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Localisation of degradative enzymes in white-rot decay of lignocellulose

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Abstract

The use of immunogold-cytochemical labelling techniques in electron microscopy of wood infected by basidiomycete fungi has assisted in the elucidation of the localisation of enzymes which degrade lignocellulose. The use of specific immunocytochemical techniques is discussed with respect to the authenticity and accuracy of the methods, the use of adequate controls in the gold-labelling procedure, and the immunospecificity of the antibodies.

Localisation of the lignin-degrading enzymes, lignin-peroxidase and laccase, has shown that these enzymes do not bind to wood cell walls unless the process of decay has already commenced. Similarly localisation of cellulases Endoglucanase II (EGII) and Cellobiohydrolase I (CBHI) has shown that these enzymes only bind to exposed ends of cellulose fibrils and to partially degraded areas of the wood cell wall. β-Glucosidase is always immobilised within the extracellular polysaccharide layer surrounding fungal hyphae.

This review postulates that there is regulation of the release sequence of these lignocellulolytic enzymes defining the spatial arrangement between the hyphae and the wood cell wall. This hypothesis is presented diagrammatically.

Introduction

An understanding of the mode of action of the lignocellulose degrading enzymes secreted by the basidiomycete fungi causing white-rot would be of great interest to agriculturalists, horticulturalists, conservationists and the construction industry. Large and increasing amounts of lignocellulose are available in the form of agricultural and crop residues. The efficient degradation of these residues would not only remove the potential pollution problem caused by bulk storage or burning but provide a suitable substrate for the growth of edible fungi and a possible alternative to peat for horticulture. The breakdown of lignin may also make it possible to convert some waste into animal feeds.

Several different species of micro-organism invade wood but the wood-rotting fungi cause most damage by colonising timber or infecting standing trees (Levy & Dickinson 1981). The wood-rotting basidiomycetes causing white-rot decay are able to degrade all the components of the wood cell wall including the highly recalcitrant polymer, lignin (Kirk et al. 1977).

These fungi have been widely studied using biochemical, cellular and biotechnological approaches

and the enzymes they produce have been isolated and characterised for their ability to degrade lignocellulose. Until recently the cellular distribution and localisation of these enzymes has not been understood. However, the advent of immunogold cytochemistry in electron microscopy has made it possible to determine their precise location.

This paper reviews the relatively small amount of electron microscopical immuno-cytochemistry in this field so far and describes the methodology. It concludes with a proposal for the degradation mechanisms combining the known biochemistry with the localisation data available.

Lignocellulolytic enzymes

Earlier studies led to the postulation that lignin breakdown was caused by secretion of extracellular enzymes from the hyphae of wood colonising fungi, though evidence of the involvement of specific proteins was not apparent (Crawford & Crawford 1980). In 1983, a peroxidase from *Phanerochaete chrysosporium* was isolated which cleaved milled wood lignin to smaller molecular weight fractions and oxidised veratryl alcohol to veratraldehyde (Tien & Kirk 1983; Glenn et al. 1983). Although peroxidases had been isolated from *Trametes (Coriolus) versicolor* previously, their role in lignin degradation was not demonstrated (Lobarzewski et al. 1982; Evans et al. 1984). Lignin peroxidases were very effective in degrading non-phenolic lignin model compounds eg. dimers, whereas manganese-dependent peroxidases attacked lignin models bearing free phenolic groups (Schoemaker & Leisola 1990). Evidence for their continued attack on polymeric lignin was less clear as re-polymerisation of lignin fragments was observed in vitro experiments (Haemmerli et al. 1986).

Laccase, a polyphenol oxidase produced by many wood-rotting fungi has also been implicated as a lignin degrading enzyme (Ander & Eriksson 1976). In vitro experiments have shown that it acts primarily in a polymerising mode though there is evidence that in conditions of low water activity or in the presence of hydrogen peroxide laccase, like peroxidase, is effective in degrading polymeric lignin (Evans 1985a; Dordick et al. 1986). Its depolymerising activity on lignin-model phenolic compounds has been clearly demonstrated (Kawai et al. 1988).

Cellulolytic enzymes have been much studied in *Sporotrichum pulverulentum* (imperfect form of *P. chrysosporium*) and *T. versicolor* (Eriksson & Pettersson 1975; Pettersson & Porath 1963; Evans 1985b). A complex of endocellulases, exocellulases and β-glucosidases have been isolated and characterised with hydrolytic activities, effecting cleavage of cellulose microfibrils. In addition, two oxidative enzymes, cellobiose quinone oxidoreductase and cellobiose oxidase have been characterised from cultures of *P. chrysosporium.* These proteins are involved in the final stages of cellulose depolymerisation and may link phenolic oxidation with cellulose degradation (Westermark & Eriksson 1974; Ayers et al. 1978; Morpeth & Jones 1986). Enzymes degrading hemicellulose have been isolated from a few basidiomycetes, but relatively little is known about these enzymes in white-rots. Demonstration that individual enzymes cleave lignin and cellulose in vitro is essential to understanding the enzymic mode of action on lignin and cellulose. However, lignocellulose within a wood cell wall is a highly integrated complex substrate bound together to produce a barrier impermeable to pests and pathogens and their enzymes, while permeable to water and other small molecular weight compounds (Cowling 1975).

There has been much interest in the theory of a small molecular weight mediator which could penetrate the pores of the wood cell wall and effect lignin degradation at some distance from the hyphae (Harvey et al. 1986). Such a molecule could be veratryl alcohol, a secondary metabolite secreted by the hyphae of *P. chrysosporium,* which is capable of reacting with lignin peroxidase to produce a cation radical. Diffusion of the cation radical of veratryl alcohol into the wood cells could effect breakdown and open up the pore size of the cells sufficiently to allow the enzymes to penetrate directly. The major question mark over this hypothesis is whether the cation radical has a sufficiently long half-life to survive diffusion and penetration.

Electron microscopic techniques

A new approach to solve the problem of whether lignocellulotytic enzymes penetrated into wood cells was required. Electron microscopy has been used extensively to elucidate the morphology of white-rot decay. It was known that although deep erosion troughs were frequently observed in the wood cell wall adjacent to hyphae lying in the cell lumen, a more diffuse thinning of the secondary wall of the cell occurred suggesting selective delignification by some species of white-rot at a distance from the hyphae in the cell lumen (Ruel et al. 1981; Blanchette 1984; Otjen & Blanchette 1986; Messner & Stachelberger 1984).

Conventional staining techniques have failed to locate specific enzymes but development of immunocytochemical labelling of ultra-thin sections of hyphae enabled individual proteins to be located using transmission electron microscopy (TEM) (Horisberger 1979; Atkey & Wood 1987). The method of choice for enzyme localisation has been a post-embedding immunogold cytochemical labelling because of the specificity, reproducibility and range of applications of the technique. Colloidal gold is a negatively charged hydrophobic colloid which will adsorb to macromolecules such as antibodies, polysaccharides and lectins via non-covalent binding. The size of the gold particles can be varied within a range of 1 to 150 nm diameter (Horisberger 1984) but all are electron dense and therefore readily visible under the TEM. The gold particles are adsorbed onto antibodies raised to specific proteins and can be used in a simple one-step labelling procedure of sections. A two-step method is however preferred, whereby the gold particles are attached to secondary antibodies or to Protein A, both of which will bind to the primary antibody raised to the protein of specific interest. As the gold granules are particulate and of a specific size eg. 10 nm or 15 nm diameter, a fine localisation and good identification of labelled structures is possible. One of the major problems encountered with this technique is that of non-specific labelling. It is necessary to use a variety of adequate controls to ensure that the protein to be localised will be the only labelled component on the sections. Straightforward controls include the omission of the primary antibody or replacement of the primary antibody with one raised to a different protein occurring in a diverse species. These controls normally present no spurious non-specific labelling, but are not sufficient to ensure that only the protein of interest is labelled by the gold-antibody complex.

All the lignocellulolytic enzymes are glycoproteins, and in raising antibodies the polysaccharide moieties are likely to be just as immuno-reactive as the protein components (Pazur 1986). Therefore it is likely that using polyclonal antibodies immunoreactivity will be apparent between polysaccharides of the correct configuration as well as with the relevant proteins. With fungal enzymes secreted from hyphae through an extracellular polysaccharide layer (slime or mucilage) it is to be expected that some wall material could attach to the glycoprotein. This would therefore give rise to labelling of fungal cell wall polysaccharides by the antibodies binding to polysaccharide moieties in addition to any protein molecules in the sections. This can be overcome by ensuring that the primary antibodies are raised to deglycosylated proteins of high purity or at least to proteins with a minimal carbohydrate component. It is also essential that the immunoreactivity of the antibody is determined against the protein to which it has been raised. Western blotting presents the most straightforward method to determine this immunoreactivity (Towbin et al. 1979). Figure 1 shows the immunoreactivity of polyclonal antibodies raised separately against lignin-peroxidase, laccase, and β-glucosidase purified from liquid cultures of *T. versicolor.*

Another cause of spurious labelling may arise from non-specific binding of the secondary antibody. This problem can be resolved by blocking as many of these sites in advance of treatment with the antibodies. Good blocking agents have been found to be bovine serum albumin, gelatine, or normal serum from a heterologous animal species to the primary antibody source. To determine if this type of non-specific labelling occurs it is necessary that sections of tissues from unrelated fungal species are processed in the same manner as the experimental sections. Only when all these controls have proved

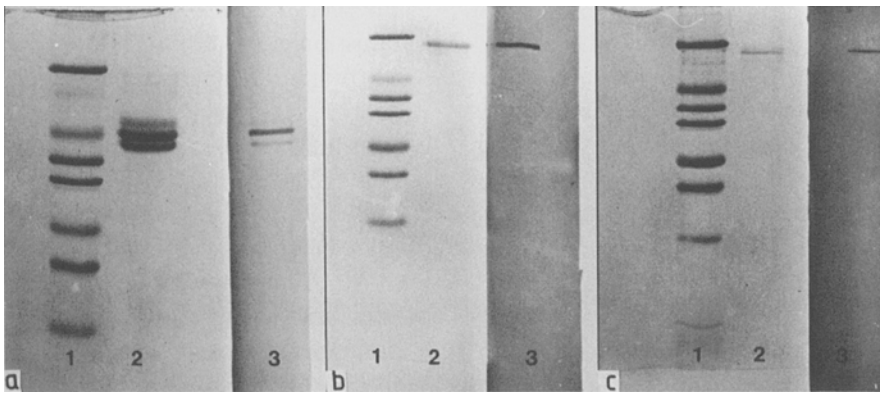

Fig. 1. Polyacrylamide gels and Western blots of a) antibodies to lignin peroxidase from *P. chrysosporium* reacting with ligninase isolated from *P. chrysosporium;* b) antibodies to laccase from *T. versicolor* reacting with purified laccase from *T. versicolor;* c) antibodies to β-glucosidase isolated from *T. versicolor* reacting with purified β-glucosidase from *T. versicolor*. Track 1 protein markers; Track 2 SDS-PAGE of isolated proteins; Track 3 Western blots.

negative in their response to the gold-labelling technique can labelling of the experimental tissues be deemed specific. Some earlier studies localising fungal enzymes had not recognised these difficulties, and some early reports of localisation may therefore require further validation.

Localisation of lignin-peroxidase

Polyclonal antibodies to lignin peroxidase were all raised in rabbits to different preparations of the antigen isolated from liquid cultures of *P. chrysosporium* as shown in Table 1. Only three different polyclonal antisera were used by the five laboratories quoted; one raised to the H8 isomer of lignin peroxidase isolated by Kirk et al. (1986), one

Table 1. Lignin peroxidase antibodies used in immunogold-labelling. Lignin peroxidase (antigen) was isolated from *Phanerochaete chrysosporium* BKM-F-1767 (ATCC 24725) in all cases.

Antigen	Antibody	Cross reactivity with antigens in other fungal species		Reference
		Positive	Negative	
Unspecified isomer	Polyclonal?	*P. chrysosporium*	*Pleurotus ostreatus* *Aspergillus fumigatus* *Candida albicans*	Garcia et al. 1987
H8 isomer	Polyclonal Kirk*	*P. chrysosporium*	*Trametes hirsuta*	Srebotnik et al 1988a
H8 isomer	Polyclonal Kirk*	*P. chrysosporium*	–	Daniels et al. 1989
(i) H8 isomer	Polyclonal Kirk*	*P. chrysosporium*	–	Blanchette et al. 1989
(ii) H2 isomer	Monoclonal Kirk*	*T. versicolor* *P. pini*	–	Blanchette et al. 1989
44KD band on SDS-PAGE gel	Polyclonal Gallagher	*P. chrysosporium* *T. versicolor*	*Agaricus bisporus* *Trichoderma viride* *Pythium oligandrum*	Gallagher et al. 1989 Gallagher 1989

* Kirk et al. 1986.

to a ligninase purified from carbon-limited cultures (Leisola et al. 1985) and one to an isomer of lignin-peroxidase isolated from nitrogen-limited cultures with a molecular weight of 44 KD on an SDS-PAGE gel (Gallagher et al. 1989). In addition a monoclonal antibody raised to the H2 isomer of lignin-peroxidase (Kirk et al. 1986) was used by Blanchette et al. (1989a).

The first attempted localisation of lignin-peroxidase in hyphae of *P. chrysosporium* used an antiserum-protein A-gold complex to visualise the enzyme molecules (Garcia et al. 1987). Protein A-marker conjugates provide visual tags based on the ability of protein A to bind specifically to rabbit immunoglobulin G molecules (replacing the secondary antibody in the procedure outlined previously). The enzyme was labelled intracellularly in the hyphae, but was not visualised extracellularly. Even when unfixed samples of *P. chrysosporium* grown on poplar sawdust were incubated with antibodies to lignin peroxidase and visualised by immunofluorescence with the secondary antibody conjugated to fluorescein isothiocyanate, no labelling of lignin-peroxidase outside the hyphae was observed (Garcia et al. 1987). In addition there appeared to be no binding of the enzyme to the sawdust particles, though binding was observed between lignin-peroxidase and milled wood lignin. The control experiments used to check the specificity of the method in these studies are shown in Table 2.

The use of the secondary antiserum-gold complex method confirmed the labelling of the same lignin peroxidase in hyphae of *P. chrysosporium* grown in liquid culture and on pine wood. It also showed that the cytoplasmic localisation was close to the plasmalemma with some binding to the membrane itself (Srebotnik et al. 1988a). Labelling of the hyphal cell wall was absent. However on changing the fixation of the sections from glutaraldehyde treatment to fixation with picric acid, extracellular lignin-peroxidase was observed in the cell wall and was found distal from the hyphae with a continuous decrease of concentration with increasing distance from the hyphae. Further work investigated the degree of penetration of lignin-peroxidase into degraded pine wood (Srebotnik et al. 1988b). By infiltrating pine wood which had been previously rotted by *P. chrysosporium* with concentrated culture fluid from a ligninolytic liquid culture, it was possible to detect sites of binding of the enzyme to the wood cell walls. Heavily degraded areas of the wood were observed to bind lignin-peroxidase in the highest density. No diffusion of the enzyme occurred into the undecayed parts of the cell wall. This observation was confirmed in birch wood degraded by *P. chrysosporium* (Daniel et al. 1989).

Further studies of birch wood infected separately with *P. chrysosporium* and *Phellinus pini,* both white-rots which selectively remove lignin from cell walls, showed lignin-peroxidase to be located both intra and extracellularly in the hyphae. It was also concentrated in the still highly lignified areas of the wood cell wall (Blanchette et al. 1989a). Protein A-gold was used in these experiments rather than the secondary antibody-gold complex. Comparable studies of beech wood rotted by *T. versicolor* have shown that lignin-peroxidase is located on the cell wall throughout the decayed secondary wall but not in the undecayed middle lamella and cell

Table 2. Summary of published experimental controls used in immunogold labelling of lignin peroxidase.

Reference	No primary antibody	Pre-immune serum	Primary antibodies raised to proteins from other sources	Antibody pre-adsorbed to lignin peroxidase
Garcia et al. 1987		+		
Srebotnik et al. 1988a, 1988b	+	+	unspecified	
Daniels et al. 1989	+		unspecified bacterial	
Blanchette et al. 1989	+	+		+
Gallagher et al. 1989	+	+	Plant phytochrome ex *Avena sativa,* mannitol dehydrogenase, White bryony mosaic virus	

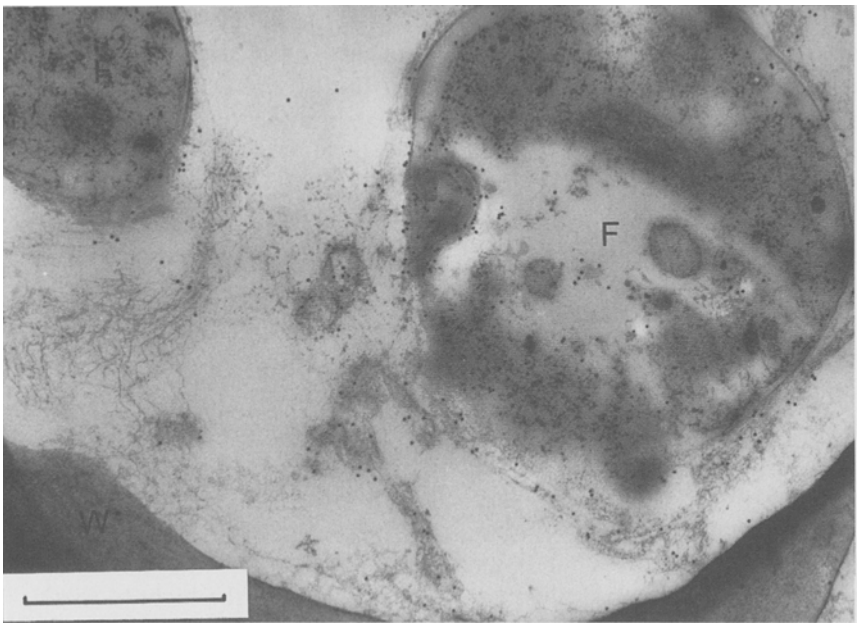

Fig. 2. Ligninase, visualised by black gold particles, localised in a hypha of *T. versicolor* in beech wood infected for 4 weeks. Bar marks equivalent to 1 μm. F = fungal cell. M = extracellular mucilage. W = wood cell wall.

corners (Gallagher et al. 1989). In hyphae of *T. versicolor* labelling of lignin-peroxidase was found to be associated with the plasmalemma and extracellular layers of the hyphal wall as shown in Fig. 2. These experiments used the secondary antibody-gold complex to visualise the enzyme and demonstrated that there is cross-reactivity between the antibodies raised to lignin-peroxidase from *P. chrysosporium* and the enzyme in other white-rot fungi. Confirmation of this cross-reactivity has been observed by the authors for several species of white rot fungi whose proteins show an immunoreactive band on Western blotting (Gallagher 1989, Evans et al. 1990). The different types of control tissues and antibodies used in the gold-labelling experiments by all workers are shown in Table 2.

These studies show that lignin peroxidase can be visualised in hyphae close to the plasmalemma, in the hyphal cell wall, in the extracellular mucilage layers and diffusing into either culture media or onto wood cell wall surfaces (Fig. 2). The highest density of enzyme molecules are usually located around areas of greatest decay within the wood cell wall and are perhaps attached to fragments of depolymerised lignin which are too small for detection by electron microscopy. Some enzyme molecules can be seen over the cell wall surface, but undecayed parts of the wall such as the middle lamella and cell corners only become labelled when access to the enzyme has been fully opened up by removal of lignin from the secondary wall.

Comparisons between observations from different laboratories is confused by the difficulties in determining the time span of the decay process within an individual cell, and the extent of decay in different types of woods. There is scarce information from biochemical measurements of enzyme activity on the distribution of lignin peroxidase in solid media as compared with liquid cultures, and intracellular versus extracellular levels in hyphae grown in liquid cultures. Until this data is available the immunogold-labelling studies will remain unconfirmed.

Binding of substrate and enzyme has been observed by immunocytochemical labelling. There appears to be a lesser degree of binding between polymeric lignin and enzyme in intact cell walls than between enzyme and isolated milled wood lignin, or between enzyme and substrate in regions of extensive wall decay. It is assumed that in the

latter region there may be release of small depolymerised lignin fragments. Differences in the structure of lignin laid down in the secondary wall and in the middle lamella have been reported (Goring 1990). Secondary wall lignin may consist of a relatively lower molecular weight polymer with the aromatic rings aligned tangentially to the secondary wall, whereas middle lamella lignin is thought to be a more random three-dimensional polymer (Goring 1990). Immunogold labelling shows that lignin-peroxidase attachment to secondary wall lignin appears to be a more commonplace event than attachment to lignin of the middle lamella. These observations give support to the biochemical studies which have shown greater degradation activity in depolymerisation of lignin model compounds than with polymeric lignin and lignocellulose. Possible reasons for this may be the inaccessibility of cell wall lignin to the enzyme, though in sections cut for microscopy lignin would be revealed within the lignocellulosic matrix sufficient to bind peroxidase if the enzyme readily bound to polymeric lignin. The concentration of the enzyme in highly degraded regions of the cell wall suggests the enzyme may bind to smaller lignin fragments within the wall compared with no binding to the larger lignin polymer in the middle lamella. The enzyme readily binds to milled wood lignin which has a molecular weight in the order of 5000 d.

Localisation of laccase

Immunogold-labelling of laccase in hyphae of *T. versicolor* has shown it to be localised mainly in the cell wall and extracellular mucilage layer around the hyphae with a small amount localised intracellularly, though unlike lignin peroxidase it is not associated with the plasma membrane (Gallagher et al. 1989). The antiserum used in these studies was raised to a highly purified laccase of molecular weight 64 Kd, isolated from *T. versicolor*. Qualitative biochemical assays measuring catechol oxidase activity, in *T. versicolor* have shown that laccase is present both intra- and extracellularly in hyphae grown on malt agar (Gallagher 1989).

In beech heartwood degraded by *T. versicolor* labelling of the wood cell wall only occurred in already degraded regions of the secondary wall, and was not visualised in the middle lamella (Gallagher et al. 1989). Also diffusion of laccase away from the hyphae through the mucilage layer into the cell lumina was observed. Although there was some over-labelling on the original micrographs, further work with reduced gold labelling has confirmed these observations as shown in Fig. 3. The control tissues and antisera used in these studies are shown in Table 3.

Table 3. Antibodies to laccase, xylanase and β-glucosidase used in immunogold labelling.

Antigen	Antibody	Cross-reactivity with antigens in other fungal species		Reference
		Positive	Negative	
Laccase A (62 KD) from *T. versicolor*	Polyclonal	*T. versicolor* *A. bisporus*	*P. chrysosporium* *P. oligandrum* *T. viride*	Gallagher et al. 1989
Xylanase from *A. pullulans*	Polyclonal	*P. chrysosporium* *T. versicolor* *P. pini* *Fomitopsis pinicola*		Blanchette et al. 1989
β-glucosidase (60 KD) from *T. versicolor*	Polyclonal	*T. versicolor* *A. bisporus*	*T. viride* *P. oligandrum*	Gallagher & Evans 1990

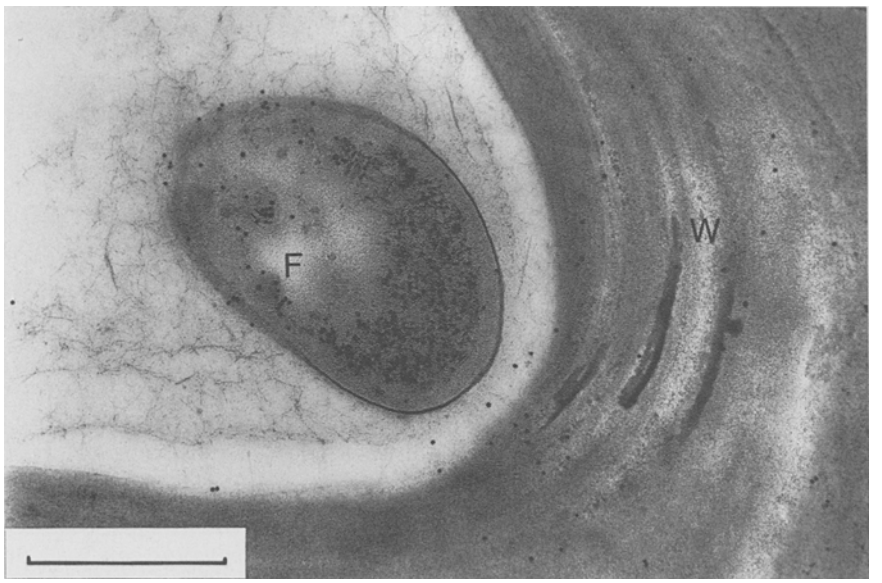

Fig. 3. Laccase localised in a hypha of *T. versicolor* in beech wood infected for 4 weeks. Bar marks equivalent to 1 μm. F = fungal cell. M = extracellular mucilage. W = wood cell wall.

Localisation of cellulases

The first studies to localise cellulases in hyphae of *P. chrysosporium* by immunogold-labelling used polyclonal antisera raised to a crude mixture of fungal glycohydrolases (Joseleau & Ruel 1989). The multi-enzyme preparation, prepared by ammonium sulphate precipitation of proteins from *P. chrysosporium,* contained both endo- and exo-glucanases with trace amounts of hemicellulases, so the antisera would contain antibodies to all these proteins. Localisation of the fungal glycohydrolases was in dense vesicles intracellularly, and appeared to diffuse from the hyphae only over a very limited distance but always within the mucilage layer secreted by the hyphae. The enzymes were localised in the wood cell walls of aspen (*Populus tremula*) rotted with *P. chrysosporium* only in regions where degradation of the wall was clearly visible.

Further independent studies to show the binding of cellulases to cellulose in wood cell walls have used colloidal gold-enzyme complexes made with proteins purified from *Trichoderma reesei* having endo-1,4-β-glucanase (EG11) and 1,4-β-D-glucan cellobiohydrolase 1 (CBH1) activities (Blanchette et al. 1989b). These complexes were used to demonstrate the extent of interaction between the enzymes and their substrate in sections of birch wood blocks degraded by *P. chrysosporium, Phellinus pini* and *T. versicolor,* respectively. In sound wood sections, both EG11 and CBH1 were localised on the sides of the microfibrillar structures with CBH1 also attached to the cut ends of the microfibrils. Little labelling was seen in the middle lamella though parts of the intercellular regions within the cell corners of the middle lamella were labelled with EG11.

In contrast, in the degraded wood sections which were well delignified and showed degradation of the middle lamella, both EG11 and CBH1 gold complexes labelled the secondary wall of the wood cells, with CBH1 having a lowest concentration of the S_2 layer near the cell lumen where the most extensive degradation had occurred. With wood degraded by *T. versicolor* which causes a non-selective decay, labelling of the wall layers with EG11 and CBH1 was greatest around sites of erosion, although a small amount of labelling occurred throughout the secondary wall layers.

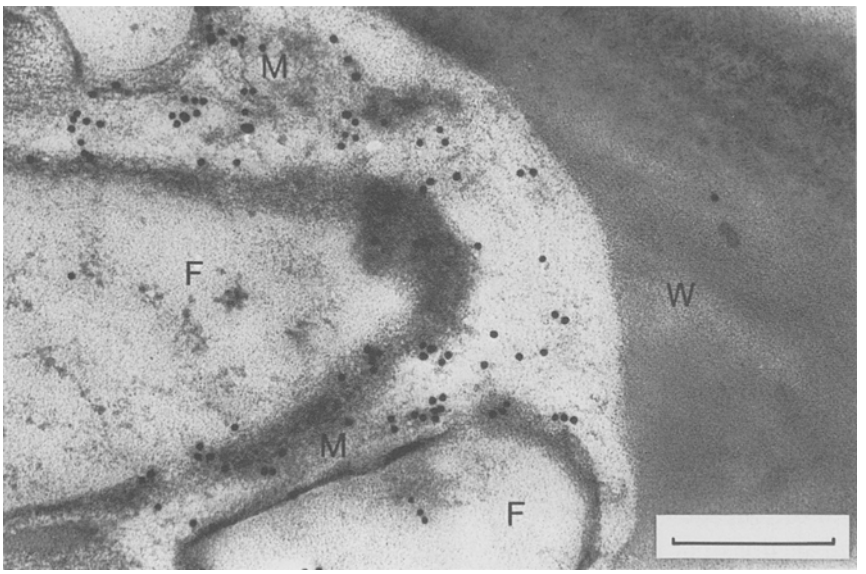

Fig. 4. β-Glucosidase localisation in a hypha of *T. versicolor* in beech wood infected for 4 weeks. Bar marks equivalent to 1 μm. F = fungal cell. M = extracellular mucilage. W = wood cell wall.

Localisation of β-glucosidase secreted by hyphae of *T. versicolor* has been shown using the immunocytochemical-gold labelling technique (Gallagher & Evans 1990). The antisera used were raised to β-glucosidase purified from cultures of *T. versicolor* and in conjunction with a gold-labelled secondary antibody demonstrated the extent of diffusion of the protein from the fungal hyphae. In all culture conditions from agitated liquid cultures to stationary wood block cultures, the enzyme was localised primarily in the hyphal cell wall and extracellular mucilage layers with some evidence of intracellular labelling in the hyphae. Labelling of β-glucosidase was not present beyond the extent of the mucilage layer, and the enzyme did not appear to be present on the microfibrillar surface even when the wood cell wall showed extensive decay, as shown in Fig. 4. There was effective immobilisation of the enzyme around the hyphae in a position that would ensure that molecules of cellobiose (1,4-β-D-glucan dimer) released by the combined action of EG11 and CBH1 on the microfibrillar cellulose were readily hydrolysed to glucose for uptake by the fungus. This pattern of localisation was confirmed by quantitative biochemical studies of enzyme activity in hyphae grown in liquid cultures in cellulose media (Gallagher & Evans 1990).

Localisation of hemicellulases

Enzyme-gold complexes were used by Ruel & Joseleau (1984) to localise hemicelluloses in plant cell walls, but the first use of immunocytochemical labelling to localise specific enzymes involved in hemicellulose degradation was reported by Blanchette et al. (1989a). Antisera to xylanase purified from *Aureobasidium pullulans* was used to detect the enzyme in sections of hyphae of *P. chrysosporium* and *T. versicolor* grown on birchwood blocks. There was considered to be high homology in the amino acid sequences of xylanases from a number of taxonomically distant sources, which would enable xylanases produced by *P. chrysosporium* and *T. versicolor* to react with the antisera (Leathers 1988). Supporting evidence for cross-reactivity using Western blotting was not reported. Xylanase was localised within the secondary wood cell wall of infected wood with gold particles found both in the decayed zones of the wood cell wall and around

the hyphae. There was no localisation of xylanase in the middle lamella where it was undecayed. In sections showing very advanced delignification, gold labelling of xylanase was not evident. Chemical analysis has shown that up to 70% of xylan is lost from decayed wood (Blanchette & Abad 1988).

Further studies of birchwood rotted by the same fungi used a xylanase-gold complex to locate xylan in the wood cell wall (Blanchette et al. 1989b). This was to determine the availability of the substrate for xylanase at varying stages of the decay process. In partially decayed wood sections the inner regions of the secondary wall were labelled, both in the S_2 and S_1 layers and in the middle lamella. Intercellular regions at cell junctions were heavily labelled in undecayed sections but little labelling occurred after extensive decay. In advanced stages of infection with *T. versicolor,* less gold labelling was observed around well-rotted areas than in unaffected parts of the cell wall. This complements the studies using antisera to xylanase to locate sites of binding between enzyme secreted by hyphae in vivo and its substrate in decayed wood, which showed the progressive removal of xylan from the secondary wall as the extent of decay increased. Table 3 shows the tissues and antisera used for these experiments.

Discussion

The immunocytochemical approach using TEM has provided some answers to the distribution of lignocellulolytic enzymes within wood decayed by white-rot basidiomycetes. It is also possible to correlate some of the findings with biochemical phenomena of lignin and cellulose degradation.

Authenticity and accuracy of the immunocytochemical techniques depends on several factors including the use of adequate controls in the gold-labelling procedure, and the immunospecificity of the antibodies to the enzymes under investigation. Polyclonal antibodies raised against glycoproteins may not be specific only to the protein moiety as the carbohydrate frequently shows as much antigenicity. The problem of such non-specific reactions can be addressed by using monoclonal instead of polyclonal antibodies, selected for their specificity to certain amino acid sequences in the protein. Alternatively, polyclonal antibodies can be raised against a synthetic polypeptide, identical to the N-terminal sequence of the protein of interest, or any region of high predicted antigenic activity if the whole sequence is known.

Not all of the studies reported in this review used antibodies specifically raised to proteins purified from the fungi under investigation, but frequently used similar enzymes from other fungi to raise the antibodies. This approach is only acceptable if a sufficiently high level of cross-reactivity between the antibody and protein of interest has been demonstrated by Western blotting or immunobinding techniques. In our experience the β-glucosidases of basidiomycete fungi show a high degree of homology in their structure as Western blotting of proteins from several white rot fungi against antibodies raised to β-glucosidase from *T. versicolor* have usually given positive results. Blanchette et al. (1989a) also commented on the wide range of fungal species in which xylanases showed homology in their amino acid sequences, though evidence of cross-immunoreactivity was not reported. It is perhaps surprising that some white rot fungi which are known to be ligninolytic do not show cross-reactivity in the immunogold-labelling studies with the antisera to lignin peroxidase from *P. chrysosporium* (Table 1), as proteins from other related species show cross-reactivity on Western blotting with a similar antibody (Evans et al. 1990). However, proteins from the ligninolytic edible mushroom, *Agaricus bisporus* do not show any cross-reactivity on Western blotting with the antibody to lignin-peroxidase from *P. chrysosporium* (Gallagher 1989) although there is strong cross-reactivity with the antibody to laccase from *T. versicolor.*

The localisation of lignin-degrading enzymes secreted from hyphae in wood cells has shown that relatively little binding of lignin peroxidase or laccase can be seen in undegraded parts of the wood cell wall. Once degradation has been initiated more of the enzyme molecules can be located in the secondary wall, while in areas of advanced decay accumulation of the enzymes is at its highest level.

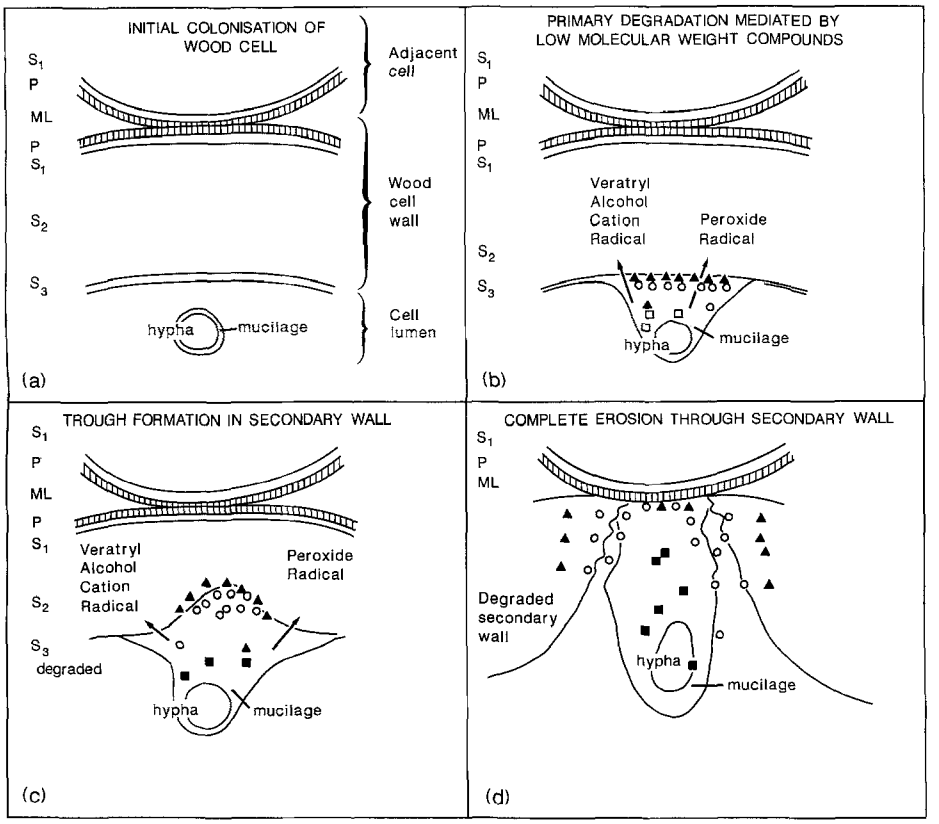

Fig. 5. Distribution of lignocellulolytic enzymes in white rot decay of wood. ML Middle lamella; P Primary wall; S_1 First layer of secondary wall; S_2 Second layer of secondary wall; S_3 Third layer of secondary wall; ▲ endo- and exoglucanases; ○ ligninases and laccase; ■ β-glucosidase.

This suggests that as lignin-peroxidase is not able to penetrate into the undegraded cell wall, it is most effective in degrading lignin fragments released from the wall at decay sites. These fragments may be of similar size to extracted milled wood lignin which is able to bind lignin peroxidase very effectively. There is little data from biochemical studies to complement and support the intra and extracellular distribution of lignin peroxidase in hyphae of white rot fungi, with most studies concentrating on enzyme activity in extracellular media in liquid cultures. For laccase the biochemical studies have given some qualitative support to the findings reported from immunogold-labelling.

Localisation of the endo- and exoglucanases secreted by white-rot fungi show that an endoglucanase is able to bind to cellulose microfibrils in the intact wood cell wall, whereas an exoglucanase binds primarily to the ends of the fibrils. As decay progresses more of the enzymes are localised in the secondary wall, and considerably fewer in the advanced stages of degradation. In contrast β-glucosidase is never found attached to the wood cell wall, but always immobilised in the mucilage layer around the hyphae. Biochemical data strongly support this observation (Gallagher & Evans 1990).

Hypothesis

These observations suggest that there is a regulation of the release sequence of the lignocellulolytic enzymes which may define their spatial arrangement between the fungal hyphae and the wood cell wall. This release pattern and localisation may enable the process of degradation to progress and be

regulated in an ordered manner in terms of cellular economy for enzyme production and nutrient uptake. A diagrammatic representation of the suggested distribution of enzymes within an infected wood cell is shown in Fig. 5.

It is postulated that none of the enzymes currently identified as being involved in the degradation of lignin or cellulose initiate the process of decay as none were localised to any significant extent in the undecayed wall and middle lamella. The initiators of both cellulose and lignin breakdown are most probably small molecular weight molecules which can readily diffuse away from the hyphae and penetrate through the pores into the lignocellulosic matrix. It has already been postulated that a mediator molecule such as the cation radical of veratryl alcohol could be involved as an agent of lignin depolymerisation, remote from the enzymes secreted by the hyphae (Harvey et al. 1986). The distribution of lignin peroxidase and laccase in infected wood sections supports the concept that they are not involved in initiating decay, except possibly at the lumen surface.

Similarly cellulose degradation may be initiated by a low molecular weight mediator which can penetrate into the pores of the cell wall matrix. The most probable candidate for this molecule is hydrogen peroxide. There is strong evidence that in the digestion of cellulose, oxidised sugars are produced and oxidation at the ends of cellulose chains occurs, by the action of peroxide radicals (Veness & Evans 1989; Vaheri 1982). We propose that free radicals from hydrogen peroxide initiate cellulose breakdown so that subsequent hydrolysis by glucanases occurs mainly in partially degraded areas. The terminal step of hydrolysis of cellobiose to glucose takes place entirely within the mucilage layer surrounding the hyphae. Generation of hydrogen peroxide for this mechanism is possible by the action of glyoxal oxidase, an extracellular enzyme considered to be the major provider of hydrogen peroxide for lignin peroxidase (Kersten 1990). Other enzymes such as glucose oxidases may also be involved but as intracellular enzymes would need to secrete the hydrogen peroxide formed, it is less probable they are involved. White rot fungi have extracellular catalase activity which may be localised to control destruction of peroxide as it diffuses from the hyphae (Veness & Evans 1989). Further work is needed to determine if this hypothetical model of enzyme localisation in vivo is correct.

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