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Structural Analysis of Water Soluble Lignin-Carbohydrate Complex(LCC) Isolated from Korean Camellia Mistletoe (*Pseudixus japonicus* Hayata)*1

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한국산 동백나무겨우살이에서 추출한 수용성 리그닌-탄수화물 복합체의 구조분석*1

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요 약

한국산 동백나무겨우살이(*Pseudixus japonicus* Hayata)에 존재하는 수용성 리그닌-탄수화물 복합체를 구성하는 다당류의 구조를 밝히고, 리그닌성분과 다당류의 결합양식을 구명하고자, 냉·온수 추출한 수용성 리그닌-탄수화물 복합체(M-LCC-WE)를 DEAE Sephadex A-50로 중성분획(M-LCC-N)과 산성분획(M-LCC-A), 나머지분획(M-LCC-R)으로 세분화한 후, M-LCC-N과 M-LCC-A에 대하여 메틸화, 아세틸화, 그리고 DDQ 산화반응을 실시하였다.

M-LCC-N을 구성하는 다당류는 (1→4) 글리코시드결합의 arabinan 과 (1→4) 나 (1→6) 글리코시드결합의 galactan과 glucan으로, M-LCC-A의 다당류는 (1→4) 글리코시드결합의 arabinan 과 (1→6) 글리코시드결합의 galactan이 다당류 주성분으로 밝혀졌으며 galacturonic acid가 결합되어 있기 때문에 산성적 성질을 나타내고 있었다.

또한 M-LCC-A에서는 galacturonic acid 의 carboxyl 그룹이 리그닌의 α-, 와 γ-위치에서 ester결합이 존재함이 확인되었다.

Keywords : *Pseudixus japonicus* H., Lignin-carbohydrate complexes(LCC), ester linkage, 2,3-dichloro-5,6-dicyano benzoquinone(DDQ), arabinan, galactan

1. INTRODUCTION

Lignin-carbohydrate complexes(LCC) is the physical and chemical association between lignin and carbohydrate in lignified plant cell walls. Lignin occurs in the cell wall of the true vascular plants, ferns and club mosses, but not in those of mosses, algae and microorganisms.

The most frequently suggested LCC structures are benzylether, ester and glycosidic linkages. From

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hardwood lignin-xylan complexes are isolated, while lignin-mannan and lignin-xylan complexes are isolated from softwood.

In 1958, Hayashi has studied the relationship between lignin and carbohydrate in wheat straw and recognized the chemical bond existing between them, resulted from that the phenolic hydroxyl content of xylo-lignin was increased before and after alkaline treatment. And the origin of lignin-carbohydrate linkage in a lignin-carbohydrate complex isolated from Spruce by a modified Bjorkman procedure was discussed by Iverson(1985).

2,3-dichloro-5,6-dicyano benzoquinone(DDQ), as the strong oxidatives, has the specific reaction on benzyl carbon and conjugated double bonds of lignin skeleton. Because DDQ oxidizes selectively benzylether and ester linkage of LCC, it is mainly utilized to investigate the structure of LCC recently. Watanabe(1989, 1991) reported that the lignin was preferably bound to at C-6 position of mannose and glucose residues and also C-2 and C-3 position of xylose in water-soluble LCC from *Pinus densiflora* with DDQ-oxidation.

Mistletoes, classified as small shrub, are the parasitic plants which live on the woody plants and synthesize the angiosperm type lignin independently and include specific sugar composition, different from its host. So far, there has been little or no published researches about lignin-carbohydrate complex and polysaccharide structure of the mistletoes in domestic country.

In this experiment, the objectives are to analyze the sugar composition in the Korean camellia mistletoe, *Pseudixus japonicus* Hayata and its host, *Camellia japonica* L, to isolate the water soluble lignin-carbohydrate complex from Korean camellia mistletoe and to examine the chemical properties, to determine the main polysaccharide structure composed of isolated water soluble lignin-carbohydrate complex, and finally to elucidate the ester linkage between lignin components and sugars.

2. MATERIALS & METHODS

2.1 Collection of materials

Korean camellia mistletoe, *Pseudixus japonicus* Hayata, used in this study was taken from *Camellia japonica* L. on Bogil Island, Wando-gun, Chollanam-do on January 4, 1994.

The materials were immediately brought to the laboratory where they were cut into small chips and dried in the air.

2.2 Isolation of water soluble lignin-carbohydrate complex

Air dried materials were milled and extracted with ethanol- benzene(1 : 2, v/v) for 48hrs, and the resultant extractive-free wood meals were depectinated by extraction with 0.25%(w/v) aqueous potassium acetate at 60℃ for 24hrs. Woody cake were recovered by filtering, washed with distilled water and acetone, and dried thoroughly at 40℃. The extractive-free and depectinated wood meals were milled by a vibratory ball mill for 48hrs and resultant powder was used in next step.

Lignin was removed by 80% aqueous dioxane extraction for 48hrs at room temperature. And then water soluble lignin-carbohydrate complex was extracted with cold water for 12hrs and sequencely hot water for 5hrs from the residual cake. Both water extracts were combined and evaporated to a small volume and poured into ethanol. The precipitates formed were recovered by centrifugation, washing with ethanol followed by petroleum ether and lyophilized to obtain water soluble lignin-carbohydrate complex as a yellowish white powder(M-LCC-WE).

Anion exchange chromatography of M-LCC-WE was carried out on a column(5 × 100cm) of DEAE-Sephadex A-50(carbonate form). M-LCC-WE was solubilized in a distilled water, applied on a column and eluted with distilled water. The eluates were concentrated to a small volume and poured into ethanol to precipitate the neutral fraction which was recov-

ered by centrifugation and lyophilized to obtain a neutral fraction(M-LCC-N). The column was next eluted with 1M ammonium carbonate solution to get acidic fraction(M-LCC-A). The column was then eluted with 10M acetic acid. The eluates were concentrated to a small volume and subjected to ethanol precipitation to obtain residual fraction(M-LCC-R) (Azuma, 1989).

2.3 Chemical analysis of each fraction

Total carbohydrate content was determined by the phenol-sulfuric acid method, using UV/VIS-spectrophotometer at the 490nm. Total uronic acid content was measured by the modified carbazole method at 525nm. Water soluble lignin content was determined at the absorbance of the 280nm, using the UV/VIS-spectrophotometer.

Each fraction was hydrolyzed with 0.5M sulfuric acid at 100℃ for 6hrs and then neutralized with barium carbonate. The precipitated barium sulfate was removed by filtration and deionized with Dowex 50 × 8(H^+ form) and evaporated to dryness. The mixtures of neutral sugars were reduced with sodium borohydride at 40℃ for 3hrs and followed by acetylated with a mixture(1:1, v/v) of pyridine : acetic anhydride at 100℃ for 1hr. The alditol acetates were separated by gas-liquid chromatography using a glass column of 3% ECNSS-M on gas chrom-Q at 190℃ (Slonecker, 1972)

2.4 Uronic acid analysis of M-LCC-A

M-LCC-A was hydrolyzed with 90% formic acid at 100℃ for 16hrs, followed by 4M TFA for additional 6hrs. The neutral monosaccharides and uronic acid were reduced with sodium borohydride and separated into two parts. One part was acetylated and the resulting alditol acetates were analyzed by GC. The other part was heated with methanol in the presence of dried Dowex 50× 8(H^+ form) for 1hr at 100℃ (methylesterification). The uronic acid was identified by comparion of the two results obtained with and without methylesterification(Sone *et al.*, 1978)

2.5 Analysis of polysaccharide structure

For methylation M-LCC-N and M-LCC-A were dissolved in dried DMSO in the ultrasonic bath and then methylsulfinyl sodium in DMSO was added. The solution was agitated in the ultrasonic bath for 1hr,and kept for further 6hrs and then methyl iodide was added dropwise with external cooling in ice water. The resulting solution was agitated in the ultrasonic bath for 30min. The resulting mixture was poured into water and extracted with chloroform five times, and the combined chloroform solution was washed with water five times and then dried with $MgSO_4$ and concentrated to dryness with evaporation(Hakamori, 1964)

The completely methylated M-LCC-N and M-LCC-A were hydrolyzed with 90% aqueous formic acid, followed by 0.5N sulfuric acid. The partially methylated monosaccharides were then reduced with sodium borohydride 40℃ for 3hrs.

The hydrolyzates were converted into alditol acetates by acethylation and separated on a column of 3% ECNSS-M on gas chrom-Q and identified through GC-MS on a SPB-1 capillary column.

ACIDIC LIGNIN-CARBOHYDRATE COMPLEX (M-LCC-A)

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DIAZOMETHANE METHYLATION (In ether, room temp. 48hrs)

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DDQ-OXIDATION (benzene : water = 18 : 1, reflux 2hrs)

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PRECIPITATION IN ETHYLALCOHOL

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ANION EXCHANGE CHROMATOGRAPHY WITH Dowex 1 × 8(AcO^- form)

Fig. 1. Procedures for benzylester and ester linkage analysis in the acidic lignin-carbohydrate complex.

2.6 Ester linkage analysis

M-LCC-A was methylated with diazomethane at room temperature for 48hrs in diethyl ether to protect carboxyl group in uronic acid and phenolic hydroxyl group(M-LCC-Am) as Fig. 1.

The Methylated M-LCC-A(M-LCC-Am) was refluxed with the same amount of DDQ for 2hrs in a benzene-water mixture(18 : 1). After the reaction, ethyl alcohol was poured into the mixture and precipitates formed was washed with the same solvent and recovered with centrifugation. The precipitates were loaded in Dowex 1×8 (AcO^- form) column chromatography and eluted with water and 10M acetic acid, successively. The acetic acid eluate was concentrated into small volume and poured into ethyl alcohol. The precipitates were obtained by centrifugation(M-LCC-Ad) and dried in vacuum(Watanabe, 1988).

After the diazomethane methylation and the DDQ-oxidation, the difference of the pH were measured three times between the reaction products.

3. RESULTS & DISCUSSION

3.1 Chemical composition of *Pseudixus japonicus* and *Camellia japonica*

The chemical compostions were shown as Table 1, in which it is indicated that ethanol-benzene extrative and ash contents of Pseudixus japonicus were higer than those of Camellia japonica.

In the *Camellia japonica*, glucose and xylose were the most abundant neutral sugar, but the major component of *Pseudixus japonicus* were arabinose, unlike its host and general woody plants(Fig. 2). These results indicated that the sugar composition were different, although *Pseudixus japonicus* lives on its host,the *Camellia japonica*. And it is implicated that the main polysaccharides of *Pseudixus japonicus* may be synthesized through its own pathway without influence of host and composed of pectic substances, such as arabinan and arabinogalactan.

Table 1. Chemical analysis of *Pseudixus japonicus* and *Camellia japonica*. (Unit : %)

Component	*Pseudixus japonicus*	*Camellia japonica*
Moisture content	13.2	7.1
Ethanol-benzene extractives	24.4	4.6
Klason lignin	31.3	31.9
Holocellulose	32.0	47.0
Ash	11.1	2.3

3.2 Isolation of water soluble lignin-carbohydrate complex and fractionantion

The yields of M-LCC-WE of *Pseudixus japonicus* were about 8.5% and this result was similar to the yield of LCC isolated from *Pinus densiflora* by Watanabe(1989). The chemical properties of each subfraction isolated from *Pseudixus japonicus* were illustrated in Table 2. The yield of acidic fraction was much higher than that of Watanabe(48.7%, 1989). High yield of M-LCC-A was expected that many uronic acid may be bound to the polysaccharide composed of M-LCC-A subfraction. According to Watanabe(1989), carboxyl group of glucuronic acid was linked to α-position and γ-position of lignin components in the LCC isolated from *Pinus densiflora*.

3.3 Neutral sugar analysis of each fraction

The main carbohydrates were separated with the above mentioned method and identified with retention time of the authentic compound. Neutral sugar components of M-LCC-WE, M-LCC-N and M-LCC-A were illustrated in Table 3. Arabinose, glucose and galactose were the most abundant neutral sugars in the fraction M-LCC-WE and arabinose in subfraction

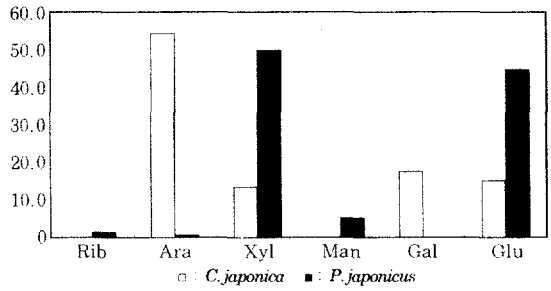

Fig. 2. Neutral Sugar content of *Pseudixus japonicus* and *Camellia japonica*.

Table 2. Chemical composition and properties of the lignin-carbohydrate complexs isolated from *Pseudixus japonicus*.
(Unit : absolute %)

Components	Lignin-Carbohydrate Complexes		
	M-LCC-WE	M-LCC-N	M-LCC-A
Recovery(%)	8.5*1	10.7*2	84.7*2
Carbohydrate Content(%)*3			
Neurtal Sugar	46.3	74.2	59.5
Uronic Acid	40.5	6.3	23.6
Lignin Content(%)*3	6.9	7.0	4.9

Notes; *1 based on the weight of extractive free mistletoe meals,
*2 based on the weight of M-LCC-WE,
*3 based on the weight of Recovery.

Table 3. Neutral sugar composition of water soluble lignin-carbohydrate complexs isolated from *Pseudixus japonicus*.
(Unit : realtive %)

Composition	Lignin-Carbohydrate Complexes		
	M-LCC-WE	M-LCC-N	M-LCC-A
Ribose	5.8	T	10.3
Arabinose	56.7	45.7	62.3
Xylose	2.6	11.1	T
Mannose	4.2	6.0	T
Galactose	16.1	8.6	27.5
Glucose	14.6	28.7	T

M-LCC-N, and arabinose and galactose were the main components in subfracton M-LCC-A. In the case of *Pseudixus japonicus*, it was expected that arabinan may be contained in the two subfraction, and M-LCC-N also includes glucan as the noncellulosic materials. M-LCC-A may be composed of arabinogalactan or galactan bound with many uronic acid through the neutral sugar analysis.

3.4 Uronic acid analysis

After the series of reaction the relative amount of sugar composition was changed and the difference was large in galactose component. Methylesterification may be caused the increase of relative amount of galactose.

Fig. 3 showed the relative sugar composition of the M-LCC-A subfraction with and without methylesterification. The increase in the relative amount of galactose may correspond to the presence of galacturonic acid.

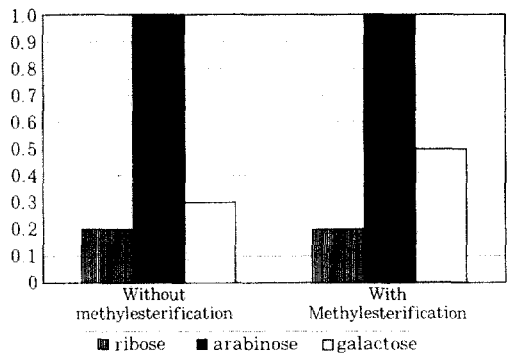

Fig. 3. Uronic acid composition of M-LCC-A.

3.5 Determination of polysaccharides structure

Table 4 and Table 5 showed the data of methylated monosaccharide of M-LCC-N and M-LCC-A by GC-MS analysis in which the ionization fragments of each peak were compared and identified with Wiley 138

library. In Table 5, the presence of 2,3,5-tri, and 3,5-di-O-methylarabinose implied (1→ 4) glycosidic linked arabinan and it was estimated that lignin or other sugar components were linked in C-2 of arabinose. 3,5-di, 3,4,5-tri-O-methylgalactose indicated (1→4) or (1→6) glycosidic linked galactan and 2,3,6-tri-O-methylglucose also indicated (1→4) glycosidic glucan as noncellulosic substances. In M-LCC-A only two peak were identified and 2,3,5-tri-O-methylarabinose and 2,3,4-tri-O-methylgalactose were regarded as (1→4) glycosidic linked arabinan and (1→6) linked galactan or heterogenious arabinogalactan mixed with the above two methylated sugars. From this results, arabinan and galactan, so-called pectic materials, may be associated with chemically active sites of lignin components of *Pseudixus japonicus*.

Table 4. Methylated monosaccharides of neutral (M-LCC-N) of lignin-carbohydrate complex isolated from *Pseudixus japonicus* (Unit : relative %)

Peak	RT	Area	Compound
1	5.24	2.6	No match Compound
2	7.01	5.2	No match Compound
3	13.23	21.9	1,4-di-O-Acetyl-2,3,5-tri-O-Methyl arabinitol
4	16.24	9.1	1,2,4-tri-O-Acetyl-3,5-di-O-Methyl arabinitol
5	17.45	11.4	3,5-di-O-methyl-1,2,4,6-tetra-O-acetylgalactitol
6	19.76	9.4	3,4,5-tri-O-Methyl-1,2,6-tri-O-acetylgalactitol
7	22.15	8.1	No match Compound
8	22.39	21.4	2,3,6-tri-O-Methyl-1,4,5-tri-O-acetylglucitol
9	25.84	10.9	1,4,5,6-tetra-O- acetyl-2,3-di-O-methylglucitol

Table 5. Methylated monosaccharides of acidic (M-LCC-A) of lignin-carbohydrate complex isolated from *Pseudixus japonicus*. (Unit : relative %)

Peak	RT	Area	Compound
1	3.12	17.3	No match Compound
2	4.11	14.2	No match Compound
3	6.89	15.6	No match Compound
4	7.54	10.1	1,4-di-O-Acetyl-2,3,5-tri-O-methylarabinitol
5	9.88	20.6	No match Compound
6	36.65	21.9	2,3,4-tri-O-methyl-1,5,6-tri-D-acetylgalactitol

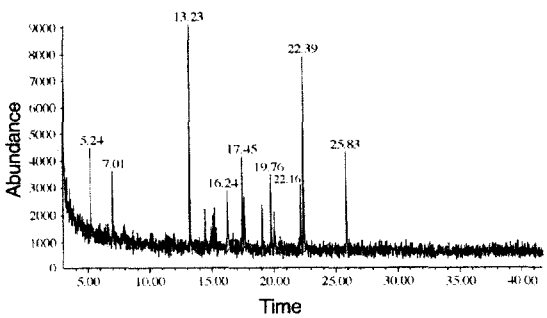

Fig. 4.Chromatogram of the hydrolysis products of the methylated M-LCC-N as their alditol acetates.

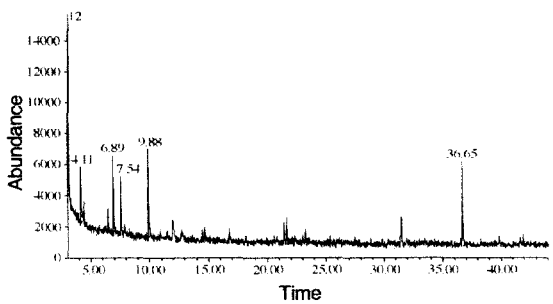

Fig. 5. Chromatogram of the hydrolysis products of the methylated M-LCC-A as their alditol acetates.

3.6 Ester linkage between lignin and carbohydrate

Due to the uronic acid, pH value of M-LCC-A indicated acidic range(pH = 5,6), but diazomethane methylation for 48 hours in ether changed the pH value from acidic to neutral range(pH = 6,9), which indicated the carboxyl groups of uronic acid were completely methylated. Because DDQ cleaved the ester linkage between lignin and uronic acid selectively, DDQ-oxidation of methylated M-LCC-A moved the pH value again back to acidic range(3,9). This return to acidic value indicated that new carboxyl groups, formed from ester linkage between uronic acid and lignin, were made by DDQ-oxidative cleavage. Judging from this results, the linkage type between galacturonic acid and lignin components may be ester linkage type in the α- or γ-position of lignin.

4. CONCLUSION

The major sugar component of *Pseudixus japonicus* Hayata was arabinose, while glucose and xylose were abundant in its host *Camellia japonica* L.

Water soluble lignin-carbohydrate complex(M-LCC-WE) was isolated from *Pseudixus japonicus* and fractionated into neutral(M-LCC-N) and acidic(M-LCC-A) subfaction with DEAE Sephadex A-50 anion exchange chromatography.

In the M-LCC-A, high content of uronic acid was identified as galacturonic acid.

The main polysaccharides of M-LCC-N consisted of (1→4) glycosidic linked arabinan and (1→4) or (1→6) glycosidic linked galactan and glucan. Those of M-LCC-A were (1→4) glycosidic linked arabinan and (1→6) linked galactan.

In the M-LCC-A fraction, carboxyl group in C-6 of galacturonic acid was associated with α- or γ-position of lignin through ester linkage.

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