Lignin of the Fiber Sclereids in the Phloem of *Larix leptolepis* Gold.*

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Abstract

The intact fiber sclereids in the phloem of *Larix leptolepis* Gord. were separated from the other phloem elements by homogenization with a polytron or with a disintegrator and isolated by a density flow method without the use of chemical treatments. The lignin in the intact fiber sclereids was degraded by thioacidolysis and the thioacidrolysates were analysed by GLC and GC/MS. The results demonstrated that the lignin in the fiber sclereids yielded more thioacidrolysates than those from the lignin in the sapwood meals. The yields of the monomers from the lignin of fiber sclereids were as high as the level derived from MWL. The lignin of the fiber sclereids is rich in β-O-4 substructures, and could be characterized to be endwise type lignin. Since the lignin in the fiber sclereids was shown to be secondary wall lignin through microscopical observation, the present results seem to support the judgement that MML is derived from the secondary walls of the xylem tracheids of softwood.

Key words: lignin, fiber sclereid, phloem, *Larix leptolepis*, thioacidolysis.

Contents

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1. Introduction

The fiber sclereid cells in the phloem of *Larix leptolepis* Gord. are known as spines. As these spines often stick into workers' fingers, their existence in the bark makes wood handling difficult. Only fiber sclereid cells can react with the phloroglucin-HCl reagent showing the presence of lignin in cells, with all other phloem elements showing no reaction at all with the reagent.

The morphological features and the formation and distribution of the fiber sclereid cells in the inner bark of *L.leptolepis* or *L.gmelinii* Gord. have been extensively studied by Imagawa et al[1,2,3,4].

The original cells of the fiber sclereids can not be distinguished from those of the other phloem elements in the first year. The original cells start differentiation, elongation and thickning not in the first year but in the spring of second year. Each cell forms dispersedly in the phloem and lignification occurs only in the secondary walls of the cells. The middle lamellae between the fiber sclereid cells and the other phloem elements do not lignify at all. Thus the lignified fiber sclereids exist independently as spines in the phloem.

The proccesses of the differentiation, thickening and lignification of the fiber sclereid cells were observed histochemically. The optical and ultraviolet microscopic observation of the proccess of lignin formation revealed clearly the concentrical accumulation of the lignin in the secondary walls of the cells. Previous topochemical studies of xylem lignins have shown that the lignins in the secondary walls, in the middle lamellae, and in the cell corners of the xylem fibers are different from each other[5]. The observation that fiber sclereids have only secondary wall lignin is significant in terms of investigations into the structure of the lignin of the fiber sclereids. If the intact fiber sclereids could be isolated and their lignin structures were determined, we would be able to get very important information on lignin structure from a topochemical view point. The information on the

lignin structures of the secondary walls of intact fiber sclereids could be used as a reference in the determination of the lignin structure for the secondary walls of xylem fibers.

Imagawa[2] has shown that fiber sclereids could be separated easily from the other phloem elements after mild alkaline hydrolysis, and Okuyama[6] was able to obtain fiber sclereids from the inner bark pulp produced by kraft cooking. The isolation of fiber sclereids dosen't seem to be so difficult compared with the difficulty of isolating intact fiber cells in the xylem. For chemical analyses including the determination of the lignin structures, it is preferred that the samples be prepared without chemical preretreatments.

This paper deals with the isolation of intact fiber sclereids from the phloem of *Larix leptolepis* Gord. without the use of chemical treatments and the characterization of the lignin in the isolated fiber sclereids through the application of thioacidolysis.

2. Experiments

2.1 Samples

A tree of *Larix leptolepis* Gord. (21 years old, 15.4 m height, 24 cm breast height diameter) was harvested from the Sapporo Experimental Tree Nursery Garden of the College Experimental Forests of Hokkaido University on October 1st, 1985. Disks, 6~8 cm thick, were cut each end of the 1 m long logs. The disks were stored in a freezer at −20℃ until they were examined.

2.2 Isolation of the fiber sclereids

After the inner bark of the disk at breast height was immersed in water, it was cut into small pieces (2x2x15mm). The pieces were placed in a beaker with water and homogenized with a Polytron using a PT352"OD"M generator shaft for first 5 minutes and a PT20"OD"S generator shaft for another 5 minutes.

The defiberized phloem was poured into a hand-made separator and the fiber sclereids were separated from the other phloem elements by the density flow method. The cells were divided into two fractions according to their density difference through the introduction of tap water into the separater with a flow rate of 300~400 ml/min. The fiber sclereids with higher densities settled to the bottoms of the containers of the separator, and the other phloem elements with lower densities floated out of the separator into a 200-mesh screen. The fraction containing the fiber sclereids was purified by eliminating the other phloem elements which contaminated the sample using a pair of tweezers under a microscope, and the fraction containing the other phloem elements was also purified by eliminating fiber sclereids it contained.

The pieces of inner bark were also disintegrated with a KRK disintegrator.

2.3 Extraction of the fiber sclereids

The fiber sclereids, the other phloem elements, the sapwood meal (40~60 mesh), and MWL (*Picea jezoensis*, as a reference) were dried in a desiccator under reduced pressures. The dried samples were powdered with a vibration ball mill. The powdered samples were extracted with ethanol/benzene, ethanol and hot water, successively, by batch systems.

2.4 Determination of Klason lignin and sugars

Klason lignins of the samples were determined according to the TAPPI standard T13m-54.

The filtrates obtained in the Klason lignin determination were used for the sugar

analysis measuring alditol acetate[7]. The dichloromethane soluble alditol acetates were analysed with a GLC using myo-innositol as an internal standard.

2.5 Thioacidolysis

The extractive free sample (100 mg) was degraded by thioacidolysis as utilized by Lapierre et al[8]. The sample was treated with 10 ml of dioxane/ethanethiol(9 : 1, v/v)-0.2*N* tetrafuruoroboronetherate in a stainless microtube (20 ml) at 100°C for 4 hours. The resulting mixture was cooled to room temperatures with tap water , and the pH of the solution was adjusted to 3~4 with sodium hydrogencarbonate. Then the solution was extracted with dichloromethane. The dichloromethane extract was dried over anhydrous sodiumsulphate and was filtrated to remove the sodiumsulphate. The dried dichloromethane soluble hydrolysates were concentrated and trimethylsilylated with *N*,*O*-bistrimethylacetoamide, trimethylchlorosilane, and pyridine (10 : 1 : 4, v/v). Ayapanin 50 γ was added as an internal standard. The solution of 0.4 μl was injected to a GLC and a GC/MS.

2.6 Conditions for GLC and GC/MS

A. Sugar analysis: a Simadzu GC-4A with a EGSS-X 3 m column, 210°C column temp., 235°C injection temp., N_2 20 ml/min, a FID detector, and a Shimadzu Chromatopack C-R1A integrator.

B. Thioacidolysis products analysis: a Shimadzu GC-5A with a methyl silicon OV-1 capirally column (0.33mmx25m), 150-280°C column temp., 5°C/min rate, N_2 40ml/min, a FID detector, and a Chromatopack Simadzu C-R3A intergrator.

C. GC/MS: a JOEL DX-300. Conditions for GLC were the same as those described in B. Ionization voltage was 70 eV.

3. Results

3.1 Isolation of the fiber sclereids

The weight of the isolated fiber sclereids founding the oven dried inner bark decreased gradually from the bottom (5.7%) to higher portions (4.8%) of the tree up to a height of 7.2m. In the parts above 7.2m, it increased as are went up (7.0% at the top of the tree).

3.2 Extracts of the fiber sclereids

The values for the ethanol/benzene, ethanol, and hot water extracts from the fiber sclereids were 7.3, 3.6, and 5.0%, respectively. The combined totals for the extracts from the fiber sclereids were lower than those from the sapwood and from the other phloem

Table 1. Extracts from the fiber sclereids and the other phloem elements in the inner bark and from the sapwood meal of *Larix leptolepis* Gord.

	Ethanol/benzene extract	Ethanol extract	Hot water extract	Total
Inner Bark				
1. Fiber sclereids	7.3	3.6	5.0	15.9
2. Other phloem elements	6.8	1.8	17.3	25.8
Sapwood meal	15.8	1.4	3.4	18.5

Percentage on the oven dried samples

elements as shown in Table 1. Although the value for the ethanol extract from the fiber sclereids was two times higher than that from the other samples, the value for the ethanol/benzene extract was half of that from the sapwood samples. The value for the hot water extract from the fiber sclereids was one point five times higher than that from the sapwood meals.

3.3 Klason lignin and sugars of the fiber sclereids

A. The Klason lignins of the fiber sclereids and the sapwood meal were almost the same, 26~29%. On the other hand, the Klason lignin from the other phloem elements was 19%, even though these tissues do not react with phloroglucin-HCl reagent (Table 2). The figures obtained for the other phloem elements do not reflect the real amount of the lignin present as pointed out by Hata[9] and Miyake[10]. This observation colloborated with the results of thioacidolysis undertaken in this study.

Table 2. Klason lignin contents of the fiber sclereids and the other phloem elements in the inner bark and of the sapwood meal of *Larix leptolepis* Gord.

	Ethanol/benzene extract	Klason lignin content*
Inner bark		
1. Fiber sclereids	(7.3)	25.9
2. Other phloem elements	(6.8)	18.8
Sapwood meal	(15.8)	29.3

* Percentage on the ethanol/benzene extract free samples

Table 3. Relative sugar composition of the fiber sclereids and the other phloem elements in the inner bark and the sapwood meal of *Larix leptolepis* Gord.

	Ar	Xyl	Man	Gal	Glu	Total sugar/sample
Inner bark						
1. Fiber sclereids	1.1	3.1	11.1	28.0	56.8	49.7
2. Other phloem elements	1.0	2.6	10.7	26.4	59.2	49.8
Sapwood meal	0.8	21.5	9.0	7.9	60.8	38.5

B. The amounts of glucose and mannose in the hydrolysates from the fiber sclereids were at the same level as those from the sapwood meals. More galactose was detected in the hydrolysate from the fiber sclereids and the other phloem elements than in those from the sapwood meals, in which xylose was predominant (Table 3).

3.4 Thioacidolysis products from the fiber sclereids

A. Yields of thioacidolysis products are shown in Table 4. The amount of the dichloromethane soluble components from MWL was 91.5%. Almost of all the lignin was degraded to the solvent soluble state. The amounts of the dichloromethane soluble components from the fiber sclereids and from the sapwood meals were 59.7 and 66.6%, respectively, and the figures were higher than those for Klason lignin content. This means that not only the lignins in the samples but also the carbohydrates turned into the solvent soluble ones.

Table 4. Yields of thioacidolysis products of the fiber sclereids and the other phloem elements in the inner bark and of sapwood of *Larix leptolepis* Gord.

	Klason lignin content	Thioacidolysis product		
		CH_2Cl_2 Sol.	H_2O Sol.	Residue
Inner bark				
1. Fiber sclereids	25.9	59.7	11.6	28.7
2. Other phloem elements	18.8	31.2	28.3	40.4
Sapwood meal	29.3	66.6	10.6	22.7
Milled wood lignin (MWL)*	—	91.5	8.5	0.0

Percentages on the extractive free samples
* *Picea jezoensis* Carr. as a reference:

B. The main peaks on GLC of the thioacidolysis products of the samples were identified by GC/MS to be as follows[5] (Fig. 1 and Table 5): (1)1,1',2-trithioethylethane, (2) 1,1',2,3-tetrathioethylpropane, (3)*threo*-1,2,3-trithioethyl-3-(4'-O-trimethylsilyl-3'-methoxyphenyl)propane, (4)*erythro*-1,2,3-trithioethyl-3-(4'-O-trimethylsilyl-3'-methoxyphenyl)propane, (5)1,2,3-trithioethyl-3-(4'-O-trimethylsilyl-3', 5'-dimethoxypheny) propane. The compounds (1) and (2) are thought to be derived from the side chains of phenylpropane units and the compounds (3)-(5) were thioacidolysate phenylpropane monomers derived from β-O-4 aryl ether substructures of the lignin.

C. The amounts of (3) and (4) from the fiber sclereids were 1.7 times more than those from the sapwood meals (Table 5).

D. The amounts of (3) and (4) for lignin from the fiber sclereids were almost same as those from the MWL (1.00) (Table 6).

Table 5. GLC analysis of the CH_2Cl_2 soluble fraction of the thioacidolysis products from the fiber sclereids and the other phloem elements of the inner bark of *Larix leptolepis* Gord.

	Peak number (Rt, min)					
	1 (6.5)	(9.3)*	2 (14.7)	3 (24.9)	4 (25.0)	5 (25.5)
Inner bark						
1. Fiber sclereids	3.60	1.00	1.36	1.01	1.05	t**
				2.06		
2. Other phloem elements	0.88	1.00	0.44	t	t	t
Sapwood meal	2.21	1.00	0.74	0.56	0.68	t
				1.24		
MWL***	0.23	1.00	t	0.70	0.86	t
				1.56		

* Internal standard: ayapanin
** t: trace amount
*** *Picea jezoensis* Carr. as a reference
1: 1,1',2-trithioethylethane; 2: 1,1',2,3-tetrathioethylpropane; 3: *threo*-1,2,3-trithioethyl-3-(4'-O-trimethylsilyl-3'-methoxyphenyl)propane; 4: *erythro*-1,2,3-trithioethyl-3-(4'-O-trimethylsilyl-3'-methoxyphenyl)propane; 5: 1,2,3-trithioethyl-3-(4'-O-trimethylsilyl-3',5'-dimethoxyphenyl)-propane

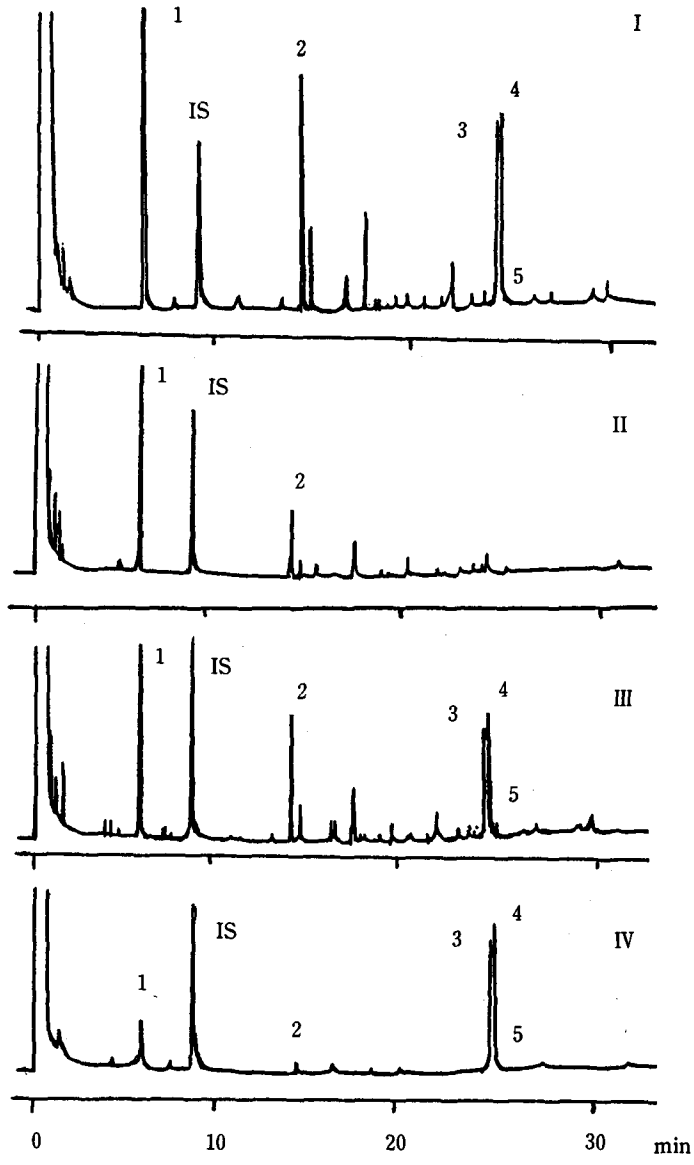

Fig. 1. GLC of the thioacidolysis products from the fiber sclereids (I), the other phloem elements (II) and the sapwood meal (III) of *Larix leptolepis* and MWL of *Picea jezoensis* (IV)

Legends

IS: Internal standard: ayapanin

1: 1,1', 2-trithioethylethane

2: 1,1', 2,3-tetrathioethylpropane

3: *threo*-1,2,3-trithioethyl-3-(4'-O-trimethylsilyl-3'-methoxyphenyl)propane

4: *erythro*-1,2,3-trithioethyl-3-(4'-O-trimethylsilyl-3'-methoxyphenyl)propane

5: 1,2,3-trithioethyl-3-(4'-O-trimethylsilyl-3'-5'-dimethoxyphenyl)propane

E. Although Klason lignin in the other phloem elements was about 20%, the thioacidolysis monomers such as (3) and (4) were not detected (Table 5). The amount of the thioacidolysis residue from the other phloem elements was very high (40.4%) compared with those of the other samples: for fiber tclereids 28.7% and for sapwood meals 22.7% (Table 4).

F. The relative amount of guiacylpropane units/lignin from the fiber sclereids was two times higher than that from the sapwood meal and was almost the same as that from the MWL (Table 6).

Table 6. Relative amount of the guaiacylpropane units from the fiber sclereids and the other phloem elements of *Larix leptolepis* Gord. degraded by thioacidolysis

	Sample (mg)	KL (%)	Lignin (mg, A)	C_6C_3 units (B)	(B)/(A)
Inner bark					
1. Fiber sclereids	100	25.9	25.9	2.06	0.079 (1.00)
2. Other phloem elements	100	18.8	—	—**	—
Sapwood meal	100	29.3	29.3	1.24	0.042 (0.50)
MWL*	20	—	20.0	1.56	0.078 (1.00)

* *Picea jezonensis* Carr. as a reference

** Trace amount of the compounds (3) and (4), which were derived from the contaminated fiber sclereids, was detected.

4. Discussion

4.1 Isolation of the fiber sclereids

The sample used in this work was harvested at the beginning of October. At this time of year, the physiological and biological activities of a tree become lower, and the tissues in the phloem become more densly packed together than is the case in the spring. Therefore the separation of the fiber sclereids from the other phloem elements in a tree, harvested in the autumn is in general more difficult than the separation of the sclereid fibers in a tree which has been harvested in an early spring.[11] In spring, the bonding of the tissues in the phloem seems to loosen because of the differentiation, elongation, and thickening of the cells. The newly formed fiber sclereids can begin to mature. The size and thickness of the mature and immature fiber sclereids differ. In this study it was found that the newly formed fiber sclereids easily floated out of the separator because of their lower densities and contaminated the other phloem elements on the 200-mesh screen. Consequently, the isolation of the other phloem elements without fiber sclereids was found to be more difficult in the spring in this study. And in autumn, because almost all of the newly formed fiber sclereids had matured and their densities had become higher than in the spring, the separation of the fiber sclereids from the other phloem elemnts was easier.

4.2 Thioacidolysis

For the determination of β-O-4 aryl ether substructures in lignin, ethanolysis has been predominantly used.[12] However, this method has several drawbacks in the determination of such substructures. The number of compounds produced through the ethanolysis products is larger than those formed by nitrobenzene oxidation or $KMnO_4$ oxidation, and the

identification of each peak in GLC can be a rather laborious task. The amount and kinds of monomers found in the ethanolysates change over the course of the reaction time because of side reactions.

Recently, Lapierre et al.[8] reported that thioacidolysis is a useful method for the determination of β-O-4 aryl ether substructures in lignin. The method has the merits that side reactions do not occur so often as in ethanolysis, and that the thioacidrolysates are limited to a few monomers. In addition, the yields of reaction products are high.

The strong nucleophilic reagent, thioethyl anion, attacks carbocation Cα of the side chains of lignin, at first, and stabilizes the reaction intermediates, hence preventing side reactions. For this reason, the number of the final reaction products is limited.

Compounds (1) and (2) were presumed to be derived from the side chains of phenylpropane based on model experiments conducted by Lapierre[8]. However, the peaks were not so big in the GLC of the thioacidolysates from the MWL as in those from the fiber sclereids, the other phloem elements, and the sapwood meal. Significantly, in the GLC of the thioacidorolysates from the other phloem elements, no peaks corresponding to phenylpropane monomers were observed in spite of the existence of peaks corresponding to compounds (1) and (2) (Fig. 1). These results indicate that compounds (1) and (2) can be derived from not only lignin side chains but also from carbohydrates. This conclusion is also supported by the finding that the amount of the solvent soluble thioacidolysis products derived from the fiber sclereids and the sapwood meals was higher than their lignin content (Table 4). The cabohydrates also turned into solvent soluble components through thioacidolysis.

4.3 The nature of the lignin in the fiber sclereids

In softwood lignin, almost all of the phenylpropane units have guaiacyl pendant group. In addition to these dominant units, a very small number of units with syringyl and *p*-hydroxyphenyl groups were found. Larger concentrations of *p*-hydroxyphenyl type monomers exist in the lignin of the cell corners, of the compound middle lamellae, or of the comperssion woods than in the lignin of normal secondary walls.

In this study, although syringyl units were detected on GC/MS of the hydrolysates as a minor peak no peaks corresponding to *p*-hydroxyphenyl units were detected. This result coincides with the fact that the lignin in the fiber sclereids is formed only in the secondary walls. Consequently, we can conclude that the lignin in the fiber sclereids is the typical secondary wall lignins.

The analysis of the thioacidolysis products indicated that the lignin in the fiber sclereids was rich in β-O-4 aryl ether substructures (Tables 5 and 6) and could be characterized as endwise lignin according to the definition by Sarkanen[13]. The present results are very interesting when seen in conjunction with the findings of the tracer experiments conducted by Terashima[14] where it was shown that noncondensed type lignin was formed predominantly in the secondary walls of the xylem fibers of wood.

4.4 The morphological origin of MWL from softwood xylem

The morphological origin of MWL from softwood fibers is still a matter of discussion. Björkman[15] has suggested that the bulk of MWL comes from the middle lamella, and Chang[16] and Sharkanen[17] have supported the concept. Whiting[18] has claimed that MWL from the black spruce *Picea mariana* originates from the secondary walls of tracheids based on his result on the rate of solvent extraction for the lignins from fractions contain-

ing either middle lamella or secondary wall prepared by the differential sedimentation of finely ground wood.

The yield of thioacidolysis monomers from the MWL of *P. jezoensis*, which was used as a reference, was twice that from the sapwood meal of *L. leptolepis*. The yield was at a level as high as that from the fiber sclereids, and the lignin was characterized to be secondary wall lignin. This indicate that the MWL of *P. jezoensis* is also rich in β-O-4 aryl ether substructures and can be characterized as an endwise type lignin. This coincides with the result reported by Matsukura[19] that the MWL of *P. jezoensis* is rich in noncondensed type lignin.

On the whole, the present results could offer positive support for the claim by Whiting[18] that MWL is derived from the secondary wall of the tracheids of black spruce.

5. Conclusion

The fiber sclereids in the phloem of *Larix leptolepis* Gord. were successfully separated from the other phloem elements by defibration of the inner bark with a Polytron followed by the application of the density flow method without the use of any chemical treatments. The lignin in the fiber sclereids was found to be rich in β-O-4 aryl ether substructures and was characterized as endwise type lignin according to Sarkanen's definition. The ratio of thioacidolysis monomers to lignin from the fiber sclereids was higher than that from the sapwood meals and was at as high a level as from MWL. Since it is known that the lignin in fiber sclereids is formed only in the secondary wall, the present results seem to collaborate the judgement that NWL is derived from secondary wall of the tracheid cells in the softwood xylem.

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要　　約

木材のある特定の細胞を熱処理を伴わずに機械的に単離することは現在のところほぼ不可能である。一方カラマツの師部細胞中に存在する特殊な細胞である厚壁細胞ファイバースクレレイドはリグニンに富み，〝トゲ〟と称されるほど硬い。また，このファイバースクレレイドは，二次壁にのみリグニンが沈積し，周囲の細胞間層にはリグニンがまったく存在しない。そのため，師部を機械的に解繊することでこれを単離することが可能と考えられた。

本研究では化学的に前処理することなしにファイバースクレレイドを機械的に単離すること，単離したファイバースクレレイドの化学成分，特にリグニンの構造について検討を加えることを目的とした。

ファイバースクレレイドは，師部組織を水溶液中でポリトロンなどのホモゲナイザーや離解機などで離解したのち，流水中で比重の差を利用して他の師部組織から分離することが出来た。

単離されたファイバースクレレイドおよびファイバースクレレイド以外の師部組織の化学分析を行い，辺材木粉，エゾマツ MWL の化学分析の結果と比較した。

その結果，次のことが判明した。

1)エタノール/ベンゼン抽出物，エタノール抽出物，熱水抽出物などにおいて，ファイバースクレレイドは特徴ある挙動を示した。

2)ファイバースクレレイドの Klason リグニン量は辺材木粉中のそれとほぼ等しかった。硫酸加水分解液中の糖の分析によると，グルコース，マンノースはファイバースクレレイドおよび辺材木粉とでほぼ等しかった。

3)チオアシドリシス分解物の GLC におけるピーク(1)～(5)は，GC/MS の解析によってそれぞれ同定した。ピーク(3)，(4)および(5)が針葉樹型リグニンに由来するフェニルプロパンモノマーであった。

4) 生成したフェニールプロパンモノマーの生成量に依ってリグニンの特徴付けを行うことが可能であった。

これらの結果をまとめると，ファイバースクレレイドのリグニンは，β-O-4-アリルエーテル結合に富んでおり，Sarkanen の定義によるエンドワイズ型リグニンが主体と成っているらしいことが推定された。今川らのファイバースクレレイドのリグニンが二次壁外層から逐次同

心円状に形成されていくという組織化学的研究結果と，そのリグニンが，エンドワイズ型構造単位に富むという本研究の化学的分析結果とは互いに相補的であり，大変興味深い。

一方，エゾマツ MWL は非縮合型結合単位に富むリグニンからなっていると報告されていたが，本研究においてもエゾマツ MWL のチオアシドリシス生成物量はファイバースクレレイドからの生成量に匹敵し，モノマーの生成量も対リグニン比で両者は類以していた。

このような本実験の結果は，針葉樹 MWL が本部仮道管細胞の二次壁に由来するのではないかとの推論にたいして，これを支持する一つの指標となるものと思われる。