apparently not being necessary for the degradation process.

Gross chemical analyses and microscopy studies of wood attacked by white rot fungi (wild types) have shown lignin and hemicellulose to be degraded before cellulose (2–4), a result consistent with the known protective action of lignin associated with cellulose microfibrils in wood. While it is not possible to ascertain whether lignin degradation occurs before cellulose and hemicellulose degradation by using the present antibody technique, the spatial distribution of lignin peroxidase in degrading cell wall regions suggests a possible localization in regions undergoing initial attack. The nature of simultaneous component degradation in wood by *P. chrysosporium* would, however, indicate that there is an association of ligninolytic, hemicellulolytic, and cellulolytic enzymes within cell wall regions undergoing degradation and that these may act in concert. Further studies with sequential immunolabeling techniques and additional antisera to both hemicellulase(s) and cellulase(s) should reveal their proximity to lignin peroxidase and are under way.

The continued labeling of the very degraded wood fragments from the lignin cultures suggests the continued presence of lignin peroxidase and, presumably, lignin. It is not known whether this residual matrix material represents undegraded or structurally modified lignin. The degraded matrix of the fragments was, however, characterized by the ultrastructural appearance of its residual components, features of which appear similar to lignin-polysaccharide complexes produced by chemical processes (6). Furthermore, the degraded matrix may contain condensed lignin resulting from the known ability of lignin peroxidase to polymerize lignin and phenolic substances in vivo (15).

At present, it is not known why the labeling of lignin peroxidase associated with degrading wood cell walls was less than that of lignin peroxidase associated with wood fragments. While the fragments were degraded under liquid culture conditions optimal for extracellular lignin peroxidase production, it should be noted that few studies have shown active white rot degradation of solid wood under totally submerged conditions in nature. Degradation of the wood fragments may therefore reflect an artificial situation in which unnaturally high lignin peroxidase levels are present. It is also possible that conditions within the solid wood blocks were not optimal for production of the enzyme and that both reduced levels (coupled with a greater surface area of the wood) and a slower process of lignin degradation occurred. Variations in the developmental phase of the hyphae with respect to peroxidase production may also be important. The possible involvement of additional ligninolytic enzymes which may not cross-react with the anti-lignin peroxidase must also be considered. The intense labeling reactions for lignin peroxidase of the birchwood fragments and the lesser but still positive results obtained with the birchwood blocks does, however, suggest at least a difference in enzyme concentration rather than any problem of accessibility of the antiserum to its antigen in the wood substrate itself.

Studies have shown (15), and it has been proposed (9, 16), that lignin can be oxidized by mediators such as cation radicals (e.g., veratryl alcohol), acitvated oxygen species, and Mn(III), suggesting that direct contact between lignin-degrading enzymes and lignin may not be necessary during wood degradation. It has further been suggested that lignin-degrading enzymes remain associated with the fungal hyphae during decay and are released only in limited amounts (10, 22). The present results show both an extracellular distribution and a possibility for close contact between lignin peroxidase and the wood substrate during lignin degradation. It has, however, still to be determined whether lignin peroxidase (or other lignin-degrading enzymes) is responsible for initial lignin attack or whether this is performed by the mediator-type reactions described above. Presumably such mediator reactions could occur within the confines of the degrading wood substance in close proximity to the enzyme progenitors.

ACKNOWLEDGMENTS

We thank T. K. Kirk for the anti-lignin peroxidase serum and I. Ternrud for the distilled veratryl alcohol.

This work was supported in part by the Swedish Council for Forestry and Agricultural Research and by Stiftelsen Nils and Dorthy Troedssons Forskningsfond. These contributions are gratefully acknowledged.

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