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ORIGINAL ARTICLE

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Delignification of cell walls of *Chamaecyparis obtusa* during alkaline nitrobenzene oxidation

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Abstract To clarify the behavior of whole lignins in wood cell walls during alkaline nitrobenzene oxidation, the delignification process from cell walls in normal and compression woods of *Chamaecyparis obtusa* Endl. (Cupressaceae) was observed using ultraviolet and transmission electron microscopies. The lignin content conspicuously decreased to around 10% after 35 min in normal wood. The lignin content in compression wood finally leveled off at aroumd 10% after 50 min. In gel filtration of oxidation products in ethyl acetate, a high molecular weight fraction was prominent in extracts from the early stage of the reaction. As the oxidation progressed, the high molecular weight fraction became less prominent in both normal and compression wood. Changes in the weights of cell wall residues during reaction indicated that approximately half of the components other than lignin were also removed from the cell walls. This shows that the majority of lignin with relatively high molecular weight is removed from the cell walls together with polysaccharides in the early stage of the reaction and that further oxidative degradation occurs in solution in later stages. Only a small amount of the lignin with low molecular weight could be analyzed by gas chromatography.

Key words Lignin distribution · Alkaline nitrobenzene oxidation · Ultraviolet microscopy · Transmission electron microscopy · Gel filtration

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Introduction

The alkaline nitrobenzene oxidation method has been widely used to characterize lignin structures. The main oxidation products are phenolic aldehydes such as *p*-hydroxybenzaldehyde, vanillin, and syringaldehyde which are derived from oxidative degradation of the corresponding 4-hydroxyphenylpropane units and their ethers, that is, the corresponding 4-*O*-alkylated (α-*O*-4 and β-*O*-4) lignin substructures.[1] Therefore, the molar ratio of the phenolic aldehydes provides information on the relative amounts of the uncondensed *p*-hydroxyphenyl-, guaiacyl-, and syringylpropane units comprising the original lignin. In addition, the yield of these aldehydes reflects the degree of condensation of the lignin, because these uncondensed structures are cleaved by the alkaline nitrobenzene oxidation leaving condensed lignins as the residue.

The degree of condensation of lignins in various morphological regions within wood cell walls has been discussed based on the yield of the phenolic aldehydes from the alkaline nitrobenzene oxidation. Using isolated fractions rich in either secondary wall or compound middle lamella from softwood tracheids,[2–5] it is reported that the lignin in the compound middle lamella is richer in the condensed structure than that in the secondary walls. Meshitsuka and Nakano[6] obtained the fraction of soft xylem tissue from birch which was rich in compound middle lamella lignin and reported that the lignin was much more condensed than that in secondary walls. Morohoshi and Sakakibara[7,8] reported that the lignin in compression wood was more condensed than that in normal wood. Saka et al.[9] studied the reactivity of bromine toward the tissue fractions of secondary wall and middle lamella and found that the secondary wall lignin was 1.7 times more reactive toward bromination than the middle lamella lignin. As the reason for such a difference in reactivity, they suggested that the middle lamella lignin may be more highly cross linked at the 3- and/or 5-positions of the aromatic ring.

The condensed structure of lignin is very important to analyze the reactivity of lignin in pulping because the de-

gree of condensation of lignin is quite different between the secondary wall and compound middle lamella. The structure is also important in understanding cell wall lignification because the lignin deposited in the early stage of lignification is more condensed than that deposited in the later stage. For a better understanding of the pulping and the cell wall lignification, it is necessary to clarify protolignin structure which contains both condensed and noncondensed structures. However, in the alkaline nitrobenzene oxidation, the degree of condensation has only been evaluated by the total yield of the phenolic aldehydes and the origin of these aldehydes has never been studied in detail. The behavior of whole lignins during the alkaline nitrobenzene oxidation is very important to understand the relationship between condensation and the total yields of phenolic aldehydes. The purpose of the present study is to clarify the behavior of whole lignins during the alkaline nitrobenzene oxidation. For this purpose, the delignification of tracheid walls during the alkaline nitrobenzene oxidation was investigated by ultraviolet (UV) and transmission electron microscopies (TEM) using samples from normal and compression woods of *Chamaecyparis obtusa* Endl. The oxidation products were analyzed by gas chromatography (GC) and gel filtration to determine the molecular weight distribution of the lignin that is removed from cell walls during reaction.

Materials and methods

Wood samples

Wood samples were obtained from normal and compression wood of hinoki (Japanese cypress, *Chamaecyparis obtusa* Endl., Cupressaceae). The normal wood sample was obtained from a straight trunk, and the compression wood sample was obtained from a trunk that was artificially bent for 1 year. Wood disks were sampled at breast height, and they contained 12 annual rings in both normal and compression wood sample trees. Small blocks were cut from the outermost sapwood. Radial sections of 200 μm thickness in normal wood and tangential sections of 200 μm thickness in compression wood were cut from the sample blocks with a sliding microtome. These sections were extracted with ethanol/toluene (1:2) solution and air-dried before use.

Alkaline nitrobenzene oxidation

Sections of approximately 50 mg were treated with a mixture of 1.5 ml of 2 N aqueous NaOH solution and 0.15 ml of nitrobenzene in a stainless steel tubing reactor at 160°C for various reaction times. The reaction mixture was rapidly cooled by immersion in crushed ice and then transferred to a liquid–liquid extractor with 10 ml of 0.1 N aqueous NaOH solution. The mixture of nitrobenzene and neutral reaction products was extracted from the alkaline reaction mixture with ethyl acetate (3 × 10 ml). The aqueous layer was acidified to pH 2–3 with 2 N aqueous HCl solution. Then the aqueous solution was mixed with 0.5 ml of 1,4-dioxane containing 0.25 mg acetovanillone as an internal standard. This solution was further extracted with ethyl acetate (3 × 10 ml). The ethyl acetate extract containing the oxidation products was washed with saturated aqueous NaCl solution, dehydrated through a column of Na_2SO_4, and concentrated with a rotary evaporator. The products were then acetylated with 2 ml of acetic anhydride/pyridine (1:1, v/v) overnight at room temperature. After removing the acetylating reagents by evaporation with ethanol, the acetylated products were analyzed by GC and gel filtration. The solid cell wall residues remaining in the aqueous layer after the extractions were used for microscopic observation and also for the measurements of lignin content and weight loss.

Determination of oxidation products using GC

The acetylated oxidation products in the samples collected after various reaction times were dissolved in 0.5 ml of acetone. Two microliters of this solution was used for quantitative determination of vanillin acetate and *p*-hydroxybenzaldehyde acetate with a gas chromatograph (Shimadzu GC-14A) with a flame ionization detector. A fused silica capillary column (Shimadzu HR-1, i.d. 0.25 mm, length 30 m) was used under the following conditions: column temperature 180°C, injection and detection temperature 250°C, and He carrier gas.

Gel filtration of oxidation products

The acetylated oxidation products were analyzed by gel filtration to detect the changes in the molecular weight distribution depending on the reaction time. A column (diameter 2.5 cm, length 100 cm) was packed with a slurry of Sephadex LH-20 in tetrahydrofuran. The products were dissolved in 0.2 ml of tetrahydrofuran and applied to the column. Separation was performed at a flow rate of 74 ml/h at room temperature, and 6-ml fractions were collected consecutively. Ultraviolet absorbance at 280 nm of the eluted solution was measured with a UV monitor. To calibrate the relationship between elution volume and molecular weight (Mw), approximately 20 mg each of vanillin acetate (Mw = 194), *p*-hydroxybenzaldehyde acetate (Mw = 164), and polystyrene (Mw = 4750) were individually dissolved in 0.2 ml of tetrahydrofuran and applied to the column. The fractions collected from the samples after the reaction for 180 min were analyzed by GC using the same procedure as described above.

Ultraviolet and transmission electron microscopies

Some of the residues were dehydrated through an ethanol series and then embedded in Spurr's low viscosity resin.[10] Thin sections (1.0 μm thick) and ultrathin sections (0.1 μm thick) were cut with a diamond knife on an ultramicrotome (Reichert-Jung Ultracut E). The thin sections were placed on quartz slides, mounted with glycerin, and then covered

with quartz coverslips. They were observed under a microscopic spectrophotometer (Carl Zeiss UMSP-80) at the wavelength of 280 nm with a wavelength width of 15 nm. The ultrathin sections were mounted on copper grids, stained with 1% freshly prepared potassium permanganate ($KMnO_4$) in 1% aqueous sodium citrate solution for 10–15 min at room temperature, and then observed under a transmission electron microscope (JEOL JEM 2000E and JEM 1220) at 100 keV. This staining has been used for investigating lignin distribution within wood cell walls. Although the specificity of the staining must be taken into consideration when cell walls are chemically modified as in the present study, this staining provides information on lignin distribution in the cell wall.

Measurement of lignin content

The cell wall residues were washed with acetone and dried. For sections that maintained their shapes in the samples reacted over short times (15, 25, and 35 min), they were frozen with liquid nitrogen and then crushed into powder with a stainless steel mortar. For the samples reacted over long times (50, 90, and 120 min), sections were macerated and were directly subjected to the measurement of lignin content without crushing. The lignin content was determined by the acetyl bromide method[11] using a portion of the sample of approximately 5 mg dry weight. Klason lignin contents were determined on untreated sections from both normal and compression wood samples, and absorptivities were calculated from the UV absorbance and the Klason lignin content. Using the absorptivities, lignin contents were calculated on the samples collected after various reaction times.

Measurement of weight loss after alkaline nitrobenzene oxidation

The cell wall residues from both normal and compression wood samples after reaction for 180 min were suspended in 0.1 N aqueous NaOH solution and then collected on glass filters (1G4). The residues on the filters were then washed with water and ethyl acetate, successively, and then dried to a constant weight in an oven at 105° ± 3°C. The weight loss was calculated from the oven-dried weights of the samples before and after the reaction. The measurements were performed on five samples for both normal and compression wood samples and the results were averaged.

Results and discussion

Delignification process from cell wall during nitrobenzene oxidation

Ultraviolet micrographs of the cell wall residues in the samples collected after various reaction times are shown in Fig. 1. In normal wood, UV absorption in the cell corner middle lamellae decreased in the sample reacted for 15 min (Fig. 1b). In the sample reacted for 25 min (Fig. 1c), there was weak UV absorption in the outer S_1 layers and very weak absorption in secondary walls. After a reaction time of more than 35 min (Fig. 1d), there was almost no UV absorption in the cell wall residues. In compression wood, the UV absorption was not apparently decreased in the sample reacted for 15 min (Fig. 1f). In the sample reacted for 25 min (Fig. 1g), UV absorption in the outer S_2 layer and compound middle lamellae decreased slightly. There was an apparent decrease in the UV absorption in the sample reacted for 35 min (Fig. 1h). In the samples reacted for more than 50 min (Fig. 1i), there was almost no UV absorption in the cell wall residues.

Transmission electron micrographs of the cell wall residues in the samples collected after various reaction times are shown in Fig. 2. In normal wood, round electron-transparent (not stained with $KMnO_4$) spaces with diameters of approximately 100–150 nm were observed in the cell corner middle lamellae in the sample reacted for 15 min (Fig. 2c). In the sample reacted for 25 min, there were very few electron-opaque substances. These were stained with $KMnO_4$, in the periphery of the primary wall in the cell corner (Fig. 2d), suggesting that almost all of the lignin was removed. In the samples reacted for more than 50 min, $KMnO_4$ staining hardly stained the residues, suggesting the absence of lignin. In compression wood, round electron-transparent spaces with diameters of approximately 100–150 nm, as in normal wood, were observed in the cell corner middle lamellae (Fig. 2g) in the sample reacted for 25 min. In the samples reacted for more than 50 min, outer S_2 layers were electron-transparent suggesting that lignin was almost completely removed. S_1 layers and inner S_2 layers were slightly stained with $KMnO_4$, suggesting that a very small amount of lignin may exist. However, it was difficult to confirm the presence of lignin because the specificity of $KMnO_4$ staining must be taken into consideration when lignin is chemically modified.

These observations indicate that almost all of the lignin was removed from the cell walls in both normal and compression wood by reaction for more than 50 min. On the other hand, in a general process of chemical analysis on woody material, the alkaline nitrobenzene oxidation is performed for 2–3 h. From UV and TEM observations, it is apprent that lignin is removed from the cell walls in the early stage of the reaction.

Changes in lignin content in the cell wall residues during nitrobenzene oxidation

To quantify the changes in lignin content in the whole cell wall residues, the lignin content was measured using the acetyl bromide method. Figure 3 shows the lignin content in the samples reacted for various times from normal and compression wood samples. The lignin content was expressed as a relative ratio to the lignin content of the control sample. The ratio rapidly decreased from the sample reacted for 25 min to that reacted for 35 min in normal wood and leveled off to around 10% in the sample reacted for

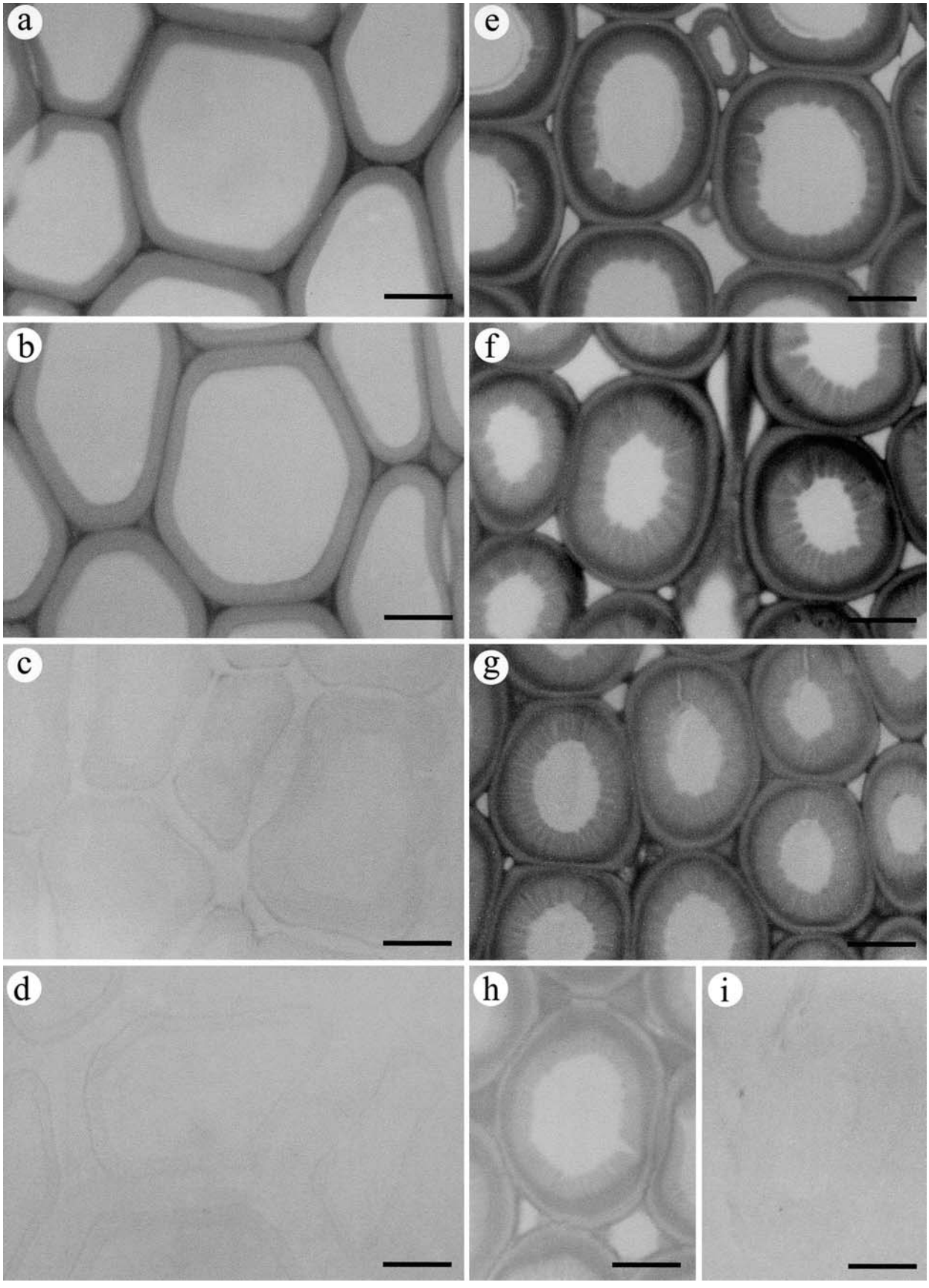

Fig. 1. Ultraviolet (UV) micrographs at 280 nm of transverse sections from normal wood (**a–d**) and compression wood (**e–i**) in the samples collected after various reaction times. **a**, **e**, control; **b**, **f**, 15 min; **c**, **g**, 25 min; **d**, **h**, 35 min; **i**, 50 min. *Bars* 10 μm

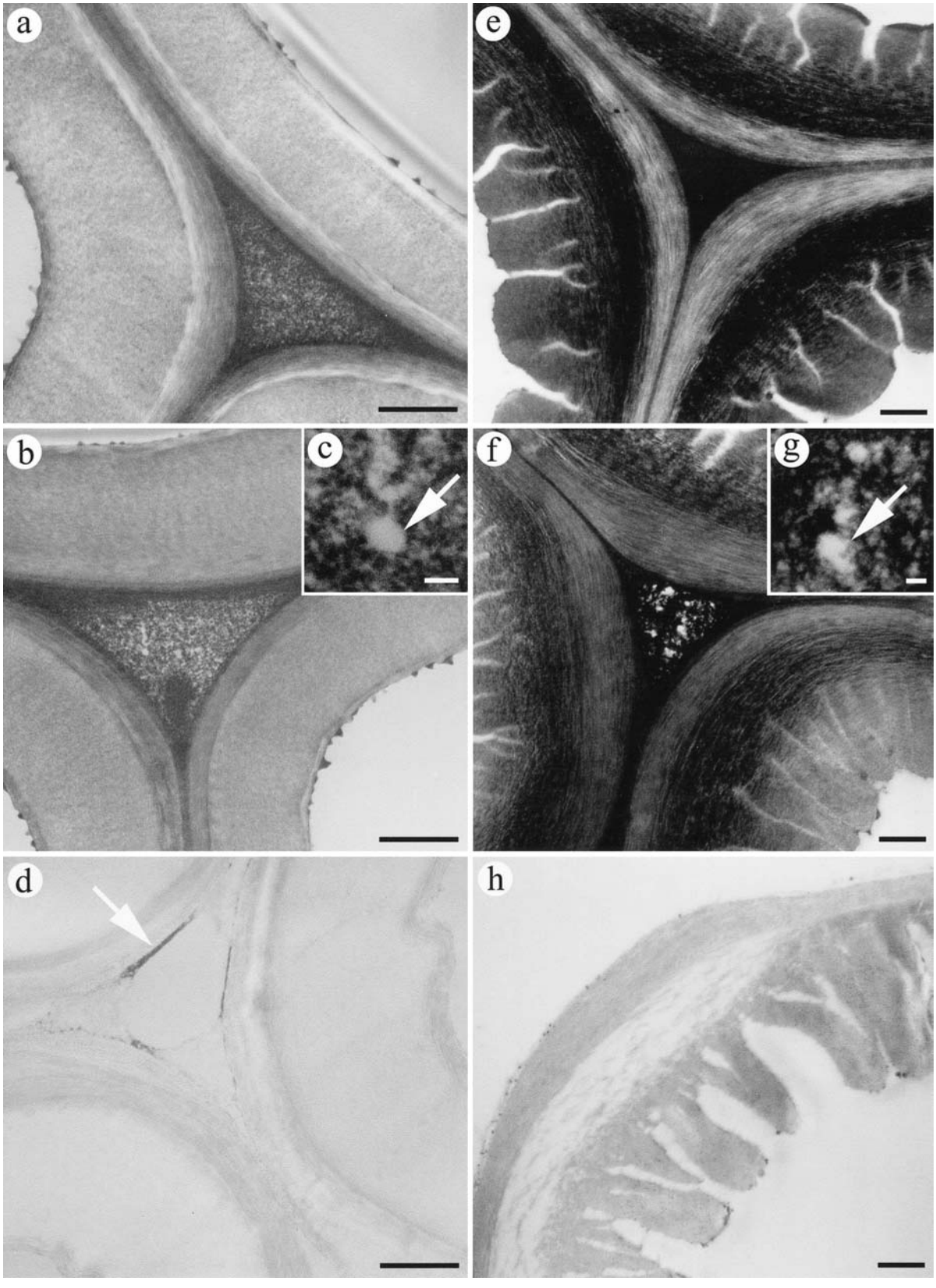

Fig. 2. Transmission electron micrographs of transverse sections from normal wood (**a–d**) and compression wood (**e–h**) stained with $KMnO_4$ from samples collected after various reaction times. **a**, **e**, control; **b**, **c**, 15 min; **d**, **f**, **g**, 25 min; **h**, 50 min; **c**, **g**, enlarged images of cell corner middle lamella in **b** and **f**, respectively. *Arrows* in **c** and **g** indicate round electron-transparent structures with diameters of approximately 100–150 nm. *Arrow* in **d** indicates electron-opaque substances found in the periphery of primary wall in the cell corner. *Black bars* 1 μm, *white bars* 0.1 μm

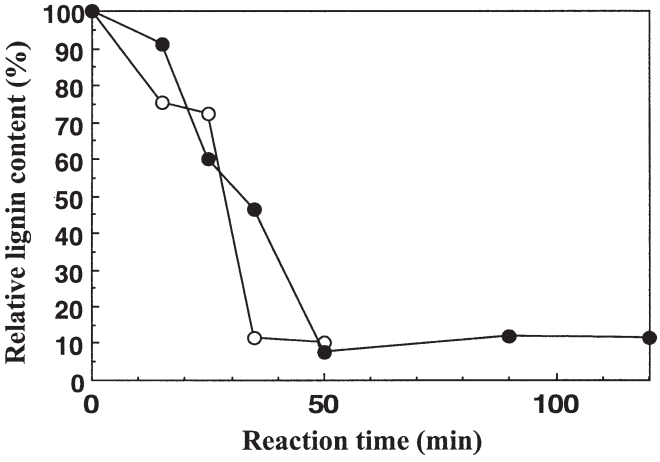

Fig. 3. Lignin content in cell wall residues from normal wood (*open circles*) and compression wood (*filled circles*) after various reaction times of alkaline nitrobenzene oxidation. Lignin content was measured using the acetyl bromide method

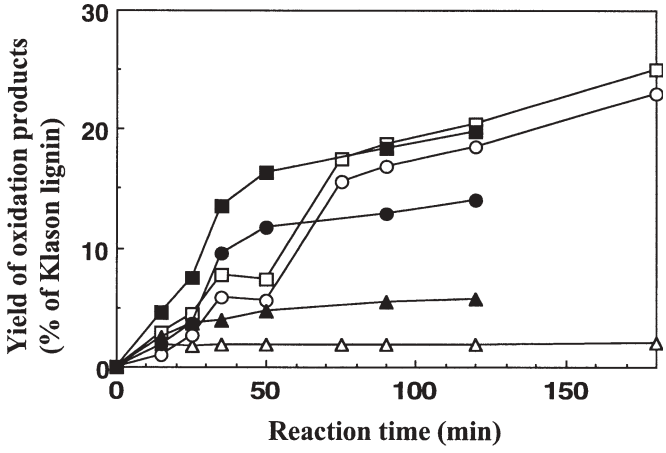

Fig. 4. Yields of *p*-hydroxybenzaldehyde (*triangles*), vanillin (*circles*), and *p*-hydroxybenzaldehyde plus vanillin (*squares*) as a function of alkaline nitrobenzene oxidation reaction time. *Open symbols*, normal wood; *filled symbols*, compression wood

50 min. In compression wood, the relative lignin content decreased more or less uniformly from the sample reacted for 15 min to that reacted for 50 min, after which it became constant at about the same level observed for normal wood. However, the leveling off of the delignification in normal wood was earlier than that in compression wood. These results were consistent with the results from UV and TEM observations of the cell wall residues. Lignin in the cell wall is removed from the cell walls in the early stages. The results that showed approximately 10% of the lignin remained in the cell wall residues after complete nitrobenzene oxidation in both normal and compression wood suggests that some lignin bonds to other components (such as polysaccharides) in the cell walls and is therefore resistant against oxidation.

Determination of oxidation products using GC

Figure 4 shows changes in the amounts of the major oxidation products, *p*-hydroxybenzaldehyde and vanillin, from the nitrobenzene oxidation. These amounts are expressed as relative ratios to the total lignin content (Klason lignin). In normal wood, the *p*-hydroxybenzaldehyde content was around 2% in the samples reacted for 15 min and kept almost the same level in the longer reactions, whereas the vanillin content increased until 35-min reaction time, decreased slightly until 50-min reaction time and then increased significantly for longer reaction times. This pause in the increase might reflect some heterogeneity of lignin against the oxidation. During this period (from 35 to 50 min), the majority of lignin was removed (Fig. 3), and therefore this result suggests that the lignin in solution oxidized but did not release aldehydes. In compression wood, the *p*-hydroxybenzaldehyde content raised appreciably until the 25 min of reaction time and then gradually increased with reaction time. The vanillin content increased markedly until 35 min of reaction time and then increased gradually for longer reaction times. Measurement of the lignin content indicated that approximately 10% of the lignin remained in the cell wall residues, 20%–25% of the lignin oxidized to yield aldehydes, and the remaining 65%–70% of the lignin was removed from the cell wall residues and existed in alkaline solution during the reaction.

Changes in molecular weight distribution of oxidation products during nitrobenzene oxidation

The results described above demonstrate that 65%–70% of the lignin exists in the alkaline solution after complete nitrobenzene oxidation. It is assumed that the majority of the lignin removed from the cell walls exists in the ethyl acetate layer. Thus changes in the molecular weight distribution of the acetylated extractives in the ethyl acetate layer were investigated using gel filtration.

The elution patterns of the acetylated oxidation products for the various reaction times are shown in Fig. 5. In both normal wood (Fig. 5a) and compression wood (Fig. 5b), UV absorbance began to be detected at an elution volume of about 140 ml in the early stage of the reaction and then decreased with reaction time. Because the void volume of this column was 140 ml as determined with polystyrene (Mw = 4750), the fraction at around 140 ml elution volume had a high molecular weight although the exact molecular weight was not determined. Thus the elution patterns suggested that lignin with high molecular weight was removed from the cell walls in the early stage of the reaction and was then oxidized in the solution throughout the reaction. This information is consistent with that obtained from UV and TEM observations. The presence of round electron-transparent spaces in cell corner regions (Fig. 2c,g) suggests that lignin is removed from the cell walls as small blocks with high molecular weight in the early stages of the reaction.

There are several reports of gel chromatography of lignins and lignin model compounds.[12–16] Connors et al.[12]

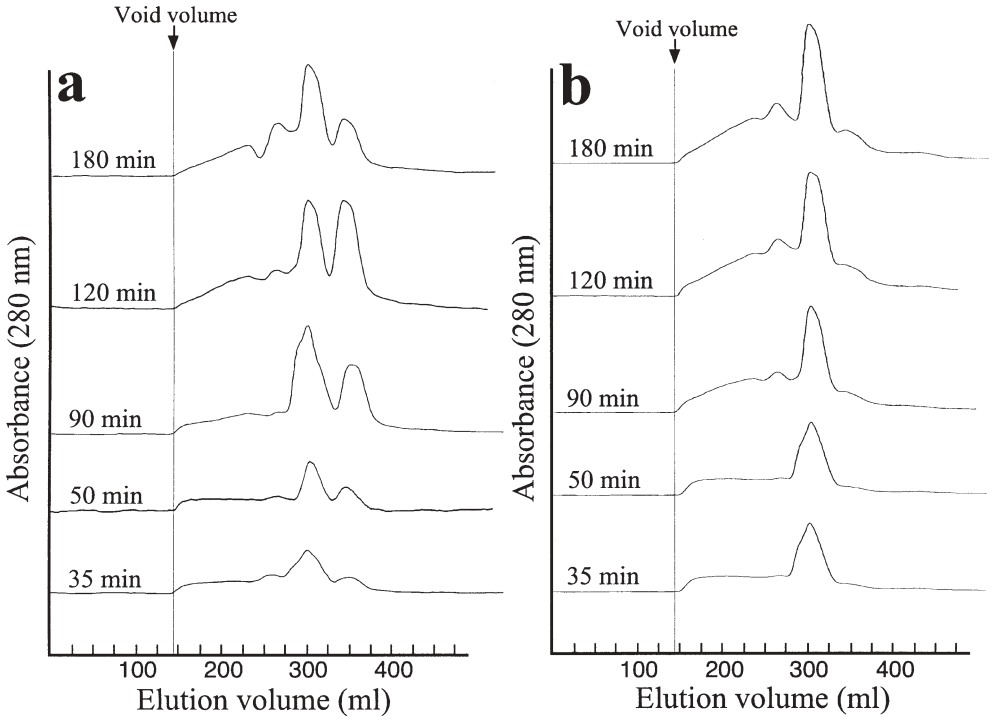

Fig. 5. Gel filtration elution patterns of acetylated oxidation products in the ethyl acetate extracts from normal wood (**a**) and compression wood (**b**) in samples collected after various alkaline nitrobenzene oxidation reaction times

examined 15 lignin model compounds with a molecular weight range of 168 to 1076 using Sephadex LH-20 with dimethylformamide as the solvent. They found a good correlation between molecular weight and elution volume. In the present study, we use tetrahydrofuran as the solvent and an exact molecular weight distribution was not determined. Elution patterns of *p*-hydroxybenzaldehyde acetate and vanillin acetate showed peaks at 380 and 310 ml, respectively (data not shown). In GC of eluted fractions, peaks were detected at more than around 300 ml of elution volume (data not shown). These data clearly show that only low molecular weight fractions can be analyzed by GC.

Weight loss after alkaline nitrobenzene oxidation

Sample weights after reaction for 180 min were 31.8% of original weight in normal wood and 21.9% in compression wood. This shows that 68.2% (in normal wood) and 78.1% (in compression wood) of original cell wall materials were removed from cell walls during the reaction. Because the Klason lignin content was 29.4% in normal wood and 38.7% in compression wood and about 10% of the Klason lignin remained in the cell wall residues in the completely oxidized samples (Fig. 3) in both cases, the cell wall materials that were removed from cell walls contained 90% of Klason lignin (26.5% in normal wood and 34.8% in compression wood). Therefore, cell wall polysaccharides, which were removed from cell walls together with lignin, account for 41.7% (in normal wood) and 43.3% (in compression wood) of original weights. These polysaccharides should exist in the aqueous and/or ethyl acetate layers. Fukushima and Terashima[17] selectively labeled *p*-hydroxyphenyl-, guaiacyl-, and syringylpropane moieties in protolignin by administration of corresponding ^{3}H-labeled monolignol glucosides to differentiating xylem of ginkgo. They fractionated nitrobenzene oxidation products from radiolabeled ginkgo wood meals and examined radioactivities of them. Their results suggested that more than 60% of lignin existed in the extracted ether layer and that 20%–30% of the radioactivity remained in the water layer after extraction with ether. Therefore, some of the polysaccharides might bind to the lignin, forming a lignin–carbohydrate complex,[18] and may be extracted with ethyl acetate or remain in the water layer. There is another possibility that the lignin removed from cell walls may reassociate with other polysaccharides in the reaction solution. Fukushima and Terashima[17] also reported that very low radioactivity was detected in residual wood meal. The results of UV and TEM in the present study shows good agreement with their result.

In conclusion, the results of the present study showed that the majority of lignin was removed from cell walls, together with a substantial amount of polysaccharides in the early stage of the reaction. Further oxidative degradation of lignin then occurred in the reaction solution as the digest continued. About 10% of the lignin remained in the cell wall residues after the reaction. The ethyl acetate extractives contained phenolic aldehydes (*p*-hydroxybenzaldehyde and vanillin) and also a relatively high molecular weight fraction. This fraction could not be analyzed by GC. To clarify the relationship between the degree of condensation of lignin and total yields of the phenolic aldehydes, the chemical structure of the high molecular weight fraction must be examined in detail. A modified nitrobenzene oxidation method using ^{1}H-NMR spectroscopy, as proposed by Katahira and Nakatsubo,[19]

will be effective in obtaining further information about the condensed structures of lignin. In addition, it is necessary to characterize lignin in water layers to clarify the behavior of whole lignins in nitrobenzene oxidations in more detail.

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