On the effect of hemicellulose removal on cellulose-lignin interactions

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SUMMARY: In a recent study, it was suggested that there could be direct associations between cellulose and lignin in mild alkaline cooked pulps. The observation was based on studies showing that the molecular straining of lignin was similar to that of cellulose. This finding has serious ramifications for technical production of pulps as it could expand on what is known about recalcitrant lignin removal during pulping. Herein, we investigate the possible interaction between cellulose and lignin discussing possible mechanisms involved at the nano- and molecular-scales, and present support for that the removal of hemicellulose by hot water extraction or mild kraft pulping causes strong interactions between lignin and cellulose.

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Introduction

The interactions between the different wood polymers in native state and how they are affected during processing are not only of fundamental but also of technical interest. In softwoods, xylan and lignin are known to be closely associated while glucomannans are associated with both cellulose (Stevanic, Salmén 2008) and lignin (Lawoko et al. 2005). These associations have been studied by 2D HSQC NMR which reveals both lignin inter-units linkages and chemical bonds between lignin and hemicelluloses (Du et al. 2014; Balakshin et al. 2007; Balakshin et al. 2011; Giummarella et al. 2016) and by dynamic FTIR spectroscopy (Salmén, Olsson 1998). However, chemical bonds between lignin and cellulose have not been unequivocally shown due to the presence of non-cellulosic glucans that are also present in wood in different forms (Fry 1989; Fry et al. 2008). Nevertheless, studies by Salmén and co-workers in the late 1990s confer the unlikelihood of direct associations between lignin and celluloses in native wood, which is reasonable given the self-association of cellulose to form nanocrystals. On the other hand, one could argue that the more unordered regions of cellulose could have associations with lignin. Evidences for such associations are however still lacking although glucans have been implicated in bonding to lignin

(Lawoko et al. 2005; Du et al. 2014; Giummarella et al. 2016; Giummarella, Lawoko 2016).

In a recent study, it was shown that in alkali pre-treated wood there is a molecular interaction between lignin and cellulose (Salmén et al. 2016). Based on this study, showing that the molecular straining of lignin was similar to that of cellulose, it was suggested that there could be direct associations between cellulose and lignin in the mildly alkaline cooked pulps. Irrespective of the mechanisms involved, this finding could in part explain recalcitrant lignin removal during pulping. In the present study, we investigated the nature of interactions between cellulose and lignin in these pre-cooked pulps. For this purpose, a recently developed protocol for Lignin Carbohydrate Complexes (LCCs) fractionation (Giummarella et al. 2016) was applied. The findings are discussed with plausible mechanisms.

Materials and methods

Materials and chemicals

All chemicals used were of analytical grade and purchased from Sigma Aldrich. Wiley meals (40 mesh) were obtained using a Wiley Mini Mill 3383-L70 (Thomas Scientific) from pre-cooked chip samples (25min. at 120°C in alkali) prepared from a mixture of 70% Norway spruce, *Picea abies* [L] Karst. (40 years old, southern Sweden) and 30% Scots pine, *Pinus sylvestris* [L]. The chips were impregnated with kraft liquor as described elsewhere (Salmén et al. 2016) and as summarized in Fig 1.

Fig 1 - Relative chemical composition of Norway spruce wood and pre-cooked kraft wood samples. The table reports the cooking conditions adopted during impregnation step. In the figure, GGM=(galacto)glucomannan, KC=pre-cooked kraft pulp.

LCCs quantitative fractionation scheme

LCC fractionation was performed as previously described by Giummarella (2016a, 2016b) and as shown in Fig 2. In summary, **W1** was extracted from ball milled acetone-extracted impregnated pulp meals (Lawoko et al. 2005) after addition of deionized water (10:1) at 80°C for 4 h and separation of the insoluble residue by centrifugation. Similarly, **P1** was obtained by addition of 20% in volume of water after complete dissolution in 1-Allyl-3-methylimidazolium chloride ([Amim]Cl)-DMSO (16 h, 70°C) of the obtained residue whereas **P2** was precipitated in ethanol by addition of three times the volume of DMSO-water ($V_{\text{DMSO}}:V_{\text{water}}=1:1$). Finally, to the remaining solution, three times its volume of water was added to obtain fractions **P3**. All fractions were washed from residual chemicals and lyophilized prior to analysis. Milled wood lignin was extracted from ball milled extractives-free impregnated pulp meals with 96% aqueous dioxane (48 h, room temperature) as reported in literature (Björkman 1956).

Carbohydrate and lignin- composition analysis

The chemical composition of monosugars in analyzed fractions was determined by acid hydrolysis (Effland 1977; Davis 1988) using HPAEC/PAD system (Dionex, Sunnyvale, CA, USA) equipped with a CarboPac PA-1 column (Azhar et al. 2015). The lignin content of each fraction was determined by Klason (Effland 1977) and acid soluble (Tappi 1985) procedures.

Fig 2 - Fractionation scheme for quantitative analysis of lignin carbohydrate complexes (LCCs) obtained from pre-cooked kraft (KC) pulp. After removal of extractives and mechanical pre-treatment, water extract (W1) hemicellulose-enriched fraction is obtained. P1, main fraction in terms of lignin and mass balance, is cellulose-based and it is the main focus of this study. Hemicellulose and lignin based P2 and P3 are obtained by selective precipitations of dissolved hemicellulose and lignin with ethanol and water, respectively, as anti-solvents.

Size Exclusion Chromatography-DMSO/0.5% LiBr

Molecular weight distributions of the samples were investigated by dissolving 5 mg of lyophilized samples, acetylated in case of P1 fractions to improve solubility, in 2 ml of DMSO+0.5% LiBr (w/w) solution. After filtration of the samples through 0.45 μm PTFE filters, Size Exclusion Chromatography (SEC) was performed with SEC 1260 Infinity (Polymer Standard Services, Germany). The detection system included a UV detector (G1314B) in series with a refractive index detector (G1362A). The mobile phase was DMSO+0.5% LiBr set to a constant flow rate of 0.5 ml/min for a total run-time of 65 minutes. The injection volume was 100 μl . The separation system consisted of PSS GRAM Precolumn, PSS GRAM 100Å and PSS GRAM 10000Å analytical columns thermostated at 60°C and connected in series. The pullulan standards with nominal masses of 708 kDa, 337 kDa, 194 kDa, 47.1 kDa, 21.1 kDa, 9.6 kDa, 6.1 kDa, 1.08 kDa and 342 Da were used for standard calibration.

2D Heteronuclear Single Quantum Coherence NMR analyses

An amount of roughly 100 mg of all the studied fractions was dissolved in 700 μl of DMSO- d_6 . Similarly, P1 fraction was dissolved in CDCl_3 after being acetylated as reported in literature (Lu, Ralph 2003). All NMR spectra were recorded at room temperature on a Bruker Avance III HD 400 MHz instrument with a BBFO probe equipped with a Z-gradient coil and. Data were processed as described elsewhere (Giummarella et al. 2016). The unsubstituted carbon 2 of aromatic groups was used as internal standard for semi-quantitative analysis (Sette et al. 2011).

Results

Mass balance and composition of LCCs enriched fraction

The extraction yields of W1 from pre-cooked kraft pulp was 9.6% which, in terms of mass and lignin balance, is approximately 40% lower than the hot water extract obtained from native spruce ($\%_{\text{wood}}=15.5\%$; $\%_{\text{wood lignin}}=7\%$) (Giummarella et al. 2016a). Mono-sugars composition of W1 fraction suggests that xylan is the main free hemicellulose, conversely to the correspondent native spruce fraction, which was, instead, enriched in mannans (Table 1). This is the consequence of galactoglucomannan degradation by peeling reaction during kraft process. The peeling reaction is less efficient in xylan because arabinose, located in carbon 3 on xylan, acts as a leaving group and the result is a stopping reaction. Cellulose-enriched fraction, P1, accounts for approximately 70% of the total mass and roughly 72% of the lignin (same fraction in native spruce contains 61% of the lignin). P2 is mainly hemicellulose-based and has similar composition of that obtained from native spruce as previously reported (Giummarella et al. 2016a).

Lastly P3, in which 90.8% of the fraction is lignin, is structurally similar to milled wood lignin as previously shown (Giummarella et al. 2016) and reported in 2D HSQC spectra (Fig 5).

Table 1 - Chemical composition and mass balance of precipitated fractions

	Mass balance	Lignin Content (Klason + ASL)		Sugar analysis by HPAEC/PAD				
				Ara	Gal	Glu	X	M
	%wood	%fraction	%wood or pulp lignin	% fraction				
Error		±0.6		±0.5	±0.6	±1.4	±1.3	±0.6
W1-KC	9.6±1	12.0	4.1	15.2	11.5	4.5	55.5	13.2
W1-native	7.7±1	11.0	3.5	4.0	6.0	16.9	16.8	56.3
P1-KC	70.2±3.5	28.2	71.7	1.6	3.2	77.5	3.9	13.8
P1-native	66±2	24.0	61.0	0.9	1.6	86.8	4.9	5.8
P2-KC	7.5±2	42.9	11.6	5.8	11.6	48.1	17.9	16.7
P2-native	9±2	44.0	14.0	2.3	5.2	56.5	14.0	22.0
P3-KC	2.2±0.5	90.8	7.2	26.6	36.1	18.4	10.0	8.9
P3-native	2.0±0.5	50.0	3.0	4.3	11.7	20.8	13.2	50.0

KC=pre-cooked kraft pulp; ASL=Acid soluble lignin; Ara=Arabinose, Gal=Galactose; Glu=Glucose; X=Xylose; M=Mannose. Data regarding native fractions can be found in previous works (Giummarella et al. 2016). In bold, the most abundant mono-sugar detected.

Interestingly, the lignin enriched fraction, *i.e.* P3, contains mainly galactose and arabinose; an indication that the side chains on the main hemicelluloses may act as linkage points to lignin. Furthermore, the presence of more glucose than mannose in the same fractions makes it more likely that the glucose linked to lignin in this fraction originates from glucan rather than mannan. Alternatively, the mannose units are more prone to peeling reactions than glucose (Casebier, Hamilton 1965) leading to the observed enrichment of glucans (*c.f.* native fraction versus pulped, Table 1).

It is worth noting that the native wood fractionation as described by Giummarella et al. (2016), Giummarella, Lawoko 2016) and the kraft pulping as described above, have one thing in common; the initial removal of glucomannan. Given the ultrastructural arrangement of polymers in the spruce fiber wall, where it is known that cellulose and glucomannan are associated (Salmén, Olsson 1998), such glucomannan removal should permit establishment of contact between retained lignin and cellulose. This may explain why the majority of the lignin is found in the cellulose rich P1 fraction (Table 1). This observation will form a key point of discussion in this paper.

Analysis of structure and molar mass

2D HSQC NMR spectroscopy was performed to decipher structural difference in lignin structures as well as LCC-linkage analysis, assigned according to previous work (Toikka et al. 1998; Du et al. 2014; Balakshin et al. 2007; Balakshin et al. 2011; Miyagawa et al. 2014) and described in Fig 3. It was applied directly on the fractions when soluble in DMSO-*d*6 and, in case of P1, on acetylated sample.

All samples analyzed were semi-quantified and compared with the fractions obtained from native spruce

(Table 2), using CH correlation of C2 of the aromatic ring as an internal standard (Sette et al. 2011).

Analysis with 2D NMR of cellulose-rich and bulk fraction (**P1**) from both native and kraft impregnated pulp shows the presence of benzyl ethers (BE) as well as phenyl glycosides (PG) linkages (Fig 4). Due to low solubility in DMSO-*d*6, this fraction was acetylated prior to NMR analysis in CDCl₃. Looking at the native P1 fraction, different benzyl ether signals are observed which could be a result of the involvement of several sugar types present in hemicelluloses, which were also present in this fraction, albeit in small amounts (Table 1). The corresponding fraction from the pulp, however, shows only one signal indicating one type of benzyl ether. This could either be a stable native form or created during pulping. The creation mechanism would involve nucleophilic addition reactions to quinone methide intermediate (Nicholson et al. 2017).

Our studies on **Milled Wood lignin** (MWL) prepared from these pulps show presence of Lignin Carbohydrates (LC) esters and phenyl glycoside. The main sugar present is xylan. Minor amounts of arabinose, galactose and mannose are present.

2D HSQC analysis of **P3** shows clearly all the common inter-unit linkages in lignin and presence of benzyl ethers. Phenyl glycosides are present in both hemicellulose enriched fraction such as **W1** and **P2**. These glycosides are most likely between lignin and xylan in accordance with published assignments (Miyagawa et al. 2014) and in agreement with the xylan content of the fractions. In general and as expected, the LC signals are weak in relation to lignin and carbohydrate signals due to their low frequency. The low expectancy of LC bonds is dictated by the competing reactions to LC bond formation during lignin biosynthesis.

Lignin Carbohydrate complexes

Inter units-linkages and end-groups in Lignin

Fig 3 - Lignin carbohydrates bonds (LCCs), inter-unit linkages and end groups in lignin detected in the 2D HSQC NMR spectra of LCCs enriched fractions obtained from pre-cooked kraft (KC) pulp

Fig 4 - 2D HSQC expanded spectra of P1 in CDCl₃ obtained from native and pre-cooked kraft (KC) pulp after acetylation. In the figure, M=Mannose in red; Glu=Glucose in brown, X=Xylose in turquoise; r=carbon terminal in reducing end; Ac=Acetylated; Ar=Aromatic. The subscripted numbers indicate the carbon number in either the aromatic or sugar ring. LCC nomenclature, lignin inter-units linkages and end group can be found in Fig 3.

Benzyl ether and esters are formed through nucleophilic addition reactions of hydroxyls in the polysaccharides to a quinone methide intermediate, an electrophile formed when a coupling involving a beta radical occurs during lignin polymerization. Other nucleophiles, such as hydroxyls groups in water and phenolic hydroxyls, compete for the electrophilic site and indeed have been shown to form the main addition products.

The dominating lignin structure is β O4, although minor amount of phenyl coumaran and resinol structures are present. Absence of acetyl groups on C2 and C3, which are present in native mannans (Giummarella et al. 2016) and xylans (Giummarella, Lawoko 2016; Giummarella, Lawoko 2017), is due to deacetylation in alkaline condition during pretreatment.

Fig 5 - 2D HSQC expanded spectrum of MWL, W1, P2, P3 in DMSO- d_6 . As in figure 4, M=Mannose in red; A=Arabinose in green; Glu=Glucose in brown; Gal=Galactose in orange; X=Xylose in turquoise; U1 and U4=Carbon number in 4-O-Methyl Glucuronic Acid;(n)r=carbon terminal in (non)-reducing end.

Table 2 - 2D HSQC NMR Linkage analysis in lignin and LC linkages in native and pre-treated spruce.

	W1		P1-Acetylated		P2		P3	
	Native	KC	Native	KC	Native	KC	Native	KC
Dissolved sugars	Glu, X, M	X	Glu, M	X	(X), Glu, M	X, M	Glu	X
Linkages analysis (%C₉ of the fraction)								
β O4	25	53	52	60	41	56	37	48
$\beta\beta$ (Resinol)	-	-	-	-	6	3	4	9
DBO	10	-	-	-	6	16	4	6
β 5	5	5	12	-	13	10	12	13
SD	-	-	-	-	2	-	2	2
β 1 (dienone)	-	-	-	-	2	-	2	2
LCC (%C₉ of the fraction)								
PG	33.1	12	9.2	D	-	D	-	2
BE	-	-	D	D	-	D	2	1
γ -esters	D	-	-	-	-	-	D	-

KC=pre-cooked kraft pulp; X=Xylans, M=Mannans; Glu= Glucans, ()=detected in acetylated samples; - =not detected; D=detected but not quantifiable; data regarding native fractions can be found in previous works (Giummarella et al. 2016a; Giummarella, Lawoko 2017). PG=Phenyl glycosides; BE=Benzyl Ethers.

Semi-quantitative NMR analysis was carried on the fractions from the pre-treated kraft spruce (KC) and compared with those from native spruce (Table 2). Looking at sugars detected, the predominance of xylans amongst the hemicelluloses, in all the kraft pulp fractions, confirms the degradation of the main hemicellulose, galactoglucomannans.

An interesting observation was made when comparing the lignin-inter-unit linkages in native wood to the kraft pre-treated fractions. The condensed structures such as $\beta 5$, $\beta \beta$ diminish in the kraft pre-treated fractions with exception of P3 while the $\beta O4$ increased. This is contrary to what is known for pulps cooked in the conventional manner where most of the lignin has been removed, *i.e.* the $\beta O4$ contents are severely diminished and the condensed structures increased, something consistent with known mechanisms (Crestini et al. 2017, Gierer 1980). Our results suggest fractionation of lignin in the sequential isolation procedure. The early removed more condensed lignin may be associated with the dissolved glucomannan. Indeed, condensed lignin structures have previously been associated with glucomannan (Lawoko et al. 2005). The alkali pretreatment, although mild, seems to play also an important role in the cleavage, to some extent, of both phenyl glycosides and benzyl ethers LC linkages.

In W1 fraction, for instance, where most of the phenyl

glycosides ends up as shown in our previous work (Giummarella et al. 2016; Giummarella, Lawoko 2017), a three-fold decrease is observed after impregnation treatment. Similarly, in P1, both LCCs signals slightly decrease. No direct conclusion on the quantification of γ -esters can be drawn, due to the region being heavily overlapped by other signals. However, the sensitivity of esters to hydrolyze under alkaline conditions is generally known.

When comparing the molecular weight of LCCs from native spruce with the corresponding ones obtained from alkaline-treated wood, it can be seen a three-fold decrease for P1 which, as earlier mentioned, is the most predominant fraction in terms of wood and lignin balance ($\approx 70\%$). A similar pattern is observable also for P2 and P3, which are both approximately 60%, lower than the relative native spruce fractions.

In the case of P1 and P2, these observations are consistent with partial alkaline hydrolysis of carbohydrates and peeling reaction of glucomannans during the mild alkaline impregnation. Looking at P3, lignin-rich fraction, a cleavage of lignin carbohydrates ester type present in the native fraction (Giummarella et al. 2016) together with peeling reaction of the carbohydrates, might explain the decrease of molecular weight during the treatment.

Fig 6 - Molar mass distribution of lyophilized fractions obtained from Norway spruce (left) and from pre-cooked kraft (KC) pulp on the right. The table reports the Number average molar mass (Mn), Weight average molar mass (Mw) and Polydispersity index (D) of the fractions according by RI detector. Y-axes, $w(\log M)$, show the mass fractions w in constant molar mass increments ($\log (M)$).

Discussion

Rationalizing the relationship between lignin and cellulose

Mechanism, nanoscale: From an ultrastructural viewpoint, glucomannans are known to be more closely associated to cellulose, and xylans are more associated to lignin (Salmén, Olsson 1998). Removal of glucomannan from cellulose crystal surface brings lignin in contact with cellulose as suggested by Salmén et al. (2016). Indeed, our studies would provide some support for this as they show the extensive removal of glucomannan. In the case of the native wood, the removal of glucomannan was a result of a step in the pre-analytical fractionation, comparable to what was achieved in the pre-cooked wood.

Mechanism, molecular-level: In the case of simple 80 °C water extraction of glucomannans, the aforementioned resultant close proximity of lignin to cellulose is likely not be followed by any chemical modifications, given that the conditions are very mild. In the case of the kraft impregnation pre-treatment, however, chemical reactions are possible; hydroxyls on cellulose surfaces can react with quinone methides produced from lignin during pulping, to form covalent bonds. Some literature exists on this molecular mechanism option (Nicholson et al. 2017). Such reactions should however be limited by the presence of hydrogen sulfide ions, which being stronger nucleophiles would result in cleavage rather than formation of LC linkages. The situation could be different in a conventional cook where the levels of reactive hydrogen sulfide ions are reduced, creating possibilities for LC bonds of the ether type to form. Such bonds have been observed in kraft lignins (Crestini et al. 2017).

Native lignin-glucan interactions: The presence of both benzyl ethers and phenyl glycosides in P1 from native spruce (Giummarella et al. 2016), as well as after mild alkaline impregnation as shown in Fig 4, cannot exclude that glucan- LCCs, if present in native wood, are stable enough during this treatment.

With the present data, however, it is not possible to determine which of the abovementioned options is effective in the two presented cases, i.e. water extraction or kraft impregnation process. All three hypotheses are supported by the experimental data but will require deeper investigations.

Conclusions

The interactions between cellulose and lignin are of technical interest as lignin removal forms a crucial part of pulp production. From the obtained data, it seems evident that some close lignin-cellulose associations may exist in these pulps as a result of hemicellulose removal. To this effect, future work will focus on determining more conclusively the nature of these interactions. These interactions might have been formed during pulping, but might also have been to some extent been created during the wood formation.

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