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Decomposition of Cellulose by *Poria Placenta:* Light and Electron Microscopy Study

By Terry L. Highley, John G. Palmer, and Lidija L. Murmanis

Forest Products Laboratory, Forest Service, U.S. Department of Agriculture, Madison, WI 53705 USA

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Decomposition of Cellulose by Poria placenta: Light and Electron Microscopy Study

Summary

The degradation of isolated cellulose by the brown-rot fungus *Poria placenta* was studied by light and electron microscopy. Hyphae grew randomly on and among cotton fibers; they did not penetrate walls of fibers and were not found in lumina. Many hyphae were supported by extracellular material reminiscent of hyphal sheaths or slime layers. The material or sheath completely surrounded the hyphal cell wall and extended a considerable distance from hyphae. completely surrounding the fibers. Intracellular products diffused from autolyzed hyphae into the sheath surrounding the hyphae. The depolymerizing agent appeared to penetrate fiber walls often at a considerable distance from the hyphae. Our observations of TEM micrographs seem to support earlier suggestions that the initial dissolution of cellulose is accomplished by a small diffusible depolymerizing agent. Furthermore, the results suggest that the depolymerizing agent may be produced intracellularly. In addition, the extracellular material or sheath that associates with mycelial hyphae may aid in concentrating and transmitting this agent to the fibers.

Schlüsselwörter (Sachgebiete)

Braunfäule
Cellulosefasern
Celluloseabbau
Cellulase
Holzzerstörung
Hyphenscheiden
Abbau-Agens
Poria placenta

Cellulose-Abbau durch Poria placenta: Licht- und elektronenmikroskopische Studie

Zusammenfassung

Der Abbau von Cellulosefasern durch den Braunfäulepilz *Poria placenta* wurde licht- und elektronenmikroskopisch untersucht. Die Hyphen wuchsen wahllos auf und zwischen den Fasern; sie vermögen nicht die Zellwände zu durchdringen und sind auch nicht in den Lumina anzutreffen. Manche Hyphen wurden durch extrazelluläres Material gefördert, das an Hyphenscheiden oder schleimige Lagen erinnerte. Dieses Material umgibt die Hyphenzellwand völlig und übenwuchert die Hyphen, indem es die Fasern ganz umschließt. Intrazelluläre Stoffe verbreiten sich von den autolysierten Hypen in die Hyphenscheiden und umschließen dabei die Hyphen. Das abbauende Agens scheint die Faserwände häufig zu durchstoßen in beträchtlicher Entfernung von den Hyphen. Die elektronenmikroskopischen Feststellungen bedeuten eine Stütze für frühere Annahmen, wonach die Initialphase des Celluloseabbaues auf der Wirkung von abbauenden, leicht eindringenden Agenzien beruht. Ferner zeigen die Resultate, daß dieses abbauende Agens intrazellulär gehildet werden kann. Weiterhin können das extrazelluläre Material oder die Scheiden, die mit dem Mycel der Hyphen verbunden sind, die Konzentration und Übertragung dieses Agens zu den Fasern fördern.

Introduction

Brown-rot fungi differ from white-rot fungi and many mold fungi in that they release only endo-1, 4-ß-glucanase activity into culture medium (Highley 1973; 1977). Thus. filtrates from brown-rot fungi will not degrade crystalline types of cellulose. The fact that brown-rot fungi lack exo-1,4-ß-glucanase activity but still vigorously decompose cellulose in wood has frustrated investigators for years.

Brown-rotters drastically reduce the degree of polymerization (DP) of cellulose with very low weight loss, and the decomposition products are readily soluble in alkali (Cowling 1961). Thus, the brown-rot fungi must produce an agent that easily penetrates the lignocellulose structure and breaks glucosidic bonds. In addition, the resulting metabolites must not diffuse from the lignocellulose structure in any quantitiy. To have this effect, an agent smaller than any known cellulase would be required. No isolated cellulase has been able to duplicate the effect of brown-rot fungi on cellulose, and the active agent has been postulated to be a substance other than an enzyme – e.g., hydrogen peroxide (Koenigs 1972; 1974).

Microscopicstudies of brown-rot attack on wood characteristically show little change in size of the fiber cell walls as polysaccharides are digested leaving a lignin framework (Fukazawa et al. 1971; Jutte and Sachs 1976). In a scanning electron microscopy (SEM) study (Blanchette et al. 1978), brown-rot decay of wood was

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evident in tracheids occupied by hyphae as well as in tracheids distal to the fungus. In another SEM study hyphae of the brown-rot fungus *Poria placenta* were found only on the tracheid cell walls, and slime layers apparently fully covered the hyphae wall (Jutte and Sachs 1976). Sheaths surrounding hyphae of Poria *vaillantii* (brown-rot) were also observed in transmission electron microscopy (TEM) by Chou and Levi (1971). Jutte and Sachs (1976) suggest that this type of sheath might act in transmitting enzymes.

Microscopic and submicroscopic studies, using a pure form of cellulose, should elucidate how brown-rot fungi degrade this substrate. The use of a pure substrate should also aid study on the growth pattern of the fungus since the effect of inhibitors and the presence of other cell wall substances - e.g., lignin - can be avoided. This investigation followed growth of the hyphae of *P. placenta* during degradation of isolated cellulose, using light and electron microscopy, and sought evidence for the mode by which this fungus degrades cellulose.

Experimental

Fungal Strain and Growth Conditions

An isolate of the brown-rot fungus *Poria placenta* (Fr.) Cke. [Mad–6981was used in this study because of the considerable amount of information available on its degradative activities in wood and on isolated cellulose.

Cellulose fibers (Hercules, cotton, Type A–600)were soaked in H_2O for 24 hours and then aligned parallel under a microscope on a thin piece of wood (approximately 1 mm x 5 mm x 5 mm). The wood with the cellulose fibers on it was then placed on pine feeder blocks inoculated with *P. placenta* in chambers patterned after the standard ASTM soil-block procedure (ASTM 1971).

Microscopy

Samples were withdrawn from chambers after 2, 4, 6, and 8 weeks incubation for observation. For SEM, samples were fixed in aqueous glutaraldehyde or in osmium tetroxide vapor, air dried, mounted on stubs. and coated with gold. For TEM, samples were fixed in formaldehyde-glutaraldehyde mixture in phosphate buffer and post-fixed in osmium tetroxide in s-collidine buffer, both buffers having pH of 7.2 (Karnovsky 1965). Samples were stained in 1.5% uranyl acetate in 30% ethanol, dehydrated in acetone series and propylene oxide. and embedded in araldite resin, epon, and dodecenyl succinic acid anhydride mixture following the procedure of Mollenhauer (1964). Embedded samples were polymerized at 70°C and sectioned with a diamond knife.

Following preliminary experimentation, the following technique proved satisfactory for reflectance light microscopy (RLM) and transmission light microscopy (TLM). Samples were fixed in formaldehyde-glutaraldehyde mixture for 2 hours and then washed sequentially in phosphate buffer and distilled water. Samples were stained for 10 minutes in 0.125% Poirrier's Blue in distilled water, washed with distilled water, and mounted in an acidified 1:1 (v/v) solution of distilled water and glycerine.

Results

Fibers from cotton and wood differ in gross structure but the cellulose in them is very similar in molecular structure (Cowling and Brown 1969). Cotton fibers consist almost entirely of crystalline microfibrils. Fig. 1 shows micrographs of typical cotton fibers not inoculated with the fungus. The fibers had been chemically treated by the manufacturer to remove impurities, and this probably accounts for the irregular surfaces observed in SEM.

Low magnification photomicrographs showed that hyphae of *P. placenta* grew randomly over and along the cotton fibers (Fig. 2a, b). Higher magnification photomicrographs (Fig. 3a, b) and electron micrographs (Figs. 4a, b; 5a, b) further revealed that hyphal growth was limited to the fiber surface and did not occur in the fiber lumen. Over the cotton fibers in culture bottles, hyphae formed a canopy that was easily removed from the cotton.

Severe degradation of many cotton fibers was observed within 2 weeks (Figs. 5b; 6a, b); at 8 weeks all fibers in the small samples were almost completely degraded. Still there was no evidence of hyphal penetration into cell walls of fibers. Different sizes of hyphae were observed (Figs. 2b; 3a; 7a), some of which may correspond to microhyphae (Chou and Levi 1971).

Fig. 1. Cotton fiber control - i.e., not inoculated. a: TEM. b: SEM.

Fig. 2. Mycelium of *Poria placenta* growing on cotton fibers. a: Random distribution of hyphae on and along fibers. b: Hyphae of variable sizes; arrows point to small hyphae, possibly microhyphae. RLM.

Fig. 3. a: Large (LH) and small (SH) hyphae growing along the surface of cotton fibers. RLM. b: Hyphae (H) covered by extensive hyphal sheath (HS) associated with cotton fiber (CF). TLM.

Fig. 4. Hyphae near and on the surface of cotton fibers (CF) enwrapped in hyphal sheath. a: Sheath (HS)) with fibrillar appearance. b: Sheath (HS) on cotton fiber with a crusty but smooth appearance; aerial hypha (H) without apparent sheath. SEM.

Many hyphae were surrounded by an extracellular material (Figs. 3b; 4a through 7b) designated as hyphal sheath (Evans et al. 1981) or slime layer (Jutte and Sachs 1976). In SEM micrographs this material often appeared to be of a granular or fibrillar nature (Fig. 4a) and apparently absent on hyphae not in contact with fibers (Fig. 4b). When one or more hyphae were in close proximity to or in contact with a fiber, the sheath (HS) appeared to cover uniformly the hyphae and some of the fiber, but the surface of the sheath appeared to be crusty at some places (Fig. 4b). The extracellular material or sheath seemed to attach hyphae to the fibers, and often several hyphae were covered by the same sheath (Fig. 5a, b). TEM micrographs also showed that sheaths completely surrounded the hyphal cell wall so that often hyphae did not directly contact the fibers. Many hyphal sheaths extended a considerable distance from hyphae and also appeared completely surrounding fibers (Figs. 5a, b: 6b). Hyphae not in contact with fibers also were wrapped in sheaths (Fig. 7a, b).

TEM micrographs showed that hyphal cells were in various stages of development ranging from young (Fig. 8a) to partially necrotic (Fig. 6a). whether or not in contact with fibers, even in the same sample. In

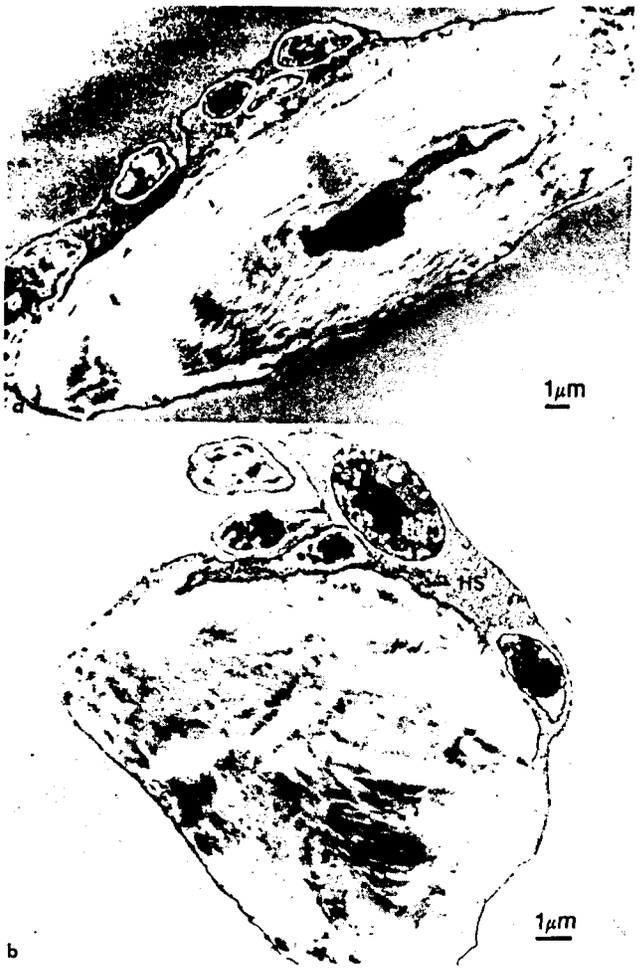

Fig. 5. Single hyphal sheath (HS) enwrapping several hyphae of various sizes plus the cotton fiber. a: Fine granular material diffused into fiber lumen. b: Advanced stage of fiber degradation. TEM.

Fig. 6. Cotton fiber degradation. a: Walls split in direction of fiber axis and transversely to axis with necrotic hypha (NH) adjacent. b: Split fiber wall and fine granular material between lamellae and within lumen; fibrillar material (F) within the sheath. TEM.

addition, autolysis of cells appeared common in this fungus, and intracellular material was released into the hyphal sheath (Figs. 7b; 8b). When in contact with the fiber, the extruded material closely surrounded the fiber (Fig. 8b). Materials of granular, fibrillar, and reticulate appearance were observed distributed throughout sheaths (Figs. 5a, b; 6b; 8b) and also occurred in lumina (Figs. 5a; 6b) and between lamellae within fiber walls (Fig. 6b).

Degradation apparently began when P. placenta became attached to the fiber, evidently by the hyphal sheath. There appeared to be a complete penetration of a depolymerizing agent throughout the fiber wall at a considerable distance from the hyphae, as evidenced by the degraded fibers (Figs. 5a; 6a, b). The walls of degraded fibers were split at the surfaces in the direction of the fiber axis as well as transversely to the axis (Fig. 6a, b). Fibrils were not separated from the main structure. There was no noticeable erosion of the fiber surface.

Discussion

The mode of growth of the brown-rot fungus *P. placenta* on cellulose fibers was different from what has been reported for most other cellulolytic organisms (Basu and Ghose 1962; Berg and Hofsten 1976; Berg et al. 1972). Hyphae of P. placenta did not penetrate to the fiber lumen but grew only on the fiber surface. Cellulolytic mold fungi (e.g., *Trichoderma* spp., Aspergillus spp.) penetrate through the fiber to the lumen (Basu and Ghose 1962; Berg and Hofsten 1976), and growth is abundant in the lumen and within the cell wall. Evidently these fungi make their way through wood by means of surface-bound enzymes (Berg and Hofsten 1976). Hyphae of the soft-rot fungus *Chaetomium indicum* penetrate into the fiber lumen as well as through the secondary wall parallel to the long axis of the cell reminiscent of the mode of attack of this fungus in wood (Basu and Ghose 1962). Bacterial attack on cotton fibers is initiated from the lumen (berg et al. 1972). The bacteria do not grow on the fiber surface but are observed between lamellae in the fiber wall. Degradation occurs where the bacteria contact the fiber, indicating degradation by means of cell-bound cellulases.

Considering cellulose degradation by typical cellulases and the mode of growth of P. placenta on cellulose fibers observed in our electron micrographs, it is difficult to visualize how this fungus could employ typical cellulolytic enzymes to depolymerize cellulose. A fun-

Fig. 7. Hyphae within sheath not in contact with cotton fibers. a: Several hyphae of variable sizes within hyphal sheath (HS). In cross section, sheath appears as if a limiting membrane were present (long arrow). b: Intracellular material (short arrows) extruded within.

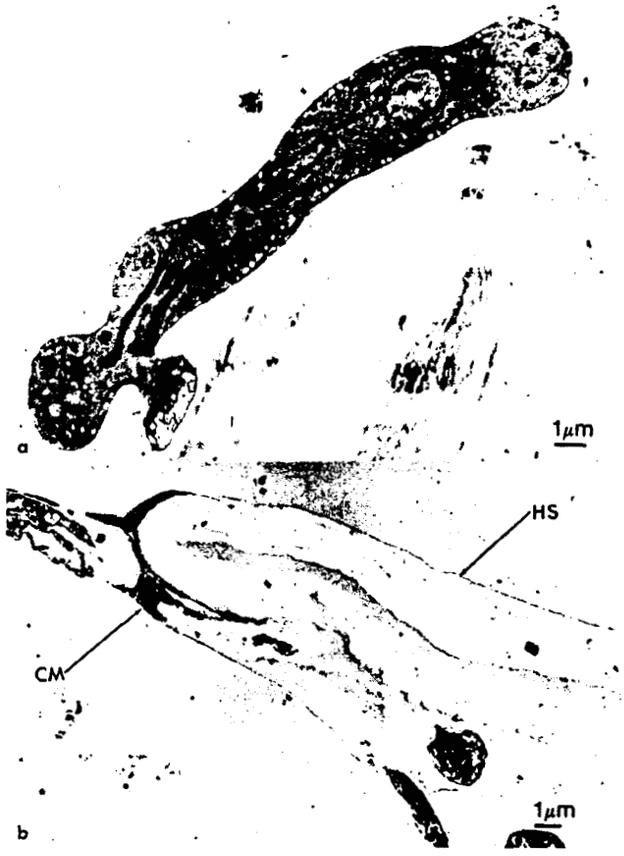

Fig. 8. Hyphal cells of different developmental stages in contact with cotton fibers. a: Young hyphae with cytoplasm rich in organelles; fiber does not appear degraded. b: Cellular material (CM) within hyphal sheath (HS) surrounding a cotton fiber. TEM.

gus growing only on the surface of a fiber would cause only localized attack on the fiber, and degradation would proceed very slowly because the cellulases are too large to penetrate the cell-wall capillaries (e.g., spaces between microfibrils and between the cellulose molecules in the amorphous regions) (Cowling 1961). It would be expected that a slow erosion of the fiber surface would occur as crystallites are removed from the surface by cellulases. We did not observe this type of attack. Electron micrographs of fibers attacked by *P. placenta* indicated that the depolymerizing agent penetrated into the fiber and acted at a distance from the hyphae, rapidly degrading the cellulose. In addition to being too large, a cellulase would not be expected to produce this type of attack because the affinity of cellulases to their substrates drastically reduces their rates of diffusion.

Granules were observed between lamellae and in lumina. The water supply in fibers was adequate for diffusion, and the possibility exists that sheath substances could diffuse and provide a catalytic or essential reagent.

It has been suggested that microhyphae penetrate the cell wall and that they act as nutrient channels by releasing enzymes (Jutte and Sachs 1976). However, we did not observe any hyphae of *P. placenta* penetrating the walls of cotton fibers.

Our results support Cowling's (1961) earlier suggestion that the depolymerizing agent of *P. placenta* is able to penetrate the submicroscopic capillary surfaces of cellulose. Microscopic examination of decayed wood by Cowling (1961) showed that weight loss due to decay could not be accounted for by formation of bore holes. Also, measurements of progressive changes, in α-, ß-, and γ-cellulose, degree of polymerization (DP) of holocellulose, and hygroscopicity of wood decayed by *P. placenta* all indicated penetration of the entire cell-wall structure. Highley (1977) also reported drastic reduction in DP of cellulose by *P. placenta* at very low weight loss. Thus there is strong evidence that the hydrolytic catalyst of *P. placenta* readily penetrates and acts within the capillaries between cellulose strands.

Cowling and Brown (1969) offer the explanation that the different mode of cellulose depolymerization by *P. placenta* may be due to the production of smaller extracellular cellulases than are reported for white-rot fungi and molds. However, recent work with isolation and purification of extracellular enzymes from *P. placenta* failed to identify a small cellulase (Wolter et al. 1980). A purified carbohydrate-degrading enzyme from this fungus exhibited only endo-1,4-ß-glucanase

activity. The enzyme was not active on insoluble celluloses such as cotton and avicel.

Another explanation, also suggested by Cowling and Brown (1969), is that the initial dissolution of cellulose and other cell-wall polysaccharides is accomplished by catalysts that are not enzyme proteins. These materials could be substantially smaller in molecular size and could, therefore, penetrate the cell-wall capillaries and act at a distance from the hyphae. Our electron micrographs support hydrolysis of cellulose at considerable distance from the hyphae and diffusion of a small depolymerizing agent throughout the fiber.

Our electron micrographs raise the possibility that autolysis of hyphae might be a key to cellulolysis by *P. placenta.* Hyphae attached to fibers in various stages of degradation were frequently autolyzed, and the autolysis products apparently diffuse into the sheath surrounding the hyphae. Evidently autolysis is a method employed by wood-decay fungi to recycle nitrogen (Levi et al. 1968). Autolysis products could be small metabolites such as H_2O_2, acids, or even small peptides that can diffuse into the cell-wall capillaries and depolymerize cellulose. The sheath may aid in transmitting and confining the depolymerizing agents to the fiber. Proctor (1941) also saw sheaths in the brown-rot fungus *Trametes serialis* and suggested that the sheaths might act in transmitting enzymes. Similar sheaths were observed by Chou and Levi (1971) in the brown-rot fungus *Poria vaillantii* and also by Jutte and Sachs (1976) in *P. placenta.*

Entrapment of the depolymerizing agent produced by brown-rot fungi in the hyphal sheath could account for the inability of extracellular preparations from brown-rot fungi to degrade cellulose. It has also been reported that the sheath-associated proteins of some fungi are much more stable than when freed from their matrix (Dickerson and Baker 1979). Thus, the sheath surrounding the hyphae of *P. placenta* might also function to protect the depolymerizing agent from destruction. (The sheath substrate could even delay chemical reactions and itself introduce such reactions among cellulose fibrils.) Direct contact between the sheathed hyphae and cellulose, therefore, seems necessary for effective cellulose degradation by brown-rot fungi such as *P. placenta.*

If the hyphal sheath is a key to cellulose degradation by brown-rot fungi, an understanding of how it is produced could provide a basis for new control procedures based on preventing sheath formation.

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