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Isolation and characterization of *O*-acetylated glucomannans from aspen and birch wood

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Abstract

O-Acetylated glucomannans were isolated from aspen and birch wood employing two different procedures and thereafter subjected to carbohydrate analysis by NMR spectroscopy and MALDI mass spectrometry. In one of the isolation procedures, acetone-extracted aspen or birch wood meal was extracted with dimethyl sulfoxide and then with hot water. Fractionation of the hemicellulose-containing extracts by size-exclusion chromatography was subsequently performed. In the other procedure, fractional precipitation with ethanol was used to isolate glucomannans from lyophilized process water produced by mechanical pulping of aspen. The aspen and birch glucomannans are *O*-acetylated at the C-2 or C-3 position of some of the mannose residues (random distribution), with a degree of acetylation of approx 0.3. In both cases the degree of polymerization was approx 16, indicating that low-molecular mass fractions of the glucomannans in hardwood have been isolated here.

Keywords: Hardwood; Glucomannan, structures; *O*-Acetylation; Hemicellulose, extraction; ^{1}H and ^{13}C NMR spectroscopy; MALDI-MS1

1. Introduction

Hemicelluloses, one of the major constituents of wood species, are present between the cellulose fibrils in the cell wall.[1,2] The glucomannans are linear polymers of (1→4)-linked β-D-mannopyranosyl and β-D-glucopyranosyl residues. Three to five percent of the dry weight of most hardwoods consists of glucomannans with a glucose-to-mannose ratio of 1:1–1:2.[1,3] Native (galacto)glucomannans from softwoods contain *O*-acetyl substituents at C-2 and C-3 of certain of the mannose residues, with a degree of acetylation (DS_{Ac}) varying between 0.17 and 0.36.[1,3]

In earlier investigations, we have detected an acetylated mannan as a minor constituent of a fraction isolated from aspen wood treated in a microwave oven.[4,5] Some water-soluble hemicelluloses are also released from wood into the process water during mechanical pulping, in connection with which the wood fibers are separated from one another by mechanical forces and the fiber wall is partially damaged.[6] The major polysaccharides solubilized from Norway spruce during thermomechanical pulping are partially acetylated galactoglucomannans.[7]

NMR spectroscopy is an important non-destructive tool for investigation of the fine structure of polysaccharides. Several different high-resolution NMR techniques can be used to examine the anomeric configuration and sequence of glycosyl residues.[8] Furthermore, in a number of studies the distribution of *O*-acetyl groups has been determined by direct NMR analysis of the polysaccharides of interest.[4,9–11]

In the present investigation glucomannans have been isolated from the two hardwood species aspen and birch. Two alternative procedures were employed to separate certain of the water-soluble glucomannans from the cell wall matrix, i.e., mechanical pulping or

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treatment with DMSO. The water-soluble polysaccharides were then further fractionated and the isolated glucomannans subsequently subjected to carbohydrate analysis, NMR spectroscopy and MALDI-MS.

2. Results and discussion

2.1. Isolation of aspen glucomannan from process water

Glucomannan was isolated by fractional precipitation with ethanol of the process water obtained from mechanical pulping of aspen. Fractional precipitation with ethanol has been used extensively for the purification of hemicelluloses.[12] Pure polysaccharides can frequently be obtained by gradual addition of ethanol to dilute aqueous solutions, provided that the difference in the solubilities of the hemicelluloses present in ethanol is sufficiently large. The other isolation procedure is shown in Fig. 1.

The polymers in the water-soluble fraction of the lyophilized process water contained relatively large amounts of glucose and mannose (Glc:Man:Xyl:Gal:Ara = 44:31:16:4:4 (mass %) determined by enz/HPAEC analysis). No mannose was detected in precipitate **1** (Glc:Man:Xyl:Gal:Ara = 40:0:39:16:5 (mass %)), while precipitate **2** (Glc:Man:Xyl:Gal:Ara = 21:47:16:10:6 (mass %)) contained relatively high amounts of mannose and glucose, as well as xylose, galactose and arabinose. Precipitate **3** (Glc:Man:Xyl:Gal:Ara = 27:59:9:2:2 (mass %)) was further enriched in mannose and glucose, at the expense of galactose and arabinose. In order to reduce the amount of xylose present, precipitate **3** was treated with enzymes that hydrolyze *O*-acetylated 4-*O*-methylglucuronoxylan. The monosaccharide content of the resulting precipitate **3ET** (yield, 29 mg) suggests that this preparation contains an almost pure glucomannan, with minor contamination by polysaccharides containing xylose, galactose and arabinose residues (Table 1).

2.2. Isolation of glucomannan from aspen

Glucomannan was isolated from an acetone extract of aspen wood meal by extraction with DMSO and then with hot water (Fig. 1). The extraction procedure employed was a modification of the one described earlier.[13] The extraction yield (calculated on the basis of the amount of mannose extracted and the total amount of mannose in the wood) was approx 4%, indicating that most of the glucomannan was not solubilized from the wood meal employing this procedure.

The carbohydrate compositions of the extracts thus obtained were determined by enzymatic hydrolysis and subsequent capillary zone electrophoresis (CZE).[14] The relative carbohydrate compositions of the polysaccha-

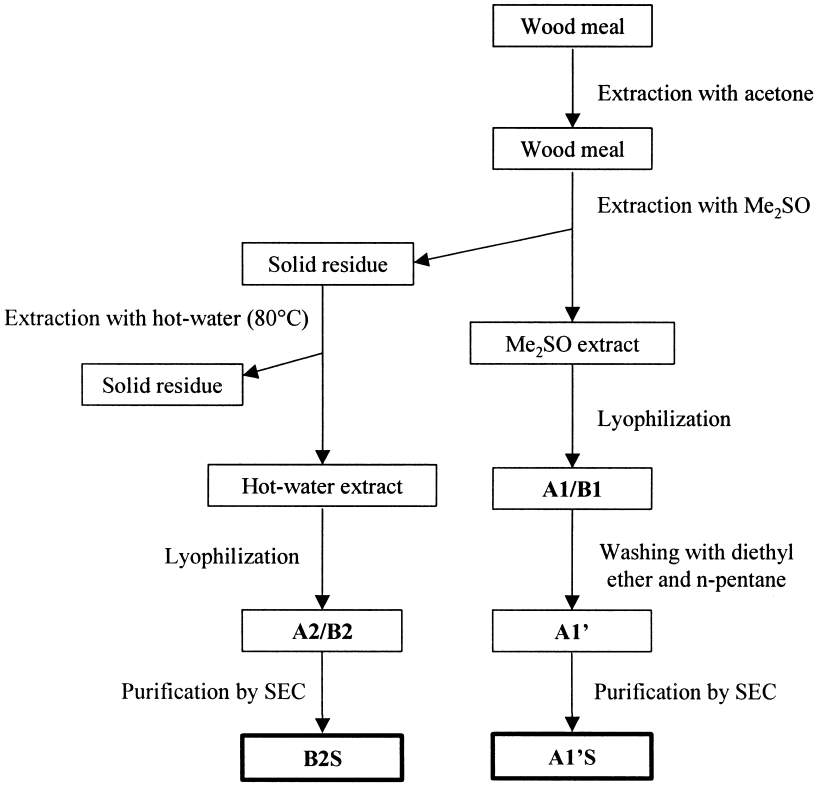

Fig. 1. Isolation of *O*-acetylated glucomannans from wood meal. **A** and **B** denote aspen and birch, respectively.

Table 1
Carbohydrate compositions, degrees of acetylation and degrees of polymerization for the glucomannans isolated from aspen and birch wood

Wood sample	Starting material	Sample	Analytical procedure	Relative carbohydrate content (% [a])					DS_{Ac} [b]	DP_p [c]
				Glc	Man	Xyl	Gal	Ara		
Aspen	Process water	**3ET**	enz/HPAEC	31	61	2	3	3	– [d]	–
			NMR [e]	35	65	n.d. [f]	n.d.	n.d.	0.3	16
			SEC/MALDI-MS	–	–	–	–	–	0.3	18
	DMSO extract	**A1′S**	TFA/CZE [g]	41	55	2	1	1	–	–
			SEC/MALDI-MS	–	–	–	–	–	0.4	17
Birch	Water extract	**B2S**	TFA/CZE [g]	28	68	1	1	2	–	–
			NMR [e]	32	68	n.d.	n.d.	n.d.	0.2	14
			SEC/MALDI-MS	–	–	–	–	–	0.3	16

[a] Mass %.
[b] DS_{Ac}, degree of substitution with acetyl groups.
[c] DP_p, peak-average degree of polymerization; defined as the number of hexoses in the backbone of the glucomannan.
[d] –, not applicable
[e] Determined by integration of quantitative 1D NMR spectra.
[f] n.d., not detectable
[g] The levels of 4-*O*-methylglucuronic acid and galacturonic acid were below the limits of detection.

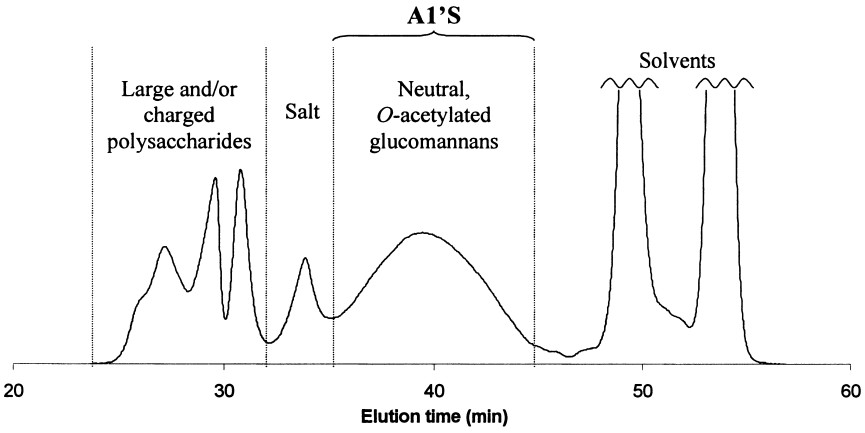

Fig. 2. SEC chromatogram of aspen extract **A1′**. Fraction **A1′S** was collected for further analysis.

rides in **A1′** and **A2** were Glc:Man:Xyl:Gal:Ara = 66:20:7:5:3 (mass %) and Glc:Man:Xyl:Gal:Ara:MeGlcA:GalA = 24:6:24:23:5:12:6 (mass %), respectively.

The DMSO extract (**A1′**) contained the major portion of the hemicelluloses extracted, as well as the highest relative amount of glucomannan and was therefore used for further purification. The hemicelluloses in extract **A1′** were fractionated by SEC employing elution with water (Figs. 1 and 2). Charged and neutral polysaccharides can be separated employing size-exclusion chromatography (SEC) with a mobile phase of low ionic strength.[5,15] Negatively charged polysaccharides, such as 4-*O*-methylglucuronoxylan pass rapidly through an aqueous SEC system, due to the repelling force exerted by the negatively charged packing material. In this manner charged polysaccharides are separated primarily on the basis of their charge, but also according to size, whereas neutral polysaccharides (e.g., *O*-acetylated glucomannans) are separated on the basis of their size alone. Fraction **A1′S** was identified on the basis of MALDI analysis, together with our previous experience of the separation of hemicelluloses by the SEC system used.[5]

A1′S was found to consist primarily of glucomannan (Table 1), with minor contamination by polysaccharides containing xylose, galactose and arabinose residues.

2.3. Primary structure of aspen glucomannan

The signals at about 2.2 ppm in the ^{1}H NMR spectrum obtained with precipitate **3ET** indicated that the oligo- and polysaccharides present are acetylated (Fig. 3). The chemical shifts and three-bond coupling constants of the major signals (4.75 and 4.53 ppm) in the fingerprint region between 4.4 and 5.6 ppm could be assigned to the anomeric protons of →4)-β-D-Man*p*-(1→ and →4)-β-D-Glc*p*-(1→, thereby providing evidence for the presence of a linear β-(1→4)-linked glucomannan.[11,16] Several other groups of resonances were also observed in this fingerprint region (Fig. 4).

These additional resonances were assigned on the basis of phase-sensitive COSY, TOCSY and HSQC experiments (Tables 2 and 3). The protons in the fingerprint region could be separated into anomeric and other ring protons on the basis of the HSQC spectrum (Fig. 5), since the corresponding carbon atoms resonate at characteristic frequencies. The anomeric and other ring carbon atoms resonate at 90–105 and 70–80 ppm, respectively (Table 3 and Fig. 5). Two kinds of *O*-acetylated residues could be identified, both exhibiting $^3J_{1,2}$ and $^3J_{2,3}$ values of 1 and 3 Hz, respectively (Table 2), which are characteristic for β-(1→4)-linked mannopyranosyl residues. All of the *O*-acetyl groups present in the glucomannan were attached at C-2 and C-3 of mannopyranosyl residues. No indication of *O*-acetylated glucose residues was observed. The ^{1}H and ^{13}C NMR spectra and chemical shifts reported here are all in good agreement with corresponding spectra and values published earlier for *O*-acetylated glucomannan from fibre flax and *O*-acetylated (galacto)glucomannan from spruce.[10,11]

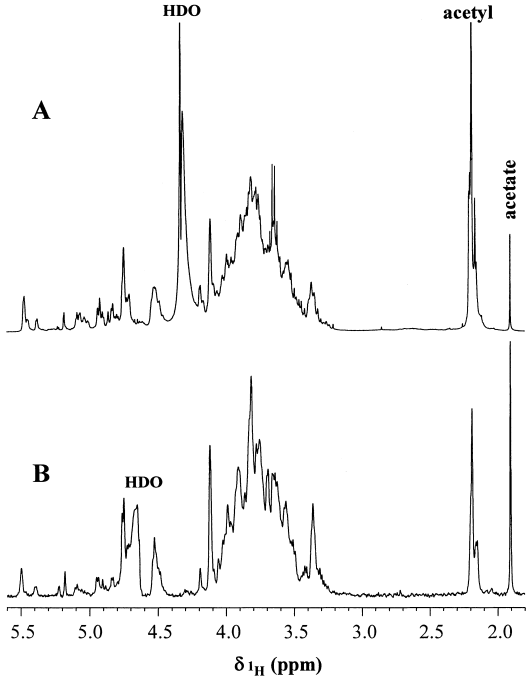

Fig. 3. ^{1}H NMR spectra of glucomannans isolated from the two hardwood species. (A) Precipitate **3ET** isolated from process water obtained from mechanical pulping of aspen; and (B) fraction **B2S** isolated from birch. These spectra were obtained at 70 and 35 °C, respectively.

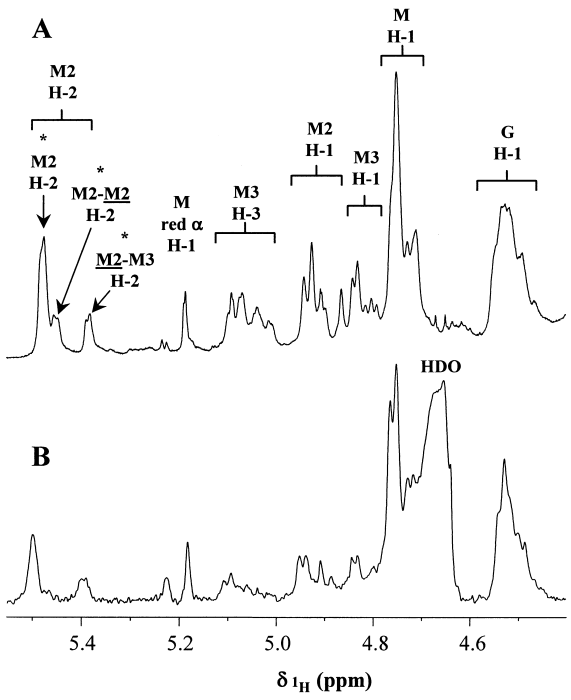

Fig. 4. Fingerprint regions of the ^{1}H NMR spectra of glucomannans isolated from aspen (A) and birch (B). These spectra were obtained at 70 and 35 °C, respectively. See footnote [a] in Table 2 for an explanation of the peak designations employed. M2–M2 and M2–M3 refer to the values for M2 within the sequences →4)[2-*O*-Ac]-β-D-Man*p*-(1→4)[2-*O*-Ac]-β-D-Man*p*-(1→ and →4)[2-*O*-Ac]-β-D-Man*p*-(1→4)[3-*O*-Ac]-β-D-Man*p*-(1→, respectively. * Assignment was made using literature data.[10]

On the basis of this NMR analysis, it can be concluded that precipitate **3ET** consists of a linear β-(1→4)-linked glucomannan which is *O*-acetylated at the C-2 and C-3 positions of certain of the mannose residues. Partially acetylated glucomannans are stored in many monocotyledons, and especially in the bulbs and tubers of *Amaryllidaceae* (konjac mannan) and *Liliaceae*.[17–19] Similar polysaccharides have been found in flax bast fibers, Eremurus roots and *Asparagus officinalis* seeds as well.[10,20,21]

2.4. Further characterization of aspen glucomannan

The glucose-to-mannose ratios of 1:1.8–2 and 1:1.3 for the glucomannans present in precipitate **3ET** and the purified extract **A1′S** (Table 1), respectively, are in good agreement with corresponding values for hardwood glucomannans reported previously.[1,22]

On the basis of MALDI mass spectra for fractions obtained from **A1′S** by SEC, it can be concluded that the dominant polysaccharide present has a backbone consisting of hexose residues substituted with acetyl groups (Fig. 6(A and B)). Deacetylation allows identification of the repeating unit in the backbone (Fig. 6(A and B)). The mean molecular mass, indicated as the peak-average degree of polymerization (DP_p), was approx 17 and 18 for the hemicelluloses in the purified extract **A1′S** and the precipitate **3ET**, respectively (Table 1). In the proton NMR spectra, resonances originating from reducing end residues can be observed (Fig. 4). Integration of the anomeric protons results in a DP value of approx 16 for the polysaccharides present in precipitate **3ET**. The DP_p values observed here are smaller than others reported in the literature for hardwood glucomannans.[1,23,24] Because of the low yield obtained by extraction in the present study, it is likely that the polysaccharides extracted were relatively easy to solubilize and probably represent a low-molecular mass fraction of the glucomannan present in the wood.

The DS_{Ac} values obtained here (Table 1) are similar to values reported earlier, e.g., $DS_{Ac} = 0.36$ for pine glucomannan and DS_{Ac} = approx 1/3 for spruce (galacto)glucomannan.[25–27] NMR analysis reveals that

Table 2
^{1}H NMR data on the constituent monosaccharide residues of the oligo- and polysaccharides present in glucomannans isolated from aspen and birch

Wood	Sample	Residue [a]	^{1}H chemical shifts in ppm [b] ($^3J_{H,H}$ in Hz)				
			H-1	H-2	H-3	H-4	H-5, H-6, H-6′
Aspen	**3ET**	M red α	5.186 (~1)	3.99	3.90	n.d. [c]	n.d.
		M red β	4.906	4.00	n.d.	n.d.	n.d.
		M	4.752 (~1)	4.12	n.d.	n.d.	n.d.
		M	4.728 (~1)	4.06	n.d.	n.d.	n.d.
		M	4.721 (~1)	4.09	n.d.	n.d.	n.d.
		M	4.708 (~1)	4.04	n.d.	n.d.	n.d.
		M2	4.939 (~1)	5.476 (3.4)	4.01	[3.62, 3.78, 3.87, 3.92] [d]	
			4.923 (~1)				
		M2	4.904 (~1)	5.449 (3.4)	3.97	[3.53, 3.75, 3.84, 3.87]	
		M2	4.862 (~1)	5.384 (3.4)	3.98	[3.54, 3.79, 3.82, 3.87]	
		M3	4.839 (~1)	4.19 (3.2)	5.080	4.14	[3.64, 3.79, 3.94]
			4.829 (~1)				
		M3	4.809 (~1)	4.17 (3.2)	5.021	4.11	[3.57, 3.89, 3.91]
		G red α	5.227 (3.1)	3.58	n.d.	n.d.	n.d.
		G	4.533 (~8)	3.37	3.68	[3.83, 3.91]	
		G	4.50 (~8)	3.32	3.66	[3.44, 3.52, 3.90]	
		G	4.472 (~8)	3.26	3.63	n.d.	n.d.
Birch	**B2S**	M red α	5.18	3.99	n.d.	n.d.	n.d.
		M	4.76	4.12	[3.57, 3.91, 3.80]		
		M	4.73	4.06	n.d.	n.d.	n.d.
		M2	4.94	5.50	4.01	n.d.	n.d.
		M3	4.84	4.19	n.d.	n.d.	n.d.
		G	4.53	[3.36, 3.62, 3.68, 3.74]			
		G	4.49	[3.32, 3.42, 3.51]			

[a] The following designations are used: M red, non-acetylated Man at the reducing end; M, non-acetylated Man; M2, 2-*O*-acetylated Man; M3, 3-*O*-acetylated Man; G red, non-acetylated Glc at the reducing end; G, non-acetylated Glc.

[b] Relative to an internal acetone standard at 2.225 ppm (D_2O, 70 °C, pD 7.8) and acquired at 400.1 MHz. The following ^{1}H chemical shifts were observed for the CH_3 groups: 2.16, 2.17, 2.19, 2.20 and 2.21 ppm.

[c] n.d., not determined.

[d] The values in brackets are cross-peaks observed in the TOCSY spectrum that have not been assigned to a proton in the monosaccharide residue.

Table 3
^{13}C NMR data on the constituent monosaccharide residues of the oligo- and polysaccharides present in precipitate **3ET**

Residue [a]	^{13}C chemical shifts in ppm [b]		
	C-1	C-2	C-3
M red α	95.2	n.d. [c]	n.d.
M red β	95.0	n.d.	n.d.
M	101.4	71.4	n.d.
M2	100.4	72.9	n.d.
M2	100.4	73.1	n.d.
M2	100.4	73.0	n.d.
M3	100.9	70.1	74.7
G	103.8	74.3	n.d.

[a] See footnote [a] in Table 2 for an explanation of the designations used.

[b] Relative to an internal acetone standard at 31.55 ppm (D_2O, 27 °C, pD 7.8) and acquired at 100.6 MHz. The following ^{13}C chemical shifts were observed for the CH_3 groups: 21.6 and 21.8 ppm.

[c] n.d., not determined.

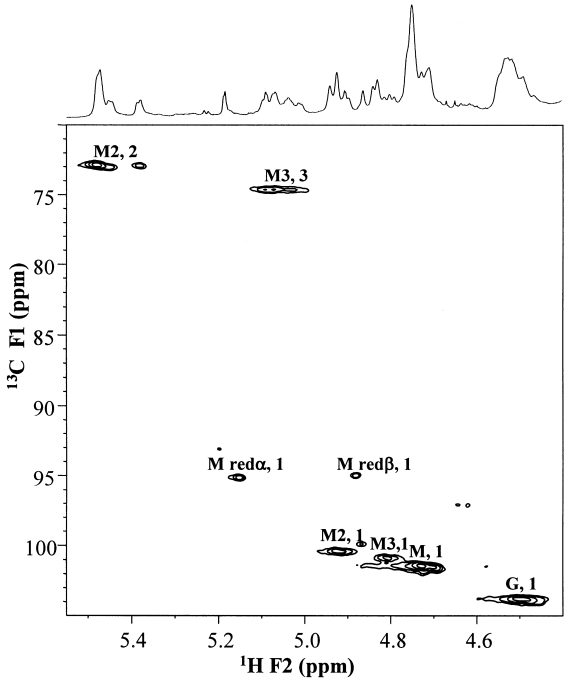

Fig. 5. Regions of the HSQC spectrum (at 27 °C) of precipitate **3ET** representing anomeric and *O*-acetylated mannose residues. See footnote [a] in Table 2 for an explanation of the peak designations employed. The last number in the cross-peak designations refers to the H- and C-atom. The spectrum illustrated at the top is the 1D ^{1}H NMR spectrum of precipitate **3ET** at 70 °C.

approx 40% of the D-mannosyl residues carry *O*-acetyl groups with an almost equal distribution between positions 2 and 3. It is unclear whether this finding reflects the distribution in the wood itself or is due to *O*-acetyl migration during the isolation procedure.[28]

2.5. Isolation of birch glucomannan

Acetone-extracted birch wood meal was extracted with DMSO and then with hot water according to the procedure illustrated in Fig. 1. Enzymatic hydrolysis and subsequent CZE analysis demonstrated that the relative carbohydrate compositions of the polysaccharides in **B1** and **B2** are Glc:Man:Xyl:Gal:Ara = 73:4:5:14:5 (mass %) and Glc:Man:Xyl:Gal:Ara:MeGlcA:GalA = 64:17:5:5:6:1:2 (mass %), respectively. In the case of birch, the largest portion of the hemicelluloses extracted was recovered in the DMSO extract (**B1**) whereas the highest relative amount of glucomannan was present in the water extract (**B2**). Therefore, the latter extract was selected for further purification. The polysaccharides in **B2** were fractionated by SEC to yield **B2S** (Figs. 1 and 2). The extraction yield, calculated from the amount of mannose extracted and the total amount of mannose in the wood, was approx 2%, indicating that the glucomannan isolated may not be representative of the total birch glucomannan. As in the case of aspen, the purified **B2S** consists primarily of glucomannan with minor contamination by polysaccharides containing xylose, galactose and arabinose residues (Table 1).

2.6. Characterization of birch glucomannan

The amount of sample obtained was sufficient to produce 1D and 2D ^{1}H NMR spectra with reasonably good signal-to-noise ratios using a 2.5 mm SEI probe at 600 MHz. The ^{1}H chemical shifts observed for the glucomannan extracted from birch are in excellent agreement with the corresponding values for aspen glucomannan (Fig. 4 and Table 2). Thus, it can be concluded that birch glucomannan also consists of linear β-(1→4)-linked glucomannan, *O*-acetylated at C-2 and C-3 of certain of the mannose residues. The glucose-to-mannose ratio of 1:2.1–2.4 indicates that the glucomannan isolated here contains somewhat more mannose than reported earlier for birch glucomannan.[1]

As in the case of aspen glucomannan, MALDI mass spectra of fractions of the purified birch glucomannan **B2S** obtained by SEC (Fig. 6(C and D)) demonstrate that the extract contains predominantly a polysaccharide with a backbone of hexose residues substituted with acetyl groups. The DP_p values of 16 and 14 obtained by MALDI and NMR analysis, respectively, are similar to the DP_p values obtained for aspen glucomannan and support the hypothesis that only low-molecular mass polysaccharides were extracted from the wood meal by the procedure employed here. The DS_{Ac} values obtained for the birch glucomannan are

somewhat smaller than those for aspen glucomannan (Table 1).

2.7. Distribution of acetyl groups

The MALDI-TOF mass spectra of aspen and birch glucomannans exhibit a considerable heterogeneity with respect to the degree of acetylation (Fig. 6). For example, peaks corresponding to the sodium adduct ions of $Hexose_{11}Acetyl_1$ (m/z 1864.8, DS_{Ac} 0.09), $Hexose_{11}Acetyl_2$ (m/z 1906.8, DS_{Ac} 0.18), $Hexose_{11}Acetyl_3$ (m/z 1948.8, DS_{Ac} 0.27) and $Hexose_{11}Acetyl_4$ (m/z 1990.8, DS_{Ac} 0.36) are observed in the spectrum of birch glucomannan (Fig. 6(C)). Earlier, DS_{Ac} values ranging from zero ($Hexose_6$) up to 0.6 ($Hexose_5Acetyl_3$) have been reported for aspen glucomannan oligosaccharides.[5]

The NMR chemical shifts of *O*-acetylated mannopyranosyl residues are differently affected by neighboring monosaccharide residues.[10] Splitting of signals in the proton spectra for both 2- and 3-*O*-acetylated mannopyranosyl residues (Fig. 4) indicates that various combinations of these sugar residues are present. Three different signals were observed for the H-2 of 2-*O*-acetylated Man (Fig. 4). Two structural fragments with *O*-acetylated mannopyranosyl residues as neighbors could be identified, →4)[2-*O*-Ac]-β-D-Man*p*-(1→4)[2-*O*-Ac]-β-D-Man*p*-(1→ and →4)[2-*O*-Ac]-β-D-Man*p*-(1→4)[3-*O*-Ac]-β-D-Man*p*-(1→. Integration of the H-2 signals of 2-*O*-acetylated Man shows that of the 2-*O*-acetylated Man approx 1/3 has *O*-acetyl-free monosaccharide neighbors and approx 2/3 has *O*-acetylated mannopyranosyl residues as neighbors. This shows that there is no simple regular distribution of the acetyl groups along the glucomannan chain. Instead, both the MALDI-MS and the NMR data indicate that the distribution is random.

Moreover, the proton NMR spectra of aspen glucomannan shown in Figs. 3 and 4 are very similar to earlier published proton NMR spectra of spruce (galacto)glucomannan.[11] This similarity of the NMR spectra and our observation reported here of an irregular distribution of the acetyl groups along the backbone are both in agreement with earlier findings on softwood (galacto)glucomannans of a random distribution of the acetyl groups along the backbone.[5,29]

3. Conclusions

New insights into the chemical structures of glucomannans present in aspen and birch wood are provided by the present study. The water-soluble glucomannans iso-

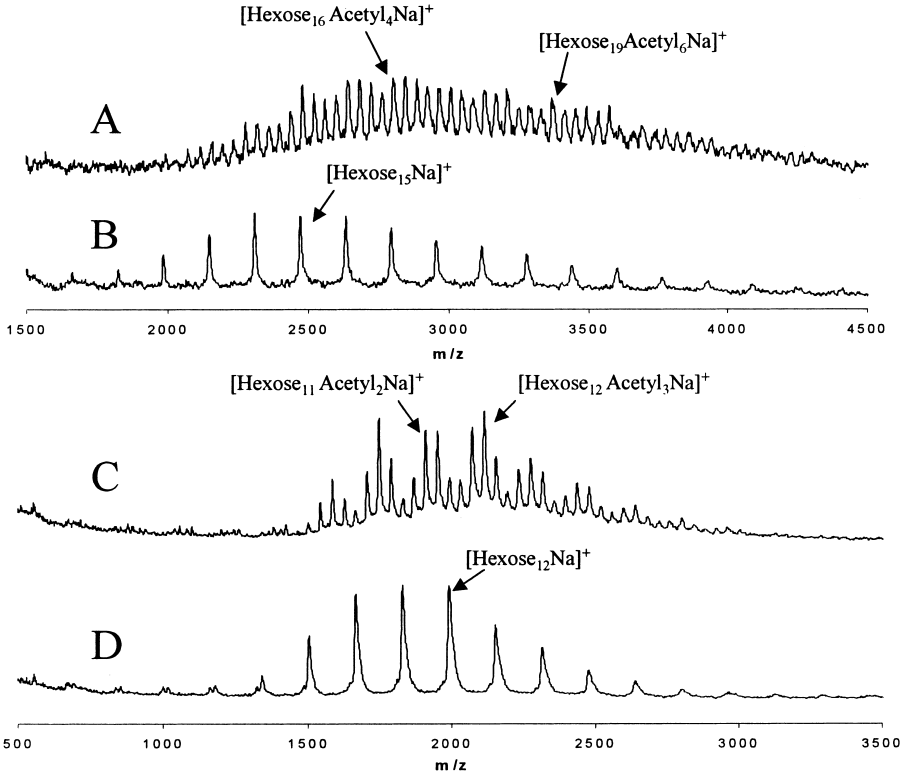

Fig. 6. MALDI-TOF mass spectra of fractions obtained by SEC of **A1′** ((A) prior to and (B) following deacetylation) and **B2** ((C) prior to and (D) following deacetylation). The peaks in (A) and (C) are separated by 42 mass units, which corresponds to an acetyl group. In (B) and (D), the distance between the peaks is 162 mass units, which corresponds to a hexose residue in the glucomannan.

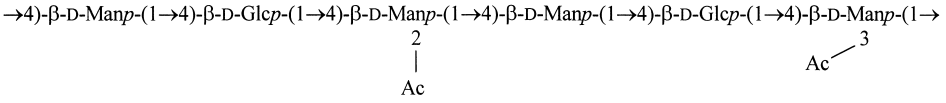

Fig. 7. Abbreviated structural formula for the *O*-acetylated glucomannan isolated from aspen and birch wood.

lated from aspen and birch wood are *O*-acetylated at the C-2 and C-3 positions of certain of their mannose residues (Fig. 7). Thus, both 4-*O*-methylglucuronoxylan and glucomannan are partially acetylated in hardwoods, whereas in softwoods only the (galacto)glucomannans are partially acetylated. The NMR and MALDI-MS data indicate that the distribution of the acetyl groups along the aspen and birch glucomannan chains is random.

4. Experimental

4.1. Materials

All reagents used were of analytical grade. The water employed as the mobile phase in the SEC system and for the preparation of solutions was ultra-purified employing a Millipore Milli-Q Plus apparatus (Millipore, Milliford, USA).

4.2. Isolation of *O*-acetylated glucomannan from process water

Process water obtained in connection with mechanical pulping of aspen wood was lyophilized and the resulting powder (1 g) suspended in 100 mL water. This suspension was mixed for 3 h at room temperature, after which it was centrifuged. The resulting supernatant was filtered through a 0.65 μm filter and then through a 0.22 μm filter in order to remove colloidal lipophilic material.

Precipitation of polysaccharides was performed in three steps. First, an equal volume (84 mL) technical EtOH (Ethanol B) was added to 84 mL of the extract and the polymers allowed to precipitate overnight at 4 °C. The resulting precipitate **1** was removed and 320 mL EtOH added to 160 mL of the supernatant and allowed to stand as before, yielding precipitate **2**. In the final precipitation, 1550 mL EtOH was added to 465 mL of the supernatant from the previous step and again allowed to stand overnight at 4 °C.

Precipitate **3** was further purified by enzymatic digestion using a combination of *Trichoderma reesei* xylanase pI 9 (5000 nkat/g xylose), β-xylosidase (1000 nkat/g xylose) and acetyl xylan esterase (500 nkat/g xylose) in 100 mM NaOAc buffer, pH 5, for 24 h at 40 °C.[30–32] After this enzymatic treatment the glucomannan was precipitated with EtOH (1:25 water–EtOH, v/v), and the resulting precipitate **3ET** (yield, 29 mg) lyophilized prior to further analysis.

4.3. Isolation of *O*-acetylated glucomannan from aspen wood

The wood meal was prepared by grinding small pieces of aspen wood until the meal could pass through mesh-20 gauze. This wood meal (11 g) was then extracted three times with 100 mL C_3H_6O at ambient temperature for a total period of 24 h. The C_3H_6O-extracted wood meal (10 g) was then extracted twice with 100 mL Me_2SO for a total period of 45 h at ambient temperature. Following the second extraction, the residue was washed twice with 20 mL Me_2SO and, subsequently, five times with 20 mL water. The extracts and the Me_2SO used for washing were combined and lyphilized to obtain **A1** (Fig. 1). **A1** was washed with Et_2O and n-C_5H_{12} (approx 2.5 mL of each) and thereafter designated **A1′**.

The wood meal residue remaining after the Me_2SO extractions was extracted twice with 250 mL hot water (80–85 °C) for 30 min. After the second of these extractions, the residue was washed with a small amount of cold water. The extracts and wash water were combined and lyophilized to obtain **A2** (Fig. 1). For final purification, **A1′** was dissolved in water to give a concentration of 10 mg/mL (the carbohydrate concentration was approx 2 mg/mL, as calculated from the carbohydrate yield obtained from enzymatic hydrolysis and subsequent CZE analysis); injected into the aqueous SEC system (see SEC/MALDI-MS); and the glucomannan (fraction **A1′S**, Fig. 2) collected.

4.4. Isolation of birch glucomannan

Birch glucomannan was isolated from C_3H_6O-extracted birch wood meal employing the same extraction procedures as in the case of aspen wood meal (Fig. 1). Extract **B2** was further purified by SEC, in the same manner as extract **A1′** (Fig. 2), and fraction **B2S** subsequently lyophilized.

4.5. Carbohydrate analysis employing enz/HPAEC

A small quantity of wet precipitate (10–20 mg) was collected after each step in the fractional precipitation of process water with EtOH; solubilized in 0.15 mL 100 mM NaOAc buffer (pH 5); and subsequently incubated with a complete enzyme mixture in order to hydrolyze

all of the polysaccharides in the sample completely to monosaccharides.[33] The composition of the resulting monosaccharide mixture was analyzed by high-performance anion-exchange chromatography (HPAEC) using Dionex DX 500 in a Carbo-Pac PA-1 column (Dionex Corp, USA) and pulsed amperometric detection (PAD, Dionex ED 40).[34]

4.6. Carbohydrate analysis utilizing enz/CZE

The carbohydrate compositions of the extracts were determined using enzymatic hydrolysis and subsequent analysis by CZE, as described previously.[14] A P/ACE System MDQ from Beckman was used for the CZE analyses.

4.7. Carbohydrate analysis by TFA/CZE

Approximately 0.2 mg of **A1′S** or **B2S** was dissolved in 50 μL 2 M TFA. This solution was then maintained at 80 °C for 3 h, after which the solvent was evaporated under a flow of nitrogen gas and the sample was dissolved in a AcONa buffer (50 mM, pH 4.07) and derivatized for CZE analysis.[14]

4.8. SEC/MALDI-MS

The SEC system consisted of three columns (Ultrahydrogel 500, 250 and 120, Waters Assoc. USA), linked in series, with a refractive index (RI) detector (Waters 410 differential refractometer, Waters Assoc. USA). Water was employed as the eluent and pumped through this system at a rate of 0.6 mL/min. Each sample was dissolved in water to obtain a concentration of approx 2 mg/mL and filtered (pore diameter 0.2 μm, Advantec MFS, Inc., USA) prior to injection of 100 μL into the SEC system. Fractions (100 μL) were collected for MALDI analysis in order to obtain narrow molecular weight distributions of polysaccharides. To this end, the solvent was evaporated from the fractions under a gentle stream of nitrogen gas prior to analysis.

In connection with MALDI-MS analysis, a saturated, aq soln of 2,5-dihydroxybenzoic acid (10 μL) was added to each dried SEC fraction. An aliquot of this sample-matrix mixture (0.5 μL) was co-crystallized on the surface of the MALDI probe by evaporation of the solvent under a vacuum. A Nafion film was then applied to the probe surface in order to enhance the quality of the mass spectra.[35] To enhance the signal-to-noise ratio, each mass spectrum was obtained by summing the responses to approx 60 laser pulses of 1–3 μJ. A Hewlett Packard G2025A MALDI-TOF apparatus was used to perform these analyses.

In order to calibrate the aq SEC system in terms of absolute molecular mass, the peak-average molar masses (M_p) of a number of individual fractions were determined from their mass spectra. The logarithm of the M_p value of each fraction exhibited a linear correlation to its elution time. This calibration of the SEC system, allowed M_p to be determined, as described in more detail elsewhere.[5] The peak-average degree of polymerization (DP_p), defined as the number of hexose residues in the backbone of the glucomannan, was subsequently calculated from the M_p value.

4.9. Determination of DS_{Ac} values by SEC/MALDI-MS

The DS_{Ac} values for isolated aspen and birch glucomannan were calculated from MALDI mass spectra of SEC fractions by first determining the acetylgroup-to-hexose ratio for each individual peak in the mass spectrum and thereafter calculating a overall mean DS_{Ac} value for the fraction. The values reported are the mean values for three SEC fractions from each species of wood.

4.10. Deacetylation of *O*-acetylated glucomannans in SEC fractions

O-Acetyl groups were removed from the glucomannan backbone employing alkaline hydrolysis. This hydrolysis was accomplished by adding four or five drops of aq NH_3 solution (min 25%) to each SEC fraction and maintaining this mixture at 80 °C for 10–15 min. The solvent was then evaporated from the fractions under a stream of nitrogen gas.

4.11. NMR spectroscopy

For NMR analysis, 3.7 mg of precipitate **3ET** was dissolved in 0.37 mL D_2O (99.9 atom % D), after which the pD was found to be 6. Shigemi NMR tubes (BMS-005B) were employed to obtain the ^1H and ^{13}C NMR spectra at 400.13 and 100.61 MHz, respectively, using a Bruker DPX 400 MHz spectrometer. The birch glucomannan sample (approx 10 μg) was dissolved in approx 40 μL D_2O (99.96 atom % D) and subsequently transferred to a 1.7-mm capillary tube, which was subsequently positioned in the probehead (2.5 mm SEI). ^1H NMR spectra were obtained employing a Bruker DRX600 MHz spectrometer operating at 600.13 MHz.

1D ^1H NMR spectra were recorded using a 90° pulse, a spectral width of 4000 (aspen) or 4800 (birch) Hz and a repetition time of 19 (aspen) or 8 (birch) s. Spectra were obtained with a probe temperature of 27, 35 or 70 °C. The chemical shifts are reported relative to an internal C_3H_6O standard at 2.225 and 31.55 ppm for ^1H and ^{13}C NMR spectra, respectively.

The relative amounts of acetyl and sugar residues were determined by integration of the signals in the fingerprint region and DS_{Ac} subsequently calculated.

Similar DS_{Ac} values were obtained by integration of the signals assigned to acetyl groups at 2.2 ppm and all carbohydrate signals.

Standard pulse sequences and phase cyclings were employed to perform 2D phase-sensitive ^1H,^1H-correlated spectroscopy (COSY) and total correlation spectroscopy (TOCSY) ($\tau_{mix} = 0.14$ s). A spectral width of 2000 (aspen) or 3000 (birch) Hz was utilized in both dimensions and the relaxation delay was 2.5 s. The proton-detected heteronuclear single quantum (HSQC) spectrum was acquired over a t_1 spectral width of 11,000 Hz and a t_2 width of 1850 Hz, with a 2048×1024 matrix (zero-filled to 2048 in t_2) and 44 transients per increment.[36–38] The delay between transients was 2.5 s and the delay for polarization transfer was set to correspond to an estimated average $^1H-^{13}C$ coupling constant of 150 Hz. Data processing was performed using standard Bruker XWIN-NMR software.

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