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Cell wall alterations in loblolly pine wood decayed by the white-rot fungus, *Ceriporiopsis subvermispora*

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Abstract

Ultrastructural, immunocytochemical and UV absorption spectroscopy techniques were used to elucidate the progressive changes that occur within woody cell walls during decay by *Ceriporiopsis subvermispora.* After only 2 weeks of incubation, uranyl acetate staining revealed a diffuse electron dense zone in the secondary wall near hyphae and around the outer circumference of the wall. The extent of cell wall staining increased with longer fungal incubation. No staining occurred in sound unaltered cell walls. Proteins of varying molecular weights (insulin, 5730 Da; myoglobin, 17600 Da; ovalbumin, 44287 Da) were infiltrated into sound and decayed wood followed by immunogold labelling and transmission electron microscopy. Insulin readily penetrated into the outer most regions of secondary walls of wood cells after 2 weeks of decay. Myoglobin was first observed to penetrate cell walls at 4 weeks of degradation and ovalbumin was found after 8 weeks in wood with advanced stages of decay where extensive cell wall disruption was evident. None of the proteins used were localized within cell walls of untreated, control wood samples. UV microspectrophotometry demonstrated a progressive loss of absorbance at 240 and 280 nm within the secondary walls and middle lamellae at various sampling times throughout the duration of the decay study.© 1997 Elsevier Science B.V.

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1. Introduction

White-rot is a type of wood decay caused by basidiomycetes that can degrade substantial

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amounts of lignin. A great deal of diversity exists among the many thousands of fungal species that cause white-rots, and many different forms of lignin degradation occur (Blanchette, 1991, 1995). Some species have the capacity to preferentially remove lignin whereas others remove lignin along with varying amounts of cellulose and hemicellulose (Eriksson et al., 1990). Fungi that can selectively delignify wood have been of interest to researchers because of their potential for removing lignin from lignocellulosic materials in general and for use in biological pulping processes (Akhtar et al., 1997; Blanchette, 1995; Blanchette et al., 1988; Kirk et al., 1993; Zadrazil, 1985). Unfortunately, the exceedingly long incubation times needed to achieve extensive lignin removal from wood does not support their immediate use for industrial delignification processes where large volumes of wood must be treated on a daily basis. However, recent investigations have shown that significant benefits can be realized when these fungi are used to inoculate wood chips for relatively short incubation times (Akhtar, 1994; Akhtar et al., 1992, 1993; Kirk et al., 1993; Messner and Srebotnik, 1994). When *Phanerochaete chrysosporium* or *Ceriporiopsis subvermispora* was used to pretreat wood chips for 2 weeks, energy consumption was reduced and paper quality improved significantly during refiner mechanical pulping (Akhtar et al., 1992, 1993, 1995). Pretreatment of wood with white-rot fungi before kraft and sulfite pulping processes has also shown benefits by reducing the chemical cooking time and increasing the amount of pulp produced (Messner and Srebotnik, 1994). Fungi identified as selective lignin degraders have been shown to be the most successful organisms to use for these processes even though little to no lignin was removed from the wood during the relatively short treatments. The mechanisms responsible for changes in woody ceil walls observed during incipient stages of white rot degradation are not well understood,

Evidence that substantial alterations have occurred within the cell walls of wood used in biopulping experiments was first recognized when refined fibers were evaluated after Simons' staining (Blanchette et al., 1992a). This differential stain caused the cut ends of fibers previously treated with white-rot fungi to stain yellow-orange in comparison to fibers from untreated wood that stained blue. The difference in staining patterns appear due to an increased porosity of the cell wall (Yu et al., 1995). Recent investigation has also shown that the amount of energy saved during mechanical pulping is correlated significantly with the amount of yellow-orange staining observed after treatment with Simons' stain (Akhtar et al., 1995).

The role of degradative enzymes during the early stages of decay by white-rot fungi is uncertain. Studies have clearly shown the localization of laccase, lignin peroxidase and manganese peroxidase using antisera and immunogold labelling techniques within degraded cell walls during advanced stages of degradation (Blanchette et al., 1989; Daniel et al., 1990, 1991; Nicole et al., 1992; Srebotnik et al., 1988b). These enzymes, however, do not diffuse readily into sound, unaltered woody cell walls and also have not been found within cell walls with incipient stages of decay. In a study by Srebotnik et al. (1988b), concentrated culture filtrates from *Phanerochaete chrysosporium* were used to infiltrate sound and decayed wood. Immunogold labelling of lignin peroxidase showed no penetration of the enzyme into sound wood or cell walls exhibiting early to moderate stages of decay. These previous studies have demonstrated that large molecular weight proteins, such as degradative enzymes with molecular weights greater than 40 000 Da, apparently cannot move through the small pore structure found in sound wood. It is only after substantial cell wall disruption has occurred that enzymes may be immunocytologically observed deep within the secondary walls or middle lamellae. Cell wall void volume and size has been studied by Flournoy et al. (1993), and maximum pore diameters of 2 nm (20 Å) have been reported for sound wood. After 40% weight loss by a simultaneous white-rot (i.e. all wall components degraded), pore sizes were only 2–5 nm (20-50 Å). These investigators conclude that the small increase in pore size would only allow ligninolytic enzymes to penetrate a fraction of the new cell wall volume. Similar results have been found for

decay of cell walls by brown-rot fungi (Flournoy et al., 1991).

To obtain a better understanding of the changes occurring in wood after decay by white-rot fungi, ultrastructural studies were completed on wood incubated with *C. subvermispora* for 2 – 8 weeks. In addition to various cell wall staining procedures, immunocytochemical studies were done to localize enzymes of various molecular weights that were infiltrated into the wood. The use of marker proteins to evaluate changes in cell wall porosity of brown-rotted wood and sulphite pulp has been used previously with success (Srebotnik and Messner, 1990, 1991) and were utilized in these studies reported here. The loss of lignin and other aromatic compounds from cell walls was determined using UV absorption microspectrophotometry (Akin et al., 1993).

2. Materials and methods

2.1. *Organism and growth conditions*

Ceriporiopsis subvermispora strain CZ-3 was selected because of its ability to rapidly colonize wood and selectively degrade lignin (Blanchette et al., 1992b), as well as its beneficial effects of saving refiner energy and improving paper quality when used in biopulping experiments (Akhtar, 1994; Akhtar et al., 1993). The isolate was obtained from the Center for Forest Mycology Research at the USDA Forest Products Laboratory, Madison, Wisconsin. Freshly cut loblolly pine *(Pinus taeda)* from the Talladega National Forest, Talladega, Alabama was debarked and chipped into approximately 16-mm size wood chips. Chips were kept frozen until used. Inoculum preparation, chip sterilization and bioreactor inoculation were carried out as previously described (Akhtar et al., 1992). Wood chips were incubated in bioreactors at 27°C and 65% relative humidity. Samples of wood chips were removed for analyses at 2, 4, 6 and 8 week intervals. Noninoculated wood chips similarly incubated in bioreactors were used as controls.

2.2. *Sample preparation*

Small segments of inoculated and noninoculated wood chips were prepared for transmission electron microscopy using two methods: (i) uranyl acetate staining and (ii) infiltration with marker proteins followed by immunogold labeling.

2.3. *Uranyl acetate staining*

Small segments of wood were fixed in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.0) for 25 h, washed in buffer and treated with 1% uranyl acetate (aqueous) for an additional hour. Samples were dehydrated through an ethanol series and embedded in Quetol at 74°C. Sections were cut and post stained in 5% uranyl acetate for 30 min.

2.4. *Immunolabeling of marker proteins*

Protein solutions were prepared by dissolving 10 mg of insulin in 0.5 μl HCI (pH 3.0) and 0.5 μl of 0.2 M NaH_2PO_4 (pH 4.45), 20 mg of myoglobin in 1 μl of 0.1 M NaH_2PO_4 (pH 7.2), or 150 mg ovalbumin in 1 μl of 0.1 M NaH_2PO_4 (pH 7.2). Proteins were obtained from Sigma, St. Louis, MO. Small segments of wood chips, approximately 1 mm^3, were placed into the protein solution at low vacuum for 30 min to insure penetration. Samples in solution were then placed on a rotating wheel for 60 min and stored for 12 h at 4°C. The samples were fixed with 1% glutaraldehyde for 2 h and rinsed thoroughly in distilled water. Wood segments were dehydrated through an ethanol series and embedded in L.R. White resin for 48 h at 45°C (Blanchette and Abad, 1992). Primary antibodies for insulin, myoglobin and ovalbumin as well as gold labelled protein A were obtained from Sigma, St. Louis, MO. Cut sections were incubated in the primary antibody for 2 h, rinsed in buffer and labelled with Protein A as previously described (Blanchette and Abad, 1992). Sections were observed and photographed with an Hitachi H-600 transmission electron microscope.

2.5. UV absorption microspectrophotometry

Segments of wood fixed in glutaraldehyde and embedded in L.R. White resin were sectioned at a thickness of 2 μm. A computer-controlled Zeiss UMSP-80 Microspectrophotometry system was used in the ultraviolet (UV) absorption mode (Akin and Hartley, 1992; Akin and Rigsby, 1992). UV spectra were collected, evaluated, and displayed with Zeiss Lambda Scan software dedicated to the system. Transmitted illumination was provided by a high pressure xenon lamp (XBO 75 W) with a connecting grating monochromator and a bandwidth of 5 nm. A 32 × quartz lens with a final aperture diameter of 1.56 μm, which was delimited within about 1/3 of the area of a field-limiting diaphragm to reduce stray light, was positioned over layers of cell walls. Absorbance of transmitted UV illumination, which was scanned from 230 to 350 nm at 2 nm increments, was measured. The system was standardized off the plastic used for embedding at 350 nm.

Thin sections of loblolly pine wood chips that were degraded by *C. subvermispora* CZ-3 for 2, 4, 6, or 8 weeks, and untreated controls were analyzed by UV absorption microspectrophotometry. The aperture for collecting UV illumination was placed over the secondary layer or over the middle lamella of cell walls. Spectra of different cell walls were taken within a section, and two sections were analyzed giving at least 4 spectra for each position of the cell wall for each treatment. Spectra in Fig. 17 and Fig. 18 are averages for treatments.

3. Results

3.1. Transmission electron microscopy -uranyl acetate staining

Untreated wood chips fixed with glutaraldehyde followed by uranyl acetate staining were sectioned and did not reveal electron dense staining within secondary walls (Fig. 1). The intact lignocellulose matrix of the secondary wall showed no reaction to the uranyl acetate treatment. Sections of wood chips removed after 2 weeks of incubation with *C. subvermispora* had distinct electron dense zones within the outer edges of secondary walls (Fig. 2). These areas were associated with cells where fungal hyphae were present in lumina. Tracheids adjacent to ray parenchyma were well colonized by the fungus and had appreciable amounts of electron density in the secondary wall near the cell lumina. Although only 1 or 2 hyphae were evident within each cell lumen, an electron dense zone extended around the circumference of the secondary wall. Fewer hyphae were found in tracheids at a distance from ray parenchyma cells. Sections examined from wood chips incubated for 4, 6 and 8 weeks showed an increasing zone of electron density within secondary walls (Fig. 3 Fig. 4. Fig. 6). In some sections, the altered zone reacting to uranyl acetate staining was unevenly distributed within the tracheid walls (Fig. 3 and Fig. 5), whereas other cells exhibited a relatively uniform zone of electron density throughout the secondary wall (Fig. 4). In areas where staining occurred deep within the secondary wall, the greatest electron density appeared at the advancing front of the altered zone (Fig. 5 and Fig. 6).

3.2. Marker enzyme infiltration

3.2.1. Insulin

Immunogold labelling of insulin that had been infiltrated into wood samples before preparing for transmission electron microscopy showed no penetration of the protein into cell walls of sound, untreated wood (photos not shown). In tracheids from wood incubated for 2 weeks, gold particles were evident within the secondary wall in a narrow band around the circumference of the cell (Fig. 7). Only slight amounts of nonspecific labelling occurred deep within the secondary walls. The extent of cell wall infiltration by insulin varied among cells colonized by the fungus (Fig. 7 and Fig. 8). The penetration of insulin increased within secondary walls after 4 and 6 weeks of incubation (Fig. 9). Many tracheid walls were extensively labelled with gold particles and some cells exhibited gold labelling throughout the S_2 layer of the secondary wall (Fig. 10).

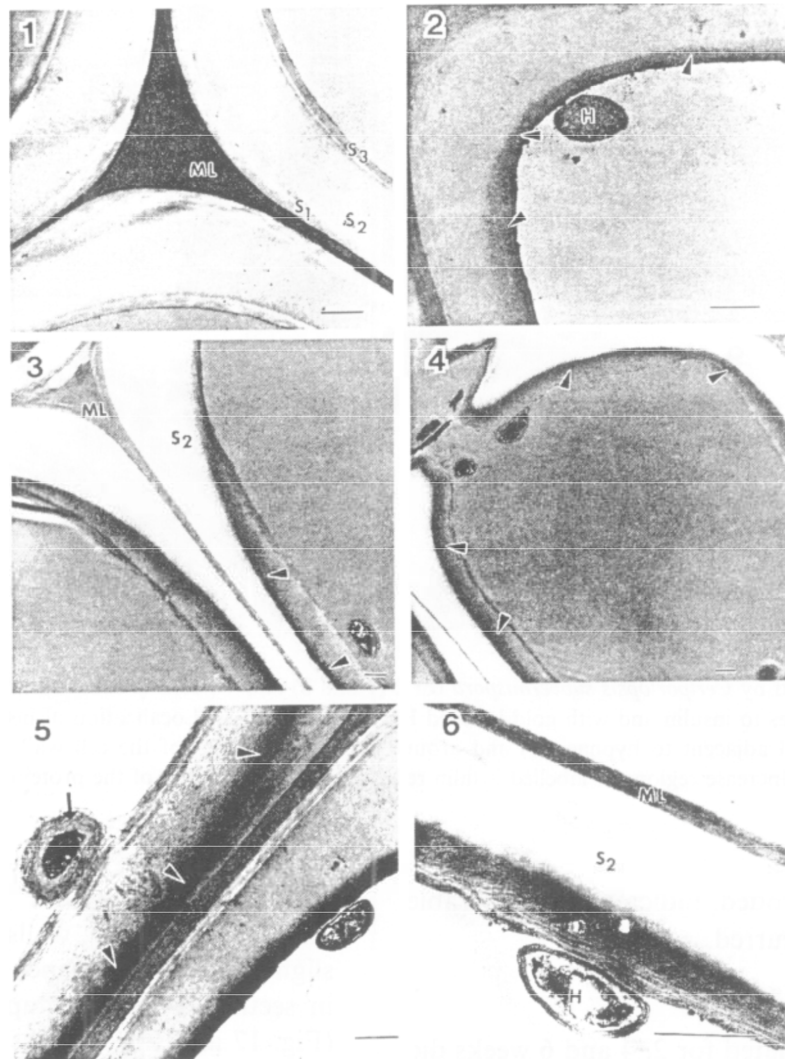
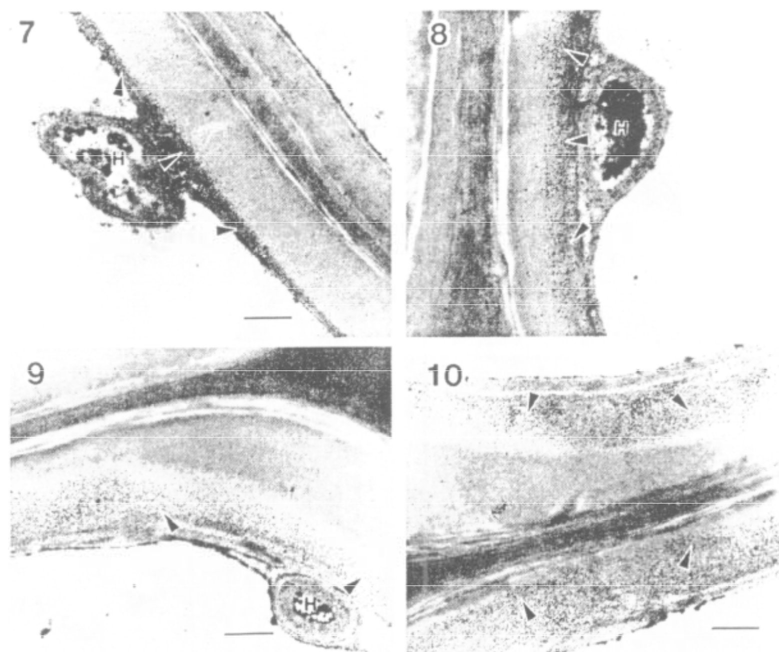


Fig. 1-6. Sections from sound, unaltered wood (1) and wood decayed by *Cerioporiopsis subvermispora* for 2 (2) and 4 weeks (3-6) after fixation and staining with uranyl acetate. 1. Cell walls of sound tracheids showing middle lamella (ml) and secondary wall layers (S_1, S_2, and S_3). No electron dense staining was evident within secondary walls. 2. An electron dense zone (arrowheads) is present within the tracheid wall near hypha (H). 3. Cell walls with dark stain are evident beneath hyphae. 4. A diffuse electron dense zone (arrowheads) is seen affecting the entire circumference of the secondary wall. 5. In some cells, a dark zone of stain is found deep within the secondary wall (arrowheads) and appears to be at the advancing front of cell wall alteration. Some hyphae in these regions contain thick extracellular sheaths (arrow). 6. Enlarged section of tracheid wall showing stained secondary wall adjacent to hypha (H). Bar= 1 μm.

3.2.2. Myoglobin

Untreated wood chips infiltrated with myoglobin and processed for immunogold labelling show a slight residue of protein in cell lumina but no infiltration within the secondary wall (Fig. 11). No labelling was observed within cell walls incubated for 2 weeks with *C. subvermispora* although some cells had a considerable amount of protein in cell lumina adhering to wall surfaces (Fig. 12). After 4 weeks of incubation, penetration of myoglobin was evident within some tracheid walls. Gold labelling in these cells occurred in a zone extending toward the middle lamella (Fig. 13). Labelling of myoglobin in secondary walls was also evident after 8 weeks of incubation (Fig. 14). Significant alterations of cell walls was apparent in areas of the wood after 8 weeks of decay. Secondary walls were often swollen and some



Figs. 7–10. Wood decayed by *Ceriporiopsis subvermispora* for 2 (7 and 8) and 4 weeks (9 and 10), infiltrated with insulin, sectioned and treated with antibodies to insulin and with gold labelled Protein A (7 and 8). Localization of insulin is found in a narrow zone within the secondary wall adjacent to hyphae (H) and around the circumference of the cell wall as evidenced by gold labelling (arrowheads) (9 and 10). Increase regions of labelled insulin reflect greater penetration of the protein into the cell wall. Bar= 1 μm.

parts of cells were eroded, indicating considerable degradation had occurred.

3.2.3. Ovalbumin

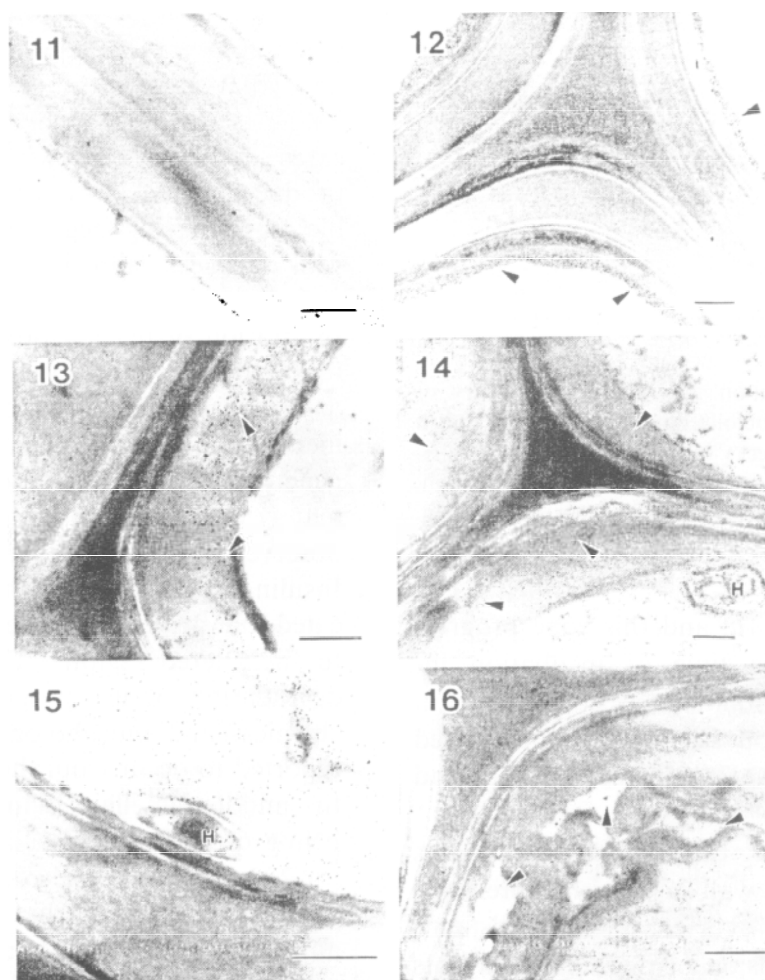
Wood chips inoculated for 2, 4 and 6 weeks did not show appreciable amounts of gold labeling within cell walls as compared to untreated, control wood chips (Fig. 15). Gold labelling was only evident within disrupted and swollen regions of secondary wall layers after 8 weeks of fungal treatment. Voids within cell walls and visibly altered regions of the cell were the only areas where ovalbumin readily infiltrated into the walls (Fig. 16).

3.3. UV absorption microspectrophotometry

The UV absorption spectral patterns for middle lamellae and secondary walls of cells are shown in Fig. 17 and Fig. 18. Two major peaks of UV absorption are evident at 240 and 280 nm. Changes in absorption at 240 and 280 nm occurred after treatment by *C. subvermispora* in both the secondary walls and middle lamellae. A slight loss of absorbance at 280 nm was apparent in sections of wood chips incubated for 2 weeks (Fig. 17 and Fig. 18). As the length of incubation increased to 4 and 6 weeks, the loss in absorption became more pronounced. Very low levels of absorption were observed in sections from wood chips treated for 8 weeks with *C. subvermispora.* A progressive loss of UV absorption was evident in the secondary wall and middle lamella regions examined over the duration of the study. Losses of UV absorbance appeared greater after 4 and 6 weeks in the secondary wall than the middle lamella.

4. Discussion

Changes within cell walls of loblolly pine wood chips were evident within 2 weeks after treatment with *C. subvermispora.* Morphological alterations of the cell wall were not detected but increased



Figs. 11-16. Immunocytochemical localization of myoglobin (1 1- 14) in control, unaltered wood (11) and wood decayed for 2 (12), 4 (13) and 8 weeks (14); and ovalbumin infiltrated into wood after 6 (15) and 8 weeks (16) of incubation with *Ceriporiopsis subuermispora.* 11, No gold labelling of myoglobin is evident in untreated tracheids. 12, Residual myoglobin retained in cell lumina is present along the surface of the cell wall (arrowheads) but no penetration of myoglobin occurs into the secondary wall (arrowheads). 13 and 14, Gold particles indicate myoglobin is present within the cell wall. Gold labelling is evident throughout secondary walls in cells with advanced stages of fungal attack. 15, Little to no labelling occurred in cell walls incubated for up to 6 weeks although labelling of residual protein in cell lumina was observed. 16, Disrupted cell walls from wood with advanced decay showing gold labelled ovalbumin within altered regions (arrowheads). Hypha (H) are present in some micrographs. Bar = 1 μm..

staining with uranyl acetate indicated secondary wall permeability had changed. Zones of increased electron density occurred throughout the outer circumference of the secondary cell wall layer even though many cells contained only one hypha within the cell lumen. Previous investigators, using uranyl acetate as a post stain for sections of wood or other lignocellulosic materials after incubation with white rot fungi, have also reported increased staining of secondary walls during advanced stages of delignification (Akin et al., 1993; Blanchette and Reid, 1986). In our study, sample preparation methods improved infiltration of uranyl acetate and allowed for longer reaction with the wood resulting in greater staining of altered cell wall regions (Blanchette, 1995). A distinct zone of affected wall material was clearly evident after only 2 weeks of incuba-

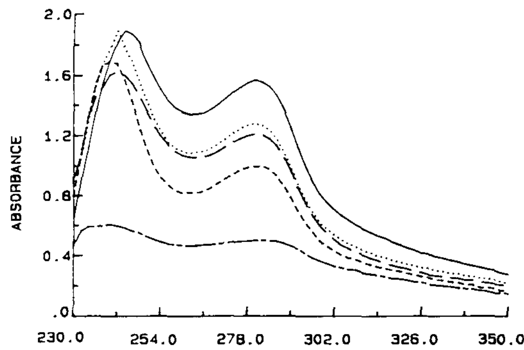


Fig. 17. Ultraviolet absorption microspectrophotometry of middle lamellae regions in loblolly pine cell walls undegraded and degraded for various weeks with *Ceriporiopsis subvermispora* CZ-3. (——) = undegraded, (¨) = two weeks degradation, (- - -) = 4 weeks degradation, (– – –) = 6 weeks degradation, (— — —) = 8 weeks degradation.

tion by *C. subvermispora,* and this zone progressively enlarged as the time of incubation increased. Since no staining was evident in unaltered, control wood samples, the inoculated wood appeared to have greater accessibility and reactivity to the uranyl acetate.

The extensive staining of secondary wall layers around the entire cell wall indicated appreciable wall changes occurred at a considerable distance from the hypha. Diffusion of degradative agents along the surface of the wall in cell lumina may be facilitated by extracellular sheaths produced by the fungus. Sheaths, often composed of β -l,3-glucans, appear to be produced during early stages of wood colonization, and facilitate wood degradation (Blanchette et al,, 1989; Green et al., 1992; Nicole et al., 1995; Ruel and Joseleau, 1991). The extensive network of extracellular sheath material produced by *C. subvermispoira* could be responsible for directing degradative agents to tracheid wall surfaces allowing rapid and widespread changes to be initiated early in the decay process. Infiltration of marker proteins of increasing sizes followed by immunocytochemical labeling clearly demonstrated that porosity of the secondary wall increased during incipient stages of decay. The penetration of insulin into the cell wall approximately corresponded to the electron dense zones observed with walls treated with uranyl acetate. Insulin migration into the secondary wall indicated that proteins of at least 5700 Da have access to the altered regions. The process of white-rot degradation occurs progressively over time and chemical and morphological changes have been reported to occur from the secondary wall toward the middle lamellae (Blanchette et al., 1987). Labeling of infiltrated insulin within wood decayed for 2, 4 and 6 weeks also demonstrates a region of increased alteration that starts in the cell wall adjacent to the cell lumen and moves to the middle lamellae and cell corner regions (Figs. 7–10). Increases in cell wall porosity have been difficult to document during the incipient stages of decay, but previous investigation has shown that a loss of cell wall rigidity and a general swelling of the secondary wall occurs after moderate delignification (Sachs et al., 1991). Similar changes within delignified fibers have been commonly reported in wood with advanced stages of decay (Adaskaveg et al., 1990; Blanchette, 1991; Otjen and Blanchette, 1986).

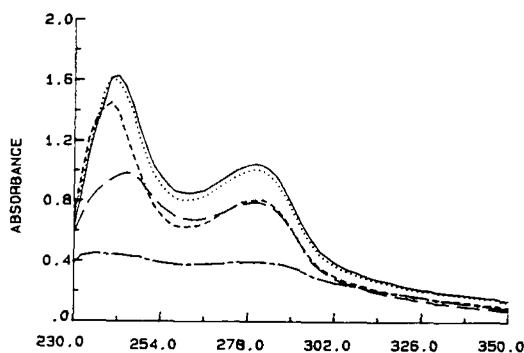


Fig. 18. Ultraviolet absorption microspectrophotometry of secondary wall layers of loblolly pine cell walls undegraded and degraded for various weeks with *Ceriporiopsis subvermispora* CZ-3. (——) = undegraded, (¨) = two weeks degradation, (- - -) = 4 weeks degradation, (– – –) = 6 weeks degradation, (— — —) = 8 weeks degradation.

Proteins with molecular weights that approximate myoglobin (17 600 Da), such as xylanase, are apparently excluded from penetrating the secondary wall until appreciable cell wall alteration has occurred. In our study, 4 weeks of decay were needed before myoglobin was evident within the secondary wall (Figs. 12–14). Larger proteins, such as ovalbumin (44 287 Da) with a molecular weight that approximates that of lignin peroxidase, manganese peroxidase and other

degradative enzymes, do not readily move into the cell wall until very advanced stages of degradation where cell wall disruption is present.

Similar results have been reported by Srebotnik et al. (1988a) using lignin peroxidase to infiltrate wood decayed by *P. chrysosporium.* No diffusion of enzyme occurred within cell walls until very advanced stages of degradation were evident. Additional evidence has been recently provided by Messner and Srebotnik (1994) that indicates relatively large molecular weight proteins do not penetrate delignified fibers until the cell wall structure has started to deteriorate. These studies strongly suggest that only low molecular mass agents are involved during the early stages of white-rot attack. The results presented in our study also suggest that the first degradative agents appear to be low molecular mass agents that readily diffuse into the wood. As decay progresses, the porosity of the cell wall continually increases allowing proteins of greater molecular mass to penetrate. Although relatively large molecular mass proteins do not appear deep within the cell wall during early stages of selective delignification, these various degradative enzymes may be active at cell wall surfaces. The thinning and general erosion of the cell wall by nonselective white-rot fungi involves an array of lignin- and cellulose-degrading enzymes that are readily produced extracellularly (Blanchette et al., 1989; Blanchette, 1991). These enzymes undoubtedly are active along hyphal sheaths in contact with the woody cell wall. The production and penetration of microhyphae and extracellular fungal sheaths by some white-rot fungi within woody cell walls suggests degradative processes in these instances may be different, and large molecular mass proteins appear to have early access into degraded areas within cell walls (Nicole et al., 1995). Since white-rot fungi are such a large and diverse group of organisms, many different methods of cell wall attack are likely to be found. Additional cytochemical investigation with different white-rot fungi and wood substrates, especially during the early stages of colonization, will be of great value to help further our understanding of these different degradation processes.

Ultraviolet microscopy has been used extensively to study the distribution of lignin and other phenolic compounds in woody plants (Lin and Dence, 1992) and forages (Akin et al., 1993; Hartley et al., 1990). UV microspectrophotometry was ideally suited for the investigations presented here so that direct observations could be made on various regions of the cell wall. Although no attempt was made to quantify losses of lignin and other phenolic compounds from the UV spectra obtained, qualitative differences were clearly evident as the incubation of wood chips with *C. subvermispora* progressed. UV absorbance maxima at approximately 240 and 280 nm were evident in the secondary wall as well as compound middle lamella. Losses of UV absorbance were relatively slight after 2 weeks of degradation but continually became more pronounced after 4, 6 and 8 weeks. These results demonstrate that some modification of cell wall phenolics is evident even after 2 weeks of incubation with *C. subvermispora.* These changes are apparently occurring throughout the cell wall since changes within the middle lamellae were commonly observed. The delignification process appears to be well underway after 4 weeks and losses, reflected by low UV absorbance in both the middle lamella and secondary wall layers, were extensive after 8 weeks of decay.

The diffuse attack by *C. subvermispora* on woody cell walls was clearly evident by the effects observed on the middle lamellae assuming that the 1.56 μm aperature used for the analysis delimited only the middle lamella region (Fig. 17). This region of the cell wall, containing the greatest concentrations of lignin, had appreciable losses of UV absorbance during the earliest stages of degradation examined. The agents responsible for this widespread attack of the cell wall would need to be of very low molecular mass to migrate sufficiently into the lignocellulosic matrix and induce this early stage of alteration. This appears to occur even before the staining patterns induced by uranyl acetate have reached the middle lamella. The microspectrophotometry methods presented here in combination with ultrastructural observations are extremely useful to evaluate the changes

that occur during lignin degradation, and help provide a comprehensive understanding of the progressive sequence of events that are initiated in wood by white-rot fungi. The results demonstrate that important changes occur within woody cell walls during incipient stages of degradation and these early events contribute significantly to the benefits realized during biological pulping processes.

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