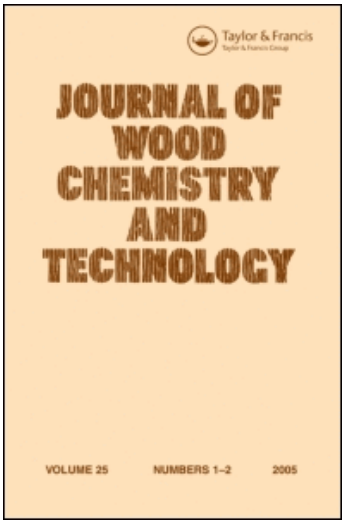

Journal of Wood Chemistry and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597282

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To cite this Article Wong, Ken K. Y. , Yokota, Shinso , Saddler, John N. and Jong, de(1996) 'Enzymic Hydrolysis Of Lignin—Carbohydrate Complexes Isolated From Kraft Pulp', Journal of Wood Chemistry and Technology, 16: 2, 121 — 138
To link to this Article: DOI: 10.1080/02773819608545814
URL: http://dx.doi.org/10.1080/02773819608545814

JOURNAL OF WOOD CHEMISTRY AND TECHNOLOGY, 16(2), 121-138 (1996)

ENZYMIC HYDROLYSIS OF LIGNIN—CARBOHYDRATE COMPLEXES ISOLATED FROM KRAFT PULP

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ABSTRACT

Four carbohydrate samples extracted from kraft pulps are used as model substrates for studying the mechanism by which xylanase enhances subsequent bleaching of kraft pulp. Fourier transform infrared spectroscopy confirms that small amounts of aromatic molecules, probably lignin, remain associated with these carbohydrate samples. When the extracts are hydrolyzed with xylanase or acid, size exclusion chromatography shows a decrease in the molecular mass of their UV-absorbing constituents, as well as their carbohydrate constituents as determined by pulsed amperometric detection. The results are consistent with the hypothesis that xylanase prebleaching hydrolyzes the xylan portion of lignin-carbohydrate complexes to leave smaller lignin-containing macromolecules in pulp fiber, thus facilitating the removal of lignin components by bleaching chemicals.

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Abbreviations: DMSO, dimethyl sulfoxide; DRIFT, diffuse reflectance FT-IR; FT-IR, Fourier transform infrared; LCC, lignin-carbohydrate complex; MM, molecular mass; PAD, pulsed amperometric detector; SEC, size exclusion chromatography; TAPPI, Trade Association of the Pulp and Paper Industry (Atlanta, GA, USA).

INTRODUCTION

After decades of discussion, the existence of covalent linkages between carbohydrate and lignin molecules, forming lignin–carbohydrate complexes (LCC's) in plant cell walls and woody tissue, has largely been accepted[1–3]. Recent work has used chemical and spectroscopic analyses to confirm the presence of lignin in carbohydrate samples and examine their association[4–6]. Another approach for verifying the persistent association of lignin with carbohydrate is the chromatographic analysis of the products from the enzymic hydrolysis of putative LCC's[7]. Size exclusion chromatography (SEC) would also provide an estimate of the molecular mass (MM) of the molecules, and two methods have been used to continuously monitor the elution of lignin and carbohydrate components independently[8,9]. Recently, the use of a pulsed amperometric detector (PAD) has been suggested as a convenient method for monitoring the carbohydrate component during SEC of LCC's. Although selectivity was not achieved for materials having an apparent MM of less than 0.5 kDa[10], this novel method could provide useful information on the nature of LCC's.

Further knowledge of LCC's may have important implications for current trends in the manufacture of pulp and paper, where technologies are being implemented to reduce the environmental impact of pulp mills[11]. This is because LCC's are considered to be one of the major impediments to the removal of residual lignin from kraft pulp[12]. Traditionally, chlorine–containing chemicals have been used to selectively remove the residual lignin and thus increase pulp brightness to the levels desired in fine paper products. Unfortunately, these reagents generate organochlorine compounds, whose discharge in mill effluents has come under critical scrutiny[13].

In Canada, xylanase prebleaching is a biotechnological alternative that has been implemented in six kraft mills to reduce the use of chlorine–containing chemicals[14]. However, it remains unclear how this enzymic pretreatment facilitates subsequent chemical bleaching of the kraft pulp. The hypothesis that LCC's constitute the target substrate for xylanase prebleaching would imply that the enzyme

reduces the overall size of the LCC's in kraft pulp. Our initial work has involved isolating xylan–containing fractions from kraft pulps and examining the effects that hydrolysis has on the MM distribution of their UV–absorbing constituents[15]. The present work uses PAD to complete the analysis, and Fourier transform infrared (FT–IR) spectroscopy to characterize the lignin and carbohydrate components in the extracts. FT–IR spectroscopy is a convenient analysis that warrants consideration because it is the basis of methods proposed for quantifying the composition of wood and wood pulp[16–18].

EXPERIMENTAL

Preparation of Samples

Kraft pulps derived from trembling aspen and black spruce, and their corresponding black liquors and wood chips, were kindly provided by M.G. Paice (Pulp and Paper Research Institute of Canada, Pointe–Claire). The kraft lignin was prepared from the black liquor, and the enzyme lignin, alkaline extract and dimethyl sulfoxide (DMSO) extract were prepared from the kraft pulps as previously described[15]. Pulp meals (25 g samples), freeze–dried samples that have been prepared using a Wiley mill fitted with a 0.5 mm screen, were further ground in a 15 × 17 cm ball mill. The mill was flushed with nitrogen, sealed and rotated at a rate of 100 r.p.m. for 24 h at 4 °C. The composition of the samples were determined using methods reported previously[15] and the results are summarized on Table 1.

FT–IR Spectroscopy

The carbohydrate and lignin preparations and the pulp meals were powders that could be directly used in the analysis, whereas wood was first ground to 40 mesh. About 12 mg of sample were mixed with 300 mg of KCl powder and ground thoroughly in a mortar. FT–IR

TABLE 1

The composition[a] and the yield of lignin and carbohydrate samples isolated from kraft pulp and black liquor derived from aspen and spruce wood.

species	sample[b]	abbrev.	yield[c]	carbohydrate (%)						lignin (%)		
				ara	gal	glu	man	xyl	glcA	acid insol.	acid soluble	kappa no.[d]
aspen	wood	AW	–	0.4	0.4	46.2	1.3	16.2	–	21.4	3.0	–
	kraft pulp	AKP	–	trace	0	69.7	0.7	15.5	–	1.2	0.8	11.0
	alkaline extract	AHE	16.6	0.8	0.1	0.9	3.4	65.8	7.3	2.7	2.4	29.0
	DMSO extract	ADE	13.1	0.2	0	1.4	4.6	72.8	6.6	2.4	3.1	48.2
	kraft lignin	AKL	–	0.2	0.1	0	0.2	2.3	–	89.3	4.7	–
	enzyme lignin	AEL	0.9	0.3	0.6	1.3	0.8	4.8	–	78.2	3.1	–
spruce	wood	SW	–	1.1	1.7	43.9	11.0	5.9	–	28.3	0.3	–
	kraft pulp	SKP	–	0.4	0.1	78.2	7.1	7.8	–	4.1	0.5	27.6
	alkaline extract	SHE	9.9	2.0	0.3	6.3	20.0	39.5	3.7	6.4	1.9	58.2
	DMSO extract	SDE	4.6	4.1	0	2.5	2.6	66.0	5.6	7.1	2.6	46.5
	kraft lignin	SKL	–	0.3	0.5	0.1	0.2	1.2	–	91.5	3.1	–
	enzyme lignin	SEL	4.9	0.2	1.9	1.8	2.5	1.3	–	80.7	1.8	–

[a] as determined in the sulfuric acid hydrolysate of the sample[15], monosaccharides are expressed in their anhydro-form (ara = arabinose, gal = galactose, glu = glucose, man = mannose, xyl = xylose, glcA = methyl-glucuronic acid)

[b] enzyme lignin, alkaline extract and DMSO extract were prepared from the kraft pulp

[c] yield from kraft pulp

[d] microkappa number determined by permanganate oxidation (TAPPI Useful Method UM246, 1991)

spectra (256 scans, 4 cm^{-1}) were determined by the diffuse reflectance method (DRIFT) using a Perkin-Elmer 1600 instrument (Norwalk, CT, USA), and the maximal absorbance was less than 0.65 AU for all samples analyzed. All spectra were baseline-corrected and normalized, and the average of three spectra was used as the representative spectrum for each sample. The second derivative spectra was used to improve the resolution of certain absorption bands[19].

Enzyme and Acid Hydrolysis of Carbohydrate Extracts

Enzyme hydrolysis was carried out using the 22 kDa xylanase from *Trichoderma harzianum* E58[20] at a loading that was based on the xylan content of the substrate and equivalent to 200 $nkat \cdot g^{-1}$ pulp at 10 % consistency, which were the conditions previously used for the xylanase prebleaching of kraft pulp[21]. It was carried out in 50 mM sodium citrate buffer, pH 5.3, at 50 °C for 1 h, and an equal volume of 1 M NaOH was added to stop the reaction. Boiled xylanase was used as the control. Acid hydrolysis was carried out in 2 N trifluoroacetic acid, at 80 °C for 3 h. The acid hydrolyzed samples were dried using a SpeedVac concentrator (Savant, Farmingdale, NY, USA).

Size Exclusion Chromatography

The MM distribution of carbohydrate and lignin macromolecules was determined using Toyopearl HW-55S and HW-50S resins (TosoHaas, Montgomeryville, PA, USA) packed in series in 0.5 × 20 cm HR columns (Pharmacia, Uppsala, Sweden)[10]. The eluent, 1 M NaOH, was run at a flow rate of 0.05 $ml \cdot min^{-1}$ using a Dionex DX500 HPLC system (Sunnyvale, CA, USA). All samples were prepared at a concentration of 1 $mg \cdot ml^{-1}$ in 1 M NaOH and filtered through 0.45 µm HV filters (Millipore, Bedford, MA, USA), and a volume of 20 µL was loaded using a SpectraSYSTEM AS3500 autoinjector (Spectra-Physics, Fremont, CA, USA). Elution of the samples was monitored using a

Dionex AD20 absorbance detector set at low for 280 nm, and a Dionex ED40 electrochemical detector with a gold electrode and parameters set for detection of sugars as recommended by the manufacturer. The MM standards used were xylose, cellobiose and dextrans with M_n (number average molecular mass) of 5.5, 24.5, 37.6 and 195.3 kDa (Pharmacia), with a linear calibration found between 0.34–37.6 kDa.

RESULTS

Carbohydrate Extracts from Kraft Pulp

The extraction of aspen kraft pulp with DMSO and NaOH yielded carbohydrate extracts containing, respectively, 13 and 17 % of the starting material (Table 1), while that of spruce kraft pulp yielded 5 and 10 %. These carbohydrate extracts were predominantly composed of xylan, with the NaOH extract from spruce kraft pulp also containing substantial amounts of glucomannan. Sugar analyses indicated that the carbohydrate extracts constituted 60–70 % of the xylan in aspen kraft pulp, and 40–50 % of that in spruce kraft pulp. The extracts from aspen kraft pulp contained less lignin than those from spruce kraft pulp, although permanganate oxidation indicated relatively high amounts of lignin were found in the DMSO extract from aspen kraft pulp.

FT-IR Analyses

The most prominent absorption band of the various carbohydrate extracts from kraft pulp was at wavenumbers 1041–1050 cm^{-1} (Fig. 1) where absorbance could be ascribed to C–O stretch in the carbohydrate. The spectrum of these extracts resembled the spectrum published for xylan samples[19,22–24], and it is clearly distinct from the spectrum of cellulose for wavenumbers from 900–1200 cm^{-1}. The alkaline extract from spruce kraft pulp, the spruce kraft pulp and the spruce wood all showed an absorption band at 810–813 cm^{-1}, which could be attributed

to galactoglucomannan[19,24]. This band was much less pronounced in the corresponding DMSO extract, confirming the results of sugar analyses which indicated that there was little glucomannan present (Table 1). All four extracts were similar in having an absorption band at 896–898 cm^{-1}, which should be pronounced in hemicelluloses. However, none of the aforementioned absorption bands was indicative of lignin[25,26]. In this range of low wavenumbers, the lignin samples derived from aspen had a prominent band at 831–833 cm^{-1} that is typical of syringyl lignins, while those from spruce had relatively more intense absorption at 816–818, 855–858, 1270 and 1450–1452 cm^{-1} that are typical of guaiacyl lignins (Figs. 1 & 2)[26]. In comparison to absorbances observed in the kraft lignins, the higher absorbances found in the enzyme lignins around 1034 and 1087 cm^{-1} were indicative of their higher carbohydrate content.

For the higher wavenumbers ranging from 1500–4400 cm^{-1}, the spectrum of the alkaline extracts was similar to that of their corresponding kraft pulps, lacking the separate C–H stretch bands seen in the lignin samples. The spectrum of the corresponding wood samples differed by having prominent absorption bands at 1504–1512, 1663–1664, and 1742 cm^{-1} (Fig. 1). These bands could be ascribed to vibrations in aromatic skeletons, C=O stretch in aryl ketones, and ester groups, respectively[25,26]. Although the signal was weak, the band near 1664 cm^{-1} was detectable in both extracts from spruce kraft pulp but only in the alkaline extract from the aspen kraft pulp. However, the second derivative spectra showed that all four carbohydrate extracts had an absorption band at wavenumbers 1512–1514 cm^{-1} (Fig. 2). This latter absorption band, indicative of the aromatic skeleton[26], could be clearly seen in both the enzyme and kraft lignins. The enzyme lignins also had a prominent band at 1663–1666 cm^{-1} which was not seen in the kraft lignins, indicating that the former had more conjugated para-substituted aryl ketones[26].

The DMSO extracts differed from the kraft pulps and the NaOH extracts in having strong absorption at wavenumber 1722–1724 cm^{-1} instead of 1594–1602 cm^{-1}. One explanation for this difference would be that the former band is attributed to C=O stretching in glucuronoxylan

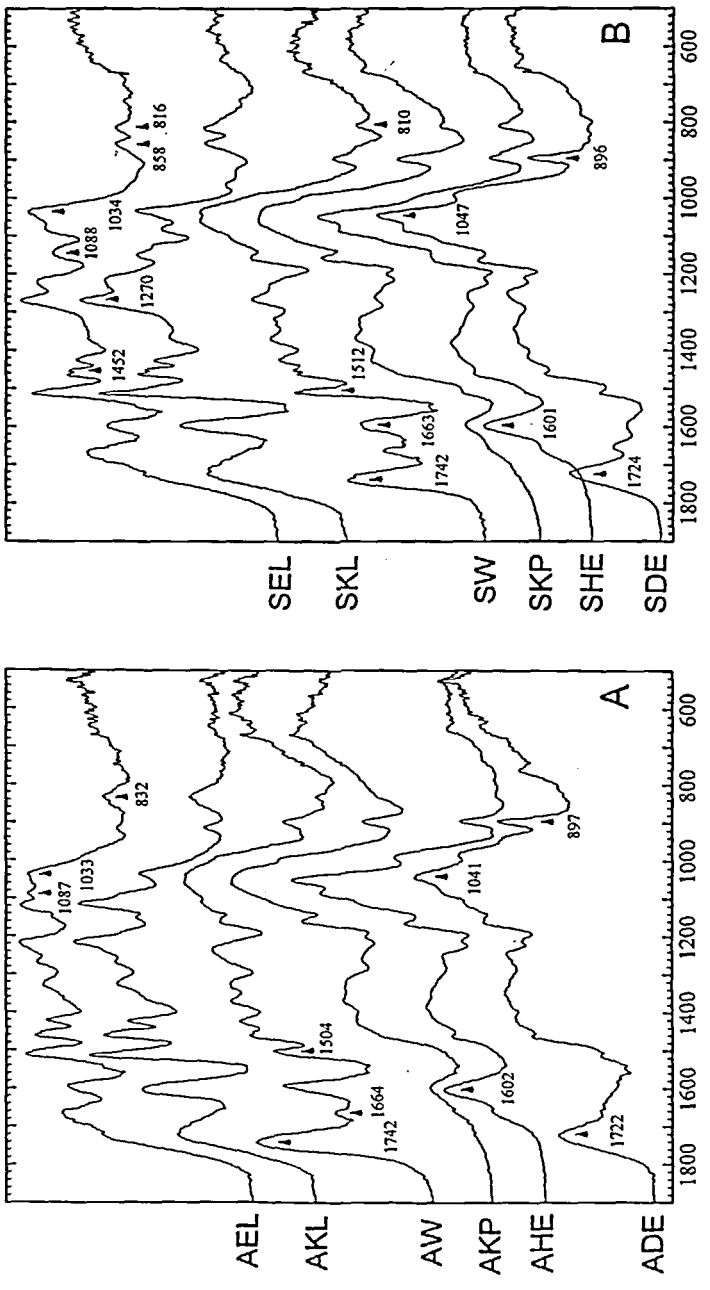

FIGURE 1. FT–IR absorbance spectra of lignin and carbohydrate samples isolated from kraft pulp, and its black liquor, derived from aspen (A) and spruce (B). Only the spectra for wavenumbers from 500–2000 cm^{-1} are shown. The sample names are listed on Table 1.

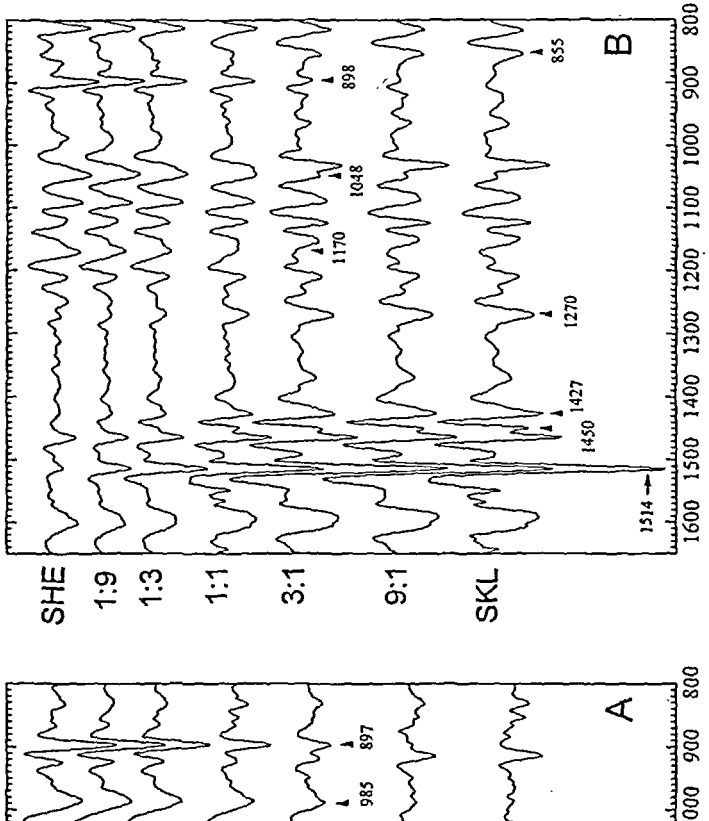

FIGURE 2. The second derivative FT–IR spectra of mixtures containing kraft lignin and alkaline extract from kraft pulp derived from aspen (A) and spruce (B). Only the spectra for wavenumbers from 800–1650 cm^{-1} are shown.

and the latter to its COO^- ion in the salt form[22]. This possibility was examined by determining the FT–IR spectrum of the two kraft pulps and NaOH extracts after washing with acid. In this experiment, 0.25 g of kraft pulp was washed twice with 25 mL of 1 % HCl and then at least five times with water, while the alkaline extracts were similarly washed with acid and water in 75 % ethanol. For all four samples, an absorption band at 1722 cm^{-1} appeared after acid washing, with a decrease in absorbance around 1600 cm^{-1}.

When the spectrum of an 1:1 mixture of the alkaline extract and kraft lignin was examined, it was found to be nearly identical to the spectrum obtained by arithmetically averaging the spectra of the individual components (data not shown). As more kraft lignin was added to the alkaline extract, the absorption bands indicative of aromatic units became more prominent in the second derivative spectra (Fig. 2). At least three bands were highly dependent on lignin concentrations ($r^2 > 0.98$), those at wavenumbers 1514–16, 1424–1427 and 1270 cm^{-1}. Bands at 1169–1170, 985 (for aspen samples only) and 897–898 cm^{-1} were highly dependent on carbohydrate concentrations ($r^2 > 0.99$), but not the band at 1048–1050 cm^{-1} that is characteristic of xylan. However, when the standard curves generated from this data were used to estimate the amount of lignin in the kraft pulps, the DMSO extracts or the enzyme lignins, the results did not agree with those obtained by determining acid insoluble and acid soluble lignin (data not shown).

Molecular Mass Distribution Analyses

For both pulps, the UV–absorbing material in the alkaline extract was larger than that in the DMSO extract. Most of the carbohydrate constituents, that registered in the PAD, co-eluted with the UV–absorbing constituents (Figs. 3 & 4). However, the two chromatograms for each sample were not identical. For example, the UV–absorbing materials were apparently smaller than the materials detected by PAD in the DMSO extract from aspen kraft pulp (Figs. 3C & 3D), whereas the

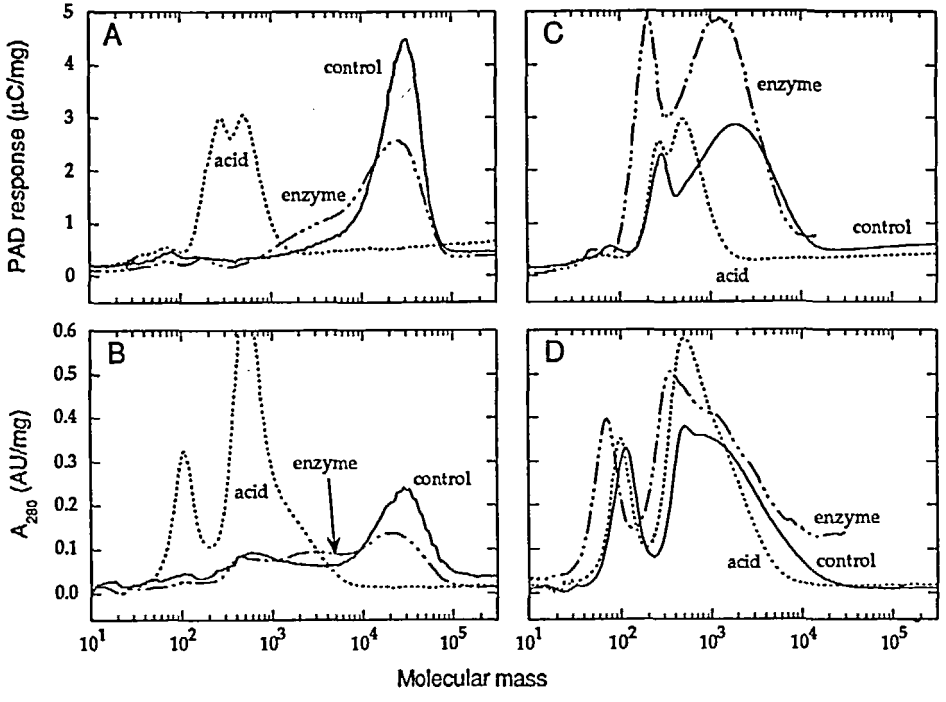

FIGURE 3. The molecular mass of the alkaline (A, B) and DMSO (C, D) extract from aspen kraft pulp after control (——), xylanase (—··—) and acid (·····) treatments. (A, C) Carbohydrate as monitored using PAD. (B, D) Lignin as monitored by UV absorbance.

opposite occurred in the corresponding extract from spruce kraft pulp (Figs. 4C & 4D). Furthermore, peaks seen in the UV chromatograms with MM < 1000 were not detected by PAD in the alkaline extracts (Figs. 3A, 3B, 4A & 4B), and those in the DMSO extracts did not co-elute with the peaks detected by PAD (Figs. 3C, 3D, 4C & 4D).

For all four carbohydrate extracts from kraft pulp, the treatment of these xylan-containing polymers with xylanase decreased the MM of the UV-absorbing material as well the molecules detected by PAD. This was most evident in the extracts from spruce kraft pulp (Fig. 4) and least evident in the DMSO extract from aspen kraft pulp (Figs. 3C &

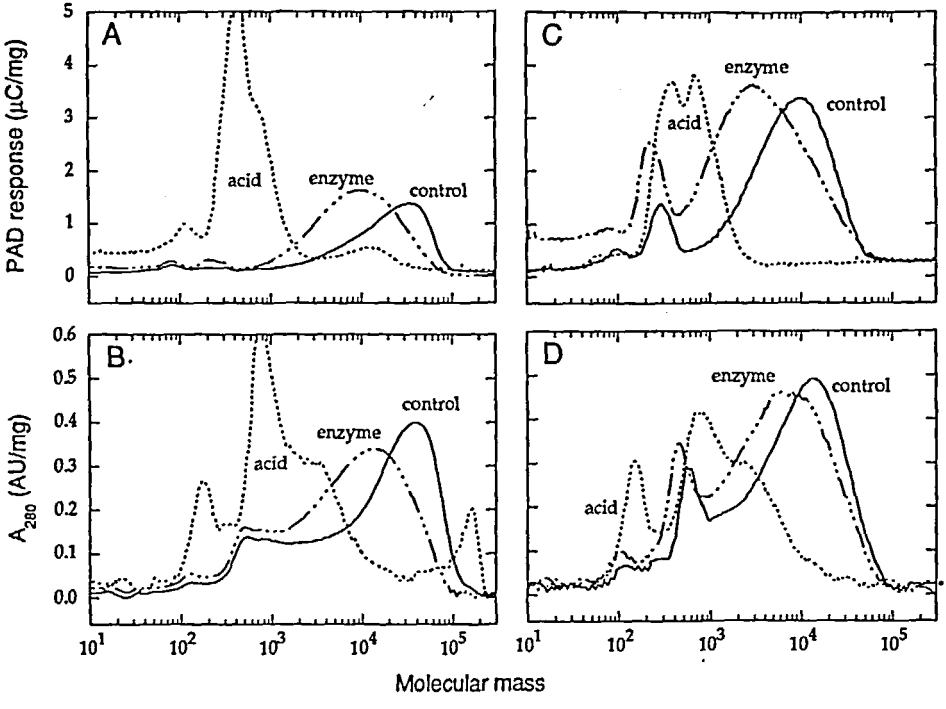

FIGURE 4. The molecular mass of the alkaline (A, B) and DMSO (C, D) extract from spruce kraft pulp after control (——), xylanase (—·—) and acid (·····) treatments. (A, C) Carbohydrate as monitored using PAD. (B, D) Lignin as monitored by UV absorbance.

3D). In the latter case, the relatively low MM's of the UV-absorbing material in the starting material may have contributed to the small effect achieved by xylanase. In none of the cases was there evidence that enzymic treatment produced small sugar molecules such as xylose.

After the hydrolysis of the carbohydrate extracts with acid, sugar analyses using anion-exchange HPLC[20] showed that essentially all of the xylosyl constituents were found as monosaccharides, with an exception being the alkaline extract from aspen kraft pulp where more than 80 % of the xylan was nevertheless hydrolyzed to monomeric units (data not shown). SEC indicated that, in nearly all cases, the UV-

absorbing and PAD-positive components generated from the polymers by acid hydrolysis were smaller than those generated by enzymic hydrolysis. The PAD detected two peaks corresponding to MM of 0.25–0.4 and 0.5–0.7 kDa, respectively. The former peak was likely composed of monosaccharides eluting in a SEC system which had a separation range from 0.34–37.6 kDa. The latter peak seemed to co-elute with the major peak of UV-absorbing material that was generated by acid hydrolysis. In general, acid hydrolysis also yielded slightly larger UV-absorbing components, which appeared as a shoulder (MM = 1–7 kDa) on the main peak in the chromatogram, as well as small UV-absorbing components (0.1–0.2 kDa). Only in the case of the alkaline extract from spruce kraft pulp was there evidence for condensation reactions that generated UV-absorbing components with MM of about 200 kDa.

DISCUSSION

The persistence of sugar molecules in lignin preparations, as well as lignin molecules in carbohydrate preparations, has often been used as evidence for the existence of lignin–carbohydrate linkages[5–8,12]. Other evidence includes the detection of phenolic units, such as ferulic acid and coumaric acid, on oligosaccharides isolated from plant cell walls[1–3]. These analyses involve breaking up the polymers into fragments that are more amenable to chemical analysis. The non-destructive analytical techniques presently available rely mainly on spectroscopy, chromatography and their combination.

For both the carbohydrate and lignin components of wood and wood pulp, most of the FT–IR absorption bands have been assigned empirically[22–27]. Although this information has not been frequently used for the analysis of putative LCC's, our results indicate that FT–IR spectroscopy provides information on both the carbohydrate and lignin components in samples of varying purity. For example, the loss of the absorption band at 1742 cm^{-1} in kraft pulps agreed with the general consensus that kraft pulping conditions eliminate most of the ester bonds, such as those linking acetyl substituents to hardwood

glucuronoxylan and softwood galactoglucomannan. Among the other substituents on hemicellulose, it has been reported that 10–30 % of the glucuronosyl residues survive kraft cooking[28] and their COO^- group occur in the salt form[22]. Our chemical and spectroscopic analyses also confirmed the presence of glucuronosyl residues. For the lignin samples, absorption bands near 816, 832, 858, 1270 and 1452 cm^{-1} could be used to distinguish hardwood and softwood lignins, while that near 1664 cm^{-1} could be used to distinguish enzyme and kraft lignin.

Concerning the possible existence of LCC's in the carbohydrate extracts, the presence of absorption bands near 1512 and 1664 cm^{-1} indicated that small amounts of aromatic structures were present. Although these aromatic molecules are usually considered to be lignin–derived, they may be partly constituted of sugars that have undergone modification under alkaline conditions and/or high temperatures[29,30]. Other analytical methods, such as pyrolysis gas chromatography–mass spectroscopy[30], could be useful for estimating the contribution of carbohydrate–derived chromophores to the aromatic compounds detected by FT–IR spectroscopy.

The mixing of lignin samples into carbohydrate extracts demonstrated the contribution of lignin to the aromatic signals. This study also suggested that it might be possible to use FT–IR to estimate the relative amounts of carbohydrate and lignin in a sample that contains more than 5 % of either component. Such a method could be very useful in research on putative LCC's, on the solubilization of lignin–carbohydrate fragments from lignified cell walls, and on sub-fractions of carbohydrate and lignin preparations. However, our results indicate that caution is required for the comparison of different samples because the magnitude of the signals appears to be dependent on the nature of both the polysaccharides and the lignins.

Our previous work used SEC to show that the UV–absorbing constituents of the carbohydrate extracted from kraft pulps differed in their MM distribution[15]. In the present work, the chromatograms obtained using PAD indicated that the alkaline extracts contain larger macromolecules of carbohydrate than those in the DMSO extracts. For the extracts from spruce kraft pulp, it is possible that the alkali had

extracted more of the larger macromolecules and consequently provided higher yields of extract than did the DMSO. For the extracts from aspen kraft pulps, the high yield of DMSO extract with much smaller MM was more likely the result of the ball milling of the pulp, which was carried out prior to DMSO extraction[15].

After acid and enzymic hydrolysis of the carbohydrate extracts, both the PAD–positive and UV–absorbing components in the pulp extracts showed a lower MM distribution. The acid hydrolysates suggested that part of the lignin was attached to the xylan because relatively large UV–absorbing components remained in all samples after the carbohydrates were essentially hydrolyzed to completion. The MM of these UV–absorbing components ranged from 1–7 kDa in most cases, within the range found for the enzyme lignin isolated from the pulps[15]. In the cases where xylanase was used to partially hydrolyze the xylan in the extracts, a large portion of the UV–absorbing components continued to co-elute with PAD–positive components. Since physical adsorption of lignin to carbohydrate was not evident during chromatographic elution in 1 M NaOH[10], the xylanase hydrolysates also suggested that part of the lignin was attached to the xylan in these samples. Taken together, the spectroscopic and chromatographic data indicate that LCC's are present in the carbohydrate extracted from kraft pulps.

The mechanism of xylanase prebleaching of kraft pulp may therefore involve the release of lignin–carbohydrate fragments from LCC's . It would appear that the materials that are solubilized [21,31] or those that become extractable [32,33] from pulp after xylanase treatment should be examined for the presence of fragments from LCC's . The occurrence of these fragments does not mean that the hydrolysis of LCC's is the sole mechanism with which xylanase enhances pulp bleaching. The contributions of other phenomena [34,35] still require consideration. The examination of model pulps prepared by depositing xylan, lignin and their combination on cellulose fiber is one approach presently used in our laboratories to examine the contribution of xylan–derived chromophores and lignin entrapment.

CONCLUSION

The results of the present study are consistent with the hypothesis that LCC's are present in kraft pulps. Xylanase treatment can reduce the overall MM of LCC's because the enzyme reduced the MM of both the UV–absorbing and the PAD–positive components in carbohydrate preparations isolated from kraft pulp. The presence of lignin in these UV–absorbing components was suggested by the aromatic structures detected by FT–IR spectroscopy and by the relatively large UV–absorbing components that remain after acid hydrolysis of the carbohydrate components. Therefore, the importance of the release of lignin–carbohydrate fragments from LCC's during xylanase prebleaching needs further consideration.

ACKNOWLEDGMENTS

The authors thank the Pulp and Paper Research Institute of Canada (Vancouver) for the use of their FT–IR spectrophotometer and ball mill. We also thank Denys F. Leclerc (Pulp and Paper Research Institute, Vancouver) for reviewing this manuscript. The work was partly financed by the Science Council of British Columbia and the Natural Sciences and Engineering Research Council of Canada.

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