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Ultrastructure of steam-exploded wood

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Summary. The ultrastructure of steam-exploded wood from the softwood *Pinus radiata* D. Don was examined by electron microscopy in order to determine the reasons for increased porosity and enhanced susceptibility to enzymatic hydrolysis. Ultrastructural changes were observed in the form of lignin redistribution and swelling of the cellulose framework. Lignin showed evidence of melting, having contracted into well defined agglomerates suspended in a web of cellulose. Using lanthanum and gold tracers of known particle size the pores in the microfibrillar cell wall have been examined. Cellulose regions were shown to contain numerous pores greater than 2 nm, while lignin agglomerates did not contain such pores. Treatment with NaOH resulted in lignin being smeared over the porous cell wall material – hence blocking pores and reducing digestibility.

Introduction

Steam explosion has been proposed as a method of enhancing the susceptibility of lignocellulosic materials to enzymatic hydrolysis in order to produce sugars (glucose, mannose, xylose) which can be converted to liquid fuels by fermentation (Puri, Mamers 1983; Vallander, Eriksson 1983; Mackie et al. 1985; Grous et al. 1986). Low molecular weight lignins are also produced which may be suitable for high value utilisation (Hemmingson 1985; see also: Bioprocessing Technology 8 (12) 1986; Scientiae 27 (3) 1986; Bioprocessing Technology 9 (12) 1987; for editorial articles on this subject).

Steam explosion is a process whereby biomass is treated with high pressure steam followed by explosive release of pressure (Foody 1982). The addition of sulphur dioxide to the substrate is recommended to enhance the effectiveness of the process especially for softwoods (Mamers, Menz 1984; Mackie et al. 1985; Hemmingson 1986; Clark, Mackie 1987). Steam explosion results in the hydrolysis of hemicellulose within the wood and the resulting sugars can subsequently be washed out in water, leaving a residue of alpha-cellulose and lignin (Puri, Mamers 1983).

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This report examines ultrastructural changes caused during steam explosion of *Pinus radiata* and relates these changes to increased porosity and enhanced susceptibility to enzymatic hydrolysis.

Materials and methods

Substrates

Pinus radiata wood chips which had been treated with SO_2 (2.55% w/v) were steamed at 215 °C for 3 min. Following explosion, the solid residue was washed in water. Part of this solid material was then treated with 20 ml 0.4% w/v NaOH g^{-1} solid (to study the effects of partial lignin removal). The characteristics of these materials are given in Table 1. Details of the analytical procedures used are given in Wong et al. (in press).

Steam-exploded wood was digested with a mixture of *Trichoderma reesei* cellulase and *Aspergillus niger* cellobiase in citrate buffer, pH 4.8, at 50 °C for 24 h (Wong et al. in press).

Electron microscopy

Steam-exploded wood was prepared for scanning electron microscopy (SEM) by placing a drop of dilute suspension on a piece of coverslip glass and allowing it to air dry. The specimen was then mounted on a stub using conductive thermoplastic adhesive and coated with gold in a Polaron E 5000 sputter coating unit before examination using a Phillips PSEM 500 scanning electron microscope.

Substrates were also prepared for transmission electron microscopy (TEM) as follows:

Steam-exploded substrates were dehydrated in an ethanol series and infiltrated with LR White acrylic resin (London Resin Co.) in gelatin capsules at 4 °C for

Table 1. Characteristics of original wood and steam-exploded wood substrates

	Original wood %	Water washed %	NaOH washed %
Lignin	26.2	44.4 (26.6)[a]	36.4
Glucose	48.1	56.8 (34.0)	66.8
Mannose	11.9	0	0
Xylose	6.0	0	0
Galactose	3.2	0	0
Arabinose	1.8	0	0
Accessible pore volume[b]		0.92 ml/g	0.34 ml/g
Digestion yield[c]		84	29

[a] Figures in parentheses refer to composition as a percentage of the original OD wood
[b] Using a 5 nm dextran probe (Wong et al., in press)
[c] 24 h digestion

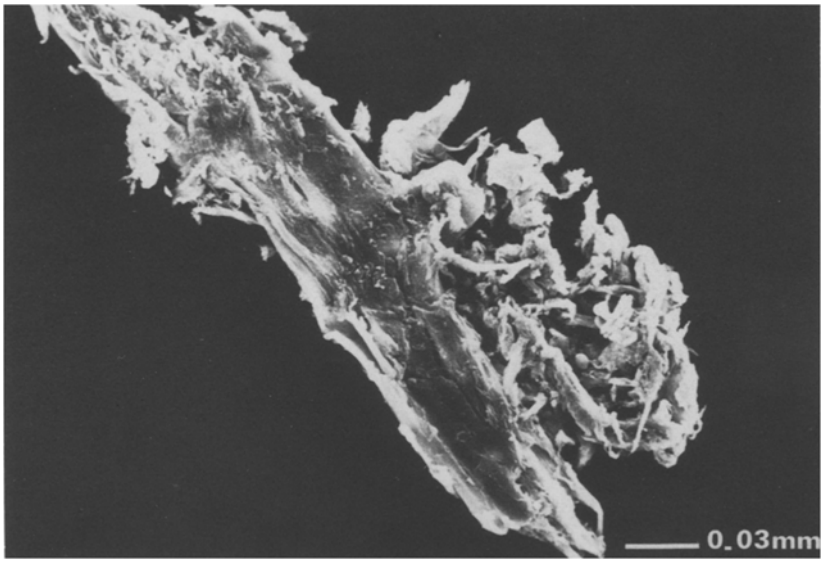

Fig. 1. A shive and cell wall fragments produced by steam explosion (SEM)

5 days. The resin was polymerised at 60 °C for 24 h. In order to localise the porous areas of the substrate, lanthanum and gold tracers were used. The presence of either of these tracers inside the substrate indicates the presence of pores with a minimum diameter of 2 nm for lanthanum and 5 nm for gold. Colloidal lanthanum treated substrates were prepared by treating with colloidal lanthanum at 4 °C for 24 h (Lewis, Knight 1977) followed by dehydration and resin infiltration as above. Colloidal gold substrates were prepared by treating with colloidal gold with a mean particle size of 5.3 nm (95% range 3.3–7.8 nm) (Janssen Life Sciences Products) at 4 °C for 24 h followed by dehydration and resin infiltration as above.

Specimens were sectioned with a diamond knife using an LKB ultramicrotome. Sections were placed on grids covered with a thin carbon support film and stained with uranyl acetate/lead citrate (30 min/5 min) or with $KMnO_4$ (2 min). Specimens treated with colloidal lanthanum prior to embedding were examined unstained at 40 kV. Stained specimens were examined at 60 or 80 kV using a Philips EM 300 transmission electron microscope.

Results and discussion

Steam-exploded *Pinus radiata,* as prepared in this work, is highly digestible by cellulase enzymes (Clark, Mackie 1987). Table 1 allows a comparison of the water washed steam-exploded substrate and the original wood to be made. This comparison shows:

- All the hemicelluloses and approximately 15% of the cellulose have been removed during pretreatment,
- very little lignin has been removed during steam explosion.

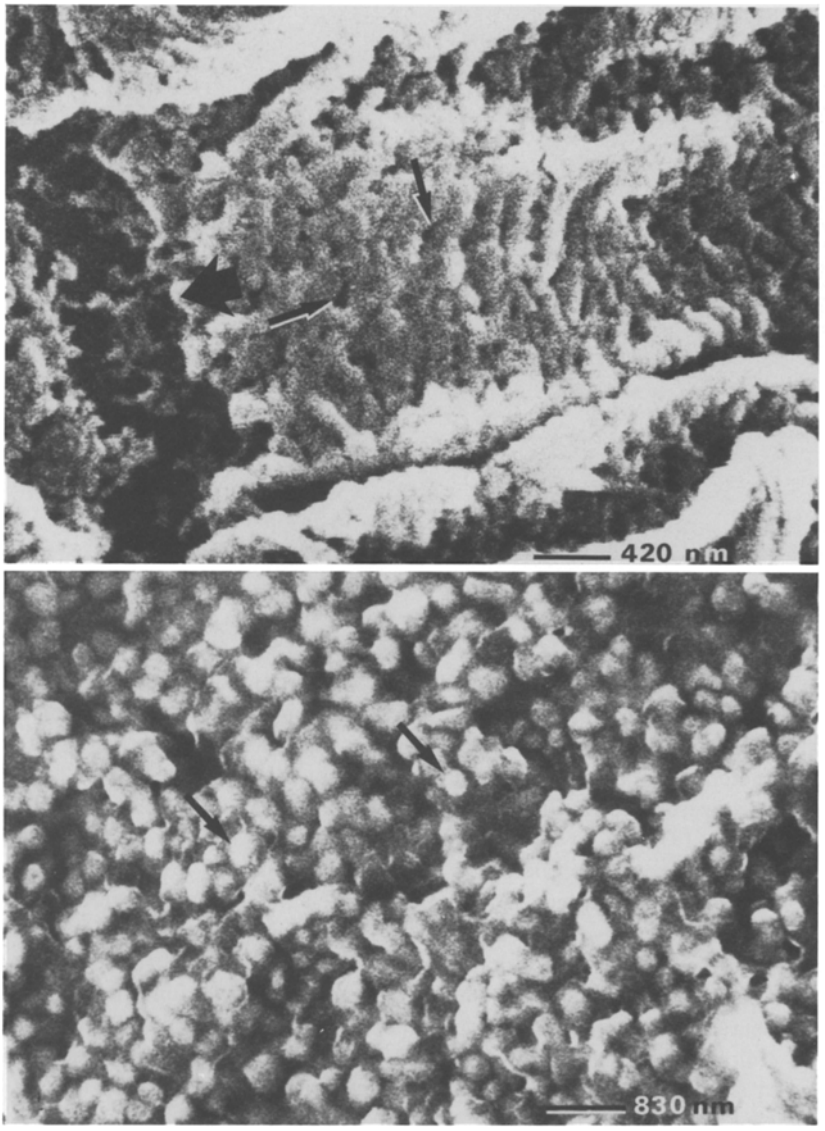

Fig. 2. **a** A close-up view of the surface of a fibre after steam explosion showing numerous relatively large pores and cracks (arrows). The less porous areas are probably middle lamella while the porous areas visible in cracks may be secondary wall (SEM); **b** a close-up view of a fracture through a cell wall fragment showing the particulate nature of the lignin (arrows) (SEM)

The pretreatment is quite severe and hence major structural changes to the woody cell wall are to be expected.

Fragments of various cell wall layers and shives in the steam-exploded material are shown in Fig. 1.

The cell wall fragments which make up most of the material are of two main types containing either middle lamella, primary wall, and S1 layer of the secondary

wall; or secondary wall only. This fragmentation apart from increasing the surface area available for enzyme attack also removes two permeability barriers which restrict access to the bulk of the cellulose in the S2 layer. These barriers are the middle lamella and the S3 layer of the secondary wall. Both have high lignin concentrations in *Pinus radiata* tracheids, 80% and 50% respectively (Donaldson 1985; Donaldson 1987) and lignin is known to prevent hydrolysis of cellulose with enzymes by shielding microfibrils and occluding pores (Cowling 1975). Hence, although steam-explosion does not remove lignin, the disruptions to the fibre structure will considerably negate its "shielding" effect.

The surface of large intact fibres contains numerous large pores (Fig. 2a) and evidence for considerable ultrastructural rearrangement is clearly visible in cell wall fragments (Fig. 2b). Fragments of cell wall show numerous particles which appear more or less spherical and of uniform size (Fig. 2b). These particles are attached to or embedded in a porous cell wall medium. Similar observations have been made by Brownell and Saddler (1987) with steam-exploded *Populus tremuloides* Mich. These authors were able to isolate the spherical particles and characterise them chemically as being primarily lignin based. These lignin particles probably form by melting at the process temperatures used (215 °C) and subsequent coalescence under the influence of surface tension as the product cools during explosion (Irvine 1985). The lignin particles are present in unexploded material, i.e. steamed only, as well as material subjected to explosion (Brownell, Saddler 1987), indicating that it is the steam treatment not the explosion which results in the changes in lignin ultrastructure. Explosion does not enhance digestibility beyond that achieved by steaming alone.

The gross physical reorganisation of lignin in steam-exploded wood discussed so far is paralleled by chemical changes. Studies on both the low molecular weight "soluble" lignin (Hemmingson 1983) and the "insoluble" residual lignin (Hemmingson, Dekker, in press) produced when *Pinus radiata* is steam exploded, have been completed. The lignin residing in the washed exploded fibre is comprised of partly depolymerised and partly repolymerised lignin (rather than of unreacted lignin). The extent of β-0-4 ether cleavage in lignin can be followed by ^{13}C NMR spectroscopy and correlates with the extent of structural breakdown of the lignocellulose.

In this study we have observed lignin redistribution within the cell wall that complements the chemical data above. In thin sections the effect of steam explosion treatment is clearly observed with dense particles of lignin suspended in a web of cellulose microfibrils (Fig. 3). Staining with $KMnO_4$ (which is specific for lignin, Bland et al. 1971) confirms the presence of lignin in discrete dense particles or agglomerates (Fig. 4).

Using cellulase enzymes a steam-exploded wood sample was prepared which comprised (essentially) only lignin (Fig. 5). The lignin agglomerates vary in density and tend to be less dense near their surfaces. The lignin particles have well-defined boundaries and the intervening areas show no trace of residual lignin, confirming that cellulose in these regions is not associated with lignin. In contrast areas of middle lamella show little evidence of lignin redistribution or increased porosity (Fig. 3A). The middle lamella would therefore continue to represent a barrier to enzyme attack if disintegration of the cell wall did not take place.

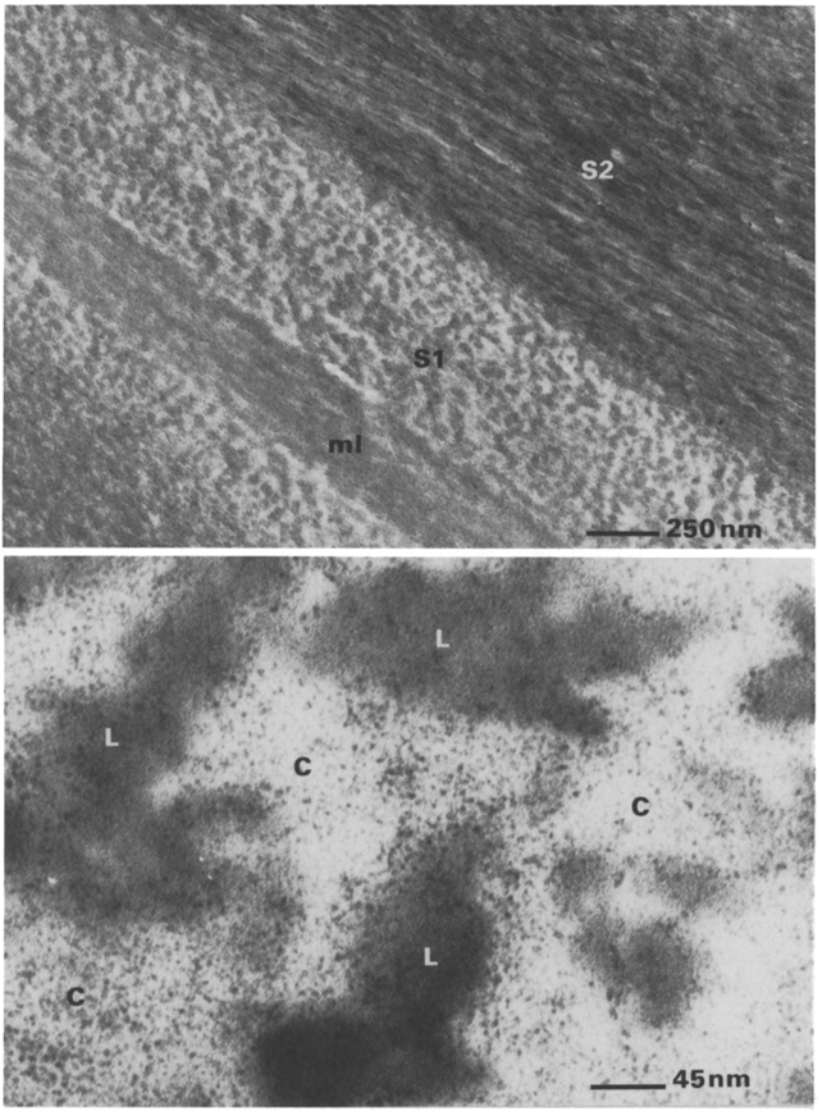

Fig. 3. **a** A sectional view through a cell wall fragment stained with uranyl acetate and lead citrate showing the middle lamella (ml), S1 and S2 layers (TEM); **b** a high magnification view of the S2 region showing dense areas of lignin (L) and porous areas of cellulose (C) microfibrils (TEM)

Treatment of steam-exploded *Pinus radiata* with both colloidal lanthanum and gold confirmed the high porosity of the microfibrillar web in which the lignin agglomerates are embedded. Lanthanum adheres to, but does not penetrate the surface of the lignin material, indicating the absence of pores larger than 2 nm (Lewis, Knight 1977). Lanthanum is evenly distributed throughout the microfibrillar cellulose regions however (Fig. 6). Colloidal gold particles are, more or less, the

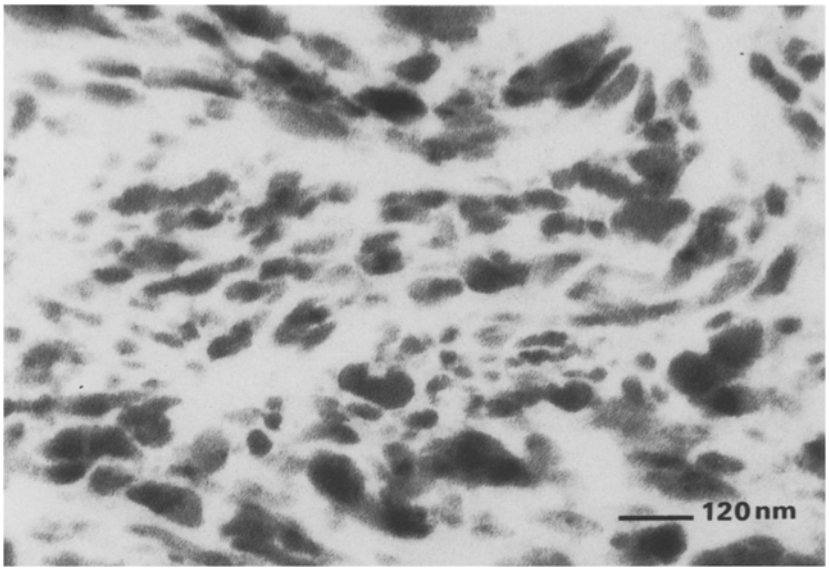

Fig. 4. Steam-exploded wood stained with $KMnO_4$ indicating the presence of lignin in the dense areas and the absence of lignin in areas occupied by unstained cellulose (TEM)

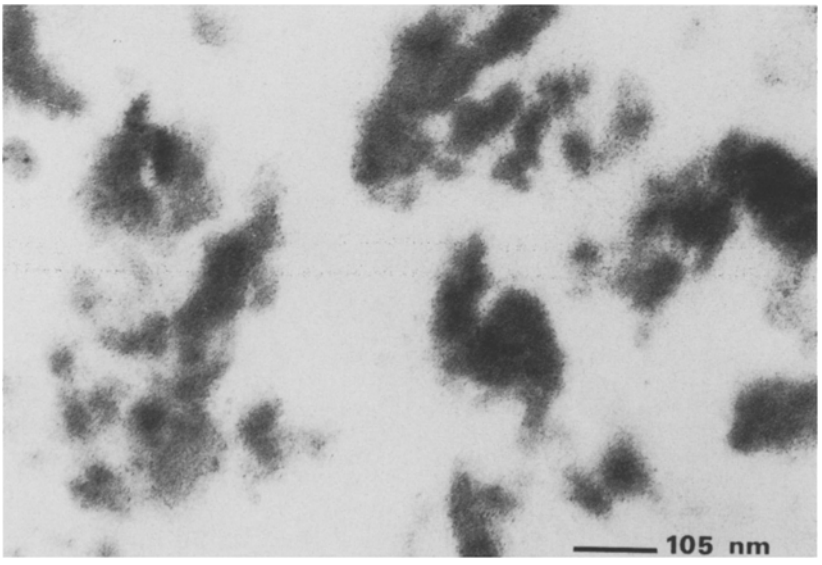

Fig. 5. Steam-exploded wood after treatment with cellulase for 24 h showing residual lignin agglomerates and the absence of cellulose microfibrils after staining with uranyl acetate and lead citrate. The particles of lignin vary in density and have well-defined edges (TEM)

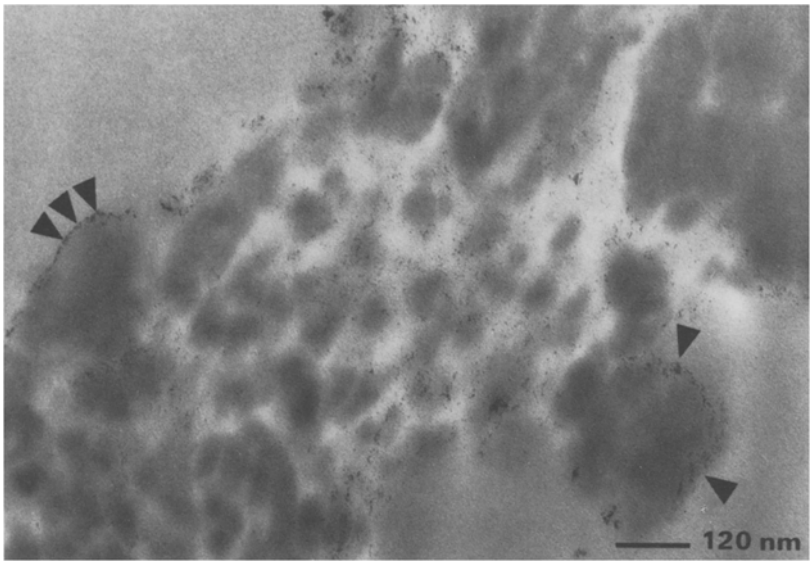

Fig. 6. Steam-exploded wood treated with colloidal lanthanum. Dense areas correspond to lignin particles while less dense areas correspond to cellulose. Lanthanum (arrows) accumulates on the surface of lignin particles, but does not penetrate them. Lanthanum particles are present throughout the cellulosic regions of the specimen (TEM)

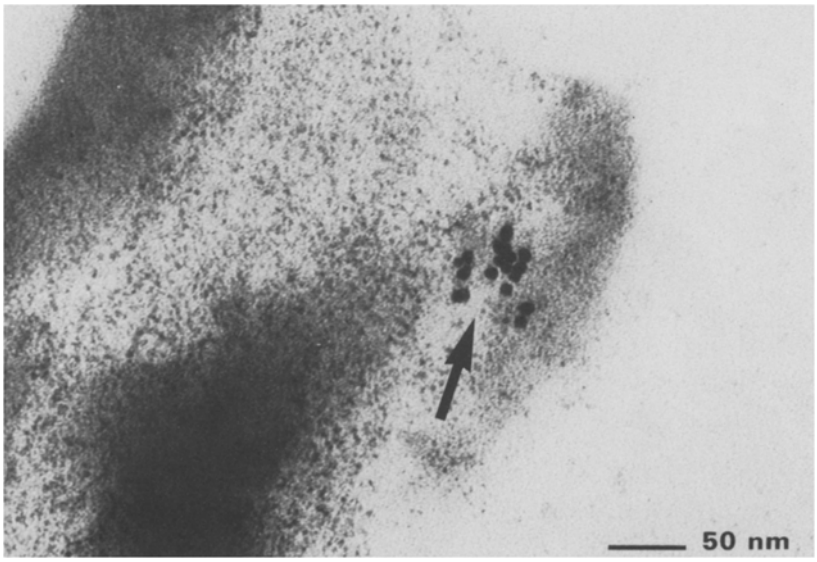

Fig. 7. Steam-exploded wood treated with colloidal gold. Gold particles have penetrated the cellulosic area of the substrate and have become entangled in the microfibrillar web (arrow) (TEM)

same size as cellulase enzymes (Cowling 1975) and the penetration of gold into the cellulosic portions of the substrates (Fig. 7) suggests that the enzyme should have ready access to these regions.

Wong et al. (in press) have observed a reduction in porosity and hence susceptibility to digestion when steam-exploded wood is treated with dilute NaOH. This effect has also been noted (Hemmingson, Dekker, in press) when acetone is used as the extracting solvent. These solvents do not remove all the lignin in steam-exploded substrates and it has been suggested that the redistribution of lignin particles during extraction may block accessible regions of the cellulose microfibrils. This is confirmed in the present study.

Figure 8 a shows a section of cell wall stained with $KMnO_4$ following NaOH extraction. In comparison with Fig. 4 (not extracted with NaOH) the lignin material is more evenly distributed throughout the cell wall. The lignin has been dispersed over a greater proportion of the cell wall material. Fig. 8 b shows lignin macromolecules produced by NaOH treatment as they diffuse away from the surface of lignin agglomerates. These lignin macromolecules appear to be similar to those described by Goring et al. (1979) in lignosulphonate with many being up to 10 nm in diameter. These lignin macromolecules may correspond to the structures observed in the methanol soluble fraction of exploded wood by Tanahashi et al. (1983).

The enhanced digestibility of steam-exploded wood is attributed to three main factors:

1. An increase in surface area caused by fragmentation of the wood.
2. Increased porosity due to lignin redistribution.
3. Increased porosity due to hydrolysis and removal of hemicellulose.

It has been shown that there is a strong positive correlation between digestibility and accessible pore volume in pretreated lignocellulosic substrates (Grethlein et al. 1984; Wong et al. in press) but the increase in accessible pore volume achieved via steam explosion or acid prehydrolysis results from simultaneous effects on both lignin and hemicelluloses so that the relative importance of these effects cannot be distinguished.

The effect of hemicellulose on the ability of cellulase enzymes to enter wood cellulose substrates has been examined by Donaldson (1988). High levels of hemicellulose in the S1 and S3 layers of the secondary walls of holocellulose fibres prevents or restricts movement of enzyme molecules into the substrate. Apart from this direct influence on the porosity of the substrate, hemicellulose may also affect the susceptibility of cellulose microfibrils to enzyme attack. Kerr and Goring (1975) have suggested that part of the hemicellulose in the secondary wall forms a thin coating on cellulose microfibrils. Removal of hemicellulose by hydrolysis during steam explosion may not only increase porosity, and hence accessibility of the substrate to cellulase, but may also increase the susceptibility of cellulose to enzyme attack by allowing direct contact between the enzyme and the cellulose surface.

Tanahashi et al. (1983) found an increase in both crystalinity and micelle width in steam exploded wood substrates. In *P. radiata* steam exploded wood, 15% of the cellulose is hydrolysed during steam treatment indicating that cellulose is also affected by the treatment. Microfibrils did not show any obvious structural changes

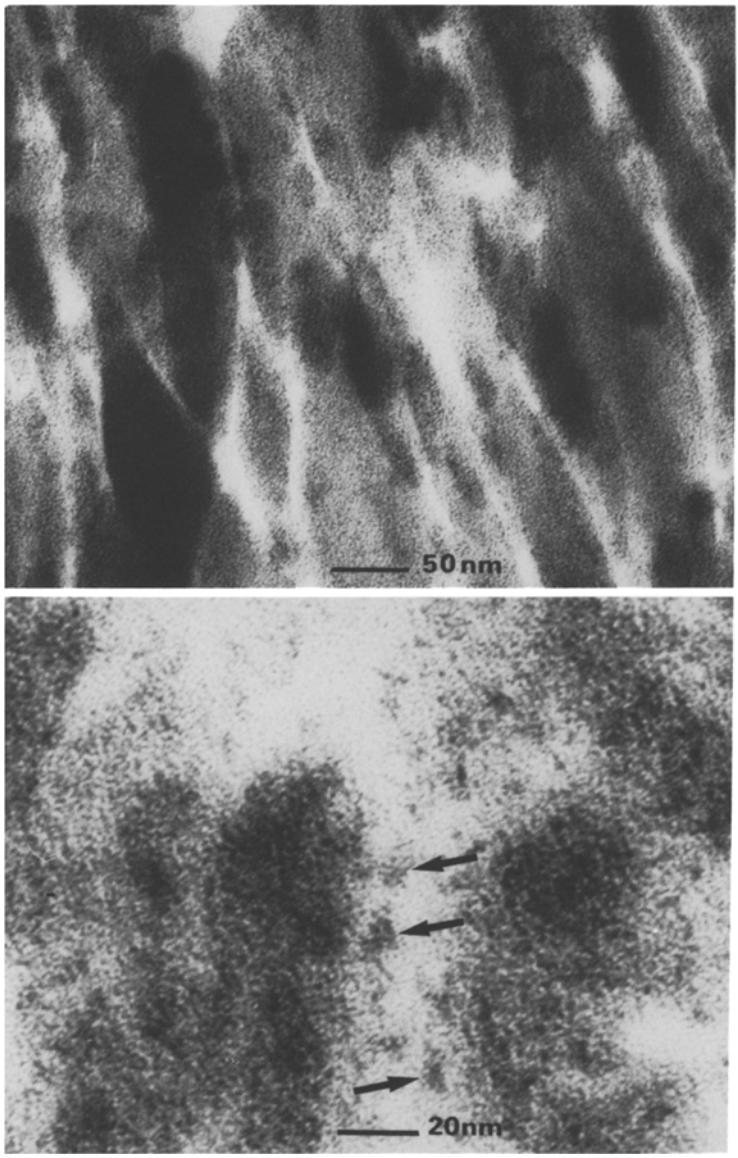

Fig. 8. **a** Steam-exploded wood treated with NaOH. Lignin agglomerates have increased in size so that they occupy a large proportion of the specimen area shown. This indicates that NaOH has partly dissolved the lignin thus reversing the effect of steaming by causing the lignin to reoccupy cellulosic areas of the specimen (TEM); **b** Steam-exploded wood treated with NaOH and stained with $KMnO_4$. Large lignin macromolecules (arrows) can be seen breaking away from the surface of lignin agglomerates which have diffuse surfaces compared to Fig. 4 and 5 (TEM)

apart from a greater interfibrillar distance as a result of stretching during reorganisation of the cell wall components. The loss of some cellulose during steaming suggests that microfibrils may be fragmented as observed by Tanahashi et al. (1983) but this could not be confirmed by observations on thin sections of *P. radiata* material. Fragmentation and changes in crystalinity of cellulose microfibrils may contribute to the increased digestability of steam exploded wood (Cowling 1975).

In conclusion, the porosity of steam-exploded wood is enhanced by a combination of lignin redistribution as a result of melting and agglomeration due to surface tension effects, the removal of hemicellulose by hydrolysis during the process, and by fragmentation of the wood to form cell wall fragments. Treatment with NaOH partially dissolves the lignin in exploded substrates resulting in redispersal of lignin, occluding pores and restricting access to cellulose microfibrils, thus reducing porosity and susceptibility to digestion with cellulase.

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