ORIGINAL ARTICLE

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Characterization of lignin–carbohydrate linkages in the residual lignins isolated from chemical pulps of spruce (*Picea abies*) and beech wood (*Fagus sylvatica*)

Abstract To investigate the linkage types between carbohydrates and lignin, residual lignins were isolated from three different unbleached pulps [kraft, alkaline sulfite anthraquinone methanol (ASAM), and soda with anthraquinone (AQ) and methanol] of spruce and beech wood and then characterized by oxidation with 2,3-Dichloro-5,6-dicyanobenzoquinone and followed by Prehm's methylation. In residual lignins, sugar moieties were bound to lignins via benzyl ether bonds. In particular, galactose and mannose are predominantly linked to lignin fragments in residual lignins of spruce wood, while xylose and galactose are favored in the formation of LC bonds in the residual lignins of beech wood. In the case of hexoses, primary hydroxyl groups (C6 position) preferentially take part in benzyl ether linkages. Hydroxyl groups in the C2 and C3 positions of xylose participate in LC bonds and a small portion of arabinose was notably connected to lignin via the C5 position. Approximately seven or eight sugars were connected in soda/AQ/methanol residual lignin per 100 C9 lignin units, while the frequencies of LC bonds in kraft and ASAM residual lignins were distinctively less at one to three sugars per 100 C9 lignin units.

Key words Spruce wood (*Picea abies*) · Beech wood (*Fagus sylvatica*) · Residual lignin · DDQ oxidation · Lignin–carbohydrate complex (LCC)

Introduction

After Björkman[1] suggested chemical association between lignin and carbohydrates (lignin carbohydrate complexes,

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LCCs) in wood cell walls, a lot of studies on structural elucidation of LCCs have been attempted in the past 30 years. At present, covalent bonds between them are accepted as a real structure in the cell walls of lignified plants. During lignin biosynthesis, hydroxyl and/or carboxyl groups in the cellulose/hemicellulose are connected to $C \pm C\alpha$ position of quinone methide intermediates by a nucleophilic addition reaction.[2]

2,3-Dichloro-5,6-dicyanobenzoquinone (DDQ) oxidatively cleaves benzyl ether and ester linkages between lignin and carbohydrates, and new hydroxyl groups become attached to the sugar moieties. The sugars and their connecting positions can be determined by methylation of the hydroxyl groups followed by analysis by gas chromatography-mass spectrometry (GC-MS).[3,4] Model experiments have suggested that LC bonds are chemically labile in acid, but the benzyl ether linkage is relative stable under alkaline pulping conditions.[5,6] It was also proposed that new ether bonds could be formed by the quinone methide intermediates during kraft pulping.[7] This is strong indication that LC bonds can play important roles for optimization of pulping conditions and bleaching processes. Thus, increasing attention has recently been given to the study of LC bonds in residual lignins and lignins in black liquor. Yamasaki et al.[8] demonstrated that lignin–hemicellulose linkages in kraft pulps may cause resistance to delignification during pulping procedures. Small amounts of carbohydrates are measured in the residual kraft lignins after isolation by enzymatic treatment.[9–11] In the residual lignins isolated from chemical pulp, arabinan and galactan components are found to be enriched. Because arabinose and galactose are attached as side chains to the main polysaccharide structures, these sugars are more likely to be connected to lignins.

In this study, six residual lignins were isolated from unbleached alkaline chemical pulps [kraft, alkaline sulfite anthraquinone methanol (ASAM), and soda/anthraquinone (AQ)/methanol (MeOH) of spruce and beech, respectively] and characterized by DDQ oxidation. This was followed by Prehm's methylation to obtain detailed evidence for chemical linkages between carbohydrates and lignins in alkaline chemical pulps and to elucidate their chemical structures.

This article discusses the behavior of LC bonds under alkaline pulping conditions by comparison with analytical results of water-soluble LCCs isolated from spruce and beech wood.

Materials and methods

Pulping and isolation of residual lignins

Pulping according to conventional kraft, ASAM, and soda/AQ/MeOH processes were carried out on a laboratory scale. The sulfite pulp was of industrial origin. Kraft pulping conditions: 22% active alkali (NaOH on oven-dried wood), 30% sulfidity, 90 min at 175°C for spruce and 165°C for beech. ASAM pulping conditions: 6.3% NaOH, 18.7% sodium sulfite, 0.1%, anthraquinone (AQ), methanol 10% (v/v), 150 min for spruce and 90 min for beech at 180°C. Soda/AQ/methanol pulping conditions: 22% NaOH, 0.1% AQ, 20% methanol, 120 min at 165°C for spruce and 160°C for beech. Liquor to wood ratio was 4:1 for all pulping processes.[12] For isolation of residual lignins, unbleached pulps produced in the laboratory were subjected to enzymatic hydrolysis.[12] After purification with dimethylacetamide (DMAC)/ether, the residual lignins were used for DDQ oxidation.

Acetylation

The residual lignins were acetylated with pyridine and acetic anhydride (1:1, v/v, 100 mg/2 ml) for 24 h at 50°C. The liquid phase was poured into cold water and the precipitate was washed thoroughly.

DDQ oxidation

DDQ oxidation was performed according to a literature method.[3,4] Acetylated lignins (150–200 mg) were reacted with an equal amount of DDQ in dichloromethane/water mixture (15–20 ml, 18/1, v/v) for 2 h at 50°C. After DDQ oxidation, a small amount of ascorbic acid was added to the reaction mixture to destroy the excess DDQ.

Prehm's methylation

The hydroxyl groups formed on the sugar moieties during DDQ oxidation were methylated with methyl trifluoromethanesulfonate and 2,6-di-(tert-butyl)pyridine in trimethyl phosphate for 3 h at 50°C under nitrogen atmosphere.[13]

Hydrolysis and reduction with NaBD$_4$

The partially methylated sugars were hydrolyzed with 2 M trifluoroacetic acid for 3 h at 100°C. After evaporation, the dried residues were partitioned with chloroform and water.

The aqueous part was recovered and concentrated to a small volume. The concentrated monomeric sugar mixtures were reduced with sodium borodeuterium (NaBD$_4$) overnight at room temperature. Through reduction with NaBD$_4$, a deuterium atom was introduced at the C1 position of sugars, which makes it possible to differentiate some symmetrical pairs within sugar derivatives.[14]

Acetylation and GC and GC-MS analysis

After addition of internal standard (*myo*-inositol), the alditol form of each sugar was acetylated with pyridine and acetic anhydride (1:1, v/v) overnight at 50°C and successively for 1 h at 100°C. The reaction mixtures were poured into water and extracted with chloroform. The organic phase was extracted with water and dried with anhydrous Na$_2$SO$_4$. The partially methylated alditol-acetates were separated by GC using a capillary column (DB 1701, 60 m × 0.25 mm i.d.) and identified by GC-MS. The temperature program for GC and GC-MS was: holding 4 min at 180°C, increased at a rate of 1.5°C/min to 265°C; the final temperature was maintained for 60 min.

Results and discussion

A gas chromatographic separation of methylated sugar derivatives is depicted in Fig. 1. The *O*-Me position in each sugar was identified by the mass spectrometric fragmentation and retention time of each methylated sugar. The MS fragmentation patterns were identical for hexoses and pentoses, respectively. For instance, three 6-*O*-Me hexoses in Fig. 1 have the same MS fragmentation. Thus, each methylated sugar derivative was identified by comparison of retention time with those of authentic sugar derivatives.[15] In Fig. 1, three 6-*O*-Me hexoses are separated very clearly at retention times between 30 and 35 min. 2-*O*-Me and 3-*O*-Me xylose showed almost at the same retention times. It was true that low concentration of methylated sugars made it difficult for exact quantification.
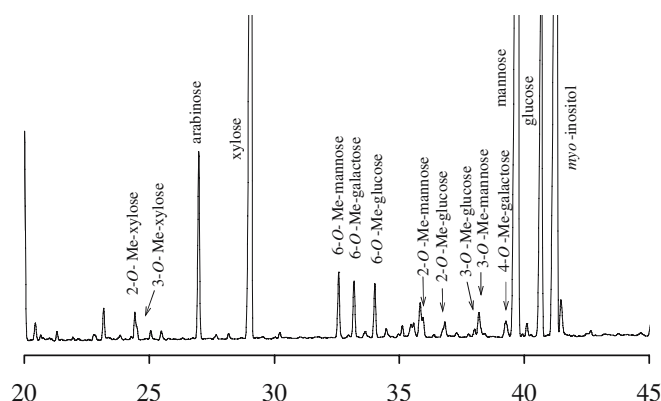

Fig. 1. Gas chromatographic separation of partially methylated sugars after 2,3-Dichloro-5,6-dicyanobenzoquinone (DDQ) oxidation and Prehm's methylation of spruce soda/anthraquinone (AQ)/MeOH residual lignin (all sugar components in alditol acetate form)

The partially methylated sugars were quantified on the basis of total contents of sugar derivatives (Table 1). The yields of methylated sugars ranged from 9% to 12.5% in spruce residual lignins based on total sugar derivatives. Residual lignins in beech wood pulps showed similar yields of methylated sugars between 8.7% and 12%. The methylated sugar derivatives, which can be released after cleavage of LC bonds, confirmed the presence of benzyl ether linkages between lignins and carbohydrates. On average, one sugar moiety out of ten on the carbohydrate chain can take part in benzyl ether linkages to lignins in the residual lignins.

Figure 2 shows the relative composition of partially methylated hexoses and pentoses. The enrichment of methylated hexoses was observed in spruce residual lignins, while pentose contributed less to LC bonds. By contrast, the content of methylated pentose was very significant in beech lignins. In particular, the pentose amounts to ca. 50% in beech soda/AQ/MeOH residual lignin, indicating that, in spite of pulping procedures, hemicellulosic sugars are still bound to residual lignins. The relative amounts of partially methylated sugars are listed in Table 2. In principle, all positions of sugars are chemically connected to Cα-position of residual lignins. In residual lignins of spruce wood pulps, a primary hydroxyl group at the C6 of mannose or galactose was assumed to the most predominant position for benzyl ether bonds. However, hydroxyl groups at the C2 of xylose and at the C6 of galactose were also noticeable in residual lignins of beech wood pulps. It was interesting to observe that the hydroxyl group at the C4 of galactose participated in the lignin–carbohydrate complex. Secondary hydroxyl groups at the C2 and C3 of hexose also took part in the LC bonds in both wood species. Due to steric preference and free mobility, the primary hydroxyl groups provided opportunity for easier approach to the Cα position of lignins.

Table 1. Yields of partially methylated sugars in the residual lignins after DDQ oxidation and Prehm's methylation

Residual lignin	Yields of partially methylated alditol acetates[a]	
	Spruce	Beech
Kraft	12.5	8.7
ASAM	11.9	12.1
Soda/AQ/MeOH	8.7	9.2
Average	11.0	9.9

DDQ, 2,3-Dichloro-5,6-dicyanobenzoquinone; ASAM, alkaline sulfite anthraquinone methanol; AQ, anthraquinone
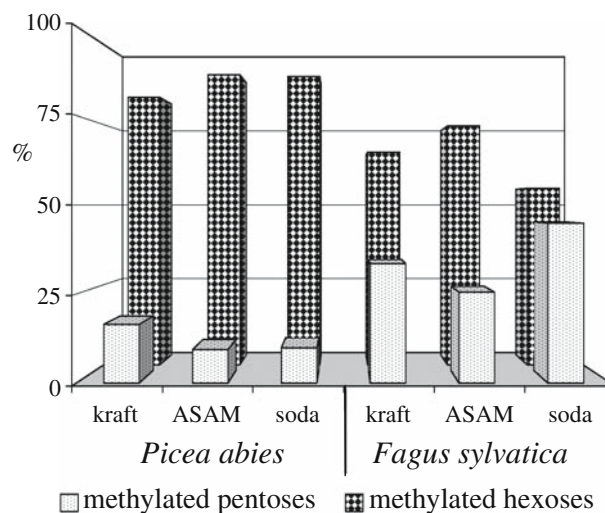[a] Yields given in mol/mol % based on total identified alditol acetates

Fig. 2. Relative composition of partially methylated pentoses and hexoses in the residual lignins isolated unbleached chemical pulps of spruce (*Picea abies*) and beech wood (*Fagus sylvatica*)

Table 2. Relative composition of partially methylated sugar derivatives released from lignin–carbohydrate bonds in the residual lignins

Partially methylated sugars (alditol acetate form)	Spruce			Beech		
	Kraft	ASAM	Soda[a]	Kraft	ASAM	Soda[a]
5-*O*-Me-arabinose	3.27	–	–	3.33	–	3.60
2-*O*-Me-xylose	11.00	6.74	7.93	27.13	19.17	41.22
3-*O*-Me-xylose	2.15	2.70	1.95	3.10	6.31	–
Σ*O*-Me-xylose	13.15	9.44	9.88	30.23	25.48	41.22
2-*O*-Me-mannose	7.81	7.83	6.21	–	–	–
3-*O*-Me-mannose	8.29	7.25	10.23	5.17	4.65	2.94
6-*O*-Me-mannose	12.12	12.30	19.75	4.14	3.07	2.84
Σ*O*-Me-mannose	28.22	27.38	36.21	9.31	7.72	5.78
2-*O*-Me-galactose	4.15	6.32	2.30	4.71	5.31	3.16
3-*O*-Me-galactose	7.02	6.07	1.61	8.16	8.88	2.94
4-*O*-Me-galactose	6.06	8.85	7.70	4.83	6.72	5.34
6-*O*-Me-galactose	16.83	18.20	18.05	19.20	17.01	16.68
Σ*O*-Me-galactose	34.06	39.44	29.66	36.90	37.92	28.12
2-*O*-Me-glucose	6.78	6.91	5.29	5.40	10.46	4.91
3-*O*-Me-glucose	2.95	2.70	2.99	1.95	2.07	4.03
6-*O*-Me-glucose	11.56	14.15	15.98	12.87	16.35	12.32
Σ*O*-Me-glucose	21.29	23.76	24.26	20.22	28.88	21.26
Total	100	100	100	100	100	100

Composition given as mol/mol %
[a] Soda with AQ and methanol

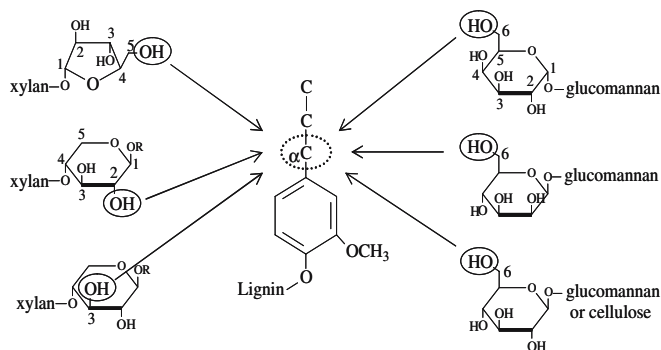**Fig. 3.** The most important binding sites in the sugar moieties of polysaccharides to the Cα position in residual lignins

Fig. 4. Comparison of relative amounts of methylated sugars between residual lignin–carbohydrate complex (LCCs) and native LCCs

The important binding sites of hexoses and pentoses are illustrated schematically in Fig. 3.

The data in Table 2 indicate that galactose is the most frequently connected sugar to lignins in spruce residual lignins. Mannose and glucose also afford several binding sites to lignins to a significant degree. In beech residual lignins, galactose and xylose are the sugars most commonly attached to lignins. Because galactose is generally present at side chains in hemicellulose structures, it could be the highest preference to LC bonds in all the residual lignins. Lignin–galactose linkages have already been investigated in kraft residual lignins by Yamasaki et al.,[8] Iversen and Westermark,[16] and Minor.[9,10] These studies found that glucomannans, in spite of their high solubility and peeling off reaction, could survive under alkaline pulping conditions. Tamminen et al.[17] also proposed lignin–galactan linkages in kraft residual lignin as well as its black liquor.

In agreement with Minor,[10] we also found in kraft and soda/AQ/MeOH residual lignins that arabinose was connected via the primary C5 position. The 5-*O*-Me arabinose was derived from arabinoxylan or pectic arabinan. According to Tamminen et al.,[17] arabinose could provide a bridging role between xylose and lignin. As can be seen in Table 2, the amount of methylated glucose was the highest in all kinds of methylated sugars in the residual lignins. According to Timell,[18] the ratio of mannose/glucose is about 3 in softwood glucomannan and ranges between 1 and 2 for hardwood glucomannan. Assuming that the opportunity of each sugar in glucomannan to connect to lignin should be equal, the amount of methylated glucose was significantly exceeded in the residual lignins. Therefore, the methylated glucose originated not only from the glucomannan but also from either cellulose or glucan. Due to high solubility of pectic substances under alkaline pulping conditions, methylated glucose could hardly be expected to be derived from glucan. Taking the above explanation into consideration, the surplus of methylated glucose appears to be derived from cellulose, indicating benzyl ether linkages between cellulose and lignin moieties. The possibility of chemical linkage between cellulose and lignin has been suggested in water-soluble native LCCs as well as kraft pulps.[15,19–21] It was demonstrated that the cellulose–lignin linkages could hin-
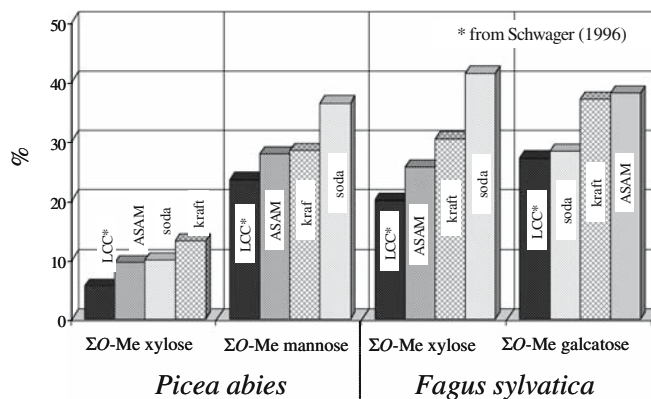
der complete separation of lignin from wood components during pulping.

The frequencies of LC bonds per C9 lignin units were calculated on the basis of experimental data obtained from total hydrolysis, C9 formula, gel permeation chromatography analysis, and DDQ oxidation.[12] In our own calculation, approximately one or three sugar moieties were linked to Cα position of lignin based on 100 C9 lignin units in kraft and ASAM residual lignins. In particular, soda/AQ/MeOH residual lignins contained about seven to eight LC bonds based on 100 C9 lignin units, which is significantly more LC bonds than expected. Under the soda pulping conditions, the benzyl ether linkages were more resistant, probably due to the lack of a strong nucleophile, like sulfide ions in kraft or sulfite ions in the ASAM process. Obst[22] and Takahashi and Koshijima[23] estimated the frequencies of LC bonds of milled wood enzyme lignin (MWEL) and native LCCs, respectively. About one LC bond was estimated for every 36 phenyl propane units in MWEL, regardless of softwood or hardwood.

In addition, the results obtained from residual lignins in this study were compared with those of water-soluble LCCs, which were isolated from spruce and beech wood.[15] As shown in Fig. 4, the hemicellulose-derived sugars, xylose and mannose in spruce wood and xylose and galactose in beech wood, are more frequently bound to lignin in residual lignins rather than in native wood LCCs. The quantitative differences in LC bond frequencies between them can be interpreted in two ways: one is the resistance of benzyl ether linkage under alkaline conditions and the other is the possible formation of new LC bonds during alkaline pulping.

Conclusions

DDQ oxidation confirmed the presence of benzyl ether linkages between lignin and carbohydrates in residual lignins isolated from unbleached chemical pulps of spruce and beech wood. Galactose, mannose, and glucose are connected frequently in spruce samples and xylose, galactose, and

glucose are preferably bonded in beech LCCs. Linkage through the primary C6 position of hexoses is the most favored. The C2 and C3 positions of xylose are also significantly involved in benzyl ether linkages, especially in beech soda/AQ/MeOH residual lignin. The most interesting finding is that cellulose could be linked to lignin in the residual lignins. Therefore, it is concluded that the resistance of residual lignins in pulps as well as the presence of carbohydrates in isolated residual lignins could be due to cellulose–lignin and hemicellulose–lignin linkages. Furthermore, comparative study between native LCCs isolated from woods and residual lignins obtained from unbleached alkaline chemical pulps makes it possible to draw the plausible explanation of two well-known suggestions: alkaline stability of benzyl ether bonds between lignin and carbohydrates and new formation of lignin–carbohydrate linkages during the alkaline pulping process.

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