APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1991, p. 374–384
0099-2240/91/020374-11$02.00/0

Vol. 57, No. 2

Involvement of an Extracellular Glucan Sheath during Degradation of *Populus* Wood by *Phanerochaete chrysosporium*

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Received 19 July 1990/Accepted 28 November 1990

Observations by transmission electron microscopy of wood samples of *Populus tremula* inoculated with the white rot fungus *Phanerochaete chrysosporium* showed that, at certain stages of their growth cycle, hyphae were encapsulated by a sheath which seems to play an active role in the wood cell wall degradation. Chemical and immunochemical techniques and ^{13}C nuclear magnetic resonance spectroscopy were applied to demonstrate the β-1,3-1,6-D-glucan nature of the sheath. Double-staining methods revealed the interaction between the extracellular peroxidases involved in lignin degradation and the glucan mucilage. The glucan was also shown to establish a material junction between the fungus and the wood cell wall. It was concluded that, by means of these interactions, the sheath provides a transient junction between the hyphae and the wood, thus establishing a point of attachment to the site of the degradation. The association of peroxidases to the glucan matrix is in favor of the role of the sheath as a supporting structure. Furthermore, that the sheath was hydrolyzed during the attack demonstrated its active role both in providing the H_2O_2 necessary to the action of peroxidases and in providing a mode of transport of the fungal enzymes to their substrates at the surface of the wood cell wall.

Many important groups of fungi, like *Basidiomycetes*, *Ascomycetes*, and *Oomycetes*, contain mixed linked β-1,3-1,6-D-glucans as part of their cell walls. Depending upon the fungal species and life cycle, the glucan may be found in the extracellular culture medium, where both its production and its degree of branching vary by culture conditions and age (2, 3). Glucans may thus be found at three different locations in fungal culture: first they may be part of the hyphal walls, they may also be excreted in the culture medium, and finally they may constitute a sheath thought to be covalently linked to the wall glucans and chitin (39). The β-linked glucans are differently soluble in alkali or dilute acids. This behavior was tentatively related to structural differences, particularly in the degree of branching of the β-1,6-linked glucosyl residues onto the main β-1,3-glucan main chain (14). The structural diversity presented by the glucans due to their origin and their location must be related to the diversity of functions that they display, such as their antitumor activity (7, 44) or their involvement as elicitors in plant pathogenic fungi interactions (1). The chemical variations in the primary structure of the glucans influence the secondary and tertiary structures of individual chains, molecular assembly, or aggregation, providing various physical properties in the solubility and gel-forming ability which in turn control the biological functions of the fungal glucans (37).

The wood-degrading white rot fungus *Phanerochaete chrysosporium* possesses β-1,3-1,6-linked glucans in its cell walls and as an extracellular sheath but can also excrete glucan in the culture medium (4). The extracellular forms of the polysaccharides are not produced at all stages of growth by the fungus and therefore are not always present. The fungus is generally grown in low-form culture flasks (4), and in these conditions several factors can affect the production of the glucan. The exopolysaccharide was not produced under high nitrogen conditions but was excreted when the glucose concentration in the medium fell below a certain level (16). The chemical features of the primary structure of the main extracellular glucan produced by *P. chrysosporium* were shown to consist of a β-1,3-linked backbone carrying single glucosyl groups attached by β-1,6 linkages to the main chain on approximately every second glucosyl residue (8). The degree of polymerization and the extent of branching of the glucan are functions of the fungal growth. It was suggested that degradation of the glucan by exo- and endoglucanases, induced during the secondary metabolism of the fungus when the glucose concentration becomes too low, was related to the formation of hydrogen peroxide (4) necessary for lignin degradation by the fungus. *P. chrysosporium* is a white rot that displays a glucan sheath surrounding living hyphae. The sheath appears to have a complex role in the support and the transport of depolymerizing enzymes in wood decay (28, 29). It could also serve in the nutrition of the fungi. A similar possibility of interaction between the glucan sheath and fungal proteins was described by Dickerson and Baker (13), who demonstrated that a relatively unspecific affinity of proteins on the wall or sheath polysaccharides could take place. The easy in vitro desorption of the enzymes from the glucan promotes noncovalent binding. Such an interaction between a β-glucosidase and the extracellular mucilage was recently described in *Coriolus versicolor* (16), and it was proposed that the immobilization of the enzyme could be the primary function of the glucan matrix.

Our previous results (21) obtained with *P. chrysosporium* K3, which has relatively high ligninolytic activity, indicated that not only cellulases but also hemicellulases and lignin peroxidases (LiP) form associations with the glucan network of the sheath of *P. chrysosporium* (36). Similarly, Daniel et al. (11, 12), by using anti-LiP antibodies on another strain of *P. chrysosporium*, mentioned an association of the lignin-degrading enzymes with an extracellular material. Also, Blanchette et al. (5), by using anti-xylanase sera to xylanases from different white and brown rot fungi, showed that these extracellular enzymes could interact with the surrounding hyphal slime layers. Here, in our present work, the glucan nature of the slime was characterized by chemical and

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immunochemical techniques. Antibodies directed against different enzymes involved in wood degradation and secreted by *P. chrysosporium* were used to study their possible extracellular location on the sheath network.

The extent of the interaction between the proteins and the glucan matrix was demonstrated by a double-staining technique which used immunocytochemical markers of the proteins together with a polysaccharide chemical staining.

MATERIALS AND METHODS

Plant material and organism. Wood samples were taken from a 20-year-old aspen tree (*Populus tremula*) harvested in France. Wood wafers (4 by 20 by 50 mm) were degraded for 6 weeks (at STFI, Stockholm, Sweden) by the wild-type strain K3 of the white-rot fungus *P. chrysosporium* as described previously (32).

Preparation of the antisera. Four of the antisera used in this work were directed against the protein of the enzyme, and one was directed against a polysaccharide component of the fungus wall. The anti-LiP antibodies were polyclonal antibodies raised in rabbits. One was a gift from E. Odier (INAPG, France) and was from LiP 9, pI 4.6 (9). The immunoglobulins G were from the third injection (incomplete Freund adjuvant), harvested at week 9.

The second anti-LiP antibody was a gift from M. L. Niku-Paavola from the Technical Research Centre of Finland, Espoo, Finland. The enzyme, LiP (L3), was isolated from the fungus *Phebia radiata* as described previously (27), and the immunoglobulins G were purified. The antiserum reacts with LiP L1 and L3. The use of this antiserum for localizing LiP from *P. chrysosporium* was justified by the results published in the literature (22, 25).

The antiserum directed against the crude enzyme extract was prepared from *P. chrysosporium* culture filtrate collected during the primary phase of metabolism. The culture filtrate was concentrated by ultrafiltration before being precipitated with ammonium sulfate. The main enzymatic activities detected by using the Somogyi-Nelson method (40) were cellulases (carboxymethyl cellulases), xylanases, and β-glucosidase. No ligninolytic activities were detected. After desalting on a UMH 10 membrane (Schleicher & Schuell), the proteins were injected in rabbits (performed in collaboration with R. Guinet, Institut Pasteur, Lyon, France). The immunoglobulins G were then purified.

The antibody directed against the β-1,3-glucan (3-*O*-β-D-glucopyranosyl-D-glucose [laminaribiose]) was a gift from M. Horisberger. It was a polyclonal antibody and was prepared as described previously (19).

Tissue preparation for transmission electron microscopy. Wood samples were fixed in different mixtures of glutaraldehyde (GA) and paraformaldehyde (PF) (0.3% GA, 1% PF; 0.5% GA, 4% PF; or 2.5% GA, 2% PF, 0.02% picric acid). All solutions were in 0.1 M phosphate buffer, pH 7.2 to 7.4. Samples were dehydrated in ethanol before being embedded in methacrylate (32) or glycol-methacrylate (41).

Chemical staining. Lignin was stained by fixation in 2.5% $KMnO_4$ solution (23) before being dehydrated and embedded in methacrylate or glycol-methacrylate. Polysaccharides were stained on thin sections by using the periodic acid-thiocarbohydrazide-silver proteinate (PATAg) method of Thiery (43) modified by Ruel et al. (35).

Immunocytochemical labeling. Antibodies were used as postembedding markers. Thin sections of decayed wood were first incubated on a drop of TBS (0.01 M Tris-phosphate saline buffer [pH 7.4], 0.5 M NaCl)–0.15 M glycine. After being rinsed in TBS, the thin sections were floated on a drop of 1% TBS–bovine serum albumin (BSA) or 5 to 10% TBS–normal goat serum before being treated with either of the antibodies adjusted to the correct dilution. Duration of contact with the primary antibody varied from 14 to 24 h.

The secondary marker, labeled with gold (10 nm in diameter), was a goat anti-rabbit antiserum or protein A (Janssen Pharmaceutics, Beerse, Belgium). It was diluted 1:10 to 1:30 in TBS-normal goat serum or TBS containing 0.1% BSA plus 0.5% gelatin from fish skin (Sigma).

Controls. The following control experiments were performed: (i) substitution of the primary antibody with preimmune (or nonimmune) rabbit serum, immunoglobulin G fraction; (ii) treatment of sections with the gold-labeled secondary antibody alone, omitting the primary antibody step; (iii) labeling with the antisera preadsorbed with their respective antigens.

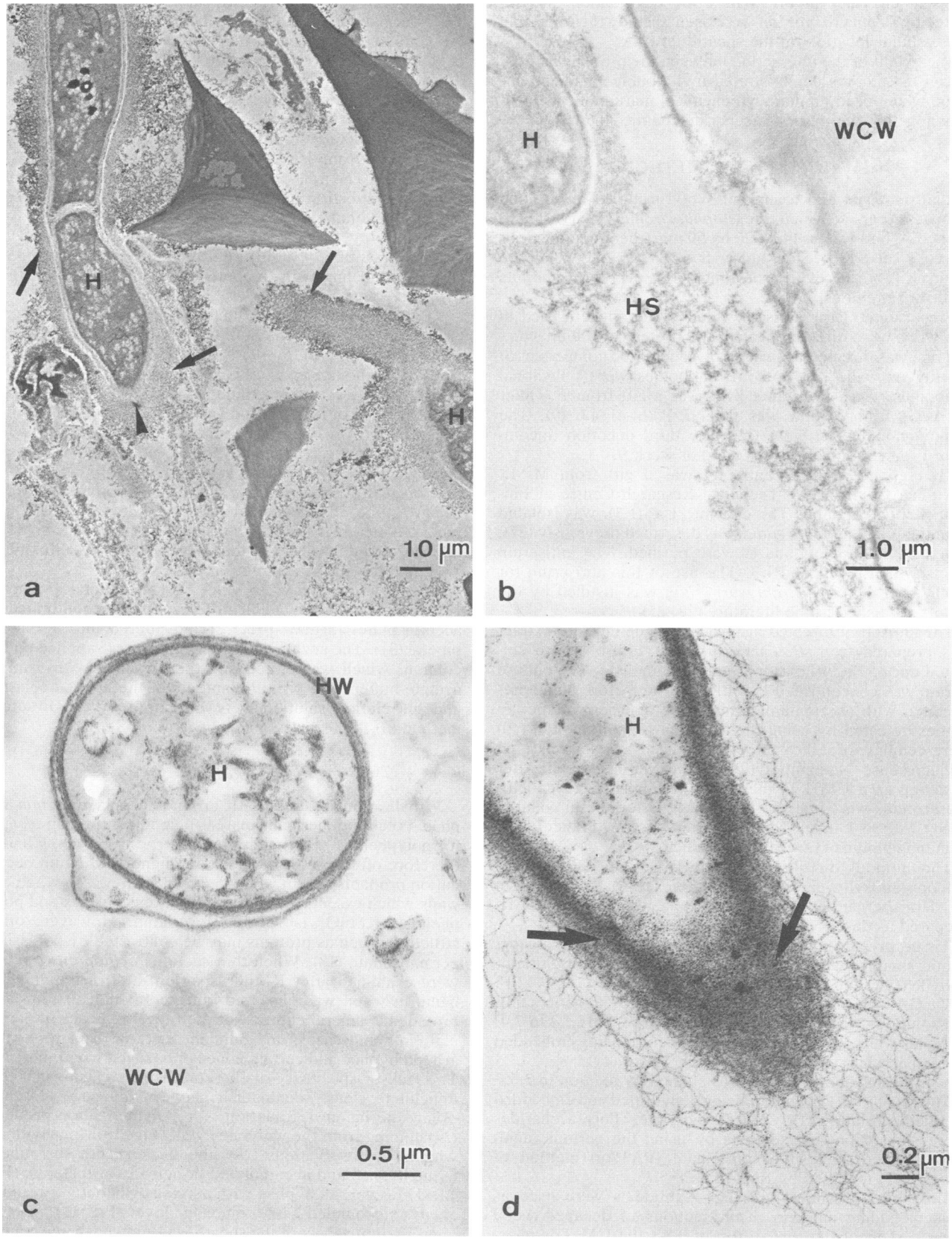
^{13}C NMR spectroscopy. The β-glucan was isolated from a crude polysaccharide fraction precipitated with ethanol from a culture filtrate of *P. chrysosporium* (a gift from A. J. Buchala, Fribourg, Switzerland). The glucan was obtained in pure form by extraction in dimethyl sulfoxide in an autoclave by the method of Buchala and Leisola (8), yielding a polymer which upon acid hydrolysis contained only D-glucose. For nuclear magnetic resonance (NMR) recording, the freeze-dried glucan was solubilized in dimethyl sulfoxide-d_6 (25 mg in 1 ml) at 70°C. The resulting viscous solution was placed in an NMR tube (10 mm in diameter), and the ^{13}C spectrum was recorded on a Brucker AM 300 spectrometer, at 75.46 MHz, taking the middle signal of the CH_3 of dimethyl sulfoxide at 39.5 ppm downfield from tetramethyl silane.

Affinity adsorption of enzymes on extracellular β-glucan. Freeze-dried purified β-glucan (25 mg) was equilibrated in acetate buffer (5 mM, pH 5.2) and poured into a Pasteur pipette (31). The enzyme mixtures (1 ml) were applied on the column, which was eluted and then washed with 5 ml of acetate buffer. The protein contents of the applied solution and the elution solution were measured by the Bradford method (6).

RESULTS

Visualization of the sheath around *P. chrysosporium* hyphae. Potassium permanganate is a good contrasting reagent for polyphenols in transmission electron microscopy and is therefore often used for visualizing lignin and lignin degradation products (33) in wood decayed by fungi. In wood cell walls which essentially consist of polysaccharides and polyphenols, $KMnO_4$ is specific for lignin. However, other structures such as proteins may be contrasted by potassium permanganate (18). When thin sections of *Populus tremula* were examined after $KMnO_4$ fixation, a strong staining of lignin in wood was observed but the $KMnO_4$ fixation also provided a general contrast of the fungal hyphae. In particular, in some cases, an abundant material became visible around hyphae and certain zones of decayed wood (Fig. 1a). The lack of specificity of the contrasting reagent makes it difficult to clearly distinguish between mucilage of fungal origin and lignin degradation or repolymerization products (34) coming from the wood cell wall (Fig. 1b). In agreement with most observations (29), the thick sheath surrounded living hyphae and accumulated principally at the apex (Fig. 1a). However, all hyphae with a living cell content were not always surrounded with a mucilage layer (Fig. 1c).

A different aspect of the sheath was shown when a general reagent for contrasting polysaccharides was applied to the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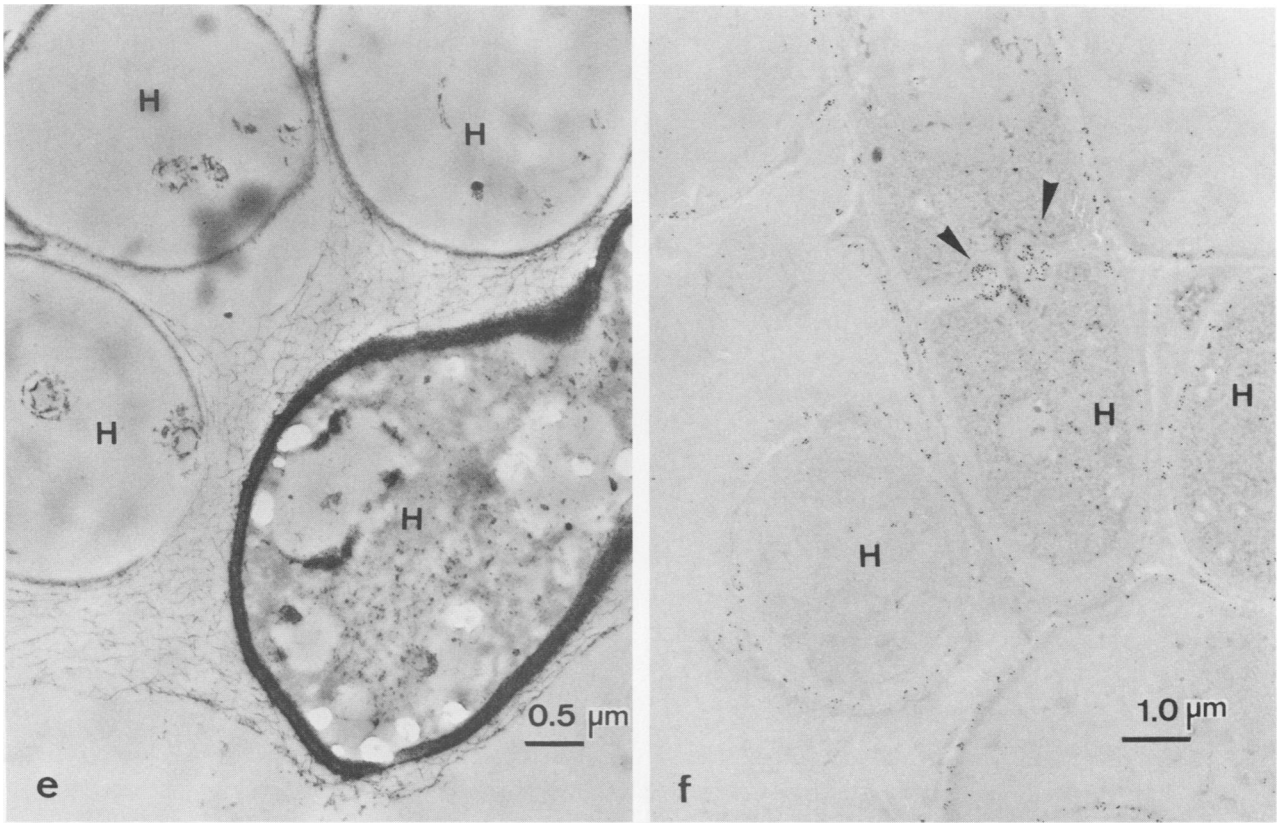


FIG. 1. Staining of decayed wood and of invading hyphae. Panels a and b were fixed with $KMnO_4$, panels c, d, and e were stained with PATAg, and panel f was stained via immunocytochemical marker. Abbreviations: H, hypha; WCW, wood cell wall; HW, hyphal wall. (a) The stain underlines the lignin part of the strongly degraded wood cell walls and contrasts with the abundant sheath (arrows) surrounding fungal hyphae and particularly the hyphal tip (arrowhead). (b) The distinction of the limit between fungal sheath and wood degradation products is not possible with certitude. (c) Hypha in the lumen of a parenchyma cell. Wood cell wall is degraded (arrows), and the hyphal wall is naked. (d) PATAg staining delineates the mesh formed by the glucan. Arrows show the intensively stained fungal wall from which the glucan originates. (e) Adhesion of living and dead hyphae via the glucan network of mucilage. (f) The anti-β-1,3-glucan antibody shows the glucan localized in the inner part of the hyphal walls and concentrated in the pore separating the hyphal tip from the next cell (arrowhead).

embedded thin sections of decayed wood. The periodic oxidation-based reaction (i.e., PATAg) of Thiery (43), although not active on 1→3-linked polysaccharides, gave a positive reaction with the β-1,3-1,6-D-glucans of *P. chrysosporium*. The polysaccharide-specific staining provided detailed images of the sheath. The glucan can be seen as a light and thin mesh anchored in the fungal cell wall from apical hypha (Fig. 1d). The mucilage seems to be mostly associated with apical hyphae which have a thick cell wall and may sometimes expand in such a way that they join together with several other hyphae, including dead ones (Fig. 1e).

Another specific staining of β-1,3-glucans was assayed on *Populus tremula* samples decayed by *P. chrysosporium*. An anti-β-1,3-glucan antibody prepared against 3-*O*-β-D-glucopyranosyl-D-glucose (laminaribiose) (19) was applied to thin sections of the wood, and the antigen-antibody complex was revealed by an immuno-gold labeling technique. This marker was specific for unsubstituted linear β-1,3-D-glucosyl sequences and failed to disclose the β-1,3-1,6-D-glucan present in the sheath. However, the wall glucan formed a specific complex with the antibody (Fig. 1f). The gold labeling was found on all of the hyphal walls, with a particularly dense marking in the septum localized on the dolipore. This suggests that a particularly high concentration of linear β-1,3-glucan structures should be present at this place.

In all micrographs visualized with either mode of staining, the sheath appeared as an amorphous network different from the membranous structures described previously (11). The $KMnO_4$ and PATAg reactivity of the slime distinguish it from the membrane structures often present in mycelium cultures of *Sporotrichum pulverulentum* (an anamorph of *P. chrysosporium*) and described previously (15).

Interaction between *P. chrysosporium* mucilage and the degraded wood cell walls. The sheath constitutes an extension of the hyphal wall and seems to have, among other functions, a role in establishing contact with the wood cell wall. Hyphal cells, agglutinated in the same mucilage material, were kept in close proximity with the wood by the interaction between the sheath and the inner surface of the wood cell wall (Fig. 2a). The unspecific $KMnO_4$ staining showed that the hyphal sheath could establish a material junction between the fungus and the wood cell wall, thus acting as an adhesive agent between the microorganism and the wood. The more selective PATAg staining revealed that the β-glucan network effectively bound to the degraded wood cell wall (Fig. 2b). Such an attachment to cellulosic material was recently demonstrated in the case of *Tricho-*

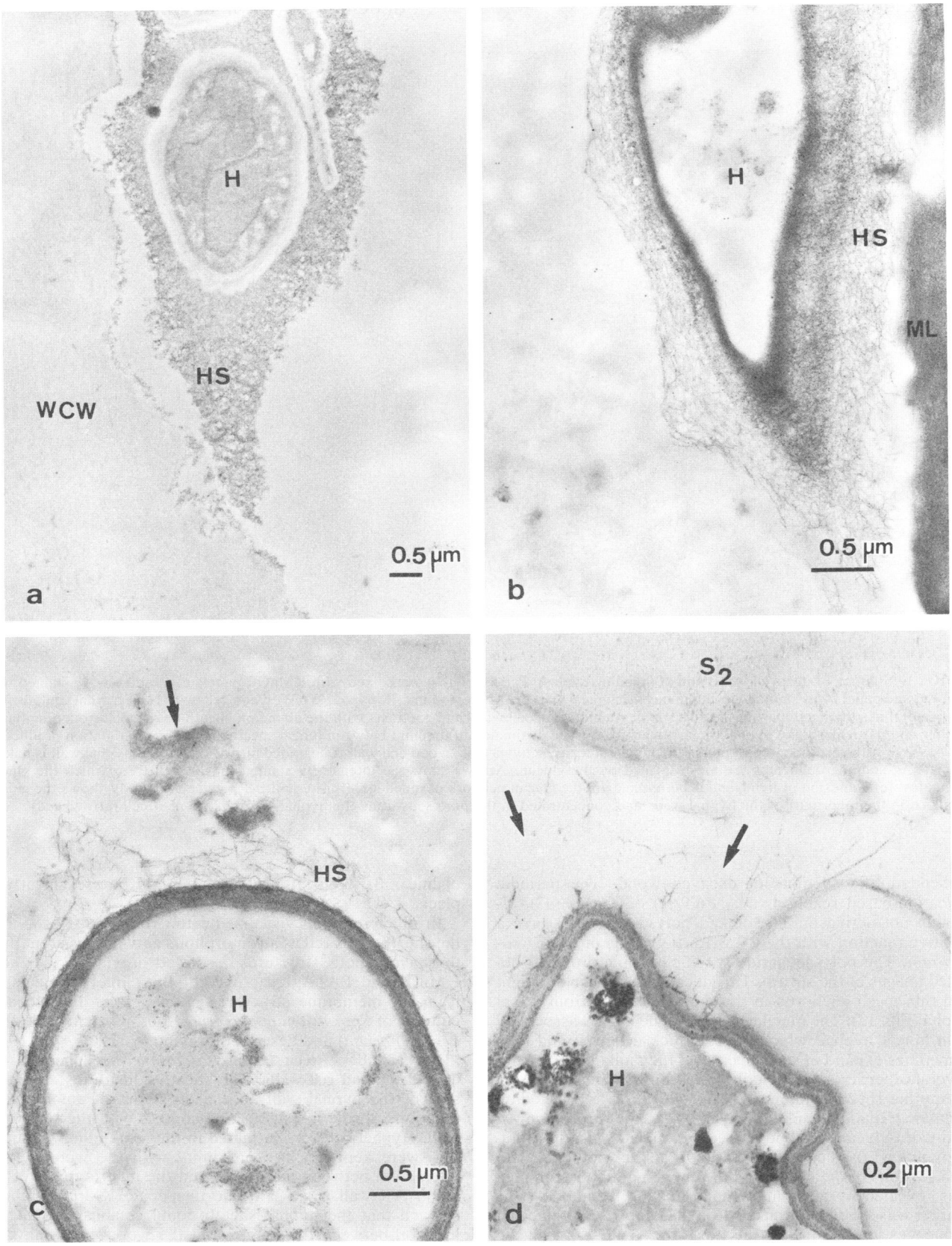


FIG. 2. Interaction between fungal mucilage and wood cell walls. Abbreviations: H, hypha; HS, hyphal sheath; WCW, wood cell wall; ML, compound middle lamella; S_2, outer layer of the secondary wall of the wood. (a) Association of the hyphal sheath with the wood cell wall ($KMnO_4$ staining). (b) PATAg staining shows the tight interaction existing between the hyphal sheath and the wood cell wall during decay. (c) Residual wood cell walls (arrow) bound to the mucilagenous hyphal sheath (PATAg staining). (d) Loosening of the PATAg-positive network of the fungal sheath (PATAg staining).

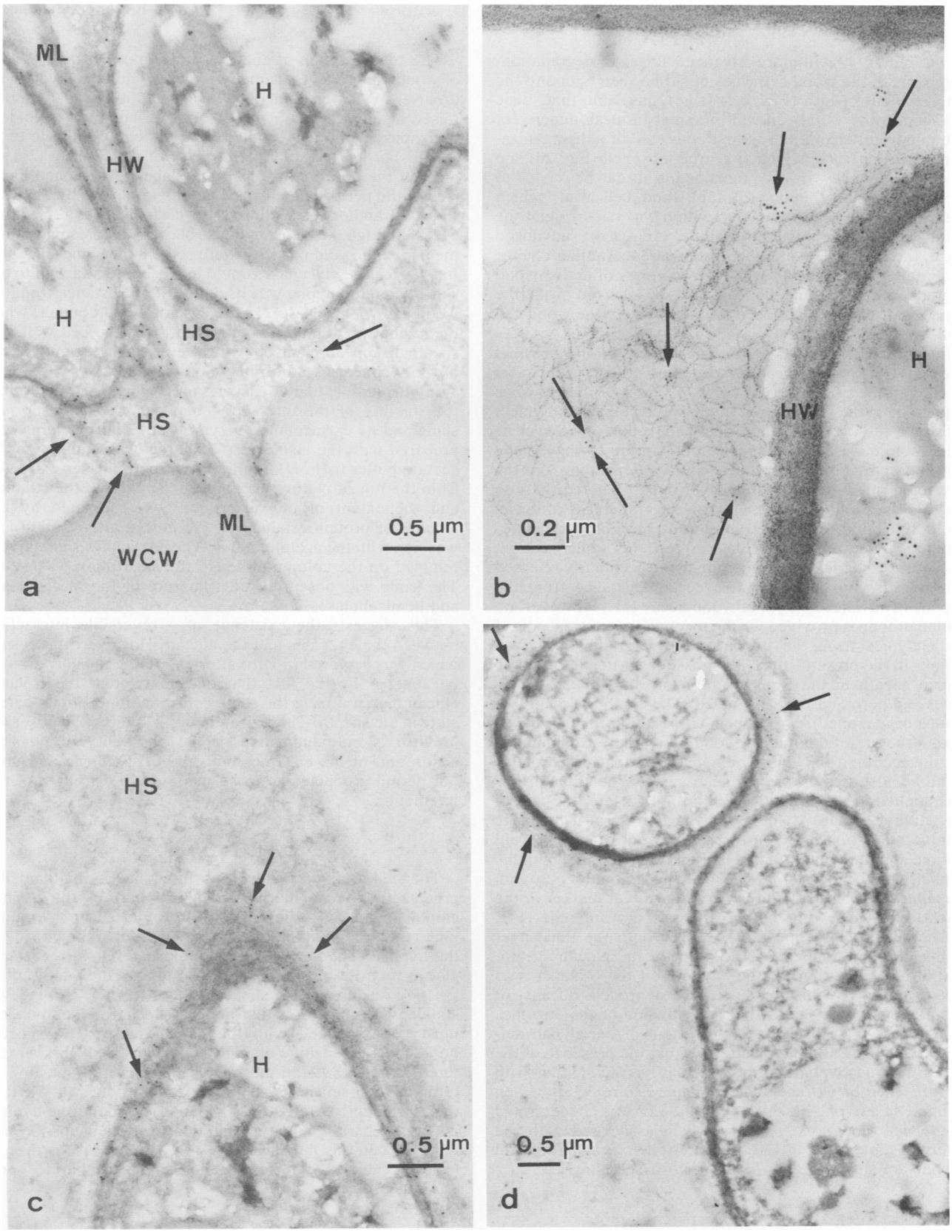


FIG. 3. Interaction of LiP with the fungal sheath. Abbreviations: H, hypha; HS, hyphal sheath; HW, hyphal wall; WCW, wood cell wall. (a) Anti-LiP detects the presence of LiP (arrows) in the mucilagenous part (HS) joining the hyphal wall to the wood cell wall. Note that LiP is also inside the fungal wall (uranyl acetate poststaining). (b) LiP (arrows) is associated with the PATAg-positive mesh of the slime (anti-LiP plus PATAg staining). (c) An abundant slime (HS) surrounds the hypha, but LiP is restricted to the hyphal wall (arrows) (anti-LiP and uranyl acetate poststaining). (d) Two morphologically active hyphae; one of them only is excreting LiP (anti-LiP and uranyl acetate poststaining).

derma reesei (42). In more advanced stages of degradation, fragments of the wood cell walls could be seen bound to the mucilagenous sheath (Fig. 2c). It is remarkable that, once attached to the wood cell wall, the glucan mesh underwent two important changes. The first consisted of a disorganization of the network, which appeared loosened (Fig. 2d) and in which only a few threads remained. This can be related to the concomitant thinning of the wood cell wall, which indicates that an intensive degradation was occurring at the point of interaction with the slime. At a more advanced stage, the mucilage eventually disappeared. Another change undergone by the sheath was that, in zones of active wood degradation, the slime appeared to be cleaved from the hypha and remained attached to places where the wood cell wall was being attacked.

Interaction between β-glucan sheath matrix and enzymes released by *P. chrysosporium*. Immuno-gold–cytochemical labeling of the principal types of *P. chrysosporium* enzymes implicated in the degradation of the wood cell wall polymers showed (21, 36) that cellulases and hemicellulases, as well as LiP, could have different localizations in the hyphae. They may be intracellular or extracellular, depending on the physiological state of the fungus. It must be noted that, on the same section of decayed wood, several stages of decay could be found. Hyphae at different physiological states could also be observed (Fig. 1e) on the same section. Microscopy studies have shown that extracellularly released peroxidases can be found at a certain distance from the hyphae in advanced stages of wood decay (11). However, no explanation was provided for the mechanisms of transport of the enzymes from the hypha to the degrading cell wall region. In the present study, the possible participation of the glucan sheath in the transportation of extracellular glycanases and peroxidases was studied. The combination of two modes of staining, i.e., by chemical reaction for the underlying structure of the mucilage and by immuno-gold labeling for the excreted enzymes, demonstrated that the proteins could be adsorbed on the β-glucan mesh. When an anti-LiP was applied in combination with uranyl acetate, the unspecific uranyl acetate staining showed the mass of mucilage making a junction between the hyphae and wood cell wall, and the anti-LiP immuno-gold marker demonstrated both the hyphal wall localization of the LiP and its interaction with the slime (Fig. 3a). When the periodate-silver marker of the glucan was used with the anti-LiP primary antibody (Fig. 3b), gold particles appeared scattered onto the thin silver grains, thereby delineating the mesh of the glucan sheath. The direct interaction of the enzyme on the β-glucan was thus evidenced by this double-staining approach. Because of this association, it seems that the diffusion of the enzymes was restricted in distance to the extent of the glucan network. However, it must be stressed that the presence of the sheath around hyphae is not necessarily concomitant with the presence of extracellular enzyme. Figure 3c shows an example of a hypha in which all of the LiP was concentrated in the wall and none of it was seen associated with the external slime. In Fig. 3d, two morphologically identical hyphae are shown, only one of which is secreting LiP.

The second group of enzymes implicated in the digestion of wood components, namely, the endo- and exoglycanohydrolases, were examined by using a polyclonal antibody raised against an extracellular enzyme complex devoid of peroxidase activities, isolated from the culture filtrate of *P. chrysosporium* in the primary phase of metabolism (21). In most of the observed samples, the labeling remained localized at the periplasmic level (Fig. 4a) within the hyphal wall (Fig. 4b) and/or intracellularly in clear vesicles (21); however, although the electron-dense sheath was present around the hypha, no gold particles were seen in the slime (Fig. 4b). In a few cases, glycanases could be observed attached to the slime when the hypha was itself at a short distance from the wood cell wall (Fig. 4c). In the case of direct contact, when the hypha was tightly associated to the wood cell wall, the sheath disappeared and enzymes were seen in the outer layer of the secondary wall of the wood (Fig. 4d).

In vitro adsorption of enzymes on β-glucan. To determine the stability of the interaction between *P. chrysosporium* slime and its extracellular enzymes, an affinity column was prepared with the insoluble β-1,3-1,6-glucan equilibrated in acetate buffer (pH 5.2). A mixture of peroxidases extracted from the fungal culture medium was applied to the column, and the extent of the adsorption was estimated by the amount of protein retained on the glucan after elution and washing with the acetate buffer. The enzyme was effectively retained on the column although the adsorption was weak. The same was observed for a mixture of fungal cellulases and hemicellulases.

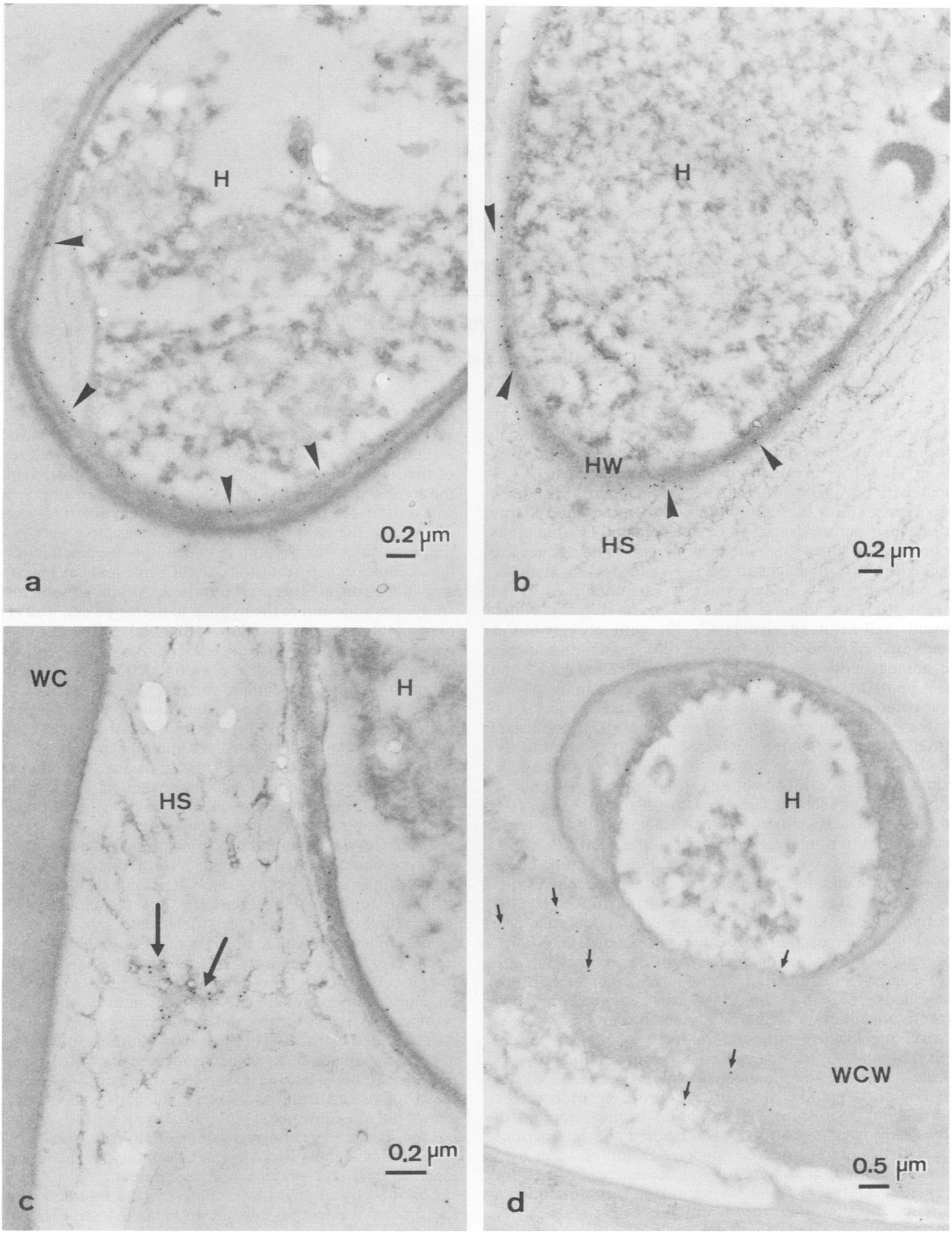
This suggests the existence of a reversible interaction between the enzymes and the glucan. However, this result, which was acquired in vitro after extracting the glucan, may be affected by the fact that once extracted, the isolated glucan may not have the same conformation as in its native state in the sheath. Therefore, the weak interaction observed in vitro is only indicative of the possibility of physical adsorption of the enzymes onto the β-1,3-glucan and does not permit a conclusion to be made on the strength of the interaction.

DISCUSSION

The cell wall of filamentous fungi has a multilamellar organization. Particularly, the apical part of the hyphae exhibits a layered structure, with the outer part corresponding to the accumulation of capsular material at the outside of the hyphae. The nature of this outer material varies from a glucan mixture of glycopeptides to glycoproteins (39). The positive reaction of the sheath to both $KMnO_4$ and PATAg stainings demonstrates that nonpolysaccharide components must be associated with the glucan mesh. The specific periodic acid-sensitive staining is due to the highly substituted structure of the glucan which carries about 30% of terminal acid-sensitive glucopyranosyl residues (4, 8). It is therefore demonstrated that the material around the hyphae is polysaccharidic in nature and that the β-glucan is forming a network in this mucilage.

A mycelium-bound extracellular polysaccharide, pro-

FIG. 4. Interaction of the glycohydrolases with the slime. Abbreviations: H, hypha; HS, hyphal sheath; HW, hyphal wall; WCW, wood cell wall. (a) Labeling with anti-CEE showing glycohydrolases inside the fungal cell, concentrated along the plasmalemma (arrowheads) (uranyl acetate poststaining). (b) Hypha with slime and glycohydrolases (arrowheads) associated with the hyphal wall (anti-CEE + PATAg staining). (c) Association of glycohydrolases (arrows) with the fungal sheath attaching the hypha to the wood cell wall (anti-CEE plus uranyl acetate poststaining). (d) Glycohydrolases (arrows) diffusing directly in the degraded wood cell wall when hypha is in direct contact with the wood (anti-CEE and uranyl acetate poststaining).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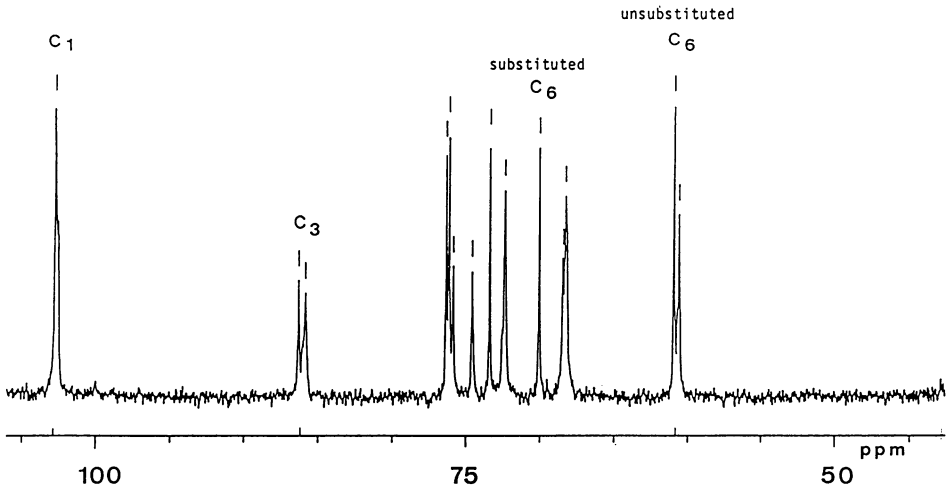

FIG. 5. ^{13}C NMR spectrum of the purified β-1,3-1,6-glucan.

duced by *P. chrysosporium* in a low-form culture flask in response to its surrounding environment, was depicted by Bes et al. (4) as a rather complex branched-chain β-1,3-glucan with occasional internal 1,6-linked glucosyl residues. Such a structure is slightly different from that of true extracellular glucan described by Buchala and Leisola (8) as a more regular glucan in which simple glucosyl residues are attached by β-1,6 linkages to the β-1,3-glucosyl main chain. The regularity of the polysaccharide was confirmed in the present study by the ^{13}C NMR spectrum (Fig. 5), which showed well-resolved signals (7, 37) with only two C-1 signals at 102.75 and 102.65 ppm, in agreement with a simple and homogeneous structure. The two signals at 85.85 and 86.35 ppm, assigned to C-3-linked carbon atoms of the unsubstituted glucosyl of the main chain and to the 1,3-1,6 substituted branch-point units, respectively (7), confirmed by their comparable intensity the regular distribution of the side chain units on one glucose residue out of two in the main chain. Additional evidence of this structure was provided by the two clearly resolved signals of unsubstituted C-6 at 60.9 and 60.6 ppm assigned to internal 1,3-linked residues and unsubstituted terminal glucosyl units, respectively (29a). The substituted C-6 gave a single signal at 70.0 ppm. These data suggest a simpler and more regular sequence than that described by Bes et al. (4). The wall glucans, i.e., the adherent glucan attached to the mycelium and the true extracellular slime, therefore show structural variations, especially in the degree of branching of the main chain with β-1,6-glucosyl substituents (30) and in the complexity of the side chains. This has important consequences for their respective properties, in particular their solubility. Consequently, the tertiary structure of the wall glucans is not necessarily the same in the wall or in the cell-bound polysaccharide where the interconnected triple helices (24, 38) can give rise to a gel-like structure. A property of complex β-1,3-glucans related to gel formation is the retention of water. This is particularly relevant when the interactions with enzymes are considered, since retention of water by a glucan would help maintain an environment well suited for enzymatic catalysis. These structural and conformational differences must account for the differences in the reactivity of the wall glucans and sheath glucans towards the different contrasting reagents demonstrated by an affinity interaction.

The physical characteristics of the sheath polysaccharide as a gel-like network must be responsible for the self-adhesion of the hyphal mycelial mats and also for the attachment of the hyphae to the wood cell wall. The fungal wall, like any cell wall or cell membrane, is an interactive surface, and it seems that the mucilage sheath takes an active part in this property. The interaction between the β-glucan and the lignocellulosic substrate (42) could represent an important step in the fungal attack on the wood. As can be seen in this study, the adhesion of the hyphae to the wood cell wall is not permanent since it was shown that the β-glucan network may be progressively hydrolyzed (Fig. 2d), leaving, attached to the inner surface of the wood cell walls, fragments carrying lignolytic enzymes. Such a role of the β-1,3-1,6-glucan is possible with the participation of β-1,3-glucanases secreted by the fungus through the porous nascent apical wall (10), which could hydrolyze the glucan network, thus making it an active element of the transient adhesion of the fungus to the wood cell wall. Consequently, the glucan sheath could have a complementary action in this mechanism as a source of reserve glucose (4).

Evidence with combined chemical and immunochemical markers demonstrated that the sheath interacts with the extracellular enzymes to which it provides a supporting structure for immobilization. The interaction with LiP is shown in Fig. 3b. However, the sheath may be present around hyphae in which LiP remained intracellular or wall bound (Fig. 3c). It seems, therefore, that the porosity of the fungal cell wall must be sufficiently modified to allow the enzymes to be excreted. The stage of maturity of the hypha is difficult to assess judging only by its morphological aspect. As illustrated by the two hyphae shown in Fig. 3d, which are apparently identical in aspect, the labeling with the anti-enzyme antibody was necessary for distinguishing a peroxidase-active cell from its inactive neighbor. All of the enzymes did not show equal affinity for the β-glucan, as shown by the images of enzyme adhesion to the sheath. The peroxidases seemed to have a higher affinity for the mucilage than did the other enzymes assayed here.

For the glycanohydrolase complex, the interaction with the glucan was more difficult to demonstrate. The most frequent localizations observed were in the wall and in the plasma membrane. It is difficult to ascertain if this situation

is general since the variations in the physiological states of fungi are numerous and the release of extracellular enzymes corresponds to an enhanced porosity of the hyphae, which is probably controlled by autolysis of the wall (10). This may explain why hyphae with the same morphological appearance seem to behave differently in the excretion of their enzymes. In cases where the extracellular hydrolases were seen adsorbed on the sheath, it should be noted that the hypha is situated in close vicinity to the wood cell wall. It seems that the polysaccharide-degrading enzymes may be transferred to the wood cell wall by a more direct contact than are the enzymes involved in lignin attack. Several authors have shown in vitro interactions between β-glucans and proteins (13, 16, 26). However, when the LiP extract and a cellulase mixture were put in contact with the extracellular β-glucan in an affinity column, only a little protein adsorption was measured. This means that a weak interaction occurred between the glucan and the enzymes, in agreement with a reversible association which is necessary for the enzyme to release from the glucan network of the slime to the wood cell wall. On the other hand, the in vitro experiment does not necessarily reflect the in vivo association of the protein on the glucan since, after extraction and isolation by freeze-drying, the resulting conformation of the glucan might differ from its native tertiary structure. It is well established that native β-1,3-D-glucans which have helical chain conformations form rather soft gels in aqueous media (17). However, during the process of their isolation which includes drying, the polymer undergoes a conformational transition and becomes insoluble. As a result, the affinity between the enzyme and the isolated glucan packed in the column may have been considerably altered from what it was in vivo with the glucan in its native conformation. The preferential affinity of LiP observed in vivo for the glucan may be related to the potential source of hydrogen peroxide represented by the oxidation of the glucose released by glucan-hydrolyzing enzymes (4). In this process, hydrogen peroxide and the peroxidases would be present at the same time and in the same location. Such associations between lignin-degrading enzymes and the fungal sheath could facilitate the interaction of lignin products detached from the wood cell wall and the peroxidases (20). Besides providing a source of metabolic glucose and H_2O_2, another interesting aspect of the hydrolysis of the β-glucan is related to its transient existence. The disappearance of the β-glucan, once attached to the wood cell wall, results in the release of the adsorbed enzymes at the site of attack of the lignocellulosic wall. Consequently, not only could the glucan matrix serve for the adsorption of the enzymes, but it also could convey the immobilized proteins to the site of wood degradation by means of its hydrolysis by β-1,3-glucanases.

ACKNOWLEDGMENTS

We thank A. J. Buchala for the gift of glucan extract, M. L. Niku-Paavola for providing the anti-LiP antibody (financed by the Technology Development Center of Finland), and E. Odier (INAPG, France) for the anti-LiP 9.

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