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Pyranose Oxidase, a Major Source of H_2O_2 during Wood Degradation by *Phanerochaete chrysosporium*, *Trametes versicolor*, and *Oudemansiella mucida*

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The production of the H_2O_2-generating enzyme pyranose oxidase (POD) (EC 1.1.3.10) (synonym, glucose 2-oxidase), two ligninolytic peroxidases, and laccase in wood decayed by three white rot fungi was investigated by correlated biochemical, immunological, and transmission electron microscopic techniques. Enzyme activities were assayed in extracts from decayed birch wood blocks obtained by a novel extraction procedure. With the coupled peroxidase-chromogen (3-dimethylaminobenzoic acid plus 3-methyl-2-benzothiazolinone hydrazone hydrochloride) spectrophotometric assay, the highest POD activities were detected in wood blocks degraded for 4 months and were for *Phanerochaete chrysosporium* (149 mU g [dry weight] of decayed wood^{-1}), *Trametes versicolor* (45 mU g^{-1}), and *Oudemansiella mucida* (1.2 mU g^{-1}), corresponding to wood dry weight losses of 74, 58, and 13%, respectively. Mn-dependent peroxidase activities in the same extracts were comparable to those of POD, while lignin peroxidase activity was below the detection limit for all fungi with the veratryl alcohol assay. Laccase activity was high with *T. versicolor* (422 mU g^{-1} after 4 months), in trace levels with *O. mucida*, and undetectable in *P. chrysosporium* extracts. Evidence for C-2 specificity of POD was shown by thin-layer chromatography detection of 2-keto-D-glucose as the reaction product. By transmission electron microscopy-immunocytochemistry, POD was found to be preferentially localized in the hyphal periplasmic space of *P. chrysosporium* and *O. mucida* and associated with membranous materials in hyphae growing within the cell lumina or cell walls of partially and highly degraded birch fibers. An extracellular distribution of POD associated with slime coating wood cell walls was also noted. The periplasmic distribution in hyphae and extracellular location of POD are consistent with the reported ultrastructural distribution of H_2O_2-dependent Mn-dependent peroxidases. This fact and the dominant presence of POD and Mn-dependent peroxidase in extracts from degraded wood suggest a cooperative role of the two enzymes during white rot decay by the test fungi.

Pyranose oxidase (POD) (pyranose:oxygen 2-oxidoreductase; EC 1.1.3.10) is an H_2O_2-producing enzyme catalyzing the C-2 oxidation of several aldopyranoses, with the preferred substrate being D-glucose (8, 21, 31, 42). The enzyme is known to be produced by a number of basidiomycetes, especially members of the order *Aphyllophorales* (41). It is a relatively large flavin adenine dinucleotide glycoprotein (M_r, ca. 300,000) but has been identified in both mycelial cell extracts and culture filtrates of laboratory cultures from several fungi, suggesting possible intra- and extracellular distributions (6, 14, 20, 34, 38, 44).

Since H_2O_2 is known to act as a cosubstrate for ligninolytic peroxidases (lignin peroxidases [LiP] and Mn-dependent peroxidases [MnP]) during lignocellulose degradation, knowledge of the source of H_2O_2 during fungal decay is of considerable interest (35). To date a number of oxidase enzymes have been postulated as possible donors of H_2O_2 during white rot decay of lignocellulose. In addition to POD (synonym, glucose 2-oxidase) (11), these enzymes include glucose 1-oxidase (24, 25), glyoxal oxidase (26), methanol oxidase (33), fatty acyl-coenzyme A oxidase (16), and aryl alcohol oxidase (synonym, veratryl alcohol oxidase) (3, 18). This last enzyme may act in concert with intracellular NADPH-dependent aryl alcohol dehydrogenase in a cyclic redox system for H_2O_2 production from aromatic substrates (17). Of these enzymes, some of the best evidence for a role in lignocellulose attack has been presented for the sugar-oxidizing enzymes and glyoxal oxidase, since they have been reported to be produced under conditions of secondary metabolism or during lignocellulose degradation when ligninolytic enzymes are being expressed (9, 11, 26). The purpose of the present study was to verify our earlier suggestions (6, 11) that POD can play an important role in the ligninolytic systems of numerous white rot fungi as a source of H_2O_2 necessary for the activity of ligninolytic peroxidases during wood decay.

The approach used to prove this hypothesis was to use (i) transmission electron microscopy (TEM)-immunocytochemical labeling of POD and (ii) biochemical analyses of POD activity in cultures of selected white rot fungi, i.e., *Phanerochaete chrysosporium*, *Trametes versicolor*, and *Oudemansiella mucida*, degrading wood substrates (birch wood blocks) under conditions similar to those in natural environments and then to correlate the results with activities of the major known lignin-degrading oxidoreductases. The three white rot fungi were chosen because of their known ligninolytic abilities and production of POD in in vitro situations. The study provides evidence which lends support for an important role of POD during wood degradation by these fungi.

MATERIALS AND METHODS

Fungal strains. *P. chrysosporium* Burds., strain K-3 (CCBAS 571), and *O. mucida* (Schrad.:Fr.) Höhn., strain III (CCBAS

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428), were obtained from the culture collection of basidiomycetes maintained at the Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague, and *T. versicolor* (L.:Fr.)Pilat, strain A361 (synonym, *Coriolus versicolor* (L.): Quel.), was obtained from the culture collection at the Department of Forest Products, Swedish University of Agricultural Sciences, Uppsala. All fungi are routinely maintained on 2.5% (wt/vol) malt extract agar plates.

Wood samples for decay, immunocytochemistry, and enzyme extraction. Three wood blocks (30 by 15 by 5 mm) of birch (*Betula verrucosa* Ehr.) were placed in 100-ml Erlenmeyer flasks containing garden loam (ca. 18 g [dry weight]). No other growth-supporting additions were used. After being autoclaved, the flasks were aseptically inoculated with a mycelial suspension (2 ml) obtained from scraping a single malt agar plate bearing actively growing cultures of individual fungi into 10 ml of sterile water and mild homogenization. The fungal wood colonization and decay proceeded at 24°C and 70% humidity for up to 4 months. Weight losses of wood blocks were determined after drying overnight at 105°C. A parallel set of wood block cultures was set up for TEM studies and enzyme assays.

POD production and purification from liquid cultures. For POD antiserum preparation, POD was purified from wet mycelial mass of *P. chrysosporium* obtained from liquid-submerged cultures grown for 12 days under conditions optimal for enzyme production (42). The three-step purification procedure consisted of hydrophobic interaction on phenyl–Sepharose CL-4B, anion-exchange chromatography on Mono Q HR 5/5, and finally gel filtration on Superose 6 HR 10/30 columns as described previously (43).

O. mucida and *T. versicolor* were also grown in liquid cultures, and crude extracts were obtained to assess cross-reactivity with the POD antiserum from *P. chrysosporium* K-3. *O. mucida* was grown under the conditions described by Volc et al. (44) (5-day-old cultures were taken), and *T. versicolor* was grown under the conditions described by Machida and Nakanishi (31) (3-day-old cultures were taken). POD activities were estimated by the 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH)–3-dimethylaminobenzoic acid (DMAB) assay (see below) on extracts from 2 g (wet weight) of washed mycelia obtained after homogenization (Ultra-Turrax homogenizer [IKA-Labortechnik, Staufen, Germany]; shaft 10N, five 1-min runs with cooling) in 7 ml of Na phosphate buffer (pH 6.5) and centrifugation (10,000 × *g*) of the resulting suspension. These extracts were used in subsequent native gradient polyacrylamide gel electrophoresis (PAGE) and Western blotting (immunoblotting) to test for immuno-cross-reactivities with the POD antiserum from *P. chrysosporium*.

Enzyme extraction from degraded wood blocks. Decayed wood blocks were scraped free of surface mycelia, wrapped in thick aluminum foil, frozen in liquid nitrogen, and crushed against a flat iron support with a hammer. The resulting macerated wood was released from the foil, and the combined material from two or three parallel blocks (according to the extent of decay) was precooled in liquid nitrogen and ground by eight 1-min runs followed by 30-s cooling periods with dry ice-ethanol in an Analytical Mill A10 (IKA-Labortechnik) equipped with a cooling thermostat. The powdered wood material corresponding to six infected wood blocks was thereafter extracted twice in 25 ml of 0.1 M Na phosphate buffer (pH 6.5) with the Ultra-Turrax homogenizer (shaft 18N, full speed, five 1-min intervals with ice bath cooling). The homogenates obtained were centrifuged for 20 min at 10,000 × *g*, and the supernatants were combined and then concentrated to 2 ml by ultrafiltration (YM 10 membrane; Amicon). Low-molecular-weight components of the extract were removed by diafiltration using the same buffer.

Enzyme assays. POD activity was assayed spectrophotometrically by measuring the production of H_2O_2 through a coupled peroxidase reaction with MBTH plus DMAB (Fluka Chemie, Buchs, Switzerland) as the chromogen (42). The reaction mixture (2.0 ml total) contained 200 μmol of Na phosphate buffer (pH 6.5), 100 μmol of D-glucose, 0.1 μmol of MBTH, 5 μmol of DMAB, 5 U of horseradish peroxidase (Sigma Chemical Co., St. Louis, Mo.), and a suitable amount of POD. The same reaction mixture without glucose was used as a reference.

MnP and laccase (benzendiol:oxygen oxidoreductase; EC 1.10.3.2) activities were assayed by using the same chromogen (MBTH plus DMAB). In the case of MnP, the reaction mixture (2.0 ml total) contained 100 μmol of succinate-lactate buffer (pH 4.5), 0.1 μmol of H_2O_2, 0.1 μmol of MBTH, 5 μmol of DMAB, 0.2 μmol of $MnSO_4$, and the enzyme extract. MnP activity was measured against a reference of the same composition but lacking Mn^{2+} (correction for possible laccase and other peroxidase activities was made). The reaction was initiated by addition of 10 μl of 10 mM H_2O_2. Laccase was determined with the same reaction mixture as for MnP except that H_2O_2 and Mn^{2+} were excluded. In the blank reference sample the enzyme extract was excluded. One unit of each of these enzyme activities was defined as the amount of activity that would give rise to an amount of the colored product of oxidized MBTH plus DMAB (590 nm, $\varepsilon = 3.2 \times 10^4$) corresponding to reduction of 1 μmol of H_2O_2 per min under the assay conditions. LiP activity was determined as the H_2O_2-dependent oxidation of veratryl alcohol (40). Catalase was assayed by measuring the rate of disappearance of H_2O_2 from the decrease in A_{240}, according to the Sigma protocol (Sigma Chemical Co.), and its activity is expressed in Sigma units. Specific enzyme activities given in Table 1 represent activities in units per gram (dry weight) of decayed wood used for the enzyme extraction. The protein content in extracts was not determined because of the low volume of final extract samples (corresponding to six wood blocks) and the triplicate measurements made for each of the enzyme activities.

Thin-layer chromatography analyses. The C-2 specificity of glucose-oxidizing activity in extracts from degraded wood blocks was confirmed by detection of 2-keto-D-glucose (D-*arabino*-2-hexosulose) by thin-layer chromatography on cellulose-coated sheets (Lucefol foils; Kavalier, Votice, Czech Republic) as described previously (41). Briefly, concentrated enzyme extracts from wood (0.25 ml) were incubated with magnetic stirring (16 h) at room temperature with 0.25 ml of 2% D-glucose in water. The reaction mixtures were then chromatographed in butylacetate-acetic acid-acetone-water (140:100:33:80), and 2-keto-D-glucose was detected by diphenylamine-aniline-phosphoric acid reagent (28), with an intense blue color indicative of 2-ketoaldoses.

Polyclonal antibodies, native PAGE, and Western blotting. POD antiserum was raised in New Zealand White rabbits as previously described (6, 23). Concentrated crude extract samples of *P. chrysosporium*, *O. mucida*, and *T. versicolor* were run on discontinuous nondenaturing 5 to 20% linear gradient PAGE gels with 3% stacking gels. Western blotting was performed with a Sigma semidry blotting apparatus with transfers (2 h at 10 V) made onto nitrocellulose membranes. For immunodetection, membranes were blocked with 3% gelatin (from porcine skin; Sigma) in Tris (10 mM)-saline (150 mM) buffer (pH 7.5) (TBS) and incubated with anti-POD (*P. chrysosporium*) antiserum (1:500 in 0.5% gelatin–TBS, 2 h at room temperature). After being washed in TBS (three times

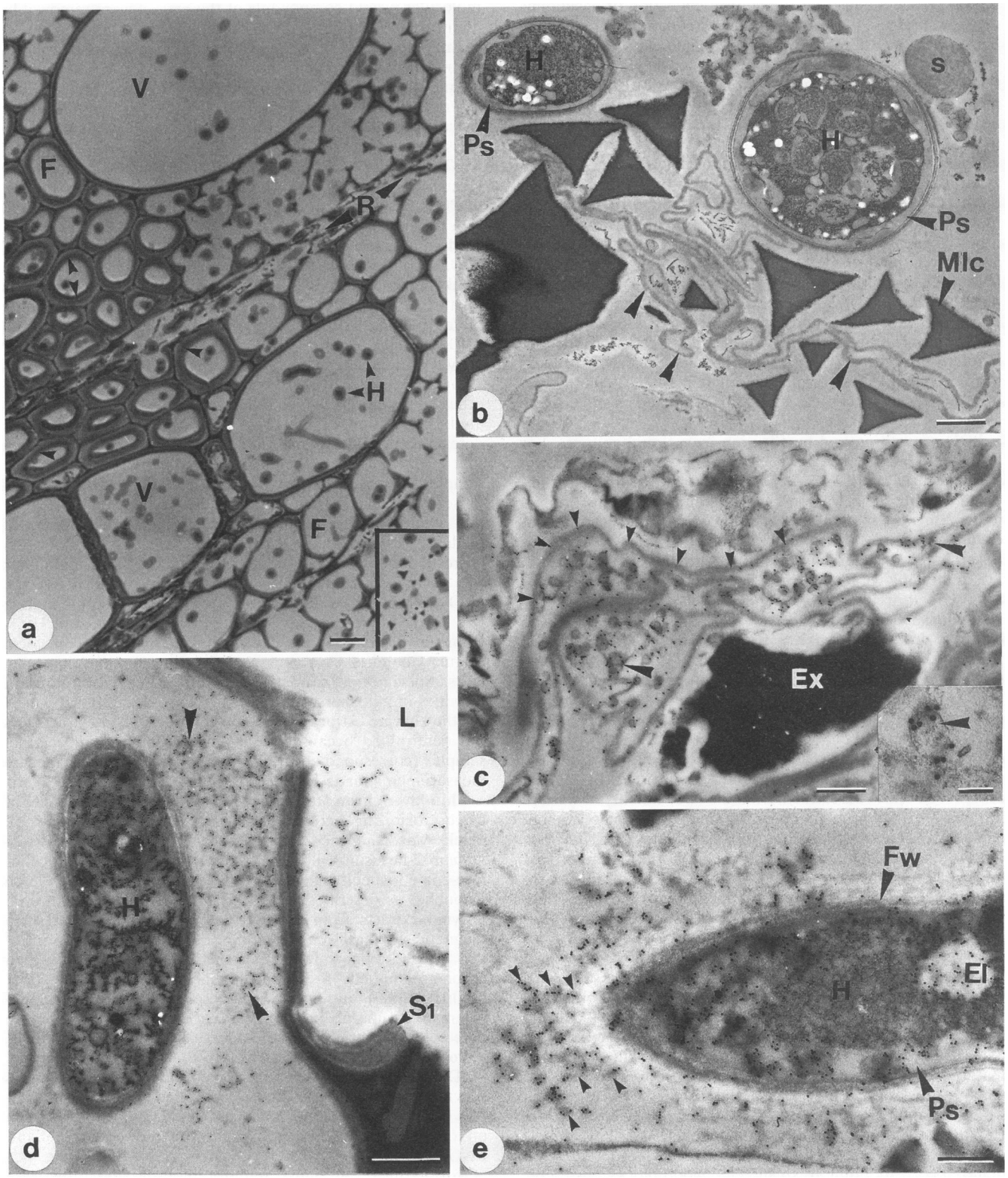


FIG. 1. Light (a) and transmission electron (b to e) micrographs showing various stages of birch cell wall degradation by *P. chrysosporium* (a and b) and the extracellular distribution of POD (c to e). (a) Light micrograph showing typical cell wall thinning and the large range of cell wall decay apparent, from early cell wall attack (top left) to where only residual middle lamella cell corners remained (enclosed region, far right). Decay zones (unlabeled arrowheads) are best seen in fibers at the early stages of decay. POD was localized in hyphae from all stages of cell wall attack. (b) Transmission electron micrograph showing advanced cell wall degradation of a region similar to that shown in panel a. Only the highly lignified middle lamella corners are remaining interspersed with hyphae, extracellular slime materials, and the remains of fungal cell wall materials (arrowheads). Hyphae were characterized by well-developed periplasmic spaces containing membranous materials. (c) Higher magnification from

for 10 min each), membranes were treated with horseradish peroxidase conjugated to pig anti-rabbit immunoglobulin G (1:1,000 in 0.5% gelatin–TBS, 2 h). Visualization was carried out with 4-chloro-1-naphthol. Activity staining of material in parallel transfers was performed with the same chromogen reagent supplemented with horseradish peroxidase (23 U/ml) and D-glucose (10 mg/ml). For all three fungi POD gave a single activity-positive band with the same mobility (not shown).

Light microscopy. For an overview of decay, semithin sections (1 to 2 μm) of resin-embedded samples were examined after being stained with 1% (wt/vol) toluidine blue in 1% (wt/vol) sodium borate.

TEM. Wood pieces for fixation were removed from wood blocks (2, 3, or 4 months) with a razor blade. Samples were fixed for 3 h in 3% (vol/vol) glutaraldehyde containing 2% (vol/vol) paraformaldehyde in 0.1 M Na cacodylate buffer (pH 7.2) at room temperature. Wood samples were then washed three times in buffer and postfixed in 1% (wt/vol) osmium tetroxide in 0.1 M buffer for 1 h at room temperature. After further washes in buffer and distilled water, samples were dehydrated in an ethanol series (20 to 100 ethanol; 10% steps, 10 min each), infiltrated with London resin (London Resin Co., Basingstoke, United Kingdom), and polymerized overnight at 60°C. Other samples were fixed for 3 h in 4% (vol/vol) paraformaldehyde containing 1% (vol/vol) glutaraldehyde in the same buffer and thereafter were dehydrated and infiltrated with resin as described above. Selected material was sectioned with a Reichert FC4 ultramicrotome, and sections were collected on nickel grids. Poststaining was conducted with 4% aqueous uranyl acetate (5 min) and lead citrate (15 min) (37). Observations were made with a Philips CM/12 STEM.

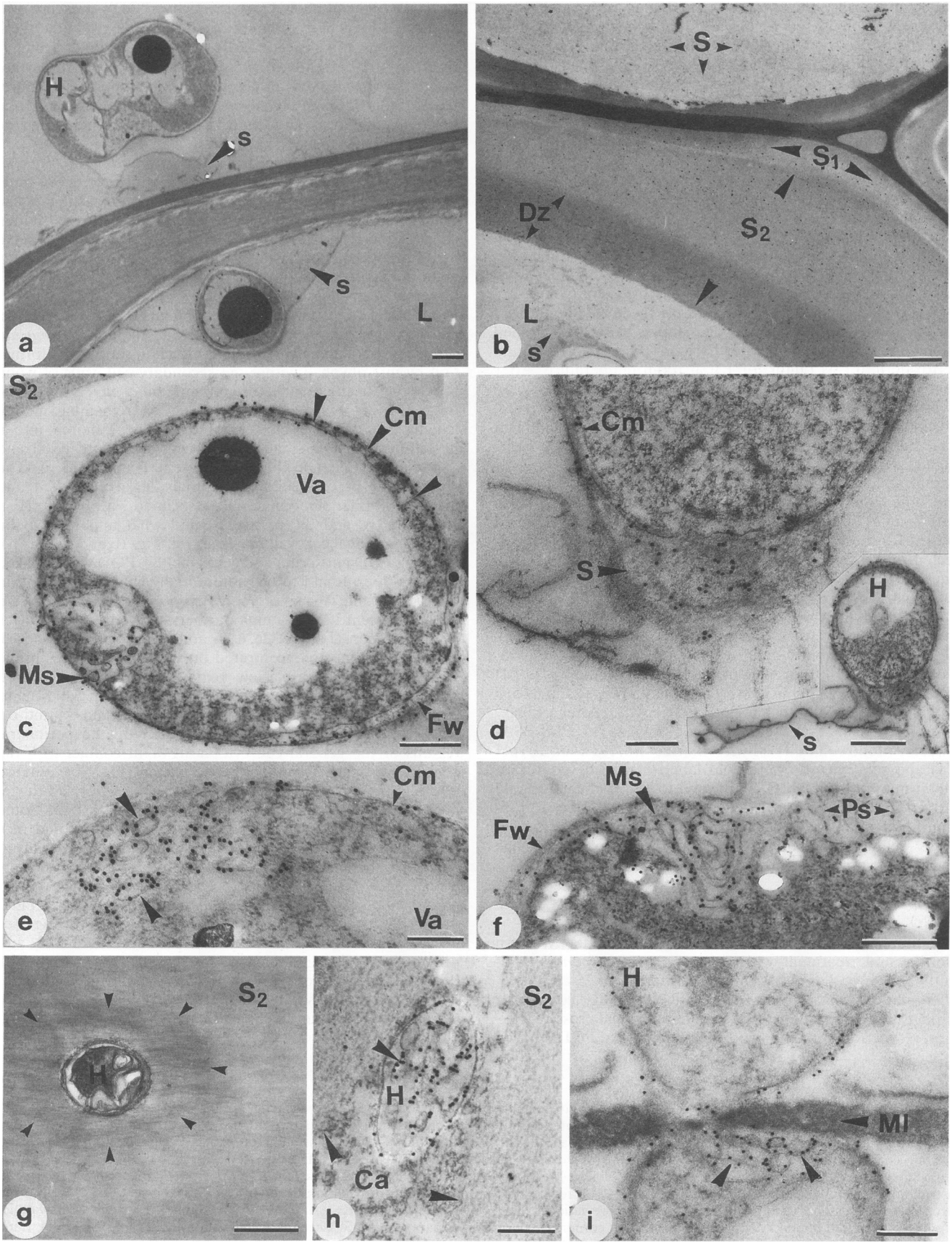
TEM-immunocytochemistry studies. Ultrathin sections for immunolabeling were processed as described previously (6). Grids bearing sections were incubated in anti-POD immunoglobulin G (1:500) in phosphate-buffered saline (pH 7.4) (PBS) containing 1% bovine serum albumin (BSA) and 0.05% (vol/vol) Tween 20 and left overnight at 4°C. After primary antiserum incubation, sections were washed in PBS-BSA-Tween 20 followed by Tris-hydrochloride–BSA–Tween 20 (pH 8.4) and subsequently were gold labeled by using goat anti-rabbit immunoglobulin G conjugated to 15-nm-diameter gold probes (B-2340; Janssen Life Science Products, Beerse, Belgium). Poststaining was as described above. Control procedures included omission of the POD primary antiserum stage and use of anti-POD preadsorbed with the POD antigen.

RESULTS

Immunological cross-reactivities of POD. After Western blotting and treatment with primary *P. chrysosporium* anti-POD and secondary antiserum, POD bands for *P. chrysosporium* and *O. mucida* showed positive staining, confirming strongly and moderately positive immunological cross-reactivities, respectively, for the two fungi. In contrast, POD from *T. versicolor* did not show cross-reactivity. POD's presence was confirmed by activity staining of corresponding parallel gels. The reasons for the non-cross-reactivity of POD from *T. versicolor* are currently unknown, although PODs from other basidiomycetes have been described as immunologically unrelated (20). Since *T. versicolor* POD did not show cross-reactivity with the antiserum, it was not included in the TEM-immunocytochemical studies.

TEM-immunocytochemistry. *P. chrysosporium* K-3, *T. versicolor*, and *O. mucida* are simultaneous white rot decay fungi and typically cause progressive cell wall thinning of the secondary cell walls of wood fibers, starting from the cell lumen (Fig. 1a and d and 2a and b). Since it was possible to observe a wide range of decay stages after 2, 3, and 4 months, only micrographs from 3-month-decay samples are shown here. All three fungi produce a narrow decay zone in which simultaneous attack of wood cell wall components takes place (Fig. 1a and 2b). In addition to causing cell wall thinning, *O. mucida* produces characteristic multiple T-branching and cavity formation by microhyphae within the secondary cell walls of wood fibers (Fig. 2g and h), a feature reminiscent of effects of soft rot fungi (7). At advanced stages of decay, remaining middle lamella cell corner regions tend to be incorporated into a hyphal-extracellular slime network in which both lysed and apparently healthy hyphae are present (Fig. 1a to c). The morphology of extracellular materials observed varied and included fibrillar slime materials associated with hyphae (Fig. 1b, d, and e and 2a, b, and d) and what appeared to be remaining fungal cell wall materials (cf. Fig. 1b and c with 3b). By using POD antiserum and gold labeling, POD was localized and was often found to be preferentially concentrated within the periplasmic space of *P. chrysosporium* and *O. mucida* hyphae present in the lumina of fibers showing various stages of degradation (Fig. 1e, 2c, e, f, and i, and 3a, b, d, and e). Labeling was most concentrated on vesicular and membrane structures within periplasmic regions, but it also occurred along the endoplasmic reticulum, cell membrane, and membrane structures in vacuolar regions (Fig. 1e and 3a and b). Such vacuolar regions were often located in the peripheral regions of the general cell cytoplasm or in contact with the periplasmic space via membrane systems (Fig. 3b). Intense labeling of membrane and vesicular structures in lysed fungal hyphae was also apparent (Fig. 1c and 3c), as was some labeling of the fungal cell wall (Fig. 1e, 2c to f, and 3a). Extracellular slime materials situated between the cell walls of degraded fibers and fungal hyphae were also labeled strongly for the presence POD (Fig. 1d and e), as were materials in lysed hyphae within the extracellular network (Fig. 1c). POD was localized in hyphae from fibers showing various stages of decay, suggesting that the enzyme is produced both early and progressively during birch wood cell wall degradation. The POD labeling pattern obtained for *O. mucida* hyphae in decayed birch was similar to that observed for *P. chrysosporium*, apart from the labeling of cavity-forming hyphae (Fig. 2h).

a region of advanced decay, showing POD associated with membranous structures (large arrowheads) present with residual fungal cell wall materials (small arrowheads). The inset shows POD associated with the membranous structures. Electron-dense extract materials present in some sections are thought to originate from wood resinous materials and their subsequent reaction with osmium tetroxide. (d) Oblique transverse section showing close proximity of a hypha with a highly degraded birch fiber and the presence of POD associated with extracellular slime materials situated between the fungus and wood cell wall. (e) Oblique longitudinal section of a hypha from the lumen of a degraded fiber, showing the spatial distribution of POD with extracellular slime materials (arrowheads) and intracellular distribution in the fungal periplasmic space as well as the electron-lucent region within the cell cytoplasm. Abbreviations: El, electron-lucent region; Ex, extract materials; F, fiber; Fw, fungal cell wall; H, hyphae; L, fiber cell lumen; Mlc, middle lamella cell corner; Ps, periplasmic space; R, ray; S_1, secondary cell wall layer; s, slime; V, vessel. Bars: a, 5.0 μm; b and d, 1.0 μm; c and e, 0.5 μm; inset to panel c, 0.1 μm.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Both the method (i.e., omission of primary antibody) and immunological (i.e., use of POD antibody preadsorbed with POD antigen) controls were negative, as was POD labeling of undegraded wood. Similar labeling patterns were achieved with both fixatives, and although a more intense labeling was obtained with samples fixed in the higher paraformaldehyde concentration, the hyphae in that case showed inferior ultrastructural preservation. The photographs presented here are therefore from samples prefixed in glutaraldehyde and paraformaldehyde and postfixed in osmium.

Enzyme activity assays. In order to increase protein yields in extracts from decayed wood blocks, we developed an extraction method enabling (i) efficient disintegration of both degraded and sound wood and (ii) release of extra- and intracellular fungal enzymes. This method made possible the detection of some ligninolytic enzymes in samples as small as a single birch wood block of 30 by 15 by 5 mm partially degraded in 1- to 2-month incubations. Specific enzyme activities are reported relative to dry weights of parallel degraded wood samples.

Results of biochemical analyses of POD and ligninolytic (per)oxidases in degraded wood blocks are summarized in Table 1. They provide strong evidence that POD is closely associated with wood decay and corroborate the above-described findings with TEM and immunogold labeling. Control extracts of noninfected wood blocks showed no activity of any of the enzymes tested. POD was detected in extracts of all the test fungi, except for an early-decay sample of *O. mucida* from the 2-month incubation period, in which only 6% dry weight loss occurred and sensitivity of the POD assay was probably insufficient. MnP activities were found in all extracts and correlated well with POD activities, while no LiP was documented with any fungus. The maximum POD activity was in 4-month *P. chrysosporium* colonized samples (74% weight loss, 149 mU g [dry weight] of decayed wood^{-1}, 67 mU ml of final extract^{-1}), whereas *O. mucida*, a much slower degrader, exerted only 1.2 mU g^{-1} at 13.5% weight loss in a 4-month sample. POD was produced in activities (milliunits gram^{-1}) comparable to those of MnP and accumulated similarly as wood decay proceeded. The activities of both enzymes were also proportional to the degrading capabilities of the test fungi (i.e., percent dry weight loss). High laccase activities (up to 421 mU g^{-1}) were revealed with only *T. versicolor*. The activity of this enzyme at pH 6.5 was negligible and did not disturb the POD assay. Traces of laccase activity were also found in *O. mucida*-degraded samples but not in *P. chrysosporium*-degraded blocks. Catalase was detected in all samples degraded by the three fungi, with the activity being the highest (201 Sigma units g^{-1}) in the 4-month *P. chrysosporium*-degraded samples.

DISCUSSION

In the process of oxidative depolymerization of both lignin and polysaccharide components during attack of wood by white rot basidiomycetes, a central role is ascribed to H_2O_2 as a cofactor of LiP and MnP and as a precursor of the highly reactive HO· radicals, which are thought to participate in fragmentation of wood polymers (2, 29, 30). One of the candidates for the major source of H_2O_2 in ligninolytic systems is the enzyme POD (42). Synthesis of the enzyme has been reported in liquid cultures of all three fungi studied here, i.e., *P. chrysosporium*, *T. versicolor*, and *O. mucida* (11, 31, 34, 44). Extracellular production of POD with *P. chrysosporium* (6) was observed by using immunogold labeling. Extracellular glucose oxidase activity has also been reported for culture filtrates of *Panus tigrinus* (14).

The hyphal periplasmic and extracellular distributions of POD reported here are similar to those reported previously for the H_2O_2-dependent LiP and MnP in *P. chrysosporium* (4, 5) and for LiP in *T. versicolor* (12), a finding consistent with a contemporary role of the enzymes during lignocellulose degradation. The periplasmic distribution of POD in *P. chrysosporium* is also consistent with that observed earlier with liquid cultures (6) and similar to that described for sites of H_2O_2 production in ligninolytic cultures of the same fungus (13). Although antibody labeling was not performed with *T. versicolor*, Highley and Muramis (19), using 3,3-diaminobenzidine, reported that sites of H_2O_2 production were located in the periplasmic space of the fungus, a spatial distribution which is also consistent with POD.

The results of TEM-immunolabeling studies and biochemical analyses of POD activity presented here indicate that under conditions simulating the process of wood decay in nature, POD can function as a constituent of the ligninolytic systems of some white rot fungi responsible for production of major quantities of H_2O_2. This view is also supported by the fact that PODs isolated from several white rot fungi (8, 11, 31, 34) show rather broad substrate specificities, acting primarily on D-glucose, D-xylose, D-mannose, D-galactose, and δ-D-gluconolactone; the former four sugars are the most abundant building units of wood polysaccharides, which considerably increases the possibilities for H_2O_2 production. Prominent localization of POD in the periplasmic space (i.e., at the cell membrane-fungal cell wall interface) of the fungal cell and its association with extracellular slime materials suggest that H_2O_2 should easily be able to penetrate into the extracellular medium, including wood cell walls.

In previous studies, POD production in *P. chrysosporium* has been correlated with idiophasic growth (11), while in *T.*

FIG. 2. Transmission electron micrographs showing aspects of birch cell wall degradation by *O. mucida* (a, b, and g) and cytoplasmic and extracellular distributions of POD in hyphae colonizing wood cells (c to f, h, and i). (a and b) Transverse sections showing *O. mucida* hyphae attached to partly degraded birch cell walls by extracellular slime and the presence of a more electron-dense staining region (decay zone) around the inner luminal cell wall, indicative of an early stage of cell wall attack. (c) Transverse section of a hypha, showing localization of POD along the fungal cell membrane, fungal cell wall, and membranous structures within a pronounced periplasmic space. (d) Higher magnification of a fungal hypha, showing association of POD with extracellular slime materials which hold the fungal cell to the fiber lumen wall. The inset shows a cross section of the entire hypha and extracellular slime arising from the hypha. (e and f) Transverse sections of hyphae showing association of POD with membranous structures (arrowheads) in the peripheral cell cytoplasm and within a pronounced periplasmic space. (g) Transverse section of birch secondary cell wall and a fine microhypha within the S_2 layer. A characteristic electron-dense staining zone (arrowheads) similar to that present along the fiber lumen (see panel b) was apparent. (h) Association of POD with membranous structures (arrowhead) in a cavity producing microhypha within the S_2 cell wall layer. (i) Hyphal penetration of a fiber middle lamella region, showing the close proximity of POD with membranous systems in the periplasmic space (arrowheads), fungal cell wall, and degrading wood cell wall. Abbreviations: Ca, cavity; Cm, cell membrane; Dz, decay zone; Fw, fungal cell wall; H, hyphae; L, fiber cell lumen; Ml, middle lamella; Ms, membrane system; Ps, periplasmic space; S_1 and S_2, secondary cell wall layers; s, slime; Va, cell vacuole. Bars: a, b, and inset to panel d, 1.0 μm; c, f, g, and i, 0.5 μm; d and e, 0.25 μm.

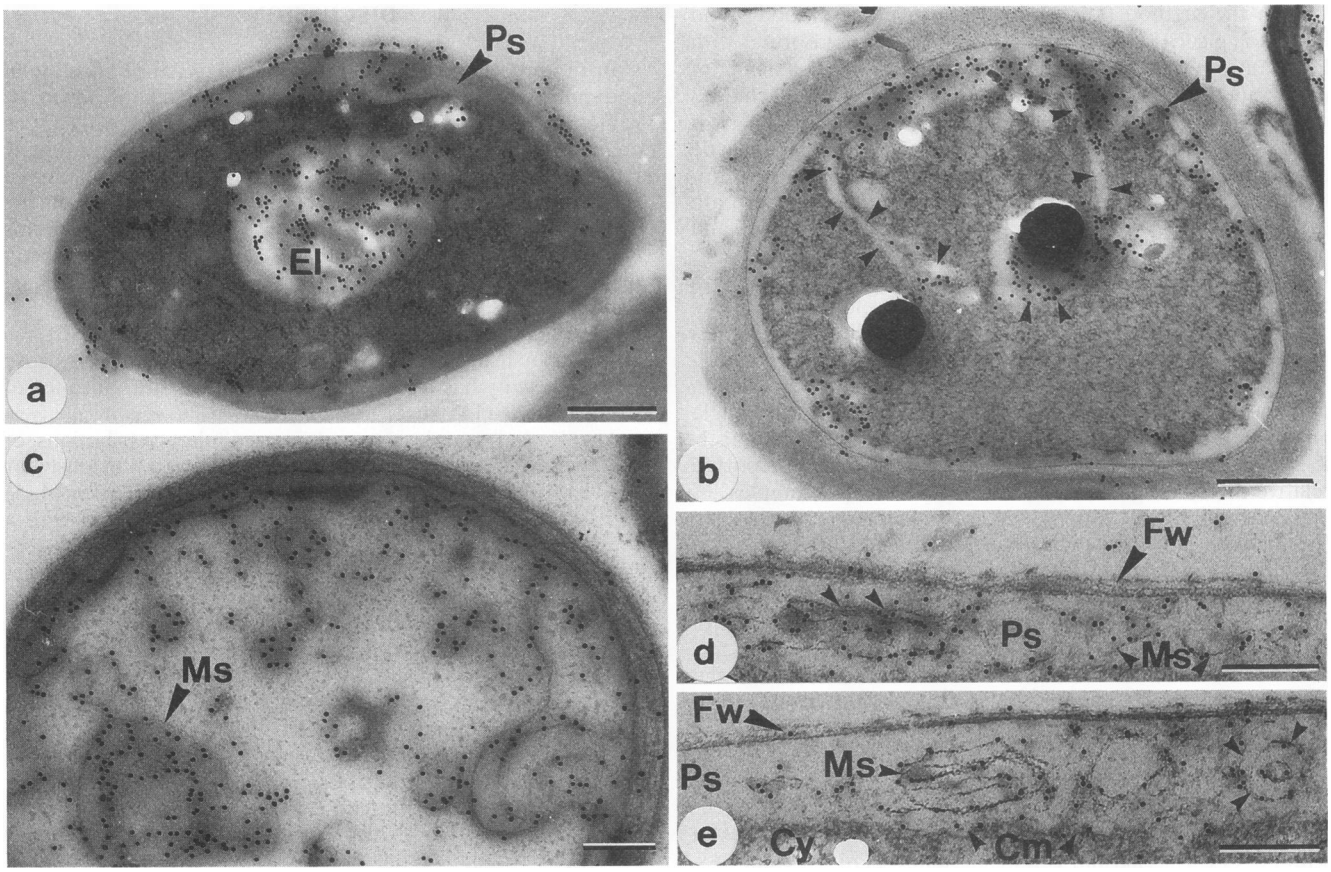

FIG. 3. Transmission electron micrographs showing aspects of the intracellular distribution of POD in *P. chrysosporium* hyphae (transverse sections) from degraded birch wood elements. (a and b) Preferential concentration of POD in the periplasmic space (Ps), along membranous systems (arrowheads), and in electron-lucent regions (El) within the more central cell cytoplasm of thick- and thin-walled hyphae. Note the similarity between the materials forming the fungal cell wall in panel b and the extracellular materials seen in Fig. 1b and c. (c) Lysed hypha, showing POD associated with membranous structures (Ms) within the disrupted cell cytoplasm. (d and e) Higher magnification showing POD associated with the cell membrane (Cm) and discrete membranous structures within a pronounced periplasmic space between the fungal cell wall (Fw) and cell cytoplasm (Cy). Bars: a, b, d, and e, 0.5 μm; c, 0.25 μm.

versicolor and *O. mucida* the enzyme is constitutive in liquid cultures (34, 44). The detection of POD at various (early and late) stages of wood cell wall decay by *P. chrysosporium* is further consistent with the well-documented nitrogen-limiting conditions for fungal growth in wood.

In addition to providing H_2O_2 for ligninolytic reactions, a possible further role of POD in *T. versicolor* could be with laccase to maintain a glucose:quinone oxidoreductase cycle in order to prevent spontaneous repolymerization of quinones formed during ligninolysis, thereby increasing lignin degradation (15, 39). In recent biobleaching studies with *T. versicolor*, increased brightness of Kraft pulps was obtained with cultures

TABLE 1. POD and (per)oxidase ligninolytic activities in extracts of birch wood degraded by the white rot fungi *P. chrysosporium*, *T. versicolor*, and *O. mucida*[a]

Fungus	Incubation time (mo)	% Weight loss	POD (mU g^{-1})[b]	MnP (mU g^{-1})[b]	Laccase (mU g^{-1})[b]	Catalase (U g^{-1})[c]
P. chrysosporium	2	43.1	4.6	48.0	NM[d]	10.9
	4	73.6	149.0	70.3	NM	200.6
T. versicolor	2	26.8	6.2	47.2	100.1	27.4
	4	58.0	45.2	115.8	421.5	5.0
O. mucida	2	6.1	NM	0.5	0.5	8.6
	4	13.1	1.2	0.7	0.2	8.1

[a] LiP activity was not measurable. See Materials and Methods for details about decayed wood extracts.
[b] Milliunits per gram (dry weight) of decayed wood.
[c] Sigma units per gram (dry weight) of decayed wood.
[d] NM, not measurable.

supplemented with glucose (27), and loss of the bleaching capability of the fungal cell extracts could be regained by reincubation with the fungus (1). Both the periplasmic distribution of POD with H_2O_2 release into the bleaching liquor and the increased bleaching obtained with D-glucose, the preferred substrate of POD (11), are consistent with an important role for POD.

Research on ligninolytic enzymes produced by wood-decaying basidiomycetes has so far mostly been performed with fungal liquid cultures grown on media optimized for lignin mineralization. Very little attention appears to have been given to cultures colonizing solid woody substrates (9, 10). Because growth conditions on these two types of media differ profoundly, the possibility that the enzyme pattern of the ligninolytic systems expressed may vary significantly in response to the culture conditions applied cannot be excluded. Accordingly, the dominant peroxidase protein identified in cultures of *P. chrysosporium* on mechanical pulp of aspen wood was an isoform of MnP different from the major MnP isoenzyme found under optimized conditions in liquid cultures (9). In addition to MnP, Western blot analyses revealed glyoxal oxidase and low levels of LiP. The latter enzyme is considered by many investigators to be the most efficient lignin-depolymerizing peroxidase attacking nonphenolic substructures of lignin. Similar to findings by others (suppression of LiP by solid substrates) (9, 32), our results indicate that MnP has a more important role in the process of fungal wood biodegradation than LiP, which was below the detection limit with all our fungi on wood blocks. Nevertheless, it is necessary to take into consideration the fact that the sensitivity of the LiP assay is lower than that of the MnP assay because of the lower (3.5 times) molar extinction coefficient of veratraldehyde compared with oxidized MBTH plus DMAB and because of the probably lower specific activity of pure LiP with veratryl alcohol compared with that of pure MnP with MBTH plus DMAB (the latter is not yet known). Peroxidase activities observed in reaction mixtures without Mn^{2+}, which were used as a reference for the MnP assay, were negligible, however. Other possible explanations for the failure to detect LiP could be the unsatisfactory extraction of the protein because of its interaction with the lignocellulose substrate (32), as recognized also with other wood-degrading enzymes (22, 36), or the function of some LiP inhibitor in the extracts (1). For example LiP was detected in fungus-colonized mechanical pulp only after affinity chromatography on conconavalin A-Sepharose (9).

Consistent with studies on liquid cultures, no laccase activity was detected in extracts of wood degraded by *P. chrysosporium*, while in extracts of wood colonized by *T. versicolor*, the enzyme exhibited the highest activities of all oxidoreductases tested. The decay capabilities of both fungi were comparable (Table 1). Considering the substantially similar levels of MnP with these two fungi, it does not seem that laccase significantly potentiates ligninolytic activity of *T. versicolor*. In wood samples (red alder wafers) decayed by the same fungus, production of laccase was also shown (10). The reason for the relatively high catalase activities, which are probably of intracellular origin, remains unclear, although location of the enzyme along the hyphal cell membrane would protect the cell cytoplasm from H_2O_2 produced in the periplasmic space.

The analytical method for preparation and evaluation of enzyme extracts from partially degraded wood blocks developed in this study provides a tool for the characterization of ligninolytic systems of wood rot fungi expressed under conditions similar to those in natural environments. It was not possible in the present study to distinguish between active extracellular secretion of enzymes and release of intracellular enzymes via hyphal lysis. However, since wood decay is a continuous process in which hyphae in fibers can show various stages of growth, development, and lysis, both extra- and intracellular enzyme release are likely to occur and contribute to decay.

This is the first demonstration for contemporary and active roles of POD and MnP during white rot decay under conditions similar to those in natural environments (only soil was used to support the fungal growth on wood). Current research based on the above-described methodological protocol is focused on comparative studies on production of H_2O_2-forming oxidases considered so far to participate in ligninolysis and on the enzyme cellobiose oxidase, which was recently revealed (30) to be a source of Fenton's reagent.

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