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Ultrastructure of wood cellulose substrates during enzymatic hydrolysis

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Summary. The ultrastructure of holocellulose and alpha-cellulose from *Pinus radiata* D. Don was examined after treatment with cellulase for either 24 hours or 7 days. A procedure for localising the enzyme *in situ* using phosphotungstic acid is described. The pattern of degradation differed between the two substrates with alpha-cellulose being much more susceptible to hydrolysis than holocellulose. In both substrates the primary wall showed evidence of hydrolysis after 24 hours and was completely hydrolysed after 7 days. In holocellulose after 7 days treatment, hydrolysis of the secondary wall was confined to localised areas of the S3 layer sometimes penetrating into the S2 region. In contrast, alpha-cellulose showed uniform degradation across the secondary wall. Enzyme did not appear to penetrate the holocellulose but full penetration occurred in alpha-cellulose, the enzyme being closely associated with individual microfibrils. The reasons for these differences in degradation pattern are discussed.

Introduction

The conversion of wood cellulose to glucose and its subsequent fermentation to ethanol for use as a liquid fuel is the subject of considerable research world wide (Wilkie 1975; Saddler et al. 1984; Takai et al. 1985). Wood in its natural state is difficult to hydrolyse with enzymes because access to the cellulose is restricted by matrix substances such as lignin and hemicellulose (Cowling 1975). Pretreatments such as delignification (Cowling 1975), vibratory ball milling (Koshijima et al. 1983), steam explosion (Mackie et al. 1985), or microwave irradiation (Azuma et al. 1985) can enhance enzymatic hydrolysis of wood based substrates (Takai et al. 1985).

The ultrastructural aspects of enzymatic hydrolysis have received little attention in the literature. Hydrolysis often begins in the vicinity of damaged parts of the fibre wall (Eriksson et al. 1969; Vevere et al. 1983). The S1 and S3 layers of the cell wall are reported to be more resistant to degradation than the S2 layer of the secondary wall or the primary wall (Boutelje, Hollmark 1972; Sinner et al. 1976).

In a study of wheat straw pulp fibre, Betrabet and Paralikar (1977) found a reduction in cellulose microfibril width from 9.8 nm to 6.6 nm following treatment with cellulase. White (1982) observed cellulase molecules bound to bacterial cellulose ribbons and noted thinning of microfibrils during hydrolysis.

This report examines ultrastructural aspects of the enzymatic hydrolysis of holocellulose and alpha-cellulose to enable understanding of the limiting factors involved in this process.

Materials and methods

Holocellulose was prepared by treating *Pinus radiata* wood chips with a 1 : 1 mixture (v/v) of glacial acetic acid and hydrogen peroxide (per-acetic holocellulose), at 80–90 °C for 6–8 hours till fully bleached. After washing, part of the holocellulose was converted to alpha-cellulose by treating with 17.5% NaOH for 1 hour at room temperature (Uprichard 1965). After washing, substrates were stored in distilled water at 4 °C until required.

Cellulase (1,4-[1,3;1,4]-β-D-Glucan 4-glucanohydrolase; EC 3.2.1.4) from *Aspergillus niger* (Sigma Chemical Co.) (1.3 Units/mg solid) was made up as a 1% (w/v) solution in citrate buffer at pH 4.8. Substrates were treated for either 24 hours or 7 days at 30 °C. For the 7 day treatment the enzyme solution was changed every other day (Vallandar, Eriksson 1983; Paralikar, Bhatawdekar 1984). In an attempt to localise enzymes *in situ* several experiments were carried out with the intention of fixing the enzyme in or on the substrate. Conventional fixation with glutaraldehyde/osmium was found to be of little use. Addition of various reagents to enzyme solutions, either in citrate buffer or in distilled water, indicated that 10% phosphotungstic acid (PTA) in 10% HCl was a useful enzyme precipitating agent and this proved useful in localising enzyme during examination of the substrate.

Substrates treated with enzyme were placed in PTA solution immediately after removal from the enzyme solution without washing. After treating for 5 minutes at room temperature specimens were then washed gently in distilled water, dehydrated in an acetone series and embedded in Spurrs resin. Control specimens with no enzyme treatment were prepared in the same way but either with or without PTA treatments.

Ultrathin sections were obtained with a diamond knife using an L.K.B. ultramicrotome and stained with either uranyl acetate/lead citrate (30 min/5 min), or $KMnO_4$/lead citrate (2 min/2 min). Sections were examined in a Philips 300 transmission electron microscope.

Results

Enzyme substrate interaction

Figure 1 a shows the inner cell wall of a holocellulose fibre. The S3 layer is stained intensely with $KMnO_4$/lead citrate, indicating high levels of acidic polysaccharides (hemicellulose) (Hoffmann, Parameswaran 1976). Figure 1 b shows a similar fibre which has been treated with cellulase for 24 hours and subsequently fixed in PTA. Enzyme is readily distinguished at the lumen boundary in the form of irregular masses bound to the wall. These structures were absent in the control specimens.

The contrast of the enzyme with $KMnO_4$/lead citrate is similar to the adjacent S3 layer of the substrate but is finer grained and lacks a fibrillar texture. Staining

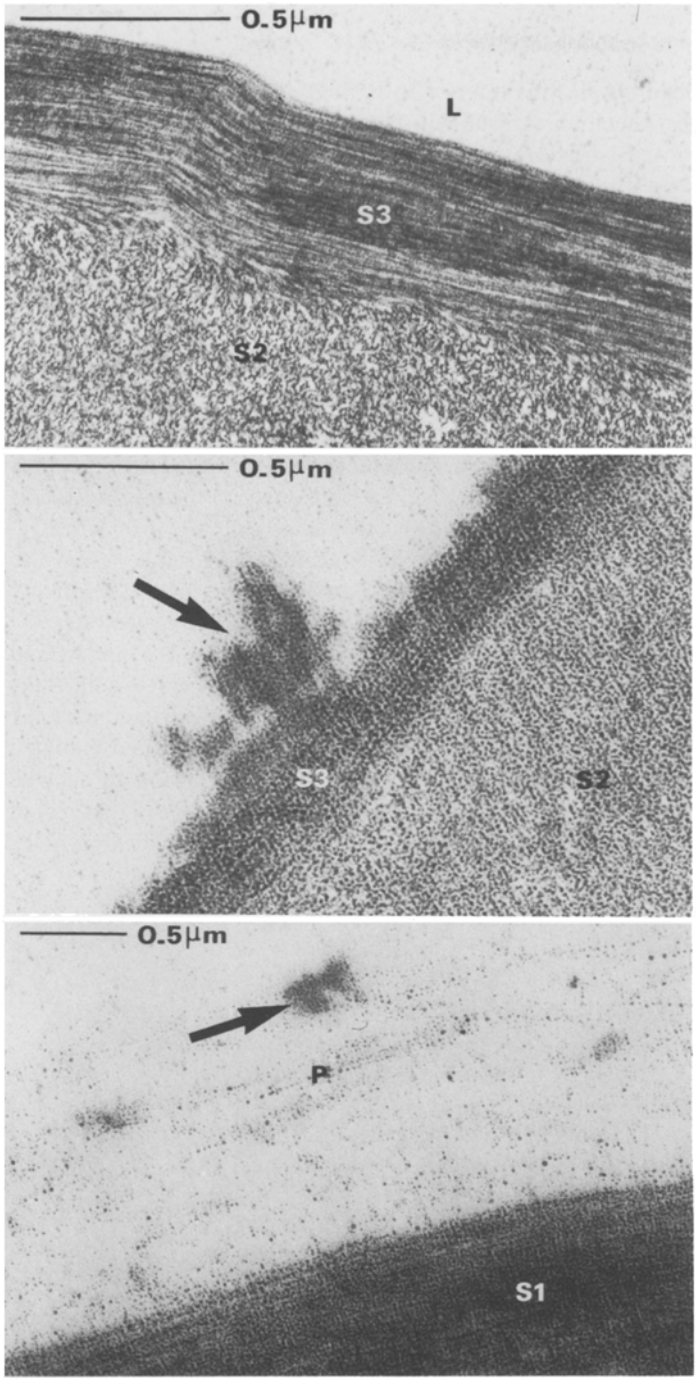

Fig. 1. **a** Holocellulose specimen with no enzyme treatment showing the clean lumen surface of the substrate. The S3 layer of the wall is densely stained with $KMnO_4$/lead citrate (L = lumen); **b** Holocellulose specimen treated with cellulase showing enzyme precipitates (arrow) bound to the lumen surface of the substrate; **c** Holocellulose specimen treated with cellulase showing cellulase precipitate within the primary wall (arrow). The densely stained S1 layer of the secondary wall is clearly distinguished from the highly porous primary wall (P)

with uranyl acetate/lead citrate was inferior to $KMnO_4$/lead citrate and the later was used for the rest of the study. Unstained sections showed only slightly greater electron density in protein structures than in adjacent substrate and background areas suggesting that only relatively small quantities of PTA are complexed with the protein. Precipitation of enzyme solution with PTA in the test tube resulted in a whitish precipitate compared to the dark brown protein. This suggests that the reaction involves a complexing between the PTA and enzyme rather than a pH dependent denaturation. Precipitation occurs in both aqueous and buffered solutions indicating that the precipitate does not involve a reaction between PTA and buffer salts.

Enzyme was bound to both inner (S3) (Fig. 1 b) and outer (primary wall) (Fig. 1 c) surfaces of the holocellulose substrate and appeared to be closely associated with microfibrils. The enzyme did not penetrate the S3 layer to any detectable level but the primary wall contained enzyme down to the level of the S1 layer of the secondary wall. The S1 layer showed intense staining with $KMnO_4$/ lead citrate indicating high levels of acidic polysaccharides in this layer also (Fig. 1 c). These observations suggest that both S1 and S3 layers of the secondary cell wall represent barriers to enzyme penetration in the holocellulose substrate.

In alpha-cellulose substrates enzyme protein was localised at the lumen and outer wall surfaces after 24 hours treatment. No enzyme was observed within the secondary wall though it would be difficult to distinguish enzyme amongst the dense microfibril network. In the primary wall, microfibrils were heavily coated with enzyme suggesting enhanced substrate affinity. The secondary wall showed uniform contrast when stained with $KMnO_4$/lead citrate indicating the removal of polysaccharides from the S1 and S3 regions as a result of treatment with NaOH.

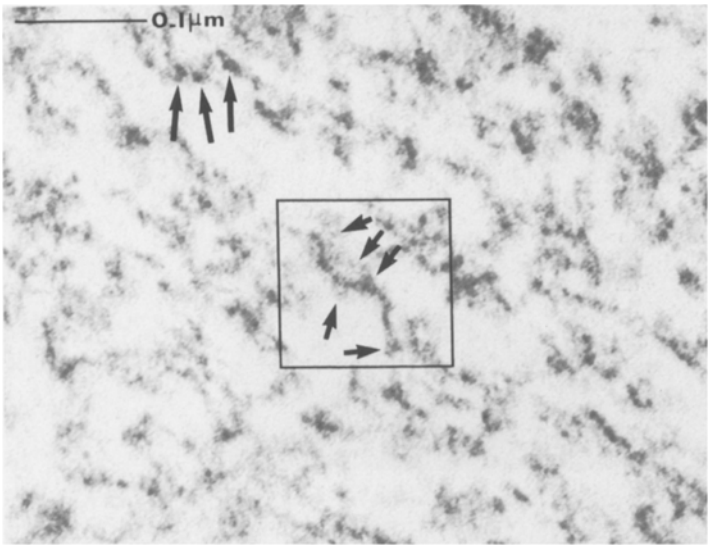

Fig. 2. An alpha-cellulose specimen showing cellulase precipitate bound to a cellulose microfibril (box) in the S2 layer of the wall (small arrows). Some microfibrils show a granular texture suggesting cleavage into shorter units by endoglucanase activity (large arrows)

After 7 days treatment with cellulase, enzyme could readily be located throughout the cell wall bound to the individual microfibrils (Fig. 2).

Topochemistry of cell wall degradation

The two substrates differed in both the location and the amount of degradation. For both substrates the primary wall had the greatest susceptibility to hydrolysis with complete solubilisation after 7 days treatment with cellulase.

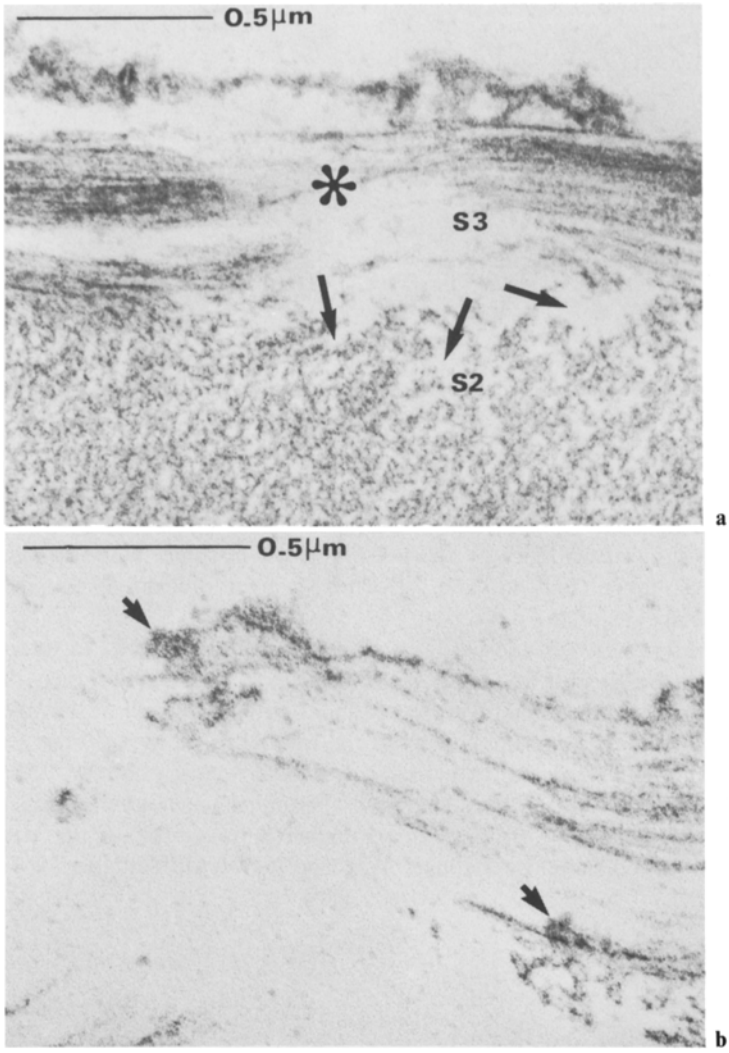

Fig. 3. **a** Holocellulose specimen treated with cellulase for 7 days showing hydrolysis of the S3 layer spreading into the adjacent S2 region (arrows); **b** Holocellulose specimen treated with cellulase for 7 days showing enzyme bound to the microfibrils of the S3 layer (arrows)

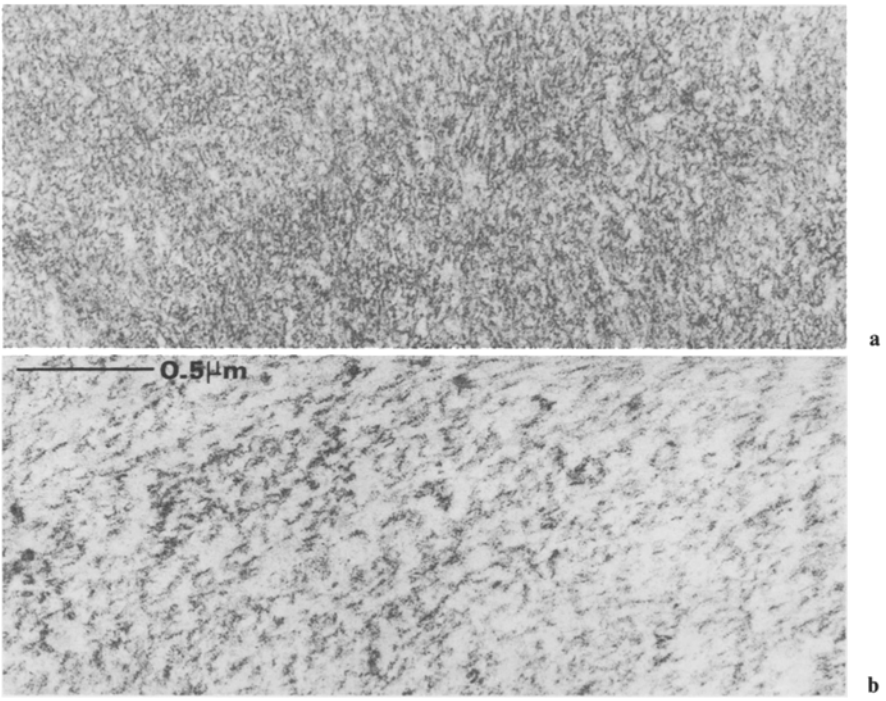

Fig. 4. a Alpha-cellulose without enzyme treatment; **b** with enzyme treatment (7 days), showing the increase in porosity as a result of hydrolysis by the cellulase enzyme

In holocellulose, no obvious degradation had occurred in any part of the cell wall after 24 hours but after 7 days small localised areas of the S3 layer of the secondary cell wall showed evidence of breakdown which in some places spread into the adjacent S2 layer (Figs. 3a and b). The S1 layer did not show any noticeable degradation even after 7 days.

In alpha-cellulose, minor wall loosening was observed after 24 hours of treatment, particularly in the S1 and S2 layers at the corners of cells. After 7 days of treatment major degradation was readily observed in all parts of the cell wall in the form of a considerable decrease in the density of the microfibril network (Figs. 4a and b). At higher magnification, individual microfibrils showed a granular effect (Fig. 2), suggesting cleavage into shorter lengths by endoglucanase activity. Intact microfibrils were approximately 5–10 nm in width which is similar to the size reported for normal wood cellulose (Hodge, Wardrop 1950; Mühlethaler 1965; Kerr, Goring 1975).

Discussion

Precipitation of cellulase in or on the substrate with PTA is a useful technique for both localising the enzyme during hydrolysis of the substrate and for determining penetration of the enzyme into the substrate. The observation that the enzyme

precipitate is always bound to the substrate suggests that affinity for the substrate is retained after reaction with PTA. However because some denaturation may have taken place, it is possible that the precipitate is inherently sticky and simply adheres to the nearest solid object. This does not detract from the usefulness of the technique as far as determining penetration of the enzyme into the substrate is concerned. The observations that cellulase material does not penetrate into holocellulose substrates is supported by the pattern of hydrolysis in these substrates which is extremely localised, occurring only at surfaces exposed to the surrounding solution. This contrasts with alpha-cellulose substrates where both location of enzyme and pattern of hydrolysis are more or less uniform throughout the substrate after 7 days treatment.

The ability of cellulase molecules to penetrate a substrate depends on the size and shape of the cellulase molecule and the size, shape and frequency of pores within the substrates (Stone, Scallan 1963; Cowling 1975; Grethlein 1985). The *Aspergillus niger* cellulase used in the present investigation has a spherical diameter of 58 Å or an ellipsoid equivalent of 32×192 Å (Petterson 1963). According to Cowling (1975), only a small fraction of the cell wall capillaries in wood cell walls are large enough to allow penetration of enzyme molecules. Delignification (Kraft, 44.5% yield) increases the number of accessible pores (Stone, Scallan 1968). Cellulase may penetrate the remaining substrate by enlargement of the smaller pores (Cowling 1975). This is illustrated in Fig. 3 a where the porosity of the S2 and S3 layers is enhanced by enzyme activity enabling further penetration into the cell wall. The rate of diffusion into the pores is also reduced because of the affinity of the cellulase for the surrounding substrate leading to blockage of pores.

Because of its high hemicellulose content indicated by $KMnO_4$/lead citrate staining, the S3 layer of the cell wall must have much smaller pores than for example the S2 layer or the primary wall. The S1 layer appears to be similar to the S3 layer in this respect. Sinner et al. (1976) were able to enhance degradation of the resistant S1 layer by pretreating with xylanase. Access to the S2 layer may be restricted by the low porosity of the S1 and S3 layers that surround it. In the primary wall which had very large pores, access is essentially unrestricted.

Hemicellulose may also interfere with access of the enzyme to the cellulose by coating microfibrils (Kerr, Goring 1975; Ruel, Barnoud 1981). For example, the degradation in the S3 region of holocellulose fibres is more restricted than in the adjacent S2 where it appears to radiate out from the site of penetration in the S3 layer (Fig. 3 a).

There is no indication of why hydrolysis of the S3 is restricted to small localised regions. Presumably these are sites which are either damaged (compression failures) (Eriksson et al. 1969), or contain less hemicellulose than adjacent regions. Katkevics et al. (1983) examined the structure of cellulose fibres from birch wood after hydrolysis with cellulase and found that hydrolysis occurred up to 7–8 μm into the secondary wall. Vevere et al. (1983), found that birch pulp fibres contain weak regions that are more susceptible to cellulase attack. Several workers have observed cracks on the surface of cotton fibres during enzyme treatment (Blum, Stahl 1952; Marsh 1957; Betrabet, Paralikar 1977; Paralikar, Bhatawdekar 1984). The S1 region of wood holocellulose does not seem to be attacked in this way confirming the earlier observation of Boutelje and Hollmark (1972).

In alpha-cellulose where most of the hemicellulose has been removed from the specimen and the wall has been swollen, access of the enzyme to the cellulose is increased because of the increase in pore size. The amount of resultant degradation also increases partly because of the exposure of naked cellulose microfibrils by the chemical pre-treatment.

Microfibrils in alpha-cellulose of radiata pine do not show obvious thinning, as reported by Betrabet and Paralikar (1978), and White (1982), but instead show a granular appearance suggesting cleavage into shorter lengths by endoglucanase activity. Some of the irregular thickening shown in Fig. 2 may represent enzyme molecules bound to the microfibril.

In conclusion hemicellulose is an important limiting factor determining the amount and pattern of enzyme attack. The high levels of hemicellulose in the S1 and S3 layers of the secondary wall limit access of the enzyme to the wall by restricting pore size and by forming a resistant coating on microfibrils. Removal of hemicellulose by chemical treatment increases access of the enzyme to the cell wall with subsequent increases in amount and uniformity of degradation.

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