APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1988, p. 62–68
0099-2240/88/010062-07$02.00/0

Vol. 54, No. 1

Delignification of Wood Chips and Pulps by Using Natural and Synthetic Porphyrins: Models of Fungal Decay†

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Received 9 June 1987/Accepted 29 September 1987

Kraft pulps, prepared from softwoods, and small chips of birch wood were treated with heme and *tert*-butyl hydroperoxide in aqueous solutions at reflux temperature. Analyses of treated pulps showed decreases in kappa number (a measure of lignin content) from about 36 to less than 2, with concomitant increases in brightness (80% increase in the better samples). Analyses of treated wood chips revealed selective delignification and removal of hemicelluloses. After 48 h of treatment, lignin losses from the wood chips approached 40%, and xylose/mannose (hemicellulose) losses approached 70%, while glucose (cellulose) losses were less than 10%. Examination of delignified chips by transmission electron microscopy showed that the removal of lignin occurred in a manner virtually indistinguishable from that seen after decay by white rot fungi. Various metalloporphyrins, which act as biomimetic catalysts, were compared to horseradish peroxidase and fungal manganese peroxidase in their abilities to oxidize syringaldazine in an organic solvent, dioxane. The metalloporphyrins and peroxidases behaved similarly, and it appeared that the activities of the peroxidases resulted from the extraction of heme into the organic phase, rather than from the activities of the enzymes themselves. We concluded that heme–*tert*-butyl hydroperoxide systems in the absence of a protein carrier mimic the decay of lignified tissues by white rot fungi.

The wood-decomposing basidiomycetes known as white rot fungi are characterized by their ability to mineralize essentially all carbonaceous components of wood to CO_2 (33). These fungi are excellent lignin degraders, often removing lignin from wood preferentially to polysaccharides (3, 4, 32; R. A. Blanchette, L. Otjen, and M. C. Carlson, Phytopathology, in press). The biochemical mechanisms of lignin degradation have been examined in only a few of the hundreds of known white rot fungi, but extensive studies of one fungus, *Phanerochaete chrysosporium*, indicate that two types of extracellular heme-containing enzymes play important roles in the decay of lignin by white rot fungi. These enzymes are peroxidases and include a group of ligninases (or "lignin peroxidases") (1, 2, 6, 10, 13, 24, 25, 35, 36) and a group of manganese peroxidases (11, 12, 21, 28, 29). Ligninases appear to initiate lignin degradation by extracting one electron at a time from methoxylated aromatic rings, forming cation radical species which undergo further nonenzymatic reactions (e.g., reactions with H_2O or O_2 or both) that lead to lignin decomposition (29, 36). Manganese peroxidases oxide Mn(II) to Mn(III), which may diffuse into wood cells and initiate additional oxidative reactions (11, 29).

Several investigators have used what they term a biomimetic approach to the study of ligninase mechanisms. Shimada et al. (34) demonstrated that a synthetic tetraphenylporphyrin iron(III) chloride in combination with iodosylbenzene or *tert*-butyl hydroperoxide (TBH) in an organic solvent would cleave the alpha-beta C-C bonds in the side chains of diarylpropane lignin model compounds in the same manner as ligninase does. Later, Habe et al. (14) found that hemin in the presence of hydrogen peroxide or TBH mimicked ligninase by catalyzing the same C-α–C-β bond cleavage of the lignin model compound 1,2-bis-(4-methoxyphenyl)propane-1,3-diol, giving a large yield of *p*-anisaldehyde (24.4%). These reactions, performed in dimethyl sulfoxide or 80% dimethyl sulfoxide in water, showed pH optima similar to that of ligninase (pH 3.0). The same investigators (15) reported that *p*-methoxyphenylethane-1,2-diol was also a product of the heme-catalyzed cleavage of 1,2-bis-(4-methoxyphenyl)propane-1,3-diol and that 83% of the incorporated oxygen came from atmospheric oxygen. A similar result was seen with ligninase from *P. chrysosporium* (19). Huynh (20) found that even nonheme single-electron oxidants (e.g., copper peroxydisulfate) mimicked ligninase in their oxidations of lignin model compounds such as veratrylglycerol-β-guaiacyl ether and veratryl alcohol.

We have taken the biomimetic approach to the next logical step. We treated wood chips and wood pulps with a variety of natural hemes and synthetic porphyrins in the presence of TBH in aqueous solution. We found that the treated wood chips were delignified extensively and were significantly depleted of their hemicelluloses. Examination of treated chips by transmission electron microscopy revealed morphological alterations of wood cells reminiscent of decay by white rot fungi. Wood pulps were bleached effectively by the same biomimetic system. After treatment, pulps were essentially lignin-free but had lost very little cellulose.

MATERIALS AND METHODS

Chemicals, enzymes, and lignocelluloses. The following chemicals were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis.: syringaldazine; TBH (70% in H_2O); hemin; hematin; copper phthalocyanine; copper phthalocyanine-3,4′,4″,4‴-tetrasulfonic acid (tetrasodium salt); 5,10,15,20-tetrakis(pentafluorophenyl)-21H,23H-porphine iron (III) chloride; 5,10,15,20-tetraphenyl-21H,23H-porphine iron (III) chloride; 5,10,15,20-tetraphenyl-21H,23H-porphine cobalt

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† Publication no. 8754 of the Idaho Agricultural Experiment Station, Moscow, and paper no. 15,461, Scientific Journal Series, Minnesota Agricultural Experiment Station, St. Paul.

TABLE 1. Bleaching of a softwood pulp with natural and synthetic porphyrins[a]

Treatment	Kappa number at pH:		
	5.5	7.0	9.5
None	35.4	35.4	35.4
TBH	12.5	11.3	10.5
TBH plus hemin	1.9	1.8	1.6
TBH plus 5,10,15,20-tetrakis-(pentafluorophenyl)-21H,23H-porphine iron (III) chloride	1.8	1.6	1.5

[a] Reaction mixtures were refluxed for 24 h and contained 250 ml of deionized H_2O, 250 mg of pulp, 2 ml of 70% TBH, and 0.4 ml of 2 mM iron porphyrin.

(II); 5,10,15,20-tetrakis(4-methoxyphenyl)-21H,23H-porphine cobalt(II); and 5,10,15,20-tetraphenyl-21H,23H-porphine zinc. Horseradish peroxidase and palmitoyl chloride were purchased from Sigma Chemical Co., St. Louis, Mo., and hemoglobin was obtained from Fluka Chemical Co., Ronkonkoma, N.Y. Manganese peroxidase was purified from culture filtrates of *P. chrysosporium* (29). Wood chips were prepared from the heartwood of birch (*Betula papyrifera*). Unbleached mixed softwood kraft pulp was obtained from a pulp mill in the northwestern United States.

Treatment of wood chips and pulps. About 0.5 g of chips (1.5 by 7 by 15 mm) was suspended in 250 ml of H_2O containing either 0.5% H_2O_2 or TBH–4 μM hemin (maintained as a 2 mM stock solution in 100% dioxane and sealed to exclude air). The suspension was boiled under reflux for a prescribed period and then was cooled. The chips were collected by filtration, washed with distilled water, and dried at 70°C to a constant weight. Pulp fibers were treated similarly, except that about 250 mg of dry pulp was added per 250 ml of reaction mixture, and the treated fibers were air dried. Various controls were run as appropriate, including treatments in the absence of hemin, TBH, or both.

Syringaldazine assays. The catalysts used to oxidize syringaldazine included natural hemes, synthetic porphyrins, and the enzymes hemoglobin, horseradish peroxidase, and manganese peroxidase. The enzymes had been derivatized before use with palmitoyl chloride as described by Dordick et al. (7) to increase their solubility in dioxane. Assay solutions consisted of the following components in 95% dioxane: 10 mM sodium acetate (pH 5.0), 0.1 mM syringaldazine, 0.5 to 10 μM catalyst, and 2 mM TBH. The increase in A_{525} was measured during the first 3 min of the reaction with a diode array spectrophotometer (HP-8451A; Hewlett-Packard Co., Palo Alto, Calif.). An extinction coefficient of 6.5×10^4 cm^{-1} M^{-1} for the syringaldazine oxidation product was used to calculate oxidation rates (16). Activities were expressed as micromoles of syringaldazine oxidized per minute per milligram of catalyst.

Microscopy. Small samples (approximately 1.5 by 7 by 15 mm) were cut from wood chips of *B. papyrifera* that had been treated as described above for 0, 12, 24, 32, or 48 h. Wood samples were then fixed in 2.0% $KMnO_4$. Unreacted fixative was removed with three changes of distilled water over a period of 3 h. Fixed wood was dehydrated through a graded Quetol 651 resin series (50, 75, and 100% Quetol 651 in distilled water) for 24 h and infiltrated for 3 days with a hard-consistency Quetol 651 medium containing 15 g of Quetol 651, 0.6 g of 2,4,6-tri(methylaminomethyl)phenol, 10 g of nadic methyl anhydride, and 20 g of nonenylsuccinic anhydride. Infiltrated samples were placed in flat molds and polymerized for 15 h at 76°C. Sections were cut with a microtome and mounted on 200-mesh copper grids for observation and photography with a transmission electron microscope (Hitachi 600; Nakaworks, Japan).

Lignin, sugar, and brightness analyses. Quantitative analyses of lignin and sugars in wood were performed as described by Effland (8) and Pettersen et al. (30). Kappa numbers (a measure of lignin content) of pulps were determined according to Technical Association of the Pulp and Paper Industry (New York, N.Y.) bulletin T-236 m-60, tentative standard 1960, and Berezins (2). Pulp brightness was determined as described by Hartler et al. (17).

RESULTS

Pulp bleaching. Both hemin and a synthetic iron porphyrin, when added with TBH, were effective pulp-bleaching agents in aqueous solution (Table 1). Our unbleached kraft pulps had initial kappa numbers of about 35, which were reduced to about 2 during 24 h of treatment with the bleaching reagent. The better bleached-pulp samples (data not shown) were up to 80% brighter than control pulps (water treatment only) and were comparable in brightness to pulps obtained after alkaline hydrogen peroxide bleaching (14). Some bleaching was observed in control reactions containing TBH without porphyrin, but the catalyst was required for the most effective decolorization of the pulp.

Activity-free heme versus protein-bound heme. Different metalloporphyrins catalyzed the oxidation of syringaldazine in dioxane-water solution when TBH was provided as an electron acceptor (Table 2). The highest oxidation rates were observed when the metalloporphyrin contained iron. Synthetic porphyrins containing metals other than iron showed less peroxidatic activity and very little pulp-bleaching capability. Only those systems that showed peroxidatic activity against syringaldazine also showed pulp-bleaching activity (Table 2).

TABLE 2. Peroxidatic activities of metalloporphyrins and related bleaching activities

Treatment	Peroxidatic activity[a]	Bleaching activity[b]
5,10,15,20-Tetrakis(pentafluorophenyl)-21H,23H-porphine iron(III) chloride	3.06	1.7
5,10,15,20-Tetraphenyl-21H-23H-porphine iron(III) chloride	0.21	8.2
Hemin [chloroprotoporphyrin IX iron (III)]	2.15	1.6
Hematin [hydroxyprotoporphyrin IX iron (III)]	1.69	5.0
Copper phthalocyanine	0.00	—
Copper phthalocyanine-3,4′,4″,4‴-tetrasulfonic acid (tetrasodium salt)	0.01	9.4
Nickel phthalocyaninetetrasulfonic acid (tetrasodium salt)	0.01	—
5,10,15,20-Tetraphenyl-21H,23H-porphine cobalt(II)	0.61	—
5,10,15,20-Tetrakis(4-methoxyphenyl)-21H,23H-porphine cobalt(II)	0.75	7.3
5,10,15,20-Tetraphenyl-21H,23H-porphine zinc	0.04	—
Manganese peroxidase (palmitoyl derivative)	0.08	
Hemoglobin (palmitoyl derivative)	0.11	—
Horseradish peroxidase (palmitoyl derivative)	0.091	—

[a] Determined by oxidation of syringaldazine in dioxane. Expressed in micromoles per milligram per minute.

[b] Values are kappa numbers after bleaching, determined in aqueous solution. Initial kappa number, 36. —, No change in kappa number relative to controls.

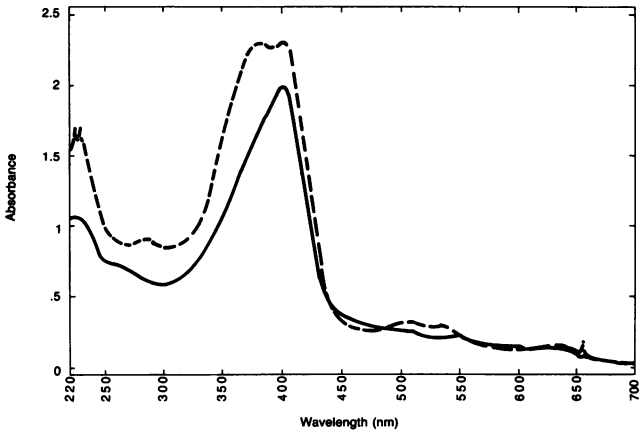

FIG. 1. Spectra of palmitoyl chloride-derivatized hemoglobin and palmitoyl chloride-derivatized horseradish peroxidase in dioxane–10 mM sodium acetate (95:5, vol/vol) (pH 5). Enzymes were derivatized in dioxane–10 mM sodium acetate (95:5, vol/vol). Proteins were added to a concentration of 0.1 mM, and palmitoyl chloride was added to a concentration of 3 mM. The mixture was stirred under anaerobic conditions at 5°C for 24 h. A precipitate was removed by centrifugation before spectra were obtained. ——, 0.1 mM horseradish peroxidase; -----, hemoglobin diluted to 0.01 mM.

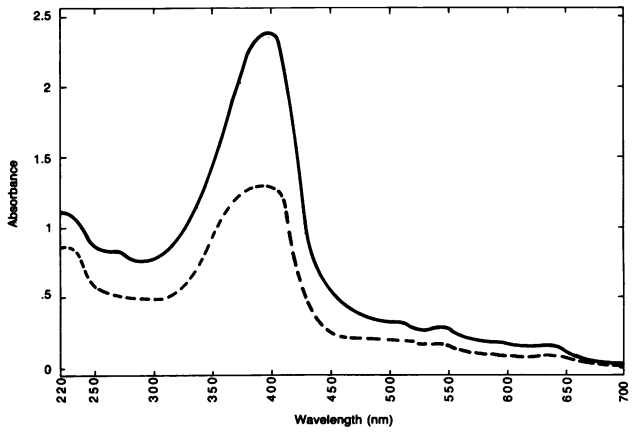

FIG. 2. Spectra of hemin and hematin in dioxane–10 mM sodium acetate (95:5, vol/vol) (pH 5). The solutions were first prepared as 2 mM hemin or hematin in pure dioxane with stirring for 24 h under anaerobic conditions. Spectra were taken after 20-fold dilutions into buffered dioxane. ——, Hematin; -----, hemin.

The enzymes horseradish peroxidase and hemoglobin had some peroxidatic activity in dioxane solution, but it appears that the activity observed was due to heme that had been removed by the solvent from the denatured enzymes and not to actual enzyme activity, as reported by Dordick et al. (7) and Kazandjian and Klibanov (22). When the palmitoyl chloride-derivatized proteins were added to buffered dioxane as described by Kazandjian and Klibanov (22), the protein moieties precipitated. However, the absorption spectra of the solutions were almost identical to those of similar solutions containing only heme (Fig. 1 and 2), with maxima at about 400 nm. There was little absorption in the 280-nm region, where proteins would be expected to absorb. The ratio A_{401}/A_{280} for the hemoglobin preparation was 2.29; for the horseradish peroxidase preparation it was 2.5. The ratios for pure hemin and pure hematin were 2.79 and 2.34, respectively. We could detect no protein in any solution by standard protein assays, including the Lowry assay and the Coomassie blue assay.

Wood chip treatment. Chips of birch wood were delignified extensively by treatment in aqueous solution with hemin and TBH. The chips were also significantly depleted of their hemicelluloses, as indicated by losses of pentoses. Cellulose losses (depletion of glucose-based polymers) were minimal. These data are summarized in Table 3.

Examination of treated wood chips by transmission electron microscopy showed that the wood cells were morphologically similar to those seen during some forms of decay by white rot fungi. Control chips (heated but with no hemin added to the treatment solution) showed normal cell walls with distinct wall layers (Fig. 3A and B). $KMnO_4$ fixation resulted in good cell wall definition and electron density. Electron-dense areas corresponded to areas rich in lignin and hemicelluloses (5, 27; Blanchette et al., in press). As expected, the greatest electron densities were in the compound middle-lamellar region (Fig. 3A and B). The secondary walls of fibers consisted of distinct S_1 and S_2 layers and a thin S_3 layer. In some fibers, the S_3 layer was not easily differentiated from the S_2 layer. Vessel walls had thicker S_1 and S_3 layers and thinner S_2 layers than did fiber cells (Fig. 3A).

After 12 h of treatment with hemin and TBH, the secondary walls of fiber cells and vessel elements were less electron dense than were similar cells from control chips (Fig. 3C and D). The S_2 layer appeared slightly swollen and larger than that in sound cell walls. The demarcation between S_1 and S_2

TABLE 3. Changes in *B. papyrifera* chips and in a softwood kraft pulp after treatment in aqueous solution

Treatment (duration [h])	% Loss in:				
	Weight	Lignin	Glucose	Xylose	Mannose
Hemin only (24)	4.0	7.5	6.9	2.2	12.7
TBH only (24)	10.1	15.8	6.9	10.8	40.1
H_2O_2 only (24)	45.7	40.6	35.9	55.9	77.9
Hemin and TBH (24)	23.4	25.6	9.8	21.6	44.6
Hemin and H_2O_2 (24)	48.7	42.5	37.5	63.0	77.9
5,10,15,20-Tetrakis(pentafluorophenyl)-21H,23H-porphine iron (III) chloride and TBH (24)	24.3	25.7	9.8	31.8	48.2
Hemin and TBH (48)	39.1	38.0	9.5	64.4	68.8
Kraft pulp after hemin and TBH[a]	19	100.0[b]	9.8	36.2, 60.2[c]	41.8, 41.6[d]

[a] Determined for purposes of comparison.
[b] Kappa number reduced from 36 to 2.
[c] Galactose.
[d] Arabinose.

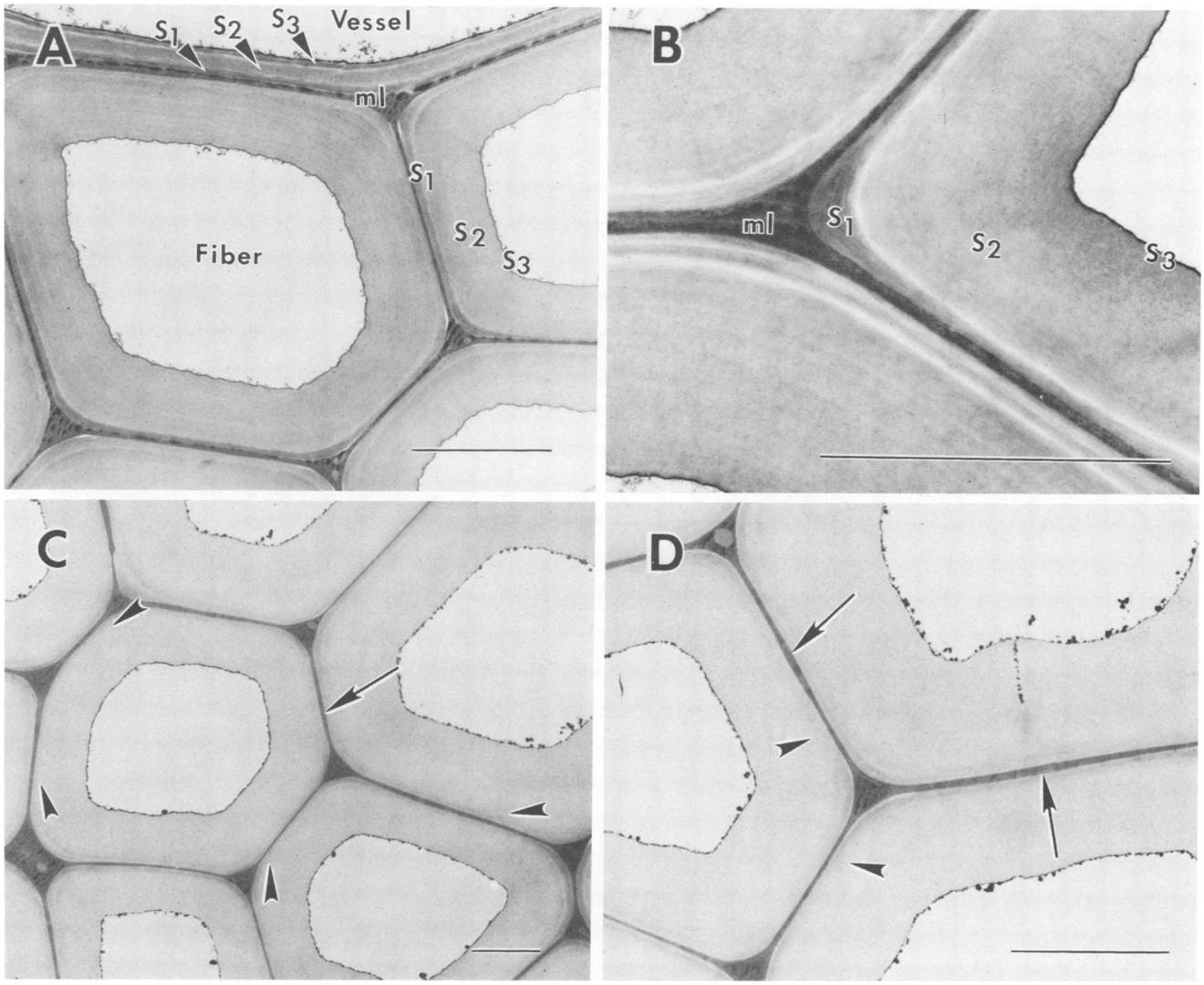

FIG. 3. Transmission electron micrographs from transverse sections of *B. papyrifera* wood fixed with $KMnO_4$. (A) Fiber and vessel cell walls of control wood chip cells are electron dense, and the compound middle lamella and secondary wall with S_1, S_2, and S_3 layers are visible. (B) Cell wall layers of three fiber cells from control wood chips. (C and D) Cells of wood chips treated for 12 h with hemin and TBH. Less electron density is apparent in the secondary wall, and cell walls have a slightly swollen appearance. The S_1 and S_2 layers do not appear distinguishable at sites between adjacent cells (arrowheads). The S_1 region near cell corners is still visible. The middle lamella between fibers is expanded in some areas (large arrows). Bar, 5 μm.

layers was no longer distinct except near the cell corners. The middle lamella was not altered in most cells, but in some areas it appeared slightly expanded (large arrows in Fig. 3C and D). This expansion also was seen primarily in the middle-lamellar region between cells but not in the cell corners.

After 24 h of treatment, the secondary wall layers of fibers and vessels were no longer easily distinguishable from one another (Fig. 4A to C). The secondary walls of vessels, fibers, and parenchyma cells had very little electron density, reflecting losses of lignin from the S_1, S_2, and S_3 regions. The extent of delignification and electron density varied depending on the area of the wood chip from which the sample was taken. Cells from the chip surface were more severely affected than were cells from areas within the chip. Cells with the least electron density were found at the surface, while cells from within the chip, after 24 h, resembled surface cells that had had 12 h of treatment. The middle lamella was in various stages of degradation. In some cells, the middle lamella remained electron dense and intact, but it did not maintain a rigid structure as it did in sound cells. In other cells, the middle lamella was less electron dense and appeared expanded (Fig. 4A). The middle lamella in these areas no longer consisted of a continuous electron-dense zone. Instead, it had a granular appearance (Fig. 4C) and was disrupted. Cells had swollen secondary walls with little electron density (Fig. 4C). The secondary walls of some cells had separations within the S_2 layer, and the cells did not maintain a normal shape.

After 32 or 48 h of treatment, secondary walls of fibers and vessels were relatively free of electron density in the S_1 and S_2 layers (Fig. 4D to F). The secondary wall was swollen, and separations were evident. Most of the cells had middle lamellae that were degraded but still maintained some electron density. The overall integrity of cells had been clearly disrupted. In cells from the surfaces of chips, middle lamellae were degraded and no longer electron dense (Fig. 4F).

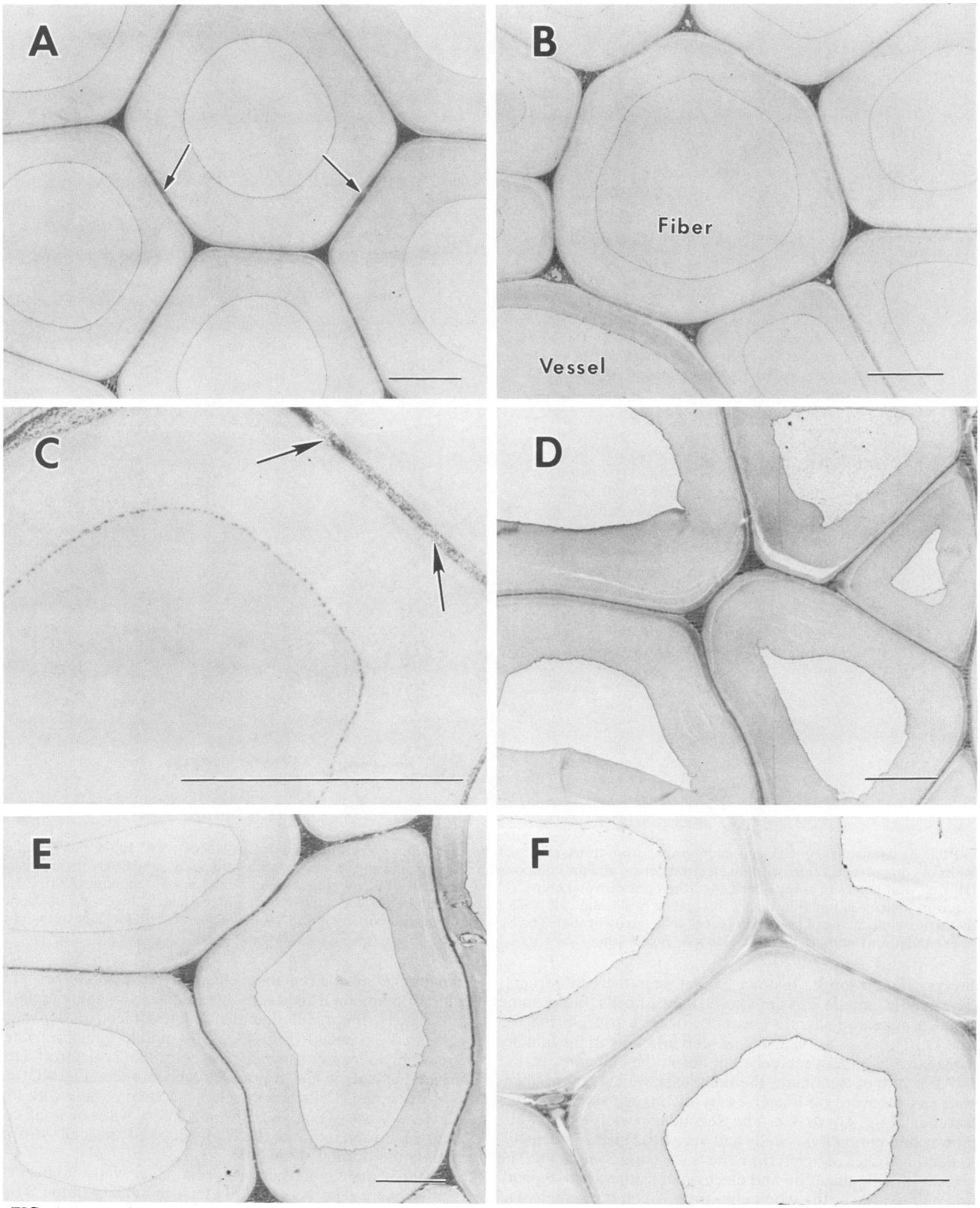

FIG. 4. Transmission electron micrographs from transverse sections of *B. papyrifera* wood fixed with $KMnO_4$ after 23 (A to C), 32 (D), and 48 (E and F) h of treatment. (A and B) Secondary walls of fiber and vessel cells show little contrast and electron density. The secondary wall layers of all cells are no longer distinct from one another, and the middle lamella is expanded in size (arrows). (C) The middle lamella in some cells is severely affected and does not have a continuous zone of electron density (arrows). A granular appearance of the degraded middle lamella is evident. (D) Swollen fiber cells are present, with separations within the secondary wall. The cells do not have the same integrity as do cells from control samples of wood. The lack of cell wall rigidity is evident in the flexible nature of the middle lamella. Bends within the middle lamella and cells with altered shapes are visible. (E) Cell walls appear weak with swollen secondary walls and expanded areas of the middle lamella. The shapes of fiber cells and vessels are distorted. (F) In cells near the surface of the wood chip, extensive loss of the middle lamella is apparent, and secondary walls are transparent. Bar, 5 μm.

DISCUSSION

Free porphyrin molecules, when dissolved in organic solvents and in the presence of peroxides, mimic the activities of heme-containing peroxidases, including ligninases (14, 34). We have confirmed this for a variety of porphyrins, both natural and synthetic, by using syringaldazine as an assay substrate for peroxidatic activity. It has been reported that peroxidases such as horseradish peroxidase actually function as holoenzymes in organic solvents when provided with peroxide (7, 22). However, our results suggest that these peroxidatic activities result from activities of heme moieties of prosthetic groups removed from the enzymes rather than from the activities of the enzymes themselves.

We have determined that free porphyrin molecules also mimic microbial activities, as is evident at the organismic level. In particular, we have shown that treatment of lignocelluloses, including whole wood, with porphyrins such as hemin in the presence of an organic peroxide results in lignocellulose degradation reminiscent of wood decay by white rot fungi. Some white rot fungi decay wood by mediating a progressive thinning of the cell walls from the S_3 layer inward toward the middle lamella, whereas others remove lignin selectively from the secondary wall and middle lamella (3, 18, 26, 31, 32). The degradative patterns observed from our treatments of wood chips with hemin and TBH in aqueous solution resemble advanced stages of decay mediated by fungi such as *Merulius tremellosus* (syn. *Phlebia tremellosus*), *Phellinus pini*, and other basidiomycetes that preferentially degrade lignin (4; Blanchette et al., in press). The progressive stages of lignin loss from the samples followed the same pattern seen with fungi. Lignin was removed throughout the secondary wall from the lumen toward the middle lamella. The middle lamella was degraded only after loss of lignin from the secondary wall. The middle-lamellar regions degraded first were those between cells. Cell corners were delignified last. Although many white rot fungi do not degrade vessel walls as readily as they do fibers (unpublished data), no differences in degradation of various cell types were observed in wood treated with hemin and TBH. The striking parallel losses of lignin and hemicelluloses that resulted from treatment of wood with hemin and TBH also resemble decay by white rot fungi (Blanchette et al., in press). Since the model system we used lacks the hemicellulases normally produced by wood-rotting fungi, it appears that, as lignin was oxidized, associated hemicelluloses were released indirectly along with lignin oxidation products. Also, the biomimetic system required only 1 to 2 days to reach the same level of wood cell delignification that usually requires 2 to 3 months of laboratory degradation by white rot fungi. This was related to the higher temperature of the biomimetic process, the ready diffusibility of the low-molecular-weight catalysts into the wood cell structure compared with fungal enzymes, and the slow growth of fungi on wood. These observations are important in comparing commercial feasibilities of fungal versus biomimetic processes for delignification of lignocelluloses.

Hydrogen peroxide, alone or in the presence of hemin, catalyzed large losses of lignin and cellulose from birch chips (Table 3). TBH, however, was less effective in delignifying chips in the absence of hemin than it was in the presence of hemin, and the TBH-based systems were less damaging to the wood cellulose than the H_2O_2-based systems were. It is clear that the choice of peroxide type is important in biomimetic systems if specific delignification is the goal.

It was somewhat surprising that the hemin-hydroperoxide process functioned so well in aqueous solution. However, close observation of the process revealed that when hemin was added first, it adsorbed tenaciously to the lignocellulosic substrate. When the peroxide was added, delignification commenced. Thus, it appears that the heme moieties were protected from auto-oxidation by adsorption to the substrate.

Our work confirms that nonenzymic heme systems function in aqueous solution as delignification and bleaching agents. We were able to bleach kraft pulps under simple conditions to yield bright pulps of potential commercial quality (Table 3). Wright and Fullerton (37) described an alkaline pulping process with synthetic hemes as catalysts but under conditions very different from our acidic process. These investigators did not compare their results to the fungal system, as we did in our work. Kirk and Yang (23) showed that cultures of wood-rotting fungi bleached kraft pulp significantly in about 8 days. Unfortunately, the pulp fiber was partly depolymerized, and significant losses of cellulose were observed. Further work is required to test bleached-pulp characteristics, such as fiber strength, but we have no reason to expect low pulp quality because in our study, the fibers appeared undamaged. The present system is too expensive for commercial use; however, the need for nonpolluting alternative methods to the use of chlorine in pulp bleaching is great (9), and further refinements of this biomimetic process may at some point yield a commercially feasible technology.

ACKNOWLEDGMENTS

This work was supported in part by ChemGen Corporation, Gaithersburg, Md., and the Idaho Agricultural Experiment Station, University of Idaho, Moscow.

We thank John Sutherland for his help in editing early drafts of this manuscript, Andre Abad for his assistance with electron microscopy, and Marilyn J. Effland (Forest Products Laboratory, Madison, Wis.) for lignin and wood sugar analyses.

LITERATURE CITED

1. **Anderson, L. A., V. Renganathan, A. A. Chiu, T. M. Loehr, and M. H. Gold.** 1985. Spectral characterization of diarylpropane oxygenase, a novel peroxide dependent, lignin-degrading heme enzyme. J. Biol. Chem. **260**:6080–6087.
2. **Berezins, V.** 1966. Micro kappa numbers. Pulp Pap. Can. **67**: 206–208.
3. **Blanchette, R. A.** 1984. Screening wood decayed by white rot fungi for preferential lignin degradation. Appl. Environ. Microbiol. **48**:647–653.
4. **Blanchette, R. A., and I. D. Reid.** 1986. Ultrastructural aspects of wood delignification by *Phlebia* (*Merulius*) *tremellosus*. Appl. Environ. Microbiol. **52**:239–245.
5. **Bland, D. E., R. C. Foster, and A. F. Logan.** 1971. The mechanism of permanganate and osmium tetraoxide fixation and the distribution of lignin in the cell wall of *Pinus radiata*. Holzforschung **25**:137–143.
6. **Buswell, J. A., B. Mollet, and E. Odier.** 1984. Lignolytic enzyme production by *Phanerochaete chrysosporium* under conditions of nitrogen sufficiency. FEMS Microbiol. Lett. **25**:295–299.
7. **Dordick, J. S., M. A. Marletta, and A. M. Klibanov.** 1986. Peroxidases depolymerize lignin in organic media but not in water. Proc. Natl. Acad. Sci. USA **83**:6255–6257.
8. **Effland, M. J.** 1977. Modified procedure to determine acid insoluble lignin in wood and pulp. TAPPI **60**(10):143–144.
9. **Eriksson, K.-E., and T. K. Kirk.** 1985. Biopulping, biobleaching and treatment of kraft effluents with white-rot fungi, p. 271–279. *In* M. Moo-Young (ed.), Comprehensive biotechnology: the principles, applications and regulations of biotechnology in industry, agriculture and medicine. The principles of biotech-

nology: engineering considerations. Pergamon Press, Inc., Elmsford, N.Y.
10. **Faison, B. D., and T. K. Kirk.** 1983. Relationship between lignin degradation and production of reduced oxygen species by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **46:** 1140–1145.
11. **Glenn, J. K., L. Akileswaran, and M. H. Gold.** 1986. Mn(II) oxidation is the principal function of the extracellular Mn-peroxidase from *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **251:**688–696.
12. **Glenn, J. K., and M. H. Gold.** 1985. Purification and characterization of an extracellular Mn(II)-dependent peroxidase from the lignin degrading basidiomycete *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **242:**329–341.
13. **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.
14. **Habe, T., M. Shimada, and T. Higuchi.** 1985. Biomimetic approach to lignin degradation. I. H_2O_2-dependent C-C bond cleavage of the lignin model compounds with a natural iron porphyrin and imidazole complex. Mokuzai Gakkaishi **31:**54–55.
15. **Habe, T., M. Shimada, T. Okamoto, B. Panijpan, and T. Higuchi.** 1985. Incorporation of dioxygen into the dehydroxylated product during the C-C single bond cleavage of 1,2-bis(*p*-methoxyphenyl) propane-1,3-diol catalysed by hemin. A novel model system for the hemoprotein ligninase. J. Chem. Soc. Chem. Commun. **1985:**1323–1324.
16. **Harkin, J. M., and J. R. Obst.** 1973. Syringaldazine, an effective reagent for detection of laccase and peroxidase in fungi. Experientia **29:**381–387.
17. **Hartler, N., E. Lindahl, C.-G. Moberg, and L. Stockman.** 1960. Peroxide bleaching of kraft pulps. TAPPI **43:**806–813.
18. **Highley, T. L., and L. L. Murmanis.** 1984. Ultrastructural aspects of cellulose decomposition by white rot fungi. Holzforschung **38:**73–78.
19. **Higuchi, T.** 1986. Catabolic pathways and role of ligninases for the degradation of lignin substrate models by white-rot fungi. Wood Res. **73:**58–81.
20. **Huynh, V.-B.** 1986. Biomimetic oxidation of lignin model compounds by simple inorganic complexes. Biochem. Biophys. Res. Commun. **139:**1104–1110.
21. **Huynh, V.-B., A. Paszczyński, P. Olson, and R. Crawford.** 1986. Oxidation of lignin model compounds by a manganese dependent enzyme from *Phanerochaete chrysosporium* as compared to chemically generated Mn(III), p. 42–45. Biotechnology in pulp and paper industry. Proceedings of the Third International Conference, Stockholm, June 16–19. Swedish Forest Products Research Laboratory, Stockholm.
22. **Kazandjian, R. Z., and A. M. Klibanov.** 1985. Regioselective oxidation of phenols catalyzed by polyphenol oxidase in chloroform. J. Am. Chem. Soc. **107:**5448–5450.
23. **Kirk, T. K., and H. H. Yang.** 1979. Partial delignification of unbleached kraft pulp with lignolytic fungi. Biotechnol. Lett. **1:**347–351.
24. **Kuila, D., M. Tien, J. A. Fee, and M. R. Ondrias.** 1985. Resonance Raman spectra of extracellular ligninase: evidence for a heme active site similar to those of peroxidases. Biochemistry **24:**3394–3397.
25. **Lobarzewski, J., and A. Paszczyński.** 1985. Lignocellulose biotransformation with immobilized cellulase, D-glucose oxidase and fungal peroxidases. Enzyme Microb. Technol. **7:**564–566.
26. **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.
27. **Parameswaran, N., and W. Liese.** 1982. Ultrastructural localization of wall components in wood cells. Holz Roh. Werkst. **40:**145–155.
28. **Paszczyński, A., V.-B. Huynh, and R. Crawford.** 1985. Enzymatic activities of an extracellular, manganese-dependent peroxidase from *Phanerochaete chrysosporium*. FEMS Microbiol. Lett. **29:**37–41.
29. **Paszczyński, A., V.-B. Huynh, and R. Crawford.** 1986. Comparison of ligninase-1 and peroxidase-M2 from the white-rot fungus *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **244:** 750–765.
30. **Pettersen, R. C., V. H. Schwandt, and M. J. Effland.** 1985. An analysis of wood sugar assay using HPLC: a comparison with paper chromatography. J. Chromatogr. Sci. **22:**478–484.
31. **Ruel, K., and F. Barnoud.** 1984. Ultrastructural aspect of wood degradation by *Sporotrichum pulverulentum*. Holzforschung **38:**61–68.
32. **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.
33. **Sarkanen, K. V., and C. H. Ludwig.** 1971. Lignins: occurrence, formation, structure, and reactions. John Wiley & Sons, Inc., New York.
34. **Shimada, M., T. Habe, T. Umezawa, T. Higuchi, and T. Okamoto.** 1984. The C-C bond cleavage of a lignin model compound, 1,2-diarylpropane-1,3-diol, with a heme-enzyme model catalyst tetraphenylporphyrinato iron(III) chloride in the presence of *tert*-butyl hydroperoxide. Biochem. Biophys. Res. Commun. **122:**1247–1252.
35. **Tien, M., and T. K. Kirk.** 1984. Lignin-degrading enzyme from *Phanerochaete chrysosporium*: purification, characterization and catalytic properties of unique H_2O_2-requiring oxygenase. Proc. Natl. Acad. Sci. USA **81:**2280–2284.
36. **Tien, M., T. K. Kirk, C. Bull, and J. A. Fee.** 1986. Steady-state and transient-state kinetic studies on the oxidation of 3,4-dimethoxybenzyl alcohol catalyzed by the ligninanse of *Phanerochaete chrysosporium* Burds. J. Biol. Chem. **261:** 1687–1693.
37. **Wright, Y., and J. Fullerton.** 1984. Redox catalysis of alkaline pulping by organometallic complexes. J. Wood Chem. Technol. **4:**61–74.