ELSEVIER

Carbohydrate Research 272 (1995) 55–71

CARBOHYDRATE RESEARCH

Characterisation of 4-deoxy-β-L-*threo*-hex-4-enopyranosyluronic acid attached to xylan in pine kraft pulp and pulping liquor by ^{1}H and ^{13}C NMR spectroscopy

Anita Teleman [a,*], Vesa Harjunpää [a], Maija Tenkanen [b], Johanna Buchert [b], Tiina Hausalo [c], Torbjörn Drakenberg [a], Tapani Vuorinen [d]

[a] *Technical Research Centre of Finland, Chemical Technology, P.O. Box 1401, FIN-02044 VTT, Finland*
[b] *Technical Research Centre of Finland, Biotechnology and Food Research, P.O. Box 1501, FIN-02044 VTT, Finland*
[c] *The Finnish Pulp and Paper Research Institute (KCL), P.O. Box 70, FIN-02151 Espoo, Finland*
[d] *Laboratory of Forest Products Chemistry, Helsinki University of Technology, FIN-02150 Espoo, Finland*

Received 27 June, 1994; accepted in revised form 20 November, 1994

Abstract

A new acidic sidegroup in xylans, from both kraft pulp and pulping liquor, was identified by NMR spectroscopy. Unmodified oligosaccharides from kraft pulp xylan were obtained by enzymatic hydrolysis with xylanase (*Trichoderma reesei*). The acidic oligosaccharides were separated from the neutral forms on an anion exchange resin. The new acidic sidegroup was identified as 4-deoxy-β-L-*threo*-hex-4-enopyranosyluronic acid (hexenuronic acid) by ^{1}H and ^{13}C NMR spectroscopy. Hexenuronic acid is a β-elimination product of 4-*O*-methylglucuronic acid and is formed during kraft pulping. HMBC and NOESY experiments showed that hexenuronic acid is attached β-(1 → 2) to xylose. The NOESY data further indicated that hexenuronic acid protrudes from the main xylan chain. The pK_a values for hexenuronic acid (3.03) and 4-*O*-methylglucuronic acid (3.14) attached (1 → 2) to xylose were determined from pH-dependent chemical shifts.

Keywords: 4-Deoxy-β-L-*threo*-hex-4-enopyranosyluronic acid; Xylan; Pine kraft pulp; Pulping liquor

* Corresponding author.

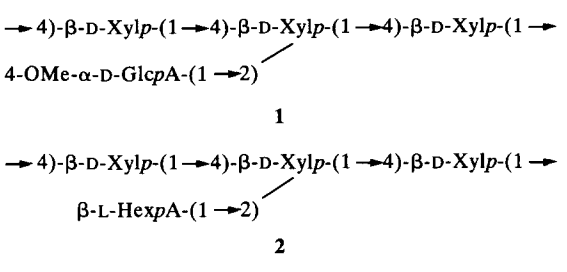
SSDI 0008-6215(95)00002-X

1. Introduction

Acetyl-4-*O*-methyl-D-glucurono- and L-arabino-4-*O*-methyl-D-glucurono-D-xylans are important constituents of hardwoods and softwoods, respectively [1]. During kraft pulping ~ 50% of the wood xylans are dissolved either through degradation or as polymers [2]. The amount of acidic groups in the residual xylans is much lower than in the native xylans. Although it is possible that xylans rich in acidic groups are preferentially dissolved, their degradation is the major cause of disappearance of 4-*O*-methylglucuronic acid groups. Quite early Clayton [3] proposed that removal of the 4-*O*-methylglucuronic acid groups could be initiated by β-elimination of methanol. Later Johansson and Samuelson [4] verified this with a dimeric model compound; (2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylitol). Their experiments clearly showed the formation of 4-deoxy-β-L-*threo*-hex-4-enopyranosyluronic acid and its slow degradation with time.

The β-elimination of natural 1,4-linked polyglycuronates (e.g., alginic and pectic acids) and related heteropolysaccharides (e.g., hyaluronic acid and heparins) is well-known and has been reviewed in detail [5]. The reaction is relatively fast because the glycosyl ion is a much better leaving group than methoxyl ion (in 4-*O*-methyl-D-glucuronic acid residues of xylans). In addition, the reaction results in readily observable chain cleavage. The esters of glycuronic acid derivatives undergo β-elimination particularly easily. During Hakomori's permethylation analysis the conditions are very suitable for β-elimination because the glycuronic acid groups are readily esterified and because the reaction conditions are strongly basic. For this reason Shimizu [6] found 4-deoxy-β-L-*threo*-4-hexenopyranosyluronic acid residues in a permethylated birch xylan and later proved that these groups were formed during permethylation [7].

Although it has been clear from the literature that 4-*O*-methyl-D-glucuronic acid residues of wood xylans (**1**) must undergo β-elimination during kraft pulping, the occurrence of 4-deoxy-β-L-*threo*-hex-4-enopyranosyluronic acid residues (**2**) in kraft pulps or in the dissolved xylans has never been verified. The most likely reason for this is that conventional pulp analysis includes an acid hydrolysis step, known to degrade this acidic residue [4,8]. Shimizu [8] detected only xylose in the hydrolysate from acid hydrolysis of 2-*O*-(4-deoxy-β-L-*threo*-hex-4-enopyranosyluronic acid)-D-xylose. Specific enzymatic hydrolysis do not suffer from these drawbacks. Here, we were able to show the existence of 4-deoxy-β-L-*threo*-hex-4-enopyranosyluronic acid groups both in the dissolved xylans and in kraft pulp, mainly by NMR spectroscopy.



Scheme 1.

2. Results and discussion

Pine chips were cooked with the conventional kraft process. In order to analyse changes in the chemical structure of dissolved softwood kraft xylans, hemicellulose fractions were precipitated from the cooking liquors at several stages of pulping. The lignin content of the precipitates was low (< 10%) which showed that the applied isolation method was selective for hemicelluloses. According to GLC analysis of the per(trimethylsilyl)ated methanolysis products, the isolated hemicelluloses were composed of arabinose, xylose, galactose, glucose, mannose and 4-*O*-methylglucuronic acid residues. Of these, xylose was the major component in all cases, which showed that the hemicelluloses were mainly xylans. Surprisingly, the content of 4-*O*-methylglucuronic acid decreased rapidly with cooking time and was negligible at the end of the cook.

^{1}H NMR spectra of the hemicellulose fractions also revealed the fast decrease in the content of 4-*O*-methylglucuronic acid residues with cooking time (Table 1). Simultaneously, the intensities of two additional, unknown proton signals (at 5.35 and 5.82 ppm in D_2O at pH 7.0) in the anomeric region increased (Fig. 1A and B). The intensities of the two signals were equal and the chemical shift of one of them (the signal at 5.82 ppm) was very sensitive to pH. These findings indicate that the unknown signals arose from acidic residues formed through conversion of 4-*O*-methylglucuronic acid residues.

The unknown signals were also present in hydrolysates obtained by treating softwood kraft pulps with an endo-1,4-β-xylanase (EC 3.2.1.8) (Fig. 1C). Xylanases are highly specific to xylan; in consequence the unknown residues must be connected to xylan. It also proves that the residues are present both in dissolved and undissolved kraft xylans. HPLC analysis of the enzymatic hydrolysate indicated several acidic oligosaccharides in addition to neutral monosaccharides and oligosaccharides (Fig. 2). For a more precise structural analysis, the acidic components of the enzymatic hydrolysate were separated from the neutral components, and the former were fractioned further by size-exclusion chromatography. The acidic oligosaccharide fraction exhibited an absorption maximum at 230 nm which is characteristic for α,β-unsaturated uronic acids [5].

Table 1
Relative amounts [a] of sugar residues for hemicellulose samples from pulp (hydrolysate and acidic oligosaccharide fraction) and pulping liquor (xylan I and II)

Sample	DP	Carbohydrate content (mol/mol%)					
		Method	HexA	MeGlcA	Ara	Xyl	Other
Xylan I [b]	[c]	^{1}H NMR	1.1	9.5	10.0	65.2	14.2
Xylan II [b]	[c]	^{1}H NMR	5.9	4.4	10.1	70.6	9.0
Hydrolysate	2.8	^{1}H NMR	4.3	0.8	7.2	87.7	0
Acidic olig. [d]	9.4	^{1}H NMR	14.2	2.1	9.6	74.1	0
Acidic olig. [d]	9.0	^{13}C NMR	14.5	1.7	9.2	74.7	0

[a] Determined by integration of anomeric protons in 1D spectra.
[b] I and II are xylans isolated from pulping liquor taken at the beginning of heating-up period (I) and at the end of the heating-up period (II).
[c] Too large to be determined by the method used.
[d] Acidic olig. = acidic oligosaccharide fraction.

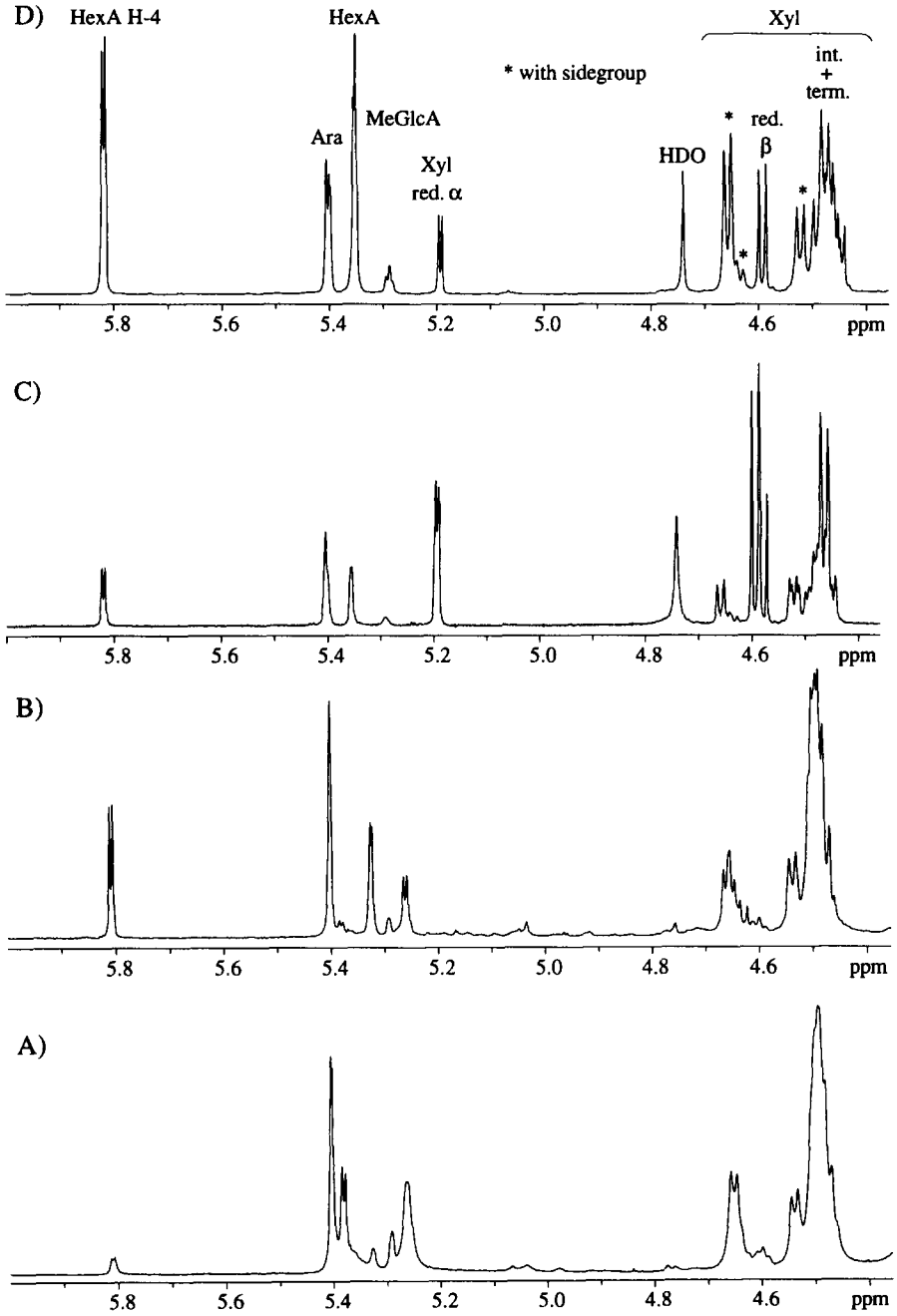


Fig. 1. (A) and (B) Proton NMR spectra at 80°C of pine xylan isolated from pulp liquor taken at beginning (A) and end (B) of the heating-up period. (C) and (D) Proton NMR spectra at 27°C of xylanase hydrolysate of pine pulp. (C) The hydrolysate before fractionation and (D) the acidic oligosaccharide fraction.

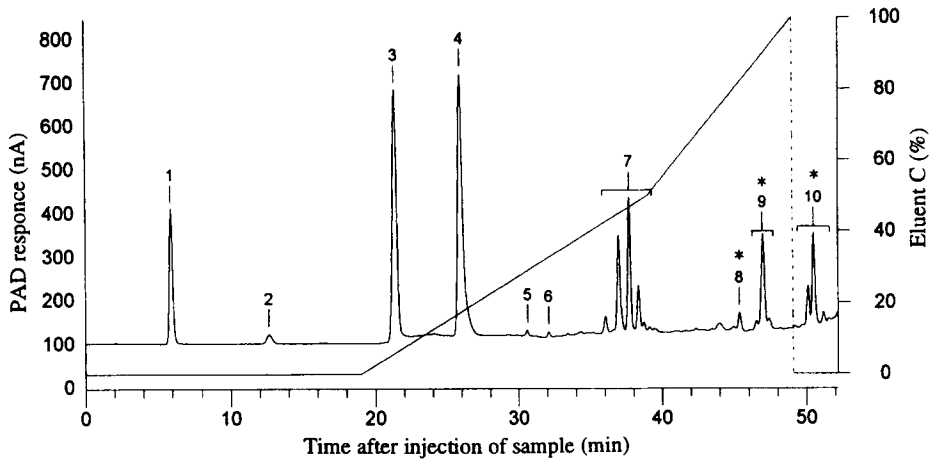


Fig. 2. Separation by HPLC of monosaccharides and oligosaccharides liberated from pine kraft pulp after a treatment with xylanase. The * indicates peaks found for the acidic oligosaccharide fraction. Peaks: 1 = fucose (internal standard); 2 = arabinose; 3 = xylose; 4 = xylobiose; 5 = xylotriose; 6 = xylotetraose; 7 = xylo-oligosaccharides substituted by arabinose; 8 = unknown; 9 = xylo-oligosaccharides substituted by hexenuronic acid; 10 = xylo-oligosaccharides substituted by two hexenuronic acids.

HPLC analysis showed the acidic oligosaccharide fraction to consist of several components, some of which contained at least two acidic residues (Fig. 2). As judged from the anomeric region of the proton NMR spectrum (Fig. 1D), the acidic oligosaccharide fraction contained at least 13 different residues. The average degree of polymerisation (DP) was ~ 9 as determined from the integral for reducing end proton resonances relative to that of all anomeric proton resonances. It was not possible to obtain complete structures for the various oligosaccharides in the acidic fraction, but it allowed characterisation of the acidic residues.

pH-effects.—Due to the pH dependence of one of the unknown resonances, ^{1}H NMR spectra of hydrolysates with the acidic oligosaccharides were recorded at several pH values. Non-linear regression fits gave pK_a values of 3.03 and 3.14 for the unknown residue (Fig. 3A) and the 4-*O*-methylglucuronic acid residue (Fig. 3B), respectively. Kohn and Kovác [9] have reported similar dissociation constants for D-glucuronic acid (pK_a 3.28), methyl 2,3,4-tri-*O*-methyl-α-D-glucopyranosiduronic acid (pK_a 3.03) and methyl 4-deoxy-β-L-*threo*-hex-4-enopyranosiduronic acid (pK_a 3.10). According to Laine et al. [10] the uronic acid component in pine kraft pulp has a pK_a of 3.4.

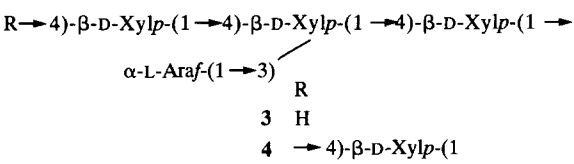
NMR chemical shifts of the acidic sugar units.—Using COSY, R-COSY and TOCSY experiments, it was possible to identify the 5.35 and 5.82 ppm resonances as belonging to a four proton spin system, 5.35, 3.79, 4.31 and 5.82 ppm (Table 2) where $^{3}J_{H1,H2} = 2.0$, $^{3}J_{H2,H3} = 6.2$ and $^{3}J_{H3,H4} = 3.4$ Hz. The ^{13}C chemical shifts of the four carbon atoms (99.01, 70.83, 66.74 and 107.85 ppm, Table 3) to which the above mentioned four protons are bound were identified from an HMQC spectrum. The two non-protonated carbon atoms (146.29 and 170.08 ppm, Table 4) were identified from HMBC 2D spectra. These ^{1}H and ^{13}C chemical shifts and $^{3}J_{H,H}$ values are consistent

with 4-deoxy-β-L-*threo*-hex-4-enopyranosyluronic acid (Fig. 4A), hereafter referred to as hexenuronic acid or HexA, a β-elimination product of the 4-*O*-methyl-α-D-gluco-pyranosyluronic acid residue. The chemical shifts reported in the present work differ somewhat from earlier reported values for hexenuronic acid [4,8,11]. This discrepancy is most likely due to different pH-values used and differences in the hexenuronic acid ring substituents. We assume that they performed their experiments under such conditions that the carboxylic group was protonated.

Starting from the anomeric proton at 5.28 ppm, the five protons in MeGlcA were identifyed using COSY, R-COSY and TOCSY experiments (Table 2). It was not possible to assign the ^{13}C resonances of MeGlcA completely, due to its low abundance. The ^{1}H and ^{13}C chemical shifts are in good agreement with earlier reported values for 4-*O*-methylglucuronoxylo-oligosaccharides [12,13].

NMR chemical shifts of the neutral sugar units.—The remaining NMR signals can clearly be divided into resonances from xyloses and arabinoses (Table 2 and 3). The proton NMR resonances were assigned starting from the anomeric protons using COSY, R-COSY and TOCSY experiments. Stereospecific assignment of the H-5_{proR} and H-5_{proS} signals in arabinose was based on relative chemical shifts (δ $5_{proR} > \delta$ 5_{proS}) and supported by the observed coupling constants ($^{3}J_{4,5proR} < {}^{3}J_{4,5proS}$) [14]. The ^{13}C NMR resonances were assigned from the proton NMR resonances by an HMQC experiment.

The chemical shifts of Ara*f* attached α-(1 → 3) to internal xylose (**3,4**) agree with earlier proton shift data for oligosaccharides from wheat endosperm arabinoxylans [15,16] and with ^{13}C chemical shifts for arabinose-containing xylo-oligosaccharides [17,18]. The average amount of arabinose is somewhat less than one per molecule (DP ~ 9). For arabinose, two different environments could be observed in about equal abundance. In the proton spectra, the two environments resulted in resolved signals only for the anomeric proton, whereas all but the C-5 carbon gave rise to resolved signals in ^{13}C spectra (Fig. 1D and 5). The resolution of the 2D spectra was not high enough to allow assignment of the different arabinose residues. However, a comparison of the chemical shifts with published data for arabinoxylans [19] indicated that the arabinose resonance at 5.400 ppm belongs to **3**, whereas the arabinose resonance at 5.395 ppm belongs to **4**.



Scheme 2.

For the xylose residues, at least ten different chemical environments were observed (see Table 2 and Fig. 6). The anomeric proton and carbon signals could be divided into four types of resonances: (i) reducing end with an equilibrium distribution between α-

and β-conformation; (ii) internal without sidegroup; (iii) internal with sidegroup; and (iv) terminal (non-reducing end). The proton signals of the terminal xylose residues were at lower frequency than the corresponding signals from the internal xylose residues, except for the two resonances at 4.46 and 4.445 ppm (Table 2). However, the chemical shift of H-4 gives the most accurate indication of whether the xylose residue is terminal (3.59–3.62 ppm) or internal (3.74–3.88 ppm) [15,16]. The introduction of a sidegroup shifts the ^{13}C resonance 3.5–5.2 ppm to higher frequency, in agreement with expected substituent α-effects [20]. Our chemical shifts for reducing end, internal without sidegroup and terminal xyloses agree with earlier published values [15–17,19].

Sequential assignments.—In Table 4 we have summarised the information from the NOESY and HMBC spectra about connections between the sugar rings. These data confirm that the HexA ring is attached to an internal xylose with a β-(1 → 2) linkage (**2** and Fig. 4). Moreover, substitution of HexA on C-2 of β-Xyl*p* shifts the C-2 signal 5.2 ppm downfield. Such a downfield shift is characteristically experienced on glycosylation by both the carbon in the aglycon and the anomeric carbon in the glycosyl residue [20,21]. The HexA anomeric carbon ^{13}C signal is split into three resonances, 98.96, 99.01 and 99.06 ppm (Fig. 5), which reflects three slightly different surroundings. Two plausible different environments of HexA are (i) HexA attached to an internal xylose adjacent to the terminal end and (ii) HexA attached to an internal xylose further removed from the terminal end. The average amount of HexA is almost 1.5 per molecule (DP ~ 9) indicating that the third environment can be caused by the presence of two HexA residues relatively close to each other in the same molecule. Another possibility for the third environment is that HexA is relatively close to α-Ara*f*.

For HexA three connectivities are observed between HexA H-1 and internal β-Xyl*p* (HexA) H-1, H-2 and H-3 (Fig. 7 and Table 4). The NOESY crosspeak between HexA H-1 and internal β Xyl*p* (HexA) H-2 is strong, and shows that these protons are close in space (< 3.0 Å). The crosspeaks with internal β-Xyl*p* (HexA) H-1 and with H-3 are weak and may be due to spin-diffusion. The NOESY connectivities between internal β-Xyl*p* (HexA) H-1 and internal β-Xyl*p* H-5_{eq}, and internal β-Xyl*p* (HexA) H-1 and internal β-Xyl*p* H-4, were both strong, whereas the crosspeak between internal β-Xyl*p* (HexA) H-1 and internal β-Xyl*p* H-5_{ax} was of medium strength (< 3.5 Å between protons). Our NOESY data agree with the left-handed three-fold helix observed for xylo-oligosaccharides in solution [15]. Thus, the HexA attached as a β-(1 → 2) sidegroup does not substantially affect the conformation of the xylan main chain.

In order to illustrate the conformation of HexA relative to the main xylan chain, a model of a HexA attached to the internal xylose of xylotriose was built and its energy minimized with the computer program InsightII (Biosym Technologies Inc.) The observed NOE distance restraints are fulfilled in the obtained structure (Fig. 4B), which, however, is only one possible conformation that satisfies the NMR data. The ring conformation of HexA in the model is $^{2}H_{1}$ [11] which according to a comparison of observed and calculated [22] $^{1}H-^{1}H$ spin–spin coupling constants is somewhat preferred over $^{1}H_{2}$, probably due to the anomeric effect.

NOESY and HMBC experiments confirmed that the arabinose units are attached via an α-(1 → 3) linkage to an internal xylose. There is no indication of doubly substituted xyloses. No sidegroup attached to a terminal or to a reducing end xylose residue was

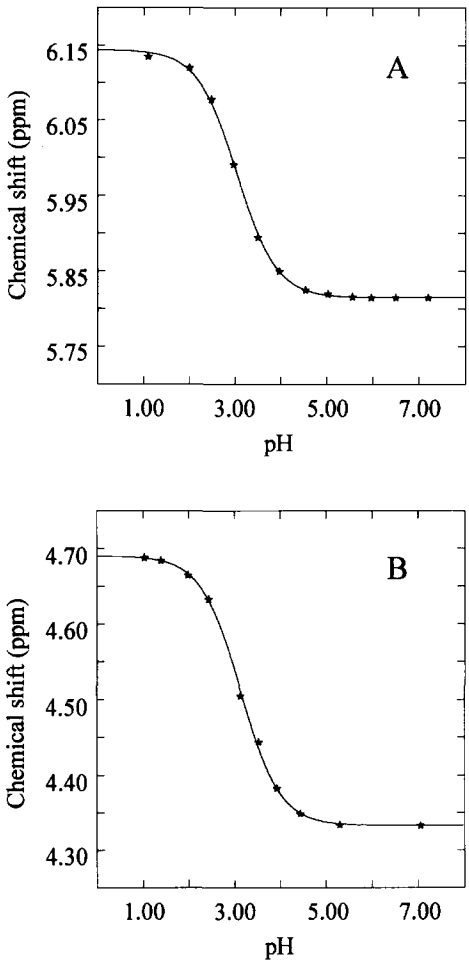


Fig. 3. pH dependence of chemical shifts of protons adjacent to the carboxylic acids. The lines are non-linear-regression fits to the experimental points. (A) H-4 of hexenuronic acid and (B) H-5 of 4-*O*-methyl-glucuronic acid.

found, either in the hydrolysate before purification or in the acidic oligosaccharide fraction. This result is in agreement with the previously reported cleavage specificity for *T. reesei* xylanase pI 5.5, which is able to cleave only β-(1 $\rightarrow$ 4) linkages between two unsubstituted xylose units [23].

3. Conclusions

HexA bound to polymeric xylan isolated from kraft pulp liquor has been identified by ^{1}H NMR. The combined use of a specific xylanase to dissolve unmodified xylo-oligo-

saccharides from the kraft pulp and 2D NMR spectroscopy has made it possible to characterise HexA attached to xylan. The characterisation was possible even for a mixture of acidic oligosaccharides.

The HexA attached as a β-(1 → 2) side-group protrudes from the main xylan chain. For MeGlcA attached in the same way, α-(1 → 2), a NOESY crosspeak between MeGlcA H-1 and β-Xyl*p* H-2 has been observed (ref. [12,13] and A. Teleman unpublished results), which indicates that MeGlcA and HexA are oriented in a similar way relative to the xylan main chain.

The xylan MeGlcA side group is degraded under alkaline conditions via β-elimination to HexA, as postulated earlier [4]. The quantitative analysis of HexA and MeGlcA during kraft pulping will be reported elsewhere.

Table 2
^{1}H NMR data for the acidic oligosaccharide fraction dissolved in D_2O at pD 7.0

Structural element[a]	Residue[b]	Chemical shift (ppm)[c]						
		H-1	H-2	H-3	H-4	H-5ax/H-5proS	H-5eq/H-5proR	OCH_3-4
(3)	α-Ara*f*-L^3	5.400	4.16	3.91	4.27	3.72	3.80	-
(4)	α-Ara*f*-L^3	5.395	4.16	3.91	4.27	3.72	3.80	-
(2)	β-HexA-L^2	5.35	3.79	4.31	5.82	-[d]	-	-
(1)	α-MeGlcA-L^2	5.28	3.58	3.76	3.22	4.34	-	3.47
	α-Xyl*p*-red.	5.19	3.55	3.76	3.74[e]	3.75[e]	3.83[e]	-
(2)	β-Xyl*p*-int.(HexA)	4.65	3.68	3.61	3.84	3.40	4.12	-
(1)	β-Xyl*p*-int.(MeGlcA)	4.62	n.d.[f]	n.d.	3.86	3.44	4.18	-
	β-Xyl*p*-red.	4.58	3.26	3.55	3.78	3.38	4.06	-
(3,4)	β-Xyl*p*-int.(Ara)	4.52	3.44	3.75	3.84	3.40	4.12	-
	β-Xyl*p*-int.	4.48	3.29	3.55	3.78	3.37	4.10	-
	β-Xyl*p*-int.	4.47	3.29	3.55	3.81	3.42	4.16	-
	β-Xyl*p*-term.	4.46	3.26	3.43	3.62	3.30	3.97	-
	β-Xyl*p*-int.	4.445	n.d.	n.d.	3.76	3.34	4.05	-
	β-Xyl*p*-term	4.438	3.25	3.42	3.60	3.28	3.92	-

[a] Structural elements are represented by short-hand notation: ○,● Xyl; △,▲ α-Ara; □,■ β-HexA; ◇,◆ α-MeGlcA; – linkage. The filled symbols represents the residue for which the chemical shifts are given on the actual line.

[b] α-Ara*f*-L^3 means arabinofuranose attached with an α-linkage to O-3 of a xylose residue; β-HexA-L^2 means hexenuronic acid attached with an β-linkage to O-2 of a xylose residue; α-MeGlcA-L^2 means 4-*O*-methylglucuronic acid attached with an α-linkage to O-2 of a xylose residue; red., int. and term. mean reducing end, internal and terminal end (non-reducing end), respectively; for the xylose residues (HexA), (MeGlcA) or (Ara) mean that hexenuronic acid, 4-*O*-methylglucuronic acid or arabinose, respectively, is attached as sidegroup to the xylose residue.

[c] Measured at 600 MHz on solutions in D_2O at 27°C and pD 7.0 relative to internal TSPA-d_4.

[d] – = not relevant.

[e] Assignment might have to be interchanged. [f] n.d. = not determined.

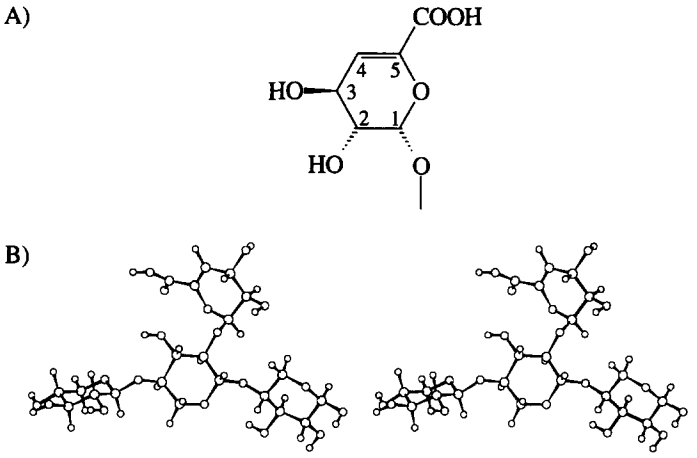


Fig. 4. (A) Conventional structure of 4-deoxy-β-L-*threo*-hex-4-enopyranosyluronic acid and (B) stereo structure of HexA attached to internal xylose of xylotriose.

Table 3
^{13}C NMR data for the acidic oligosaccharide fraction dissolved in D_2O at pD 7.0

Structural element[a]		Residue[b]	Chemical shift (ppm)[c]						
			C-1	C-2	C-3	C-4	C-5	C-6	OCH_3-4
▲	(3,4)	α-Ara*f*-L[3]	108.48 108.43	81.55 81.52	78.07 78.04	85.61 85.58	62.15	-[d] -	- -
■	(2)	β-HexA-L[2]	99.06 99.01 98.96	70.83	66.74	107.85	146.29	170.08	- - -
◆	(1)	α-MeGlcA-L[2]	98.35	n.d.[e]	n.d.	n.d.	n.d.	177.63	60.67
		α-Xyl*p*-red.	92.85	72.20	71.78	77.2	63.7	-	-
	(2)	β-Xyl*p*-int.(HexA)	102.08 102.13 102.16	78.95 78.84 78.7	72.99 72.96 72.92	77.2	63.7	- - -	- - -
◇	(1)	β-Xyl*p*-int.(MeGlcA)	102.2	n.d.	n.d.	77.2	63.7	-	-
		β-Xyl*p*-red.	97.34	74.8	74.6	77.2	63.7	-	-
△	(3,4)	β-Xyl*p*-int.(Ara)	102.6	74.11	78.12 78.07	77.2	63.7	- -	- -
		β-Xyl*p*-int	102.6	73.6	74.6	77.2	63.7	-	-
		β-Xyl*p*-term.	102.28	73.6	76.44	70.02	66.04 65.93	- -	- -

[a] Structural elements are represented by short-hand notation: ○,● Xyl; △,▲ α-Ara; □,■ β-HexA; ◇,◆ α-MeGlcA; – linkage. The filled symbols represents the residue for which the chemical shifts are given on the actual line.
[b] See Table 2 for the key.
[c] Measured at 150.9 MHz on solutions in D_2O at 27°C and pD 7.0 relative to internal dioxan (67.4 ppm).
[d] – = not relevant.
[e] n.d. = not determined.

Table 4
Summary of observed NMR connectivities at the H-1 frequency for all residues and H-4 frequency for hexenuronic acid in acidic oligosaccharide fraction observed with NOESY and HMBC methods

Proton [a]	Structural element	HMBC [b]	NOESY [b]
HexA H-1	(2)	C-2,3,5; β-Xyl*p*-int. (HexA) C-2	H-2(s[c]), 3(w); β-Xyl*p*-int. (HexA) H-1 (w), 2(s), 3(w)
HexA H-4	(2)	C-2,3,5,6	H-2(w), 3(m)
MeGlcA H-1	(1)	n.d. [d]	H-2(s); β-Xyl*p*-int. (MeGlcA) H-2(s)
Ara H-1	(3,4)	C-3 [e],4; β-Xyl*p*-int. (Ara) C-3 [e]	H-2(m), 3(w), 4(w); β-Xyl*p*-int. (Ara) H-2(w), (3s)
α-Xyl*p*-red. H-1		C-3	H-2(m), 3(w)
β-Xyl*p*-int. (HexA) H-1	(2)	C-3,5; β-Xyl*p*-int. C-4	H-2(m), 3(s), 4(m), 5_{ax}(s), 5_{eq}(m); β-Xyl*p*-int. H-4(s), 5_{ax}(m), 5_{eq}(s)
β-Xyl*p*-int. (MeGlcA) H-1	(1)	n.d.	n.d. [f]
β-Xyl*p*-red. H-1		C-3,5	H-2(w), 3(m), 5_{ax}(s)
β-Xyl*p*-int. (Ara) H-1	(3,4)	C-3,5; β-Xyl*p*-int. C-4	H-2(m), 3(s), 5_{ax}(s); β-Xyl*p*-int. H-4(s), 5_{eq}(s)
β-Xyl*p*-int. H-1		C-3,5; β-Xyl*p*-int. C-4	H-2(m), 3(s), 5_{ax}(s); β-Xyl*p*-int. H-4(s), 5_{eq}(s)
β-Xyl*p*-int. H-1	C-3,5; β-Xyl*p*-int. C-4	H-2(m), 3(s), 5_{ax}(s); β-Xyl*p*-int. H-4(s), 5_{eq}(s)	
β-Xyl*p*-term. H-1	β-Xyl*p*-int. C-4	H-2(m), 3(s), 5_{ax}(s); β-Xyl*p*-int. H-4(s), 5_{eq}(s)	
β-Xyl*p*-int. H-1	C-3,5; β-Xyl*p*-int. C-4	H-2(m), 3(s), 5_{ax}(s); β-Xyl*p*-int. H-4(s), 5_{eq}(s)	
β-Xyl*p*-term. H-1	β-Xyl*p*-int. C-4	H-2(m), 3(s), 5_{ax}(s); β-Xyl*p*-int. H-4(s), 5_{eq}(s)	

[a] See Table 2 for key.
[b] The connectivities within the ring are specified first and thereafter the connectivities with the next ring.
[c] Relative intensity of crosspeaks in NOESY spectra were determined by integration. The integrated volume has been divided by the relative abundance of the residue. w, weak; m, medium; s, strong.
[d] n.d. = not determined.
[e] Overlapping ^{13}C resonances.
[f] The weak resonances could not be separated from resonances of β-Xyl*p*-int. (HexA) H-1.

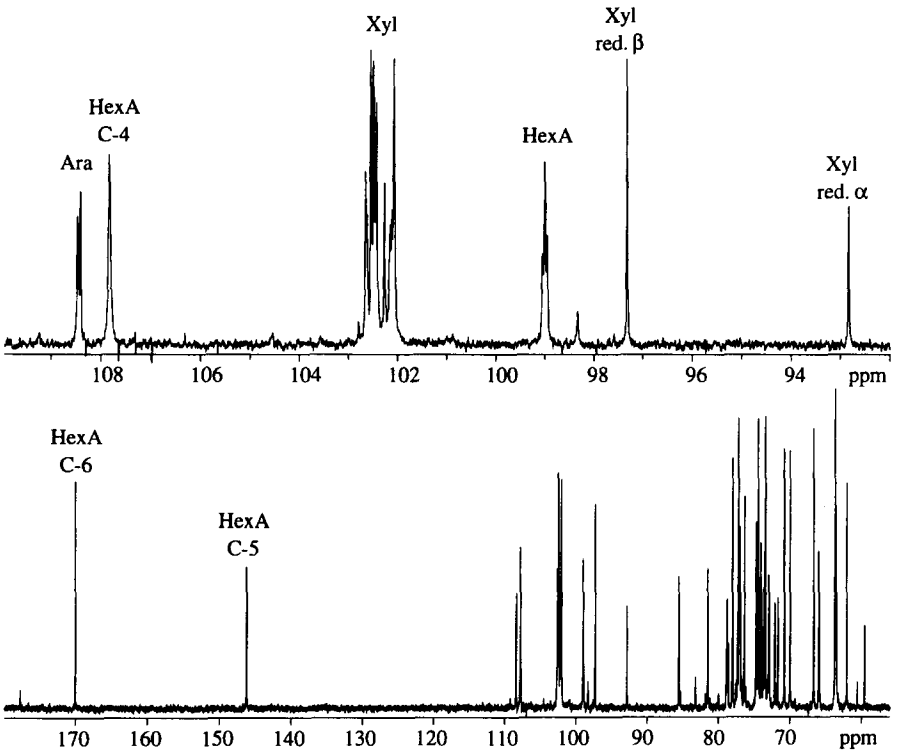


Fig. 5. ^{13}C NMR spectrum of the acidic oligosaccharide fraction.

4. Experimental

Materials.—The polymeric xylan (hemicellulose) fractions were isolated from a kraft cooking liquor of pine wood at the beginning (xylan I) and at the end (xylan II) of the heating-up period. To the cooking liquor (500 mL) were slowly added 1000 mL of 1,4-dioxane and 120 mL of glacial acetic acid under mixing. The solution was covered and allowed to stand for two days at room temperature. The clear solution was decanted and the precipitate was transferred into a centrifuge tube (40 mL). After centrifugation (15 min at 3000 rpm) the precipitate was washed in the tube successively with 20 mL portions of 1,4-dioxane-water (2:1), 1,4-dioxane, methanol and acetone. The precipitate was dried in air and analysed for sugar components by methanolysis and GLC [24]. The lignin content was determined from the specific absorption at 280 nm ($a = 28.61$ cm/g) [25].

The birch and pine kraft pulps with kappa numbers of 19.9 and 25.8, respectively, were produced on a laboratory scale. The cooking conditions for the birch kraft pulp were: temperature 165°C, effective alkali 4.5 mol/kg, sulphidity 30%; for pine kraft pulp: temperature 170°C, effective alkali 4.5 mol/kg, sulphidity 35%. The kappa

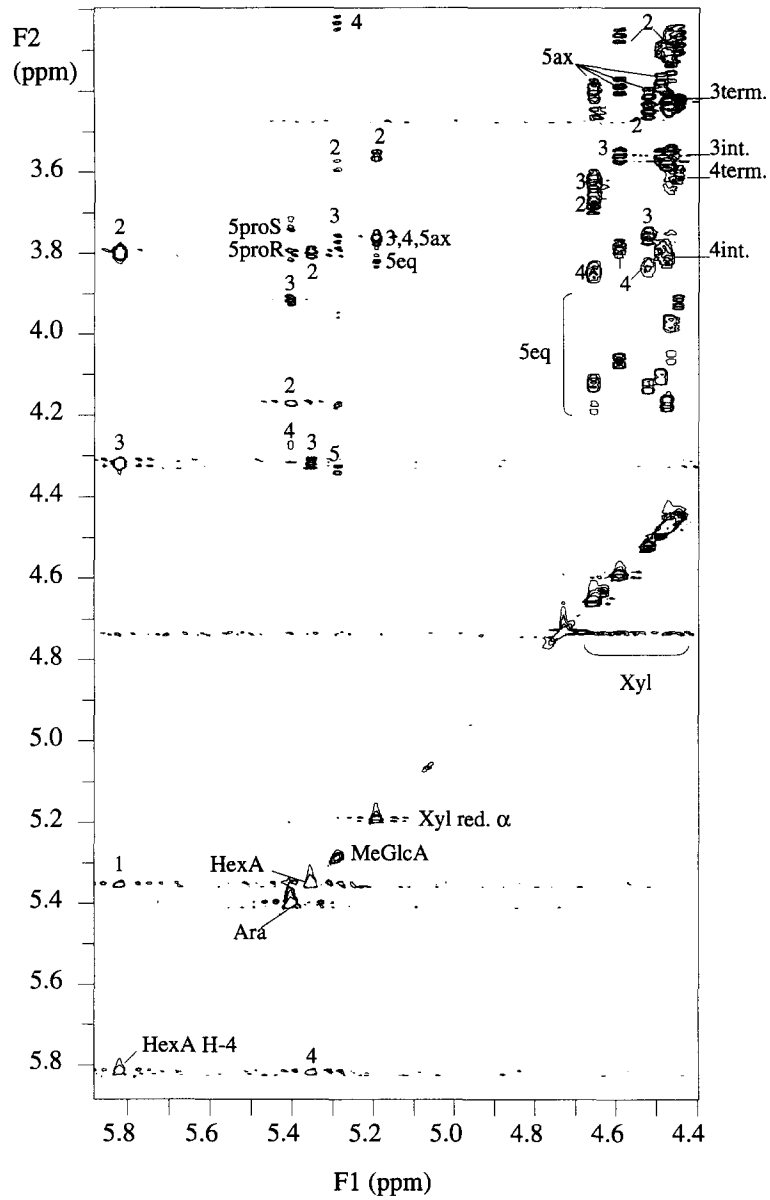


Fig. 6. TOCSY spectrum of acidic oligosaccharide fraction. Diagonal peaks of the anomeric protons and H-4 of hexenuronic acid are indicated. The numbers near cross-peaks refer to the protons of the scalar-coupling network belonging to a diagonal peak.

numbers of the pulps were determined according to SCAN C1:1977. The carbohydrate composition of the birch kraft pulp (% of dry weight) was: 72.4% glucose and 23.9% xylose; of pine kraft pulp: 81.2% glucose, 8.2% xylose, 5.9% mannose and 1.2%

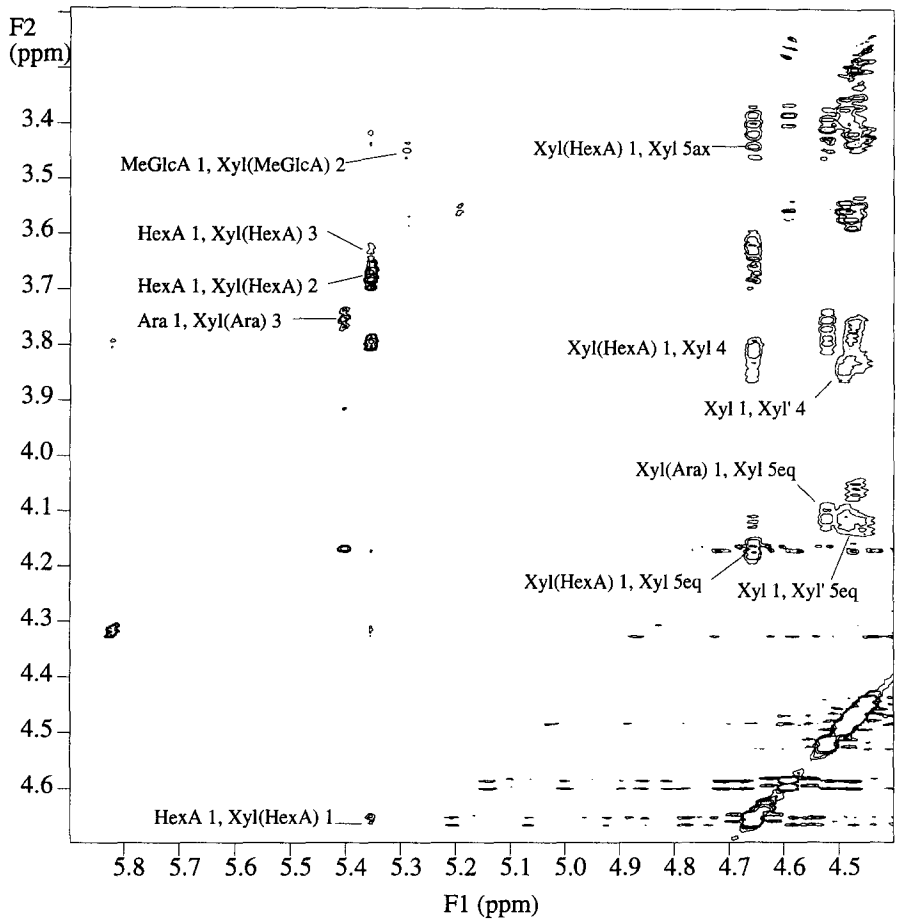


Fig. 7. NOESY spectrum of acidic oligosaccharide fraction. Only the inter-residue NOE connectivities along the H-1 frequencies are denoted.

arabinose as determined by the method of Puls et al. [26]. The amount of carboxylic acid groups was not determined. 4-*O*-Methylglucuronoxylan from birch wood was purchased from Roth (Karlsruhe, Germany). The two specific endo-β-(1 → 4) xylanases of *T. reesei* Rut C 30, with pI 5.5 and 9.0, were purified by ion-exchange chromatography as described by Tenkanen et al. [27].

HPLC analysis.—The HPLC system was a DIONEX 4500i series Chromatograph equipped with a pulsed amperometric detector (PAD-2). A CarboPac PA1 anion-exchange column (250 mm × 4 mm I.D.) was used. The eluents were: deionized water (A), 100 mM NaOH (B), 300 mM CH_3COONa-100 mM NaOH (C) and 300 mM NaOH (D). The eluent flow rate was 1.0 mL/min. The gradient program was as follows:

Time (min)	A (%)	B (%)	C (%)	D (%)	
0.0	95	5	0	0	Equilibration
15.0	95	5	0	0	Injection of sample
27.0	95	5	0	0	Start of gradient ramp 1
34.0	0	100	0	0	Start of gradient ramp 2
54.0	0	50	50	0	Start of gradient ramp 3
64.0	0	0	100	0	
64.1–67.1	0	0	0	100	Column wash, end of analysis

The detection was performed by PAD with a gold working electrode and triple-pulse amperometry with the following pulse potentials and duration's: $E_1 = 0.05$ V ($t_1 = 420$ ms), $E_2 = 0.80$ V ($t_2 = 180$ ms) and $E_3 = -0.15$ V ($t_3 = 360$ ms). A solution of 300 mM NaOH was added to the column effluent before the PAD cell at a flow rate of 0.8 mL/min.

Determination of the pK_a *values.*—The pK_a values of hexenuronic and 4-*O*-methylglucuronic acid groups were determined from oligomers obtained by treatment of birch kraft pulp and isolated birch xylan, respectively, with *T. reesei* xylanase pI 9.0 (10000 nkat/g d.w.) for 24 h at 40°C and pH 5. The hydrolysis of pulp (5% consistency) was performed in water and the pH was adjusted prior to hydrolysis with 0.5 M H_2SO_4 to pH 5. The isolated xylan was hydrolysed (1% consistency) in 50 mM sodium acetate buffer, pH 5. The pH (not corrected for isotopic effects) of the oligomer mixtures was adjusted by additions of NaOD or DCl.

Preparation of acidic oligosaccharide fraction.—Pine kraft pulp with kappa number 25.8 (37.5 g d.w., 5% consistency) was first acidified to pH 2.5 with 0.5 M H_2SO_4. After 1 h incubation at room temperature, the pulp was washed with distilled water four times and the pH was adjusted to pH 4 with 0.1 M $Ca(OH)_2$. The accessible xylan in the pulp was hydrolysed with *T. reesei* xylanase pI 5.5 (10000 nkat/g d.w.) for 24 h at 45°C. Unbuffered hydrolysate (1000 mL) containing ~1 g of neutral and acidic oligosaccharides was eluted through a column (1 × 10 cm) of Dowex 1 × 1 resin in the formate form. The neutral components were washed out with water (200 mL). The acidic oligosaccharides were eluted from the column with 50 mL of 1 M formic acid. The eluate was neutralised with sodium hydrogen carbonate (pH 7) and concentrated to a volume of ~10 mL. After centrifugation, the clear solution was eluted with water through a column (2.6 × 95 cm) of Sephadex G-10 gel at a rate of 1 mL/min. The absorbance of the eluate was followed at 254 and 280 nm. Fractions of 13.5 mL were collected and analysed for total sugar content with the phenol–sulphuric acid test. The content of hexenuronic acid groups in the fractions was evaluated from a strong ($\varepsilon = 5910$ M^{-1} cm^{-1}) specific absorption at 230 nm [5]. Fractions #13 and 14 were pooled, freeze-dried and analysed by NMR.

NMR spectroscopy.—Samples were lyophilised and redissolved in ^2H_2O (99.8 atom%, Fluka). For the acidic oligosaccharide fraction, 50 mg was dissolved in 0.71 mL ^2H_2O. pD was adjusted to 7.0 by additions of 0.2 M NaOD. ^1H and ^{13}C NMR spectra were obtained at 599.94 and 150.97 MHz, respectively, on a Varian UNITY 600 MHz spectrometer using 5 mm NMR tubes containing 0.7 mL of solution. Typical acquisition parameters were for 1D ^1H NMR (1D ^{13}C NMR) a 90° pulse of 13 μs (7 μs), a spectral width of 8000 Hz (40000 Hz) and a repetition time of 11 s for the polymers, 16 s for the

acidic oligosaccharide fraction (12 s) and 31 s for the hydrolysates. Spectra were obtained at 27°C for the acidic oligosaccharide fraction and 80°C for the polymers. The chemical shifts are reported relative to internal sodium 3-(trimethylsilyl)-3,3,2,2-tetradeuteropropionate at 0 ppm and dioxan at 67.40 ppm for ^{1}H and ^{13}C NMR spectra, respectively. The residual HDO peak was saturated continuously prior to acquisition.

Standard pulse sequences and phase cycling were utilised to obtain double quantum filtered COSY [28], R-COSY [29] ($\tau = 70$ ms) and NOESY [30] ($\tau_m = 0.5$ s) 2D spectra. The TOCSY [31,32] spectrum ($\tau_{mix} = 0.2$ s) was acquired with a sensitivity enhanced pulse sequence [33]. For all spectra hyper complex acquisition was used. A spectral width of 3500 Hz was employed in both dimensions and the relaxation delay was 2 s. For each FID 8 (16 for TOCSY) transients were acquired, the data size was 512 (1024 for COSY) in $t_1 \times 4096$ in t_2. The carrier was set on the solvent resonance and this resonance was reduced by presaturation. The phase-sensitive ^{1}H-detected HMQC [34] spectrum was acquired over a t_1 spectral window of 12000 Hz and a t_2 window of 3500 Hz with a 423×4096 matrix (zero-filled to 1024 in t_1) and 24 scans per increment. The delay between scans was 2.4 s and the delay for polarisation transfer was set for $^{1}J_{CH} = 167$ Hz. The multiple-bond ^{1}H-^{13}C shift correlation [35] (HMBC) spectra resulted from 560×4096 data matrix sizes, with 32 scans per t_1 value and a delay time between scans of 2.4 s. The spectral window was 21000 Hz for t_1 and 3500 for t_2. Two experiments were performed, one with Δ_2 50 ms and the other with Δ_2 100 ms. The raw data were processed on a SUN Sparc II using Varian Unity software. The apodization function, used for double quantum filtered COSY and relay COSY, was an unshifted sinebell in both t_1 and t_2. For TOCSY and NOESY the data sets were multiplied by shifted sine squared-bell window functions with phase shifts of 75° and 60° for t_1 and t_2, respectively. Zero-filling provided final 2D spectra of 2048×4096 data points. The HMQC and HMBC spectra were processed with a 90° phase-shifted sine squared-bell window function in both dimensions.

Acknowledgements

The authors thank Janne Laine for his help in calculations of pK_a values. This work was financed by TEKES (Technology Development Centre, Finland).

References

[1] K. Shimizu, in D.N.-S. Hon and N. Shiraishi (Eds.), *Wood and Cellulosic Chemistry*, Marcel Dekker, New York, 1991, pp 177–214.
[2] E. Sjöström, *Wood Chemistry: Fundamentals and Applications*, Academic Press, New York, 1993, pp 140–161.
[3] D.W. Clayton, *Svensk. Papperstidn.*, 66 (1963) 115–124.
[4] M.H. Johansson and O. Samuelson, *Carbohydr. Res.*, 54 (1977) 295–299.
[5] J. Kiss, *Adv. Carbohydr. Chem. Biochem.*, 29 (1974) 229–303.
[6] K. Shimizu, *Mokuzai Gakkaishi*, 22 (1976) 51.
[7] K. Shimizu, *Carbohydr. Res.*, 92 (1981) 65–74.

[8] K. Shimizu, *Carbohydr. Res.*, 92 (1981) 219–224.
[9] R. Kohn and P. Kovác, *Chem. zvesti*, 32 (1978) 478–485.
[10] J. Laine, J. Buchert, L. Viikari, and P. Stenius, submitted.
[11] J. Alföldi, R. Palovčík, C. Peciar, J. Hirsch, and P. Kováč, *Carbohydr. Res.*, 44 (1975) 133–137.
[12] F. Cavagna, H. Deger, and J. Puls, *Carbohydr. Res.*, 129 (1984) 1–8.
[13] G. Excoffier, R. Nardin, and M.R. Vignon, *Carbohydr. Res.*, 149 (1986) 319–328.
[14] G.D. Wu, A.S. Serianni, and R. Baker, *J. Org. Chem.*, 48 (1983) 1750–1757.
[15] R.A. Hoffmann, B.R. Leeflang, M.M.J. de Barse, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 221 (1991) 63–81.
[16] R.A. Hoffmann, T. Geijtenbeek, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 223 (1992) 19–44.
[17] J. Schraml, J. Hirsch. E. Petráková, E. Krahé, and C. Bliefert, *Collect. Czech. Chem. Commun.*, 49 (1984) 1605–1616.
[18] G. Annison, M. Choct, and N.W. Cheetham, *Carbohydr. Polym.*, 19 (1992) 151–159.
[19] F.J.M. Kormelink, R.A. Hoffmann, H. Gruppen, A.G.J. Voragen, J.P. Kamerling, and J.F.G Vliegenthart, *Carbohydr. Res.*, 249 (1993) 369–382.
[20] P. Kovac, J. Hirsch, A.S. Shashkov, A.I. Usov, and S.V. Yarotsky, *Carbohydr. Res.*, 85 (1980) 177–185.
[21] P.-E. Jansson, L. Kenne, and G. Widmalm, *Carbohydr. Res.*, 188 (1989) 169–191.
[22] C.A.G. Haasnoot, F.A.A.M. de Leeuw, and C. Altona, *Tetrahedron*, 36 (1980) 2783–2792.
[23] P. Biely, M. Vrsanska, L. Kremnicky, M. Tenkanen, K. Poutanen, and M. Hayn, in P. Suominen and T. Reinikainen (Eds.), Trichoderma reesei *Cellulases and other Hydrolases*, Foundation for Biotechnical and Industrial Fermentation Research, Vol. 8, 1993, pp 125–135.
[24] Y. Huang, L. Indrarti, J. Azuma, and K. Okamura, *Mokuzai Gakkaishi*, 38 (1992) 1168–1171.
[25] T. Vuorinen, *J. Wood Chem. Technol.*, 13 (1993) 97–125.
[26] J. Puls, K. Poutanen, H.-U. Körner, and L. Viikari, *Appl. Microbiol. Biotechn.*, 22 (1985) 416–423.
[27] M. Tenkanen, J. Puls, and K. Poutanen, *Enzyme Microb. Technol.*, 14 (1992) 566–574.
[28] L. Braunschweiler, G. Bodenhausen, and R.R. Ernst, *Mol. Phys.*, 48 (1983) 535–560.
[29] G. Wagner, *J. Magn. Reson.*, 55 (1983) 151–156.
[30] A. Macura and R.R. Ernst, *Mol. Phys.*, 41 (1980) 95–117.
[31] L. Braunschweiler and R.R. Ernst, *J. Magn. Reson.*, 53 (1983) 521–528.
[32] A. Bax and D.G. Davis, *J. Magn. Reson.*, 65 (1985) 355–360.
[33] J. Cavanagh and M. Rance, *J. Magn. Reson.*, 88 (1990) 72–85.
[34] M.F. Summers, L.G. Marzilli, and A. Bax, *J. Am. Chem. Soc.*, 108 (1986) 4285–4294.
[35] A. Bax and M.F. Summers, *J. Am. Chem. Soc.*, 108 (1986) 2093–2094.