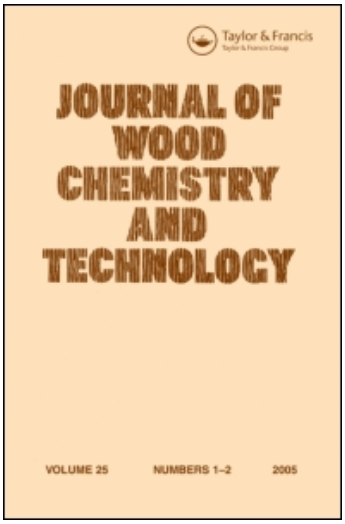

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Formation and Structure of Lignin in Monocotyledons. III. Heterogeneity of Sugarcane (*Saccharum officinarum* L.) Lignin with Respect to the Composition of Structural Units in Different Morphological Regions

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FORMATION AND STRUCTURE OF LIGNIN IN MONOCOTYLEDONS. III. HETEROGENEITY OF SUGARCANE (*Saccharum officinarum* L.) LIGNIN WITH RESPECT TO THE COMPOSITION OF STRUCTURAL UNITS IN DIFFERENT MORPHOLOGICAL REGIONS

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ABSTRACT

Heterogeneity of sugarcane lignin with respect to the composition of structural units in different morphological regions was studied by microautoradiography and some degradative analyses. Structure of the lignin differs among fiber, vessel and parenchyma. The lignin in the secondary wall of fiber is composed of syringyl(S)-, guaiacyl(G)- and *p*-hydroxyphenyl(H)-propane units with accompanying phenolic acid residues, and the proportion of these monolignols is S > G > H. The lignin in vessels of protoxylem contains more G and H units than S units, and that in vessels of metaxylem is similar to that in fibers. Phenolic acid constituent in sugarcane cell wall includes sinapic acid in addition to *p*-coumaric and ferulic acids. Ferulic acid deposits at the very early stage of lignification, and *p*-coumaric and sinapic acids increasingly deposit with the progress of lignification. Therefore, the ratio of *p*-coumaric acid or sinapic acid to ferulic acid increases with lignification. Parenchyma wall involves larger amount of phenolic acids than vascular bundle does. The lignin in parenchyma is difficult to isolate by Björkman procedure, because there is greater possibility to form cross-linkage among cell wall polymers through phenolic acids. The cross linkages involving phenolic acid ester also explains why the sugarcane lignin is easily degraded.

INTRODUCTION

Morphological heterogeneity of lignins in various woody plants has been studied extensively. However, the heterogeneous feature of lignins in monocotyledons is seldom known.

The deposition process of structural units and their distribution in different kinds of cell walls or different layers of the cell wall can be visualized by microautoradiography combined with selective labeling of a specific unit in lignin. This technique has also been applied to study of rice plants [1,2]. The results suggested that there are distinct differences in structural heterogeneity between the lignins of graminaceous plants and woody plants.

Sugarcane is a perennial, economically important crop, and bagasse is used extensively for pulping and animal feed. The knowledge of structural heterogeneity of lignin with respect to its morphological regions may contribute to comprehensive understanding of sugarcane lignin and more effective utilization of this plant.

EXPERIMENTAL

Material

Stem cuttings of sugarcane (*Saccharum officinarum* L. cultivar Ryukyu-78-16) were kindly provided by Dr. M. Hirakawa of Ryukyu University and grown on the campus of Nagoya University.

Sapwood of Japanese red pine (*Pinus densiflora* Sieb. et Zucc) and poplar (*Populus maximowiczii* × *Populus nigra*) were used to prepare milled wood lignin (MWL).

Cell Wall Preparation

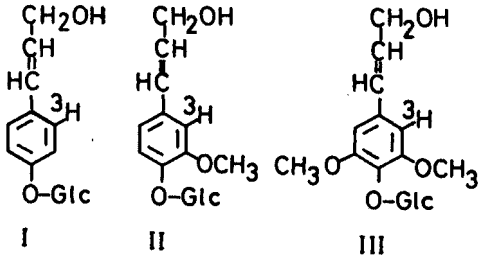
Sugarcane was harvested in middle November. The stem of 150 cm height was separated into four parts from the growing shoot tip to the bottom. Water soluble substances including sucrose was thoroughly removed by repeating pressing and washing three times. The youngest internodes (upper part) and mature internodes (lower part) were further divided into outer layer comprised epidermis and sclerenchyma, and inner layer comprised vascular bundle and parenchyma. The inner part was lightly blended in water by a Waring blender and sifted through 20 mesh. Consequently, vascular bundle remained over the sieve and parenchyma passed through the mesh. Yields of outer layer, vascular bundle-rich fraction and parenchyma-rich fraction from mature internodes were 49%, 26% and 25% respectively.

Determination of Lignin

The lignin content was determined by the improved acetyl bromide-UV procedure [3]. Extractive-free material (60 mesh, 1 mg), 25% of acetyl bromide in acetic acid (w/w, 1 mL) and 70% perchloric acid (40 μL) were placed in a glass tube and heated at 70 °C for 30 min. The mixture was shaken gently at 10 min intervals. Then the reaction mixture was transferred to a volumetric flask (10 mL) contianing 2 mL of 2 N sodium hydroxide and made up to 10 mL with acetic acid. The ultraviolet absorption spectrum was recorded and the absorbance at 290 nm was read. The lignin content was calculated by employing a absorptivity of 21.5 *l*/g.cm that is worked out of milled sugarcane vascular bundle lignin (MSL) without correction of carbohydrate. The MSL was prepared according to Björkman's procedure [4].

Syntheses of Labeled Lignin Precursors

p-Glucocoumaryl alcohol-[arom. ring-2-^{3}H] (I), coniferin-[arom. ring-2-^{3}H] (II) and syringin-[arom. ring-2-^{3}H] (III) were synthesized from 2-bromo-4-hydroxybenzaldehyde, 2-bromovanillin and 2-bromosyringaldehyde respectively, by a similar procedure described previously [5].



I II III

Labeled precursors of lignin biosynthesis

Administration of Precursors

Sugarcane was employed for administration in October was 6 months after shooting. The stem was about 2 cm in diameter and 140 cm height. The 7th and 9th internodes from the tip of growing shoot were cut out. The former is less lignified than the latter. A V-shaped groove parallel to the direction of the stem was made on each of internodes and treated with 0.1 mMol mercaptoethanol. Labeled precursors I, II and III in phosphate buffer (0.3 mg/30μL, 5.6×10^5 Bq.) were separately fed by dropping the solution to the groove (Figure 1) and allowed to metabolize for two days.

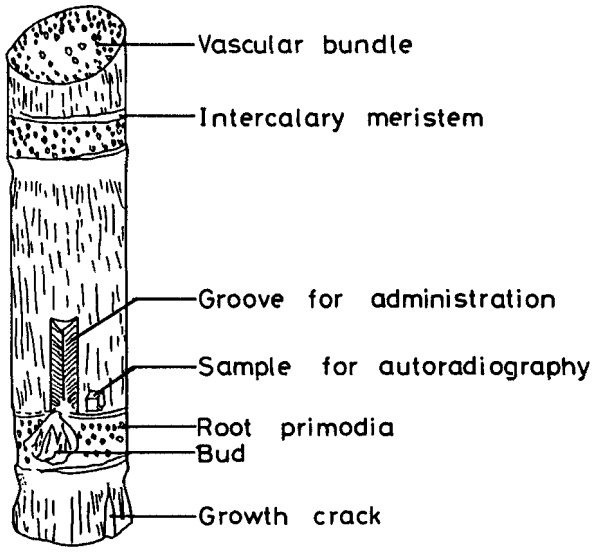


FIGURE 1. Administration of precursors to sugarcane stem

Microautoradiograph

Microautoradiograms were prepared in the same way as described in the previous paper [1].

Nitrobenzene Oxidation and Determination of Its Products

Labeled plant material was ground to pass through 60 mesh. The divided meal was extracted with ethanol/benzene (1/2) for 8 h and with water for 12 h. Extractive-free plant meal (20 mg), 2 N sodium hydroxide (300 μL), and nitrobenzene (20 μL) were placed in a small autoclave and heated at 175 °C for 3 h. Then the reaction mixture was extracted with ether (3 mL × 4), and the aqueous layer was acidified with dilute hydrochloric acid to pH 2, and extracted again with ether (3mL × 4). The ether solution was eva-

porated to dryness, and the residue was dissolved in dioxane (50 μL) containing 3,5-dihydroxybenzoic acid (0.5 mg) as an internal stantard. An aliquot (10 μL) of the solution was subjected to analysis by Shimazu LC-6A high performance liquid chromatograph (HPLC) equipped with a Unisil-pack 5C-18 column (6 × 300 mm) and a variable-wavelength UV detector. Aqueous acetonitrile (12%) containing 0.1% phosphoric acid was used as eluant at a flow rate of 1.3 mL/min. The separated components were detected at 280 nm and their radio-activities were determined with an Aloka liquid scintillation counter LSC-700.

Determination of Cell-Wall-Bound Phenolic Acids

Extractive-free meal of cell wall (20 mg) was suspended in 2 mL of 1 N sodium hydroxide and kept for 24 h with occasional stirring. The reaction mixture was acidified to pH 2 with dilute hydrochloric acid, and extracted with ether(3 mL × 4). The extract was evaporated to dryness, and the residue was dissolved in 50 μL of dioxane containing 0.3 mg of vanillin as an internal standard. An aliquot (2.5 μL) of the solution was analyzed by the same HPLC system as used for the products of nitrobenzene oxidation. The mobile phase consisted of 18% acetonitrile and 0.1% phosphoric acid at a flow rate of 1.0 mL/min. Separated components were detected at 310 nm. Authentic samples of hydroxycinnamic acids were used for determination of response factors used in the quantitative analysis.

GC-MASS Analysis

A part of acids liberated by alkali was trimethylsilylated with N,O-bis-(trimethylsilyl)-acetamide at room temperature for about 30 min, and subjected to GC-MASS analysis (OV-1701 column: 25m × 0.25mm, inj.temp.250 °C, initial temp.50 °C, rate 50 °C/min, final temp. 280 °C).

GPC Elution Profiles of Isolated Lignins and the Lignins Treated with Alkali

About 3 mg of milled pine lignin, milled poplar lignin and milled sugarcane lignin were treated with 300 μL of 1 N sodium hydroxide at room temperature for 24 h or at 170 °C for 2 h. Reaction products were neutralized with dilute hydrochloric acid and made up to 1.5 mL with dimethylformamide. An aliquot (2 μL) of the solution was applied to a Synchropack GPC 60 (300 × 7.8 mm) gel permeation chromatograph. The column was calibrated with a series of polystyrene ($\overline{Mn}$: 32500, 16700, 8900, 6200, 2855) and tetra-O-acetyl glucovanillin. The mobile phase was dimethylformamide containing 0.1 M lithium chloride [6] at a flow rate of 0.5 mL/min.

RESULTS AND DISCUSSION

Lignin Contents in Different Tissues

If it is assumed that the absorptivity of lignin involved in different tissues is the same, the lignin contents of tissue fractions will be given as shown in Table 1. Lignin contents are significantly different among tissues. Parenchyma contains smaller amount of lignin than vascular bundle. Actually, the lignin in parenchyma contains more phenolic acid residues than vascular bundle as discussed later, and has higher absorptivity. Consequently, the lignin content in parenchyma may be lower than the value shown in Table 1.

Moreover, only 0.2% of the lignin was isolated from parenchyma by Björkman's procedure [4]. This implies that the structure of lignin or the state of its occurrence in the parenchyma cell wall may be quite different from others.

TABLE 1
Lignin Content and Yileds of Milled Tissue Lignin (MTL) in Different Tissues

Tissues of Mature Internodes	Lignin Content(%)*	MTL Yield(%)**
Parenchyma	13	0.2
Vascular bundle	19	26.0
Epidermis+Sclerenchyma	22	23.0

* : Determined by improved acetyl bromide-UV procedure [3]
**: Based on lignin content

Visualization of Structural Units of Lignin in Cell Walls by Autoradiography

The lignin isolated from the epidermis and sclerenchyma of sugarcane has been studied by degradative analysis [7]. Although composition of phenolic acid residues differs between milled sugarcane lignin (MSL) and enzymatically isolated sugarcane lignin (ESL), no information about heterogeneous distribution of constituent monolignols (H, G and S units) has been obtained. This does not necessarily mean that the monolignol composition of lignin is homogeneous among different morphological regions, because the information with respect to morphological origin and degradation-resistant structure may not be obtained by degradative analysis of isolated lignin.

Microautoradiography may provide information about lignin structure with respect to morphological regions by selective labeling of individual structural unit of lignin.

p-Glucocoumaryl alcohol-[arom. ring-2-^{3}H], coniferin-[arom. ring-2-^{3}H] and syringin-[arom. ring-2-^{3}H] were used as precursors of lignin biosynthesis in present work. It is supposed that tritium in a monolignol glucoside should be incorporated into only one unit of lignin and retained in corresponding degradation product as shown in Figure 2. To confirm this point, the plant materials administered with lignin precursors I, II and III were subjected to alkaline nitrobenzene oxidation, and the chemical and

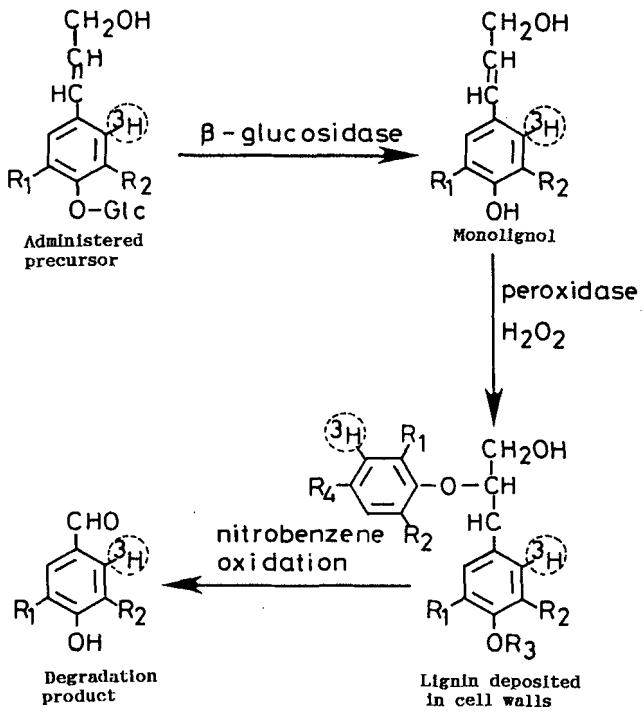


FIGURE 2. Traces of ^{3}H in lignin precursors during biosynthesis and chemical degradation

radioactive yields of degradation products were determined. Since the total radioactivity of degradation products was about 20% of the incorporated radioactivity, which is almost comparable to the chemical yield of nitrobenzene oxidation products; the selectivity in labeling H, G and S units was expressed by the percentage of the radioactivity of *p*-hydroxybenzaldehyde (H), vanillin (V) and syringaldehyde (S) relative to total one. That is,

$$\frac{\text{Specific unit-}[^{3}H]}{\text{Lignin-}[^{3}H]} = \frac{\text{Radioactivity of one degradation product}}{\text{Radioactivity of total degradation products}}$$

Precursors:

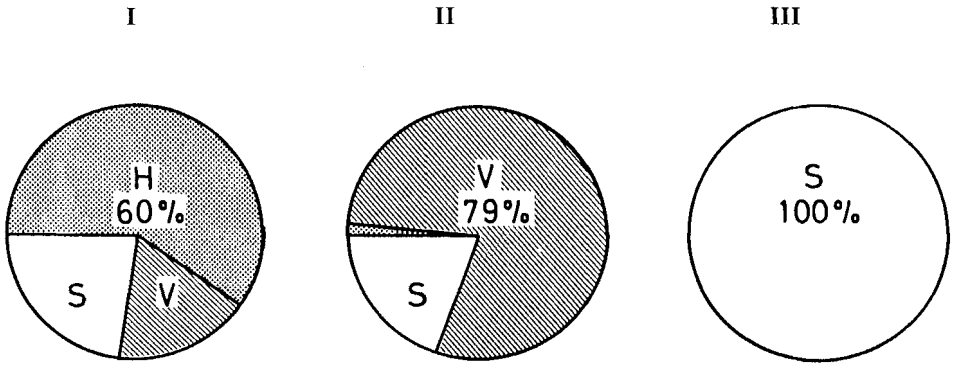


FIGURE 3. Distribution of incorporated radioactivity among building stones of sugarcane lignin

The results are shown in Figure 3. When *p*-glucocoumaryl alcohol-[arom. ring-2-^{3}H] (I) was administered, 60% of the incorporated radioactivity was retained in H units; when coniferin-[arom. ring-2-^{3}H] (II) was administered, 79% of the incorporated radioactivity belonged to the G units. In the case of administration of syringin-[arom. ring-2-^{3}H] (III), incorporated radioactivity was completely attributed to S units. These results indicate that it is possible to label three structural units in the cell wall almost selectively by administration of monolignol glucosides. Accordingly, the silver grains on each of microautoradiograms approximately represent the distribution of a specific structural unit corresponding to administered precursor.

Figure 4 shows a part of microautoradiogram of sugarcane administered with precursor I. Figure 4(a) and (b) provide a comparison of the deposition of H units at two stages of lignification. Both of the stage was considered as lignification of secondary wall of fibers. There were only a few silver grains

both in fiber and vessel walls in Fugure 4(a). With the progress of secondary wall thickening (Figure 4(b)), deposition of H unit in fibers significantly increased. A considerable amount of H unit deposited in the secondary wall of protoxylem vessel too, but scarcely in vessels of metaxylem.

Figure 5 shows the microautoradiogram of sugarcane administered with precursor II. A small amount of silver grains on microautoradiogram (a) also indicates low level of incorporation of G units at this stage of lignification, but the secondary wall of protoxylem vessel incorporated a larger amount of G units than other cell walls. At stage (b), G unit deposited much more than it did at previous stage and concentrated on the secondary walls of fibers.

Deposition of S unit in fibers (Figure 6) also greatly increased from stage (a) to (b).

Consequently, all of three structural units (H, G and S) are deposited in secondary wall of fiber. If it is taken into account that about 40% of silver grains in Figure 4 should be attributed to the deposition of other units than H, the lignin in the secondary wall of fiber is slightly S-rich polymer concomitantly containing H and G units. The lignin in the secondary wall of protoxylem vessels contains more H and G than S units.

In spite of the fact that formation of secondary wall was observed in both of the samples (a) and (b), there is a great difference in deposition of lignin units between two stages. This may be explained by the formation of multilayer [8] within fiber secondary wall, in which lignin concentration is heterogeneous.

Deposition Process and Heterogeneous Distribution of Phenolic Acid among Different Tissues

Cell wall materials of sugarcane from the different parts of the stem without distinction of parenchyma and vascular bundle were treated with alkali and liberated phenolic acids were

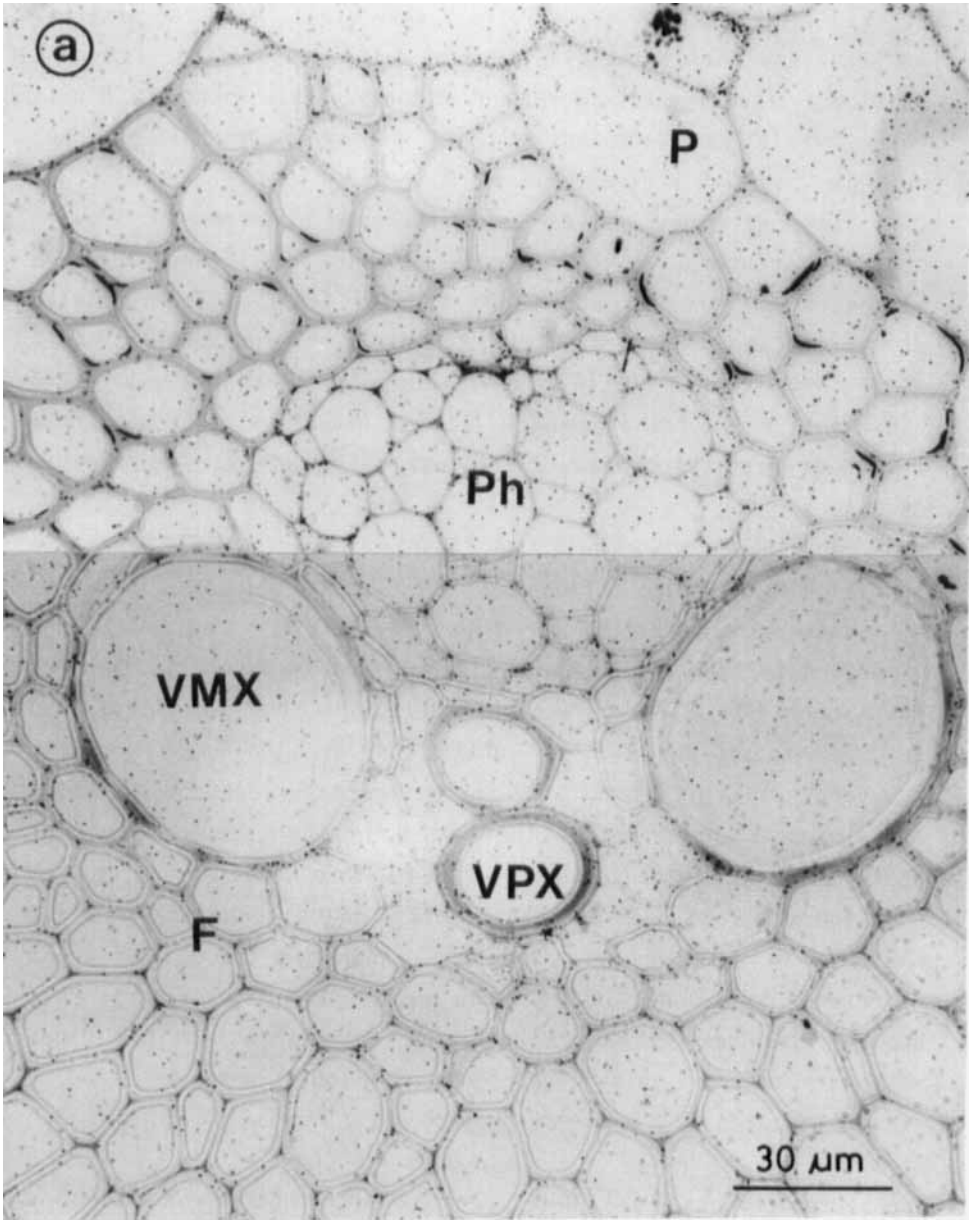


FIGURE 4(a). A part of the microautoradiogram of the 7th internode administered with *p*-glucocoumaryl alcohol-[arom. ring-2-^{3}H]; P: parenchyma; Ph: phloem; VMX: vessel of metaxylem; VPX: vessel of protoxylem; F: fiber.

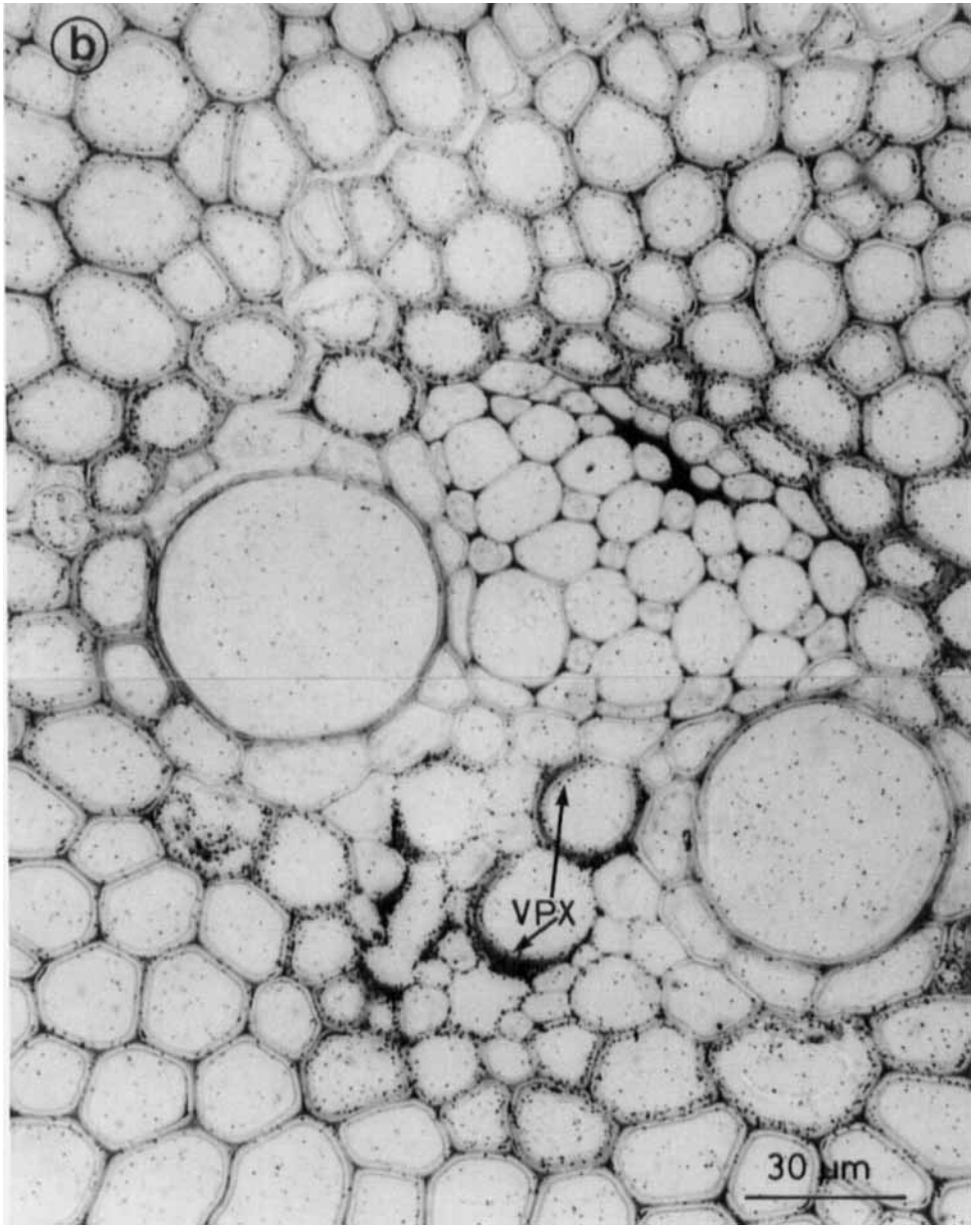


FIGURE 4(b). A part of the microautoradiogram of 9th internode administered with *p*-glucocoumaryl alcohol-[arom. ring-2-^{3}H]

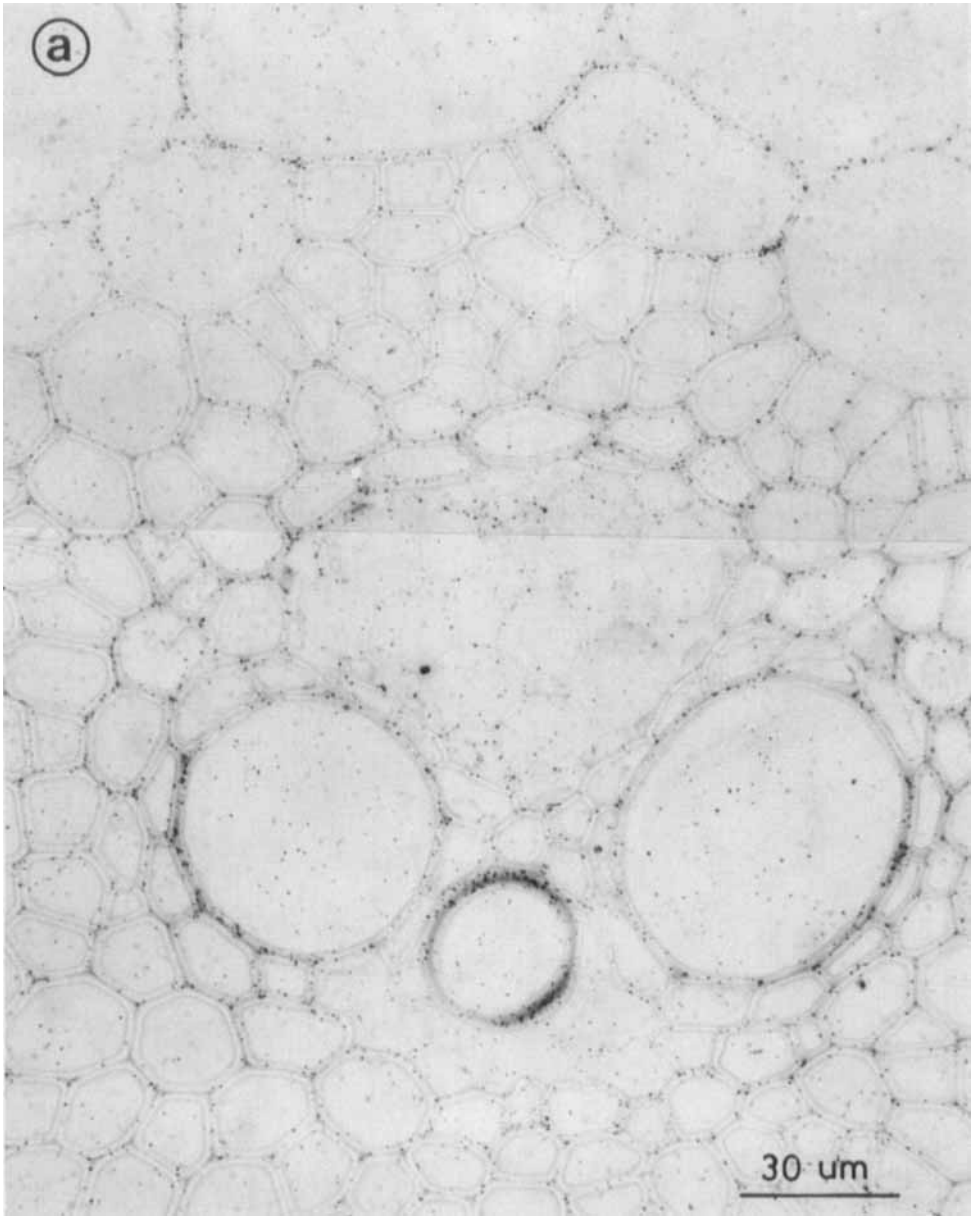


FIGURE 5(a). A part of the microautoradiogram of the 7th internode administered with coniferin-[arom. ring-2-^{3}H]

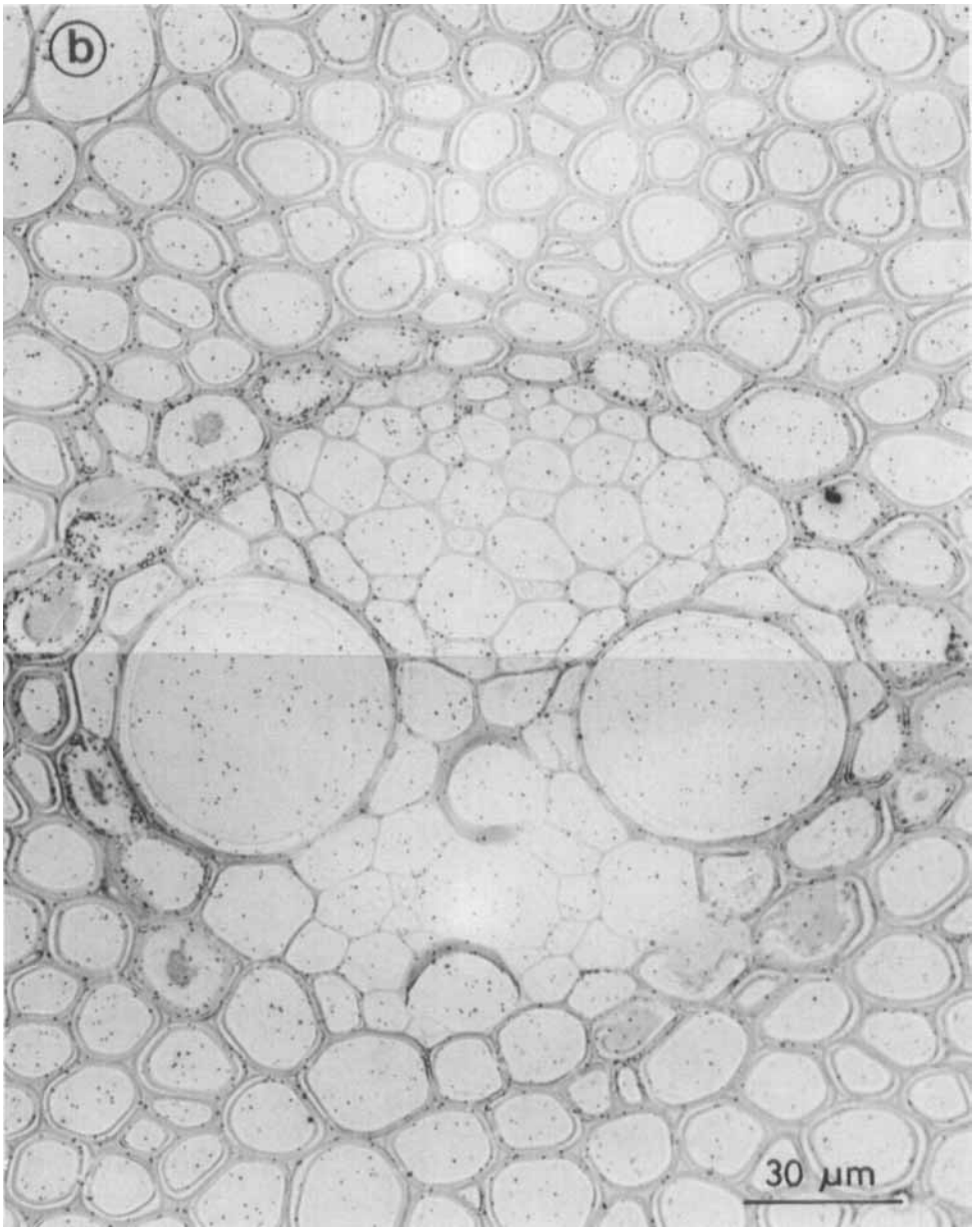


FIGURE 5(b). A part of the microautoradiogram of 9th internode administered with coniferin-[arom. ring-2-^{3}H]

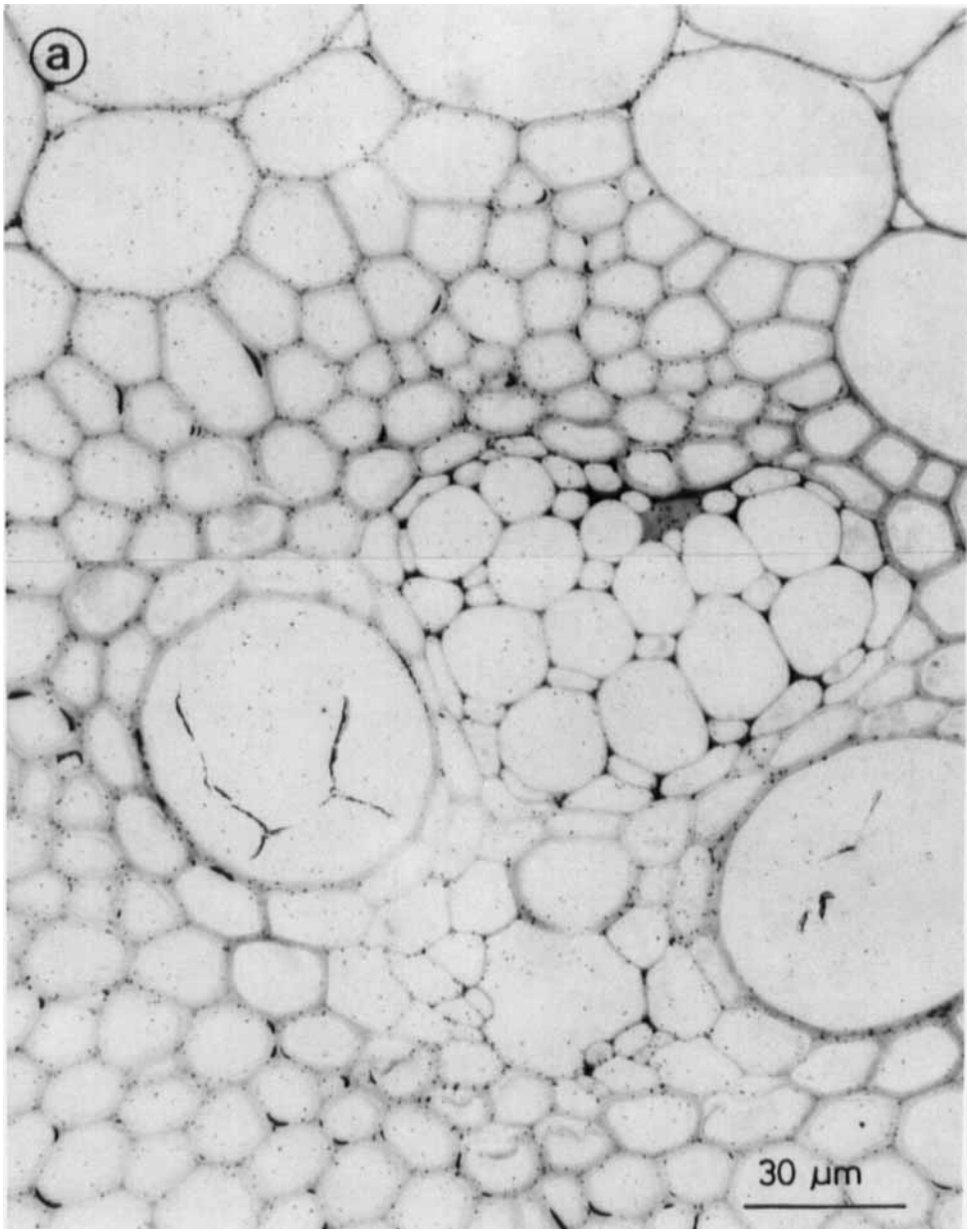


FIGURE 6(a). A part of the microautoradiogram of 7th internode administered with syringin-[arom. ring-2-^{3}H]

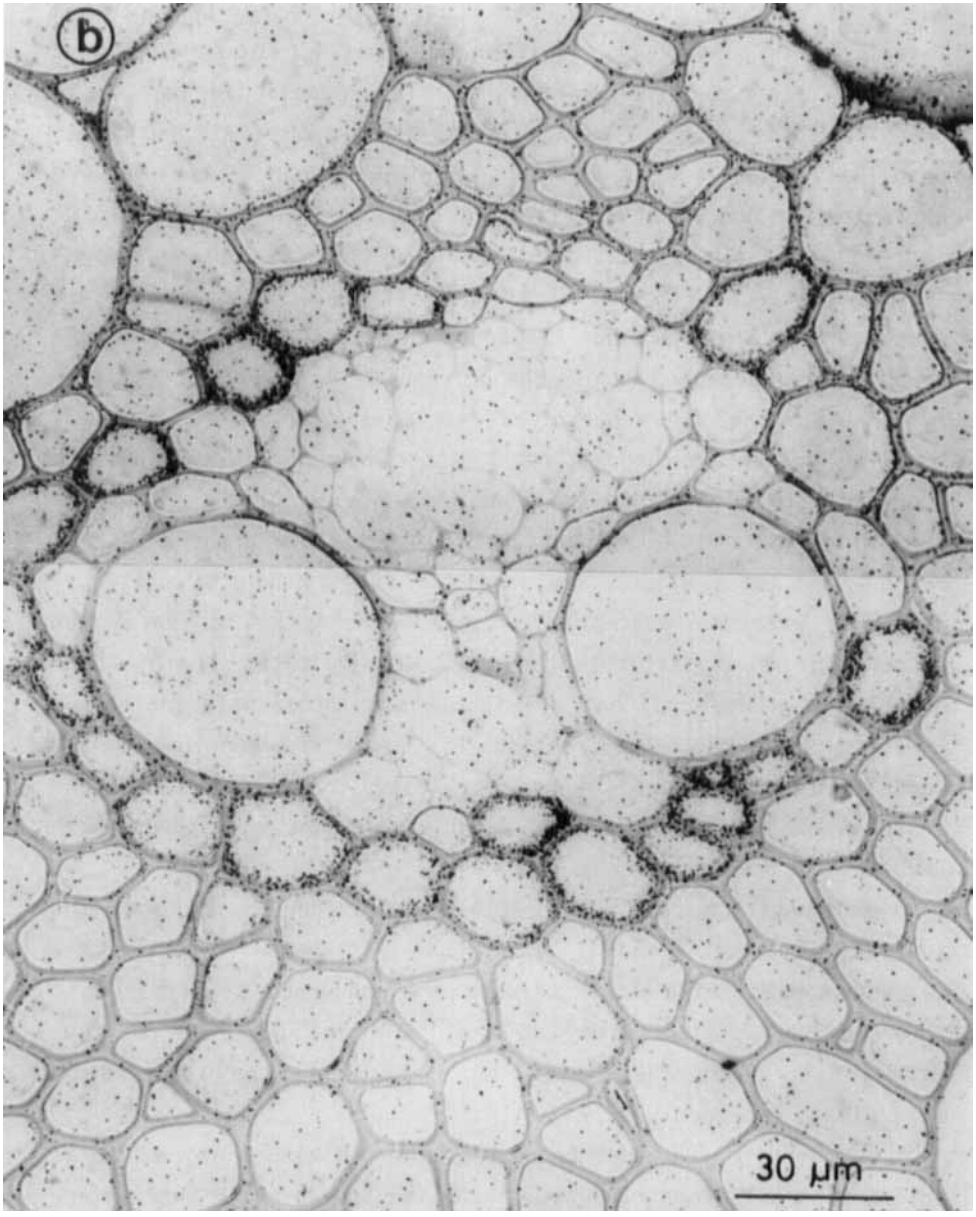


FIGURE 6(b). A part of the microautoradiogram of 9th internode administered with syringin-[arom. ring-2-^{3}H]

quantitatively determined by HPLC. In addition to *p*-coumaric and ferulic acids that occur widely, a peak was detected at the same retention time as that of authentic sinapic acid (Figure 7). To identify this compound, products of alkali hydrolysis were trimethylsilylated, and analyzed by GC-MASS spectrometry. The gas chromatogram and MASS spectrum of the compound at scan 1097 (marked with broken line) are shown in Figure 8a and 8b respectively. Molecular fragment of trimethylsilylated sinapic acid was found at M/Z 368, and other fragments such as M-15, M-30, M-45 and so on were detected too. These results provide confirmatory evidence for the occurrence of sinapic acid.

The change in composition of these phenolic acids with the progress of lignification is shown in Figure 9. With the progress of lignification, the proportion of saponifiable *p*-coumaric acid residue to total lignin increased at first, and then slightly decreased when the content of lignin reaches 20% or more. The proportion of saponifiable ferulic acid to total lignin shows decreasing trend, and that of sinapic acid constantly increased. This implies that lignification is always accompanied by the deposition of *p*-coumaric and sinapic acid. On the other hand, the reasons for decrease in the relative content of saponifiable ferulic acid may be that:

1) the deposition rate of ferulic acid residue relative to the total lignin slows down;

2) deposited ferulic acid residues are condensed by coupling to form C_5-C'_5 or etherified to form C-O-4 as suggested previously [9-12] so that the ferulic acid could not be released by mild alkali treatment.

Furthermore, in order to examine the distribution of phenolic acids among different tissues, parenchyma and vascular bundle were separated and treated with alkali. The change in the composition of phenolic acid residues in parenchyma and vascular bundle between two stages of lignification is shown in Figure 10. The parenchyma cell wall incorporated more phenolic acid ester than the cell wall of vascular bundle at both of the stages. Phenolic

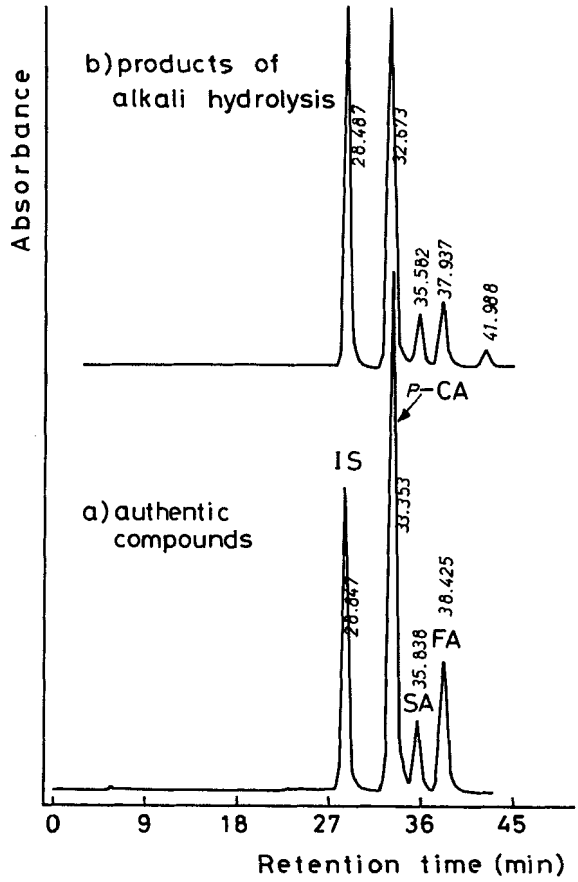


FIGURE 7. HPLC profile of a): authentic mixture of *p*-coumaric acid (*p*-CA), ferulic acid (FA) and sinapic acid (SA) and b): products of alkali hydrolysis of sugarcane cell wall materials

acid from parenchyma corresponds to about 62% of total phenolic acid in young tissue, and 58% in mature tissue. A larger proportion of ferulic acid residue than *p*-coumaric acid is involved in parenchyma of young tissue, but lower proportion in mature tissue. This supports the suggestion again that ferulic acid deposits prior to *p*-coumaric acid in parenchyma cell wall. In vascular bundle that is mainly composed of fibers, a little bit

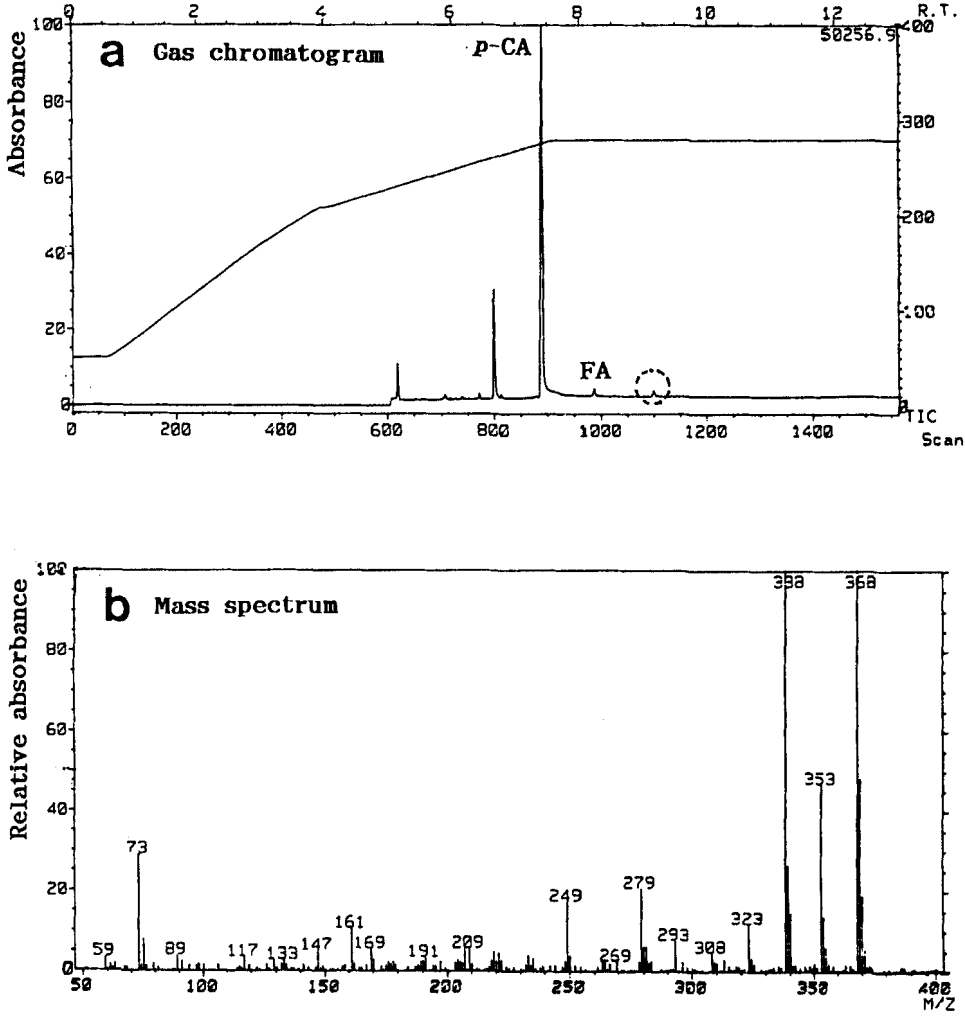


FIGURE 8. Gas chromatogram (a) of trimethylsilyl derivatives of alkali hydrolysis products of sugarcane cell wall materials and mass spectrum (b) of the component at scan 1097

greater amount of *p*-coumaric acid than ferulic acid deposits at the early stage, subsequently the amount of *p*-coumaric acid increases quickly.

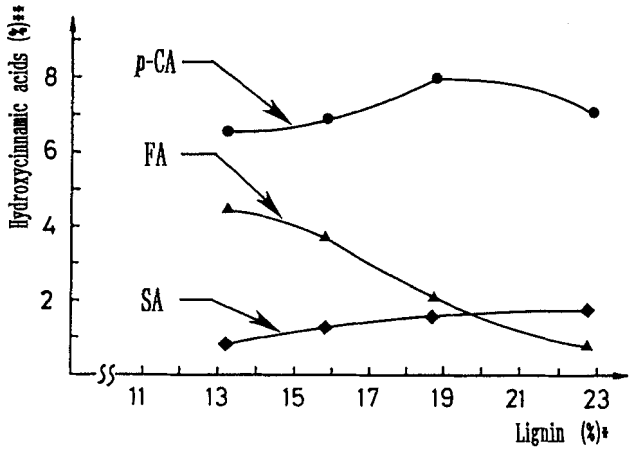


FIGURE 9. Deposition of phenolic acids with the progress of lignification; *: Based on extractive-free cell wall material; **: Based on lignin content

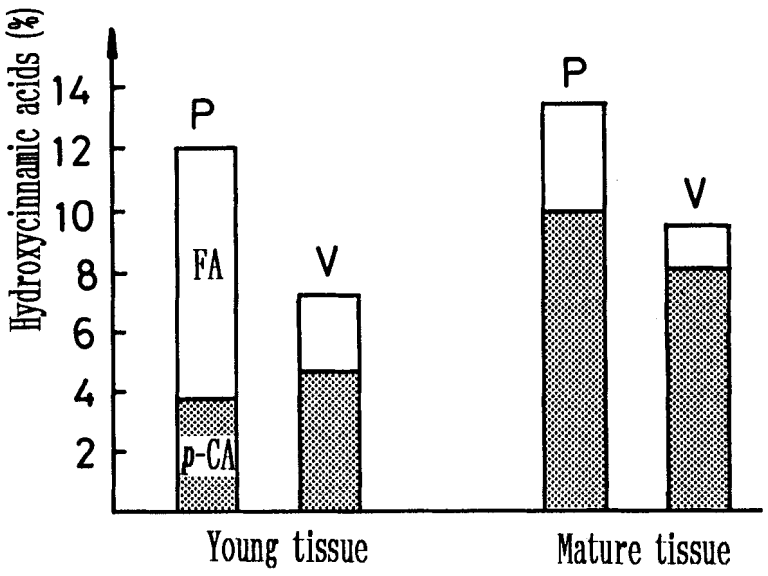


FIGURE 10. Distribution of phenolic acid residues in different tissues; *: Based on lignin content; P: parenchyma; V: vascular bundle; *p*-CA: *p*-coumaric acid; FA: ferulic acid

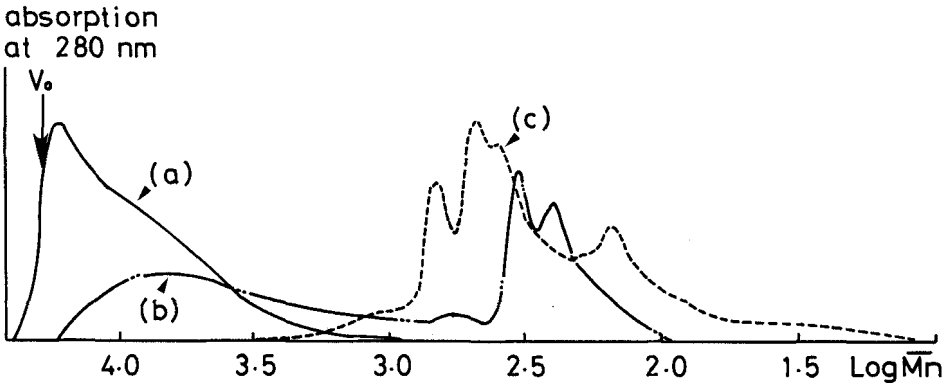


FIGURE 11. Gel permeation chromatograms of isolated lignin from *Saccharum officinarum* (a), the lignin treated with alkali at room temperature (b) and the lignin treated with alkali at 170 °C (C)

Although only saponifiable phenolic acids were dealt with in this paper, investigation by ultraviolet microscopic spectrophotometry also gave the same results as described above [8].

Effect of Cross Linking and Ester Linkage on the Degradation of Lignin Macromolecules

It has been known that graminaceous plants are delignified easily during soda pulping. The susceptibility to degradation has been ascribed to that the cell wall has high permeability of cooking reagent and the lignin contains larger amount of phenolic hydroxyl groups [13]. It is supposed that the hydrolysis of phenolic acid ester might play an important role in breakage of protolignin network. It was proved by the results from gel permeation chromatographic examination of milled sugarcane lignin (MSL) and alkali-treated MSLs. The MSL was degraded into low molecular fragments even at room temperature overnight as shown in Figure 11.

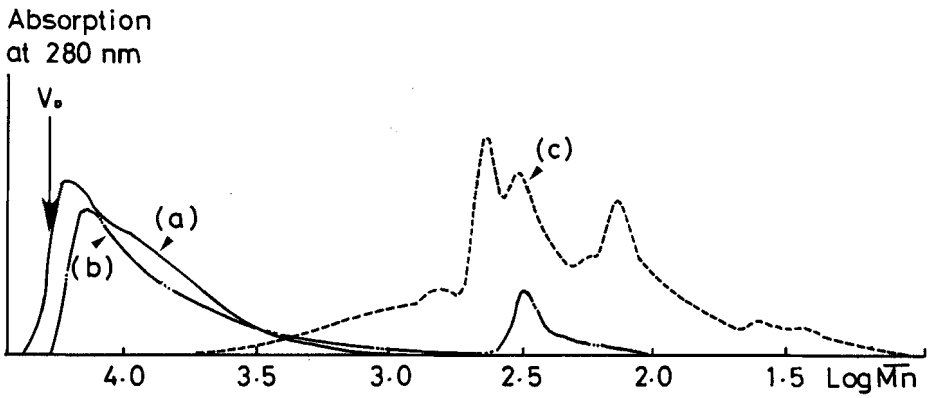


FIGURE 12. Gel permeation chromatograms of isolated lignin from *Populus maximowiczii* × *Populus nigra* (a), the lignin treated with alkali at room temperature (b) and the lignin treated with alkali at 170 °C (C)

This great change of molecular weight can not be completely explained only by the release of phenolic acid residues that are esterified to core lignin as terminal units. There must have been some cross-linkages involving ester to be cleaved. Figure 12 shows gel permeation chromatograms of the lignin isolated from poplar. In spite of the high content of *p*-hydroxybenzoic acid esters in poplar lignin, poplar MWL did not undergo depolymerization as extensively as MSL at room temperature. Pine lignin is depolymerized to a little extent under the same conditions (Figure 13). When the lignin preparations were subjected to alkali treatment at 170 °C for 2 h, those isolated from sugarcane and poplar were depolymerized to an apparent molecular weight below 1000 (Figure 11c and 12c), whereas a significant amount of lignin isolated from pine remained its molecular weight above 1000. The difference among lignin preparations in susceptibility to degradation at high temperature may be due to other bonds than ester.

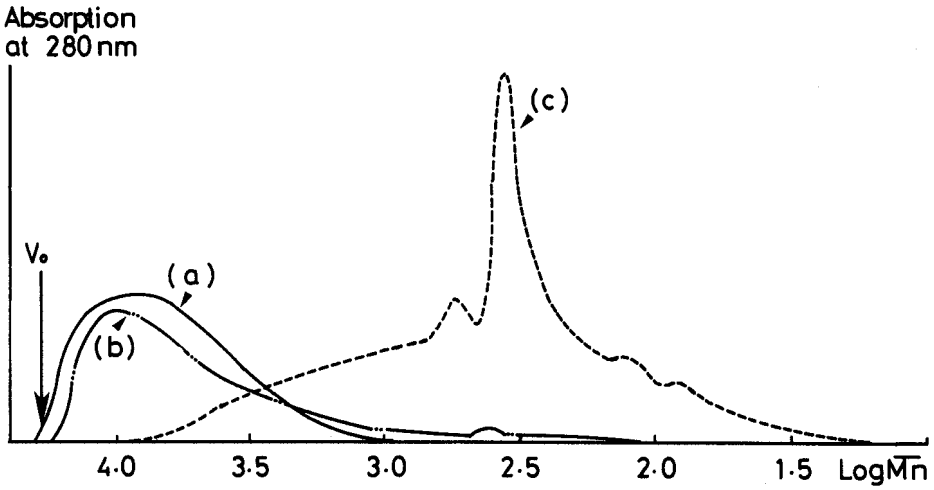


FIGURE 13. Gel permeation chromatograms of isolated lignin from *Pinus thunbergii* (a), the lignin treated with alkali at room temperature (b) and the lignin treated with alkali at 170 °C (C)

CONCLUSIONS

Sugarcane lignin is composed of *p*-hydroxyphenylpropane, guaiacylpropane, syringylpropane, *p*-coumaric acid, ferulic acid and sinapic acid moieties. Sinapic acid was first quantitatively determined. The composition of structural units varies with the stage of lignification and morphological regions.

Lignin in protoxylem vessels is composed of large amount of G and H units and small amount of S units. Lignin in secondary wall of fiber contains S, G and H units in a proportion of S > G > H.

Lignin in parenchyma wall may contain more phenolic acid residues than fiber and vessel walls. The ratio of *p*-coumaric acid to ferulic acid involved in young tissue and mature tissue is different.

It is suggested that easy depolymerization of sugarcane lignin by alkali might be attributed to the occurrence of ester linkage in lignin macromolecules.

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