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Lignin-hemicellulose complexes restrict enzymatic solubilization of mannan and xylan from dissolving pulp

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Abstract Mannan and xylan present in bleached softwood dissolving pulp were found to be partially resistant to hemicellulases even after repeated enzyme treatment. Despite the additional effect of an endoglucanase from *Gloeophyllum sepiarium*, which increased the accessibility of mannan and xylan to a mannanase from *Sclerotium rolfsii* and to a xylanase from *Thermomyces lanuginosus*, the enzyme mixture solubilized only half of the hemicellulose present in the pulp. Half of the remaining hemicellulose present in the pulp appeared to be entrapped within the cellulose matrix while the other half was associated with lignin-carbohydrate complexes. The latter hemicellulose portion was isolated and characterized. Chromatography and spectroscopic techniques revealed the presence of two types of lignin-carbohydrate complex, a galactoglucomannan-lignin complex (degree of polymerization DP 50–60) and a xylan-lignin complex (DP >200).

Introduction

Cellulose esters, ethers and viscose rayon are manufactured from dissolving pulps that are wood-derived cellulose. Commercial products made from dissolving pulps include cellophane packaging, plastics, sponges, sausage castings, man-made fibers, photographic films and cigarette filters (Hiett 1985). The solubility of the cellulose acetate in the acetate process can be affected by high concentrations of hemicellulose while even relatively small amounts of glucomannans have been found to adversely affect cellulose acetate filterability and solution haze, and cause an acetate false-body effect. Although glucuronoxylan plays a relatively minor role in influencing these properties of acetate it can severely affect the dispersion and color and can cause thermal instability. In the viscose process, high concentrations of hemicellulose can affect the viscose filterability and the xanthanation of cellulose. High hemicellulose concentrations can also adversely affect the strength of the end-product (Hinck et al. 1985).

There is an intense effort directed at the preparation of high-α dissolving pulps without detrimental effects on the pulp. In general, dissolving pulps reach a limiting α-cellulose content of about 95% (w/w), where the remaining 5% consists of hemicellulose and lignin. Discussion of the limiting α-cellulose content in the literature has centered on recalcitrant lignin-carbohydrate complexes and hemicellulose accessibility. In the last few years the potential of enzymes to solubilize the hemicellulose remaining in dissolving pulp after chemical bleaching and extraction has been assessed. Only up to 50% of the xylan present in bleached pulp could be removed by xylanases from *Aureobasidium pullulans* (Christov and Prior 1996), *Schizophyllum commune* (Paice and Jurasek 1984), *Saccharomonospora viridis* (Roberts et al. 1990) and *Trichoderma harzianum* (Senior et al. 1988). Despite the additional effect of mannanases and endoglucanases, part of the hemicellulose in the dissolving pulp seemed to be inaccessible to hemicellulases. It has been suggested that this hemicellulose portion might be modified or present as lignin-carbohydrate complexes (Gübitz et al. 1997).

The existence of covalent linkages between carbohydrates and lignin molecules in wood tissue, forming lignin-carbohydrate complexes (LCC), has been largely accepted and their association has been studied (Wong et al. 1996; Karlsson and Westermark 1996; Eriksson and Lindgren 1977). Although certain LCC in kraft pulps might be one of the target substrates for xylanases,

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other LCC remaining in pulp are not susceptible to enzymes and restrict the removal of the residual lignin during bleaching of kraft pulp (Wong et al. 1996).

In this paper we examine the nature of the residual hemicellulose fractions and their influence on the recalcitrant nature of the hemicellulose fraction of softwood dissolving pulp.

Materials and methods

Enzymes

Sclerotium rolfsii, Schizophyllum commune (BT 2115) and *Trichoderma harzianum* (ATCC 26799) were cultivated as described previously (Gübitz and Steiner 1995; Torrie et al. 1990) and the 61-kDa mannanase (*Sclerotium rolfsii*, ATCC 200224), the 45-kDa endoglucanase (*Gloeophyllum sepiarium,* MB 135) and the 26-kDa xylanase (*Thermomyces lanuginosus*, DSM 5826) were purified (Mansfield et al. 1998; Schlacher et al. 1996; Gübitz et al. 1996). The activities of xylanases and mannanases were determined using a 1% solution of birch wood xylan (Roth, Karlsruhe, Germany) and a 0.5% solution of locust bean galactomannan (Sigma) in 50 mM sodium citrate buffer, pH 4.8. Carboxymethylcellulose activity was determined in the same fashion using a 1% solution of carboxymethylcellulose and an incubation time of 30 min (Gübitz and Steiner 1995).

Pulp

Softwood-derived high-α dissolving pulp was obtained from Tembec (Temiscaming, Canada). The pulp was bleached in a chlorine/chlorine dioxide, alkaline extraction, chlorine dioxide hypochlorite sequence and contained 1.8% (w/w) xylan and 1% (w/w) mannan.

Enzyme treatment of pulp and LCC

The pulp was incubated with 5000 nkat xylanase or mannanase activity at a concentration of 5% (w/v), a pH of 4.8 (adjusted with phosphoric acid) and at 50 °C for 10 h. A blank and a control (thermally inactivated enzyme) were run at the same time. After incubation, the samples were extracted with 10% NaOH (2.5% w/v, 60 °C, 1 h) and washed. Acid hydrolysis of the freeze-dried pulp was performed at 30 °C for 60 min (72% H_2SO_4) and, after dilution to 4% H_2SO_4, the samples were autoclaved (121 °C) for 45 min. Oligosaccharides present in the supernatants were only hydrolyzed by 4% H_2SO_4 as described above. Xylose, mannose, arabinose, galactose and glucose were quantified by HPLC in a Carbopack PA-1 column from Dionex as described previously (deJong et al. 1997).

The freeze-dried LCC samples were dissolved in 50 mM citrate buffer (pH 6.0) and incubated with 10 nkat of different enzymes for 12 h at 30 °C. Subsequently oligomers were removed by ultrafiltration (two washes) with the respective molecular mass cut-off, and the carbohydrate composition was determined by HPLC after acid hydrolysis. Controls using thermally inactivated enzymes were run simultaneously. Dissolved lignin was determined by the modified Pearl-Benson method (Dence, 1992).

Isolation of hemicellulose

The pulp was hydrolyzed in a stirred ultrafiltration cell (Amicon) with a 650-Da membrane at a consistency of 10%. In the first step the pulp was incubated with 1500 nkat (activity on filter-paper) of a cellulase preparation (Celluclast, Novo) and with hemicellulases as specified above in 50 mM citrate buffer at pH 6.0 and at 30 °C. Thereafter buffer was added to the concentrated (five-fold) suspension to reach the original volume. This procedure was repeated twice till the pulp was completely solubilized after approximately 48 h. Finally, the solution was concentrated tenfold after two washes with distilled water. The concentrate was fractionated in ultrafiltration cells with molecular mass cut-offs of 30 kDa, 10 kDa and 3 kDa. The supernatants were washed twice with distilled water. A yield loss of about 10%–15% resulted from the washing steps of the fractionation procedure. Selected carbohydrate fractions were further purified by silica gel chromatography. A 1-ml sample of the concentrate was fractionated in a silica gel column (30 × 40 cm, silica gel 60, Fluka) with a mixture of distilled water, ethanol and isopropanol 3:2:1 as eluent. Subsequently the carbohydrate composition of the collected fractions (12 × 5 ml) was determined by HPLC after acid hydrolysis.

FT-IR spectroscopy

A 15-mg sample of the dried carbohydrate preparations was mixed with 300 mg KCl and ground thoroughly in a mortar. FT-IR spectra (256 scans, 4 cm^{-1}) were recorded on a Perkin-Elmer 1600 instrument (Norwalk, Conn., USA) using the diffuse reflectance accessory. All spectra were baseline-corrected and normalized. Ivory nut mannan, locust bean galactomannan (Megazyme), konjak glucomannan (Arkopharma, Carros, France), softwood galactoglucomannan from *Tsuga canadiensis* (kindly provided by J. Puls, Hamburg, Germany) and softwood lignin (obtained from P, Vancouver, Canada) were used as standards.

Size-exclusion chromatography

The molecular mass distribution of the isolated carbohydrates was determined by HPLC on Toyopearl HW-55 S and HW-50 S columns as described previously (Wong et al. 1996). Elution of the samples was monitored by UV and an electrochemical pulsed amperometric detector (PAD). Mannooligosaccharides and xylooligosaccharides from DP 2–6 (Megazyme) and dextrans (T10, T40, T70 and T500, Pharmacia) were used as standards.

Results and discussion

As mentioned earlier, the resistance of the hemicellulose in dissolving pulps towards enzymatic hydrolysis could be due either to a lack of accessibility or to the presence of LCC. To provide experimental evidence for either case, the dissolving pulp was probed by aggressive hydrolysis with hemicellulases and by complete enzymatic hydrolysis of the cellulose followed by additional treatments with various hydrolytic enzymes. It was hoped that treatment of the dissolving pulp with an agressive, crude cellulase preparation would provide almost complete hydrolysis of the substrate. This should indicate whether the residual hemicellulose remaining in the pulp after hemicellulase treatment was either entrapped within the cellulose matrix, and thus released during hydrolysis, or associated with lignin-carbohydrate complexes and therefore more resistant to hydrolytic attack.

Previous reports have indicated that generally less than half of the hemicellulose present in dissolving pulps could be solubilized using xylanases from various fungal species (Christov and Prior 1993; Roberts et al. 1990; Senior et al. 1988; Paice and Jurasek 1984). The dis-

solving pulp in this study showed similar results, the addition of various xylanase (*T. lanuginosus*), mannanase (*S. rolfsii*) and endoglucanase (*G. sepiarium*) combinations showing that a maximum of 56% of the xylan and 60% of the mannan present in the original pulp could be solubilized (Fig. 1). Other researchers (Christov and Prior 1996; Senior et al. 1988) have found that repeated enzyme treatment further increases hemicellulose removal while our results do not support these findings (data not shown). Sequential incubation of the pulp with xylanases and mannanases indicated that the mannan might be somehow shielded by xylan since preincubation of the pulp with the xylanase significantly enhanced the effect of the mannanase (Fig. 1). Edgar et al. (1998) have reported similar findings where the enzymatic removal of hemicellulose enhanced the action of an endoglucanase on kraft pulp.

Under the assumption that the remaining hemicellulose was still largely inaccessible to the enzymes, the original dissolving pulp was enzymatically hydrolyzed with the Celluclast cellulase preparation in a membrane reactor, to try to solubilize the cellulose completely, and various fractions were isolated by ultrafiltration. As shown in Table 1, nearly 95% of the pulp was hydrolyzed, leaving about 3% of the carbohydrates present in the original pulp as a recalcitrant residue retained in the supernatant of the membrane reactor. When we fractionated this supernatant according to size, mannose was the predominant sugar in the 10- to 30-kDa fraction whereas mainly xylose was found in the 3- to 10-kDa and > 30-kDa fractions (Table 1).

As indicated in Table 1, about 60% of the mannose and xylose and 75% of the galactose were liberated (LC3 + LC3–10 fraction) after complete solubilization of the cellulose, thus it is probable that more than half of the "recalcitrant" hemicellulose that was not solubilized by the hemicellulase treatment of the dissolving pulp was inaccessible to these enzymes as a result of "masking" by the cellulose component. Although about 60% of the xylose and mannan was liberated (Fig. 1), the recalcitrance of the remaining 40% of the hemicellulose portion implied that the residual hemicellulose was present in another form that could not be readily attacked by these enzymes.

The fraction above 3 kDa was not characterized further, since this fraction only contained monosaccharides and small oligosaccharides. The fractions of 3–10 kDa, 10–30 kDa and above 30 kDa were hydrolyzed a second time with hemicellulases, a cellulase/hemicellulase cocktail and culture filtrates known to contain a range of hydrolytic enzymes (Table 2). In general, the low-molecular-mass fraction (LC3–10) was significantly hydrolyzed; 81% of the glucose, 77% of the mannose and 42% of the xylose present in this fraction were hydrolyzed by the *S. rolfsii* culture filtrate, hence this fraction did not appear to be recalcitrant (Table 2). However, only 15% of the mannan present in the LC10–30 fraction could be hydrolyzed. The galactose substituents seemed to be more accessible since about 20% were removed by the *S. rolfsii* culture filtrate. Only about 12% of the xylose present in the high-molecular-mass fraction LC30 was hydrolyzed by the xylanase compared to 40% of the LC3–10 fraction. As the LC10–30 and LC30 fractions were quite resistant to enzymatic and culture filtrate treatments, these samples were prime candidates to indicate the presence of LCC and they were investigated further.

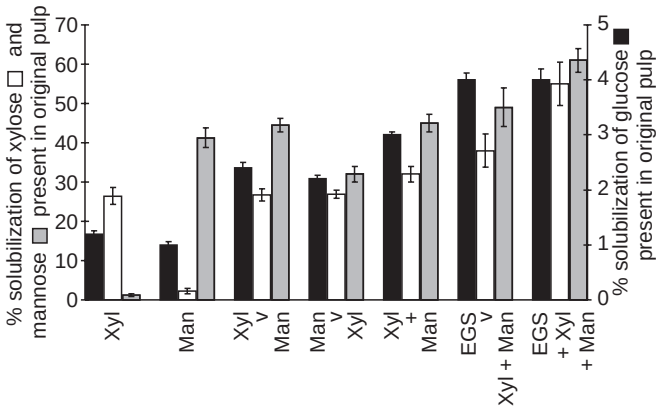

Fig. 1 Solubilization of hemicellulose from softwood dissolving pulp by combined and sequential treatment with xylanase (Xyl, *T. lanuginosus*), mannanase (Man, *S. rolfsii*) and endoglucanase (EGS, *G. sepiarium*)

The fractions of 10–30 kDa and 30 kDa were cleaned-up for spectroscopic/chromatographic analysis by liquid chromatography with silica gel as a stationary phase. Sugar analysis of the purified fractions showed that the LC10–30 fraction consisted of 52% mannose, 16% glucose, and 11% galactose. The sugar ratio

Table 1 Yield and composition of hydrolyzed dissolving pulp after ultrafiltration. *ND* not determined, *LC3* lignin-carbohydrate fraction, *L3*kDa, *LC3–10*, 3–10 kDa, *LC10–30*, 10–30 kDa, *LC30* >30 kDa

Sample	Cut-off	Composition (mg/g orignal pulp)					
		Glucose	Mannose	Xylose	Galactose	Arabinose	Lignin
Pulp		94	1.0	1.8	0.2	0.1	ND
LC3	<3	93	0.52	0.72	0.15	0.08	ND
LC3–10	3–10	0.15	0.09	0.42	0.01	0.01	0.60
LC10–30	10–30	0.09	0.25	0.08	0.05	0.00	0.17
LC30	>30	0.05	0.03	0.30	0.00	0.01	0.40

Table 2 Hydrolysis of lignin-carbohydrate samples isolated from dissolving pulp by carbohydrolases and culture filtrates from various fungi. *CC* Celluclast. The mannanase was from *S. rolfsii*, the xylanase from *T. lanuginosus*

Enzyme system	Sample composition (%)							
	LC30		LC10-30			LC3-10		
	Glc	Xyl	Glc	Man	Gal	Glc	Man	Xyl
Mannanase	0	0	5	4	3	2	45	0
Xylanase	0	12	0	0	0	4	0	40
CC + mannanase + xylanase	20	12	7	7	ND	71	49	45
Culture filtrates								
S. rolfsii	34	4	14	14	23	81	77	48
T. harzianum	41	13	11	8	14	66	58	35
S. commune	13	8	7	9	15	77	42	42
G. sepiarium	7	1	8	10	13	56	53	51

Only those sugars detected are shown.

Glc:Man:Gal of 1:2.8:0.9 supports the conclusion that the LC10–30 fraction carbohydrate is mostly a galactoglucomannan. The presence of lignin was confirmed by the characteristic IR band at 1510 cm^{-1} and contributed about 26% (w/w) of the material as determined by the Pearl-Benson method.

The improved resolution of second-derivative FT-IR over standard FT-IR spectroscopy is well suited for the elucidation of multicomponent samples. We analyzed the anomeric "fingerprint" region (950–700 cm^{-1}) of the LCC samples and those of suitable standards. Although the carbohydrate anomeric IR region is well understood (Fengel 1992; Michell 1989), it is advantageous to obtain reference spectra on the same instrument to eliminate influences due to instrumental effects (i.e., the DRIFT method), environmental effects (i.e., KCl rather than KBr in the medium) and data processing. Inspection of the second-derivative IR spectra shows that galactoglucomannan and mannan can not be in the fraction of 10–30 kDa at significant concentrations since galactoglucomannan should show a strong peak at 771 cm^{-1} and mannan should show a strong peak at 945 cm^{-1} (Fig. 2, Table 3). Of the remaining two carbohydrates, glucomannan may be present at low concentrations, as suggested by the weak 901 cm^{-1} peak, which is strong in the glucomannan standard. Galactomannan is suggested to be present by the peaks at 877 $cm^{-1}$and 827 cm^{-1}. The inclusion of lignin was evident by the characteristic out-of-plane aromatic C-H mode at 856 cm^{-1} and 817 cm^{-1}, which are characteristic of guaiacyl-based lignin; this is further shown by additional absorption bands at 1510 $cm^{-1}$and 1596 cm^{-1} (not shown) that are absent in carbohydrates. The weak anomeric IR signals obtained for the 30-kDa fraction prevented second-derivative analysis of the FT-IR spectra to elucidate the exact nature of the carbohydrate structure of this sample.

When the molecular size of the LC10–30 fraction was examined by size-exclusion chromatography, it was found that the UV-absorbing (the lignin) and PAD-positive substances (the carbohydrates) co-eluted at a retention time corresponding to molecular mass of about 10^4 Da (Fig. 3). As physical adsorption is not very likely under the elution conditions used (1 M NaOH) the lignin and hemicellulose components are probably covalently linked together. The molecular mass of 10^4 Da represents a degree of polymerisation (DP) of about 55, where the lignin contributes about 14 units. Similarly, for the LC30 fraction, the analysis gave a composition of 4% mannose, 6% glucose, 38% xylose,

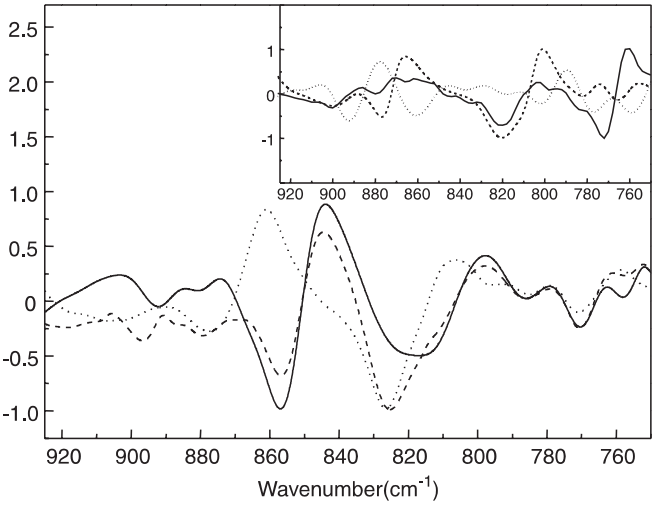

Fig. 2 Second-derivative IR spectra of galactomannan (···), softwood lignin (——) and the 10- to 30-kDa lignin-carbohydrate fraction (- - - -) isolated from softwood dissolving pulp Inset the second derivative for galactoglucomannan (——), glucomannan (···) and mannan (- - - -)

Table 3 IR bands of the isolated 10- to 30-kDa lignin-carbohydrate complex fraction and of various mannans; *s* strong, *m* medium, *vs* very strong, *sh* shoulder

Sample	Characteristic IR bands (cm^{-1})		
Galactoglucomannan		820s	771s
Galactomannan		877s	827s
Glucomannan	901s	877s	820s
Mannan	945m	877s	820s
Softwood lignin		856s	816s
Fraction of 10–30 kDa	877s	855s	827vs + 816sh

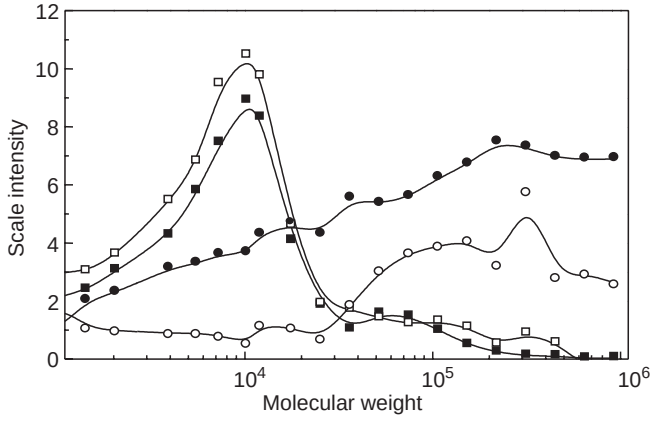

Fig. 3 Gel permeation chromatography of lignin-carbohydrate samples isolated from softwood dissolving pulp. Fractions: ■ □ LC10–30 and ●○ LC30. Monitors: ■● absorption at 280; □○ pulsed amperometric detection

2% arabinose and about 50% lignin. Size-exclusion chromatography showed that the UV-absorbing (the lignin) and PAD-positive substances (the carbohydrates) were weakly correlated. The increasing profile of the UV signal suggested that low-molecular-mass lignin was part of this fraction. It is possible that a portion of the carbohydrates was entrapped within the lignin, thus proving to be largely inaccessible to the enzymes.

Angiosperm lignin-carbohydrate complexes are known to consist of galactoglucomannan, arabino-4-*O*-methylglucuronoxylan or arabinogalactan carbohydrate moieties linked to lignin at the benzyl positions (Johnson and Overend 1991). For the softwood dissolving pulp used in this study, about 0.6% of the mass is in the form of a lignin-galactoglucomannan complex. As in Fengel's and Wegener's LCC model, the lignin appears to play a role as a cross-linking agent in the LC10–30 fraction LCC.

In contrast to lignin-galactomannan complexes, the existence of xylanase-resistant lignin-xylan complexes in both hardwood and softwood kraft pulps has previously been reported (Wang et al. 1997; Watanabe et al. 1996). However, as the xylan-containing LC30 fraction showed a weak correlation between absorption (lignin) and PAD (carbohydrates) the resistance to enzymatic hydrolysis of the LC30 was not immediately clear. Although the LC30 fraction was expected to contain lignin-arabino-4-*O*-methylglucuronxylan, the fact that size-exclusion chromatography showed a DP range above the 100–200 typically quoted for xylan (Sjöström 1993) indicated that the contribution of a xylan-lignin complex may not be significant.

The weak correlation between the PAD and UV results can probably be rationalized by two other factors. First, the LC30 fraction contains other hemicellulose components, as shown by the presence of other sugars not found in xylan, such as, glucose. These other sugars contribute to about a quarter of the total carbohydrates. Indeed, glucose showed the highest degree of hydrolysis (Table 2). Secondly, the UV monitor shows a steadily increasing lignin molecular mass profile up to DP values of a few thousand. Therefore, it is probable that the carbohydrates were entrapped within a lignin matrix, thus proving to be inaccessible. A hydrophobic lignin shell around a carbohydrate nucleus would explain the weak correlation between the PAD and UV signals and the recalcitrant nature of this fraction. If this were true, it would explain the increased effectiveness of the culture filtrates where the possible presence of lignin-degrading enzymes perhaps increased the accessibility to the carbohydrate fraction.

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