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Isolation and characterization of galactoglucomannan from spruce (*Picea abies*)

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Abstract

Water-soluble hemicelluloses were extracted from spruce chips by microwave heat fractionation. The chips were impregnated with water at different pH values. Screening of heat-fractionation conditions, i.e. impregnation medium, temperature and residence time was performed with the aim to extract *O*-acetyl-galactoglucomannan. The impregnation and heat fractionation conditions were evaluated on the basis of the yield of dissolved mannan (oligo- and polysaccharides), molecular weight of the carbohydrates and amount of dissolved lignin. Increasing temperature and residence time increases the yield of mannan and decreases the molecular weight of dissolved carbohydrates. For a structural study of the extracted carbohydrates the chips were impregnated with water and treated at 200°C for 2 min. Oligo- and polysaccharides were fractionated with preparative size-exclusion chromatography from the filtered extract.

The structure of the obtained saccharides in two fractions **8** and **9** was determined by ^{1}H NMR spectroscopy. The polysaccharides in the fractions were *O*-acetyl-galactoglucomannan with a degree of polymerization ~20 and ~11 for fractions **8** and **9**, respectively. The molar ratio for galactose:glucose:mannose was approximately 0.1:1:4. About one-third of the D-mannosyl units are substituted by *O*-acetyl groups almost equally distributed between C-2 and C-3.

Keywords: Spruce (*Picea abies*); Hemicellulose; Mannan; *O*-acetylation; Galactoglucomannan; Microwave oven; Size-exclusion chromatography; NMR spectroscopy

1. Introduction

Lignocellulose (mainly cellulose, hemicellulose and lignin) constitutes a renewable raw material widely available in the form of waste from the forest industry, energy crops, agricultural residues, straw and grass (Eriksson, 1990; Goldstein, 1981; Kuhad, Singh & Eriksson, 1997). There is an increased interest in the development of techniques for fractionation of lignocellulosic materials for example to be used in the production of environmental friendly polymers. In the current study we have heat fractionated softwood, followed by further isolation and characterization of hemicellulose. Hemicellulose is a group of heteroglycans consisting of D-xylose-, D-mannose-, L-arabinose-, D-glucose-, D-galactose- and 4-*O*-methyl-D-glucuronic acid-residues (Timell, 1967). The major hemicellulose in softwood is acetylated galactoglucomannan, which can constitute up to 20 w/w% of the dry wood. Smaller amounts of arabino-4-*O*-methylglucuronoxylan (5–10 w/w%) are also found (Timell, 1967). Lignin is an amorphous polymer built up of phenylpropane units and the amount of lignin in softwood is 26–32 w/w% of dry wood. Approximately 40–45 w/w% of wood dry weight is cellulose, a linear polymer of D-glucosyl units. (Timell, 1967).

Hemicelluloses have a lower degree of polymerization (DP) than cellulose. Acetyl-galactoglucomannan has been reported to have an approximate DP between 100–150 (Timell, 1967), equivalent to a molecular weight (M_w) around 16 000–24 000 Da. It has a backbone of β-(1 → 4)-D-Man*p* and β-(1 → 4)-D-Glc*p* residues with α-(1 → 6)-D-Gal*p* and acetyl side-groups. It is found primarily in the lignified secondary cell wall of softwoods (Meier, 1985). Two types of acetylated galactoglucomannan exist in softwood, one galactose rich (5–8 w/w% of the dry wood) and one galactose poor (10–15% of the dry wood). The molar ratio for galactose:glucose:mannose is

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approximately 1:1:3 and 0.1:1:3, respectively (Timell, 1967). The acetylations are at the hydroxyl groups on the C-2 and C-3 positions (one acetyl per 3–4 backbone hexose units).

The components in lignocellulose are tightly associated and in several processes it has been proved to be difficult to separate the lignin from hemicellulose and cellulose without modifying the hemicellulose (Puls & Schuseil, 1993). The different ways to fractionate the hemicellulose from lignocellulose include physical methods such as steam treatment (Biermann, Schultz & McGinnis, 1984; Lamptey, Robinson & Moo-young, 1985; Lipinsky, 1983; Puls, Poutanen, Körner & Viikari, 1985; Schultz, McGinnis & Biermann, 1984; Teh-An, 1996) and microwave irradiation (Azuma, Katayama & Koshijima, 1986; Azuma, Tanaka & Koshijima, 1984; Junel, 1999; Magara, Ueki, Azuma & Koshijima, 1988) and chemical methods such as the organosolv processes (Bennani, Rigal & Gaset, 1991; Paszner, Jeong, Quinde & Awardel-Karim, 1993; Sjöström, 1993). Heat treatment is often combined with the addition of chemicals, i.e. alkali or acid, which is crucial for the outcome due to different factors like solubility of the components but also chemical reactions (Junel, 1999; Lai, 1991). In the current work we have used microwave heat fractionation in order to obtain intact or fragmented *O*-acetyl-galactoglucomannan from spruce chips. The dissolved material from the heat-fractionation was size-fractionated using size-exclusion chromatography (SEC). The aim was to find conditions that allowed the isolation and characterization of lignin-free *O*-acetyl-galactoglucomannan. This initial screening of conditions was evaluated on the basis of the yield of dissolved mannan after heat-fractionation and the molecular weight (M_w) of the obtained dissolved lignin free carbohydrates (i.e. mainly hemicellulose). Samples of fractionated *O*-acetyl-galactoglucomannan were analyzed by NMR for determination of polysaccharide composition and structure.

2. Material and methods

2.1. Heat fractionation

The substrate was spruce (*Picea abies*) obtained as chips from Harry Nilsson sawmill in Hästveda, Sweden. The spruce chips were milled and then impregnated (soaked) with water, NaOH, KOH or H_2SO_4. After impregnation, 9.1 g (dry weight of the wood-material) in 100 ml water were heat treated at a predetermined temperature and residence time in a microwave oven (MLS-1200 Mega Microwave workstation from Milestone) (Junel, 1999). The treatment vessel had a volume of 350 ml and was made of Teflon. After heat treatment the insoluble material (mainly cellulose and lignin) was removed by filtration (Acrodisc® Syringer Filter, 0.2 μm, PALL Gelman Laboratory, Ann Arbor, MI USA) and the water-soluble oligomers and polymers further size fractionated by size-exclusion chromatography (SEC). The conditions for impregnation and fractionation (with pH, different temperatures and residence times) are displayed in Table 1.

2.2. Size-exclusion chromatography (SEC)

2.2.1. Molecular weight distribution

After filtration of the solution obtained from the heat fractionation, a sample of the filtrate (100 μl) was loaded on a size exclusion chromatography (SEC) system (FPLC, Pharmacia Biotech, Uppsala, Sweden) with refractive index (RI)- (Erma-inc, Tokyo, Japan) and ultra violet (UV)-detectors (Pharmacia Biotech, 280 nm). UV-absorbing materials in the samples can be expected to be mainly lignin and material detected with the RI-detector with no corresponding UV absorbance can be expected to be mainly carbohydrates (Morohoshi, 1991). The preparative and analytical separations were performed using gel filtration media from Pharmacia Biotech (Uppsala, Sweden). In the SEC

Table 1
Conditions for the fractionation in microwave oven

Impregnation medium	Concentration (%)[a]	Temperature (°C)	Time (min)	pH (after heat fractionation)
Water		170–200	2, 5, 10	3.5–4.1
		190–220	20	3.1–3.4
NaOH	0.025	190–220	5, 10, 20	3.8–4.1
	0.05	190–200	2	5.0–6.0
		180–220	5, 10	4.2–5.5
		190–220	20	3.8–4.1
	0.1	190–200	2	5.0–5.5
		180–190	5	4.9–5.2
		170–180	10	
	1	170–200	2, 5, 10	9.4–9.6
	2	170–200	2, 5, 10	12.4–12.6
KOH	0.05	170–200	2, 5, 10	4.3–4.6
H_2SO_4	0.05	190–200	2	2.3
		180–190	5	2.3
		170–180	10	2.3–2.5

[a] % w/v liquid.

system three columns were connected in series, (i) a precolumn Superdex 75 (HR 5/5), (ii) a Superdex 75 (HR 10/30), and (iii) a Superdex 200 (HR 10/30) column, (Pharmacia Biotech, Uppsala, Sweden). The molecular weight (M_w) distribution was determined using dextran (Fluka Chemie AG, Buchs, Switzerland) with M_w of 1270, 5220, 11 600, 23 800 and 48 600 Da as M_w standards. Acetone was used to determine the total volume of the columns (47 ml). Water was used as mobile phase at a flow rate of 0.5 ml/min.

2.2.2. Collection of fractions for NMR and acid hydrolysis

Fractions after SEC of the filtrate from water impregnation and heat fractionation at 200°C for 2 min (fractions **8** and **9**) were collected for further investigations. The filtrate was loaded on SEC (10 mg of material (after freeze-drying) in 500 μl) and the same fractions from 6 independent separations were pooled and lyophilized.

2.3. Analytical procedures

2.3.1. Total carbohydrate content analysis of the raw material

The composition of the spruce chips was analyzed using acid hydrolysis as described by Hägglund (1951) and analyzed by HPLC (Shimadzu, Kyoto, Japan) with an Aminex HPX-87P column (BIO-RAD, Hercules CA, USA) at 80°C giving monomeric sugar unit composition. Water was used as eluent at a flow rate of 0.4 ml/min. Lignin was determined as Klason-lignin.

2.3.2. Yield of mannan in filtrates

The filtrate after heat fractionation (1 mg dry matter) was dissolved in 0.5 M H_2SO_4 up to 4 ml and then hydrolyzed in an autoclave for 4 h at 120°C (Lai, 1991; Wright & Power, 1987). Afterwards, $Ba(OH)_2$ was added to remove the sulfate and neutralize the pH, and the solution was filtered with 0.2 μm filters to remove $BaSO_4$ (s). The sugar monomer components were determined by HPLC as described in Section 2.3.1. For determination of the monomeric sugars after acid hydrolysis of fractions from SEC, a High Performance Anionic Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) (DIONEX, Sunnyvale CA, USA) with a PA-10 column was used. The eluent was 5 mM NaOH and the flow rate 1 ml/min. With the HPLC systems mannose, xylose, glucose, galactose, arabinose and glucuronic acid were used as standards.

The amount of extracted galactoglucomannan in the filtrates after heat fractionation was estimated by analyzing the mannose content in the filtrates and in the filtrates after acid hydrolysis. Thus, to make the estimation feasible, the galactose and glucose content of heteromannans was disregarded. The total amount of mannose obtained in the filtrates after hydrolysis of oligo- and polysaccharides is denoted M_H, the mannose amount in the original filtrate is denoted M_F and the total (theoretical) mannose amount obtained from raw material analysis (described under Section 2.3.1) is denoted M_T. The yield of mannan (oligo- and polysaccharides containing mannose), expressed in % of the total amount in the raw material was calculated as $(M_H - M_F)/M_T$ (Junel, 1999).

2.3.3. Enzymatic hydrolysis

Purified endo-(1 → 4)-β-mannanase from *Aspergillus niger* (Ademark et al., 1998) was used for enzymatic hydrolysis at an enzyme loading of 10 nkat/mg substrate incubated at 40°C for 48 h at pH 5.0 in 50 mM NH_4Ac.

The purified (1 → 6)-α-galactosidase AglB from *Aspergillus niger* (Ademark Larsson, Tjerneld & Stålbrand, 2000) was used to release the galactose groups from the glucomannan main chain at an enzyme loading of 5 nkat/mg substrate incubated at 40°C for 48 h at pH 4.8 in 100 mM NaAc. Bovine serum albumin (100 μg/ml) was included in the experiment. 4.3 mg acetyl-galactoglucomannan was used as substrate. The galactose produced was analyzed using HPAEC-PAD with the PA-10 columns as described in Section 2.3.2, except that 15 mM NaOH was used as the eluent and galactose was used as standard.

2.4. NMR spectroscopy

For NMR analysis, a portion of each of the dried samples (~3.5 mg) was dissolved in 0.6 ml D_2O (99.9 atom %D, Cambridge Isotope Laboratories). The pD of these solutions, which were clear and colorless, was measured and found to be 6.5. ^1H NMR spectra were obtained at 400.13 MHz using a Bruker DPX 400 MHz spectrometer. One-dimensional (1D) ^1H NMR spectra were recorded using an 85° pulse of 7 μs, a spectral width of 4000 Hz and a repetition time of 15 s. All spectra were acquired at a probe temperature of 70°C. The chemical shifts are reported relative to an internal acetone standard at 2.225 ppm.

Standard pulse sequences and phase cyclings were employed to perform two-dimensional $^1H,^1H$-correlated spectroscopy (COSY) and total correlation spectroscopy (TOCSY) ($\tau_{mix} = 0.14$ s) experiments. A spectral width of 2000 Hz was employed in both dimensions and the relaxation delay was 2.5 s. For each FID, 12 (or 16 in the case of TOCSY) transients were acquired; the data size was 1024 (or 512 for TOCSY) in $t_1 \times 2048$ in t_2. The final size of the data matrix after Fourier transformation was 1024×2048. Data processing was performed using standard Bruker XWIN-NMR software.

3. Results and discussion

3.1. Heat fractionation with microwave oven

With the aim to find conditions allowing the isolation of intact or fragmented *O*-acetyl-galactoglucomannan free

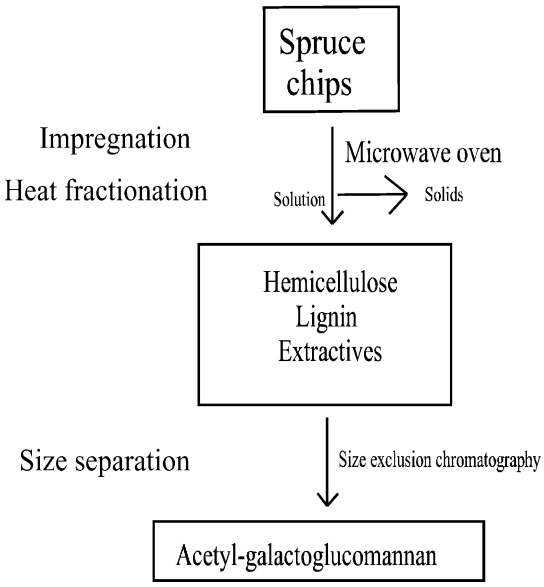

Fig. 1. The procedure to isolate *O*-acetyl-galactoglucomannan from spruce chips by microwave oven heat fractionation and subsequent fractionation by SEC.

from lignin, spruce chips were heat fractionated and further processed as schematically shown in Fig 1. First, a total carbohydrate analysis was made on the spruce chips to determine the monomeric sugar content. The contents of mannose units were 12.9%, xylose units 5%, glucose units 44.8%, galactose units 2.4%, arabinose units less than 1% and lignin residues 27.6% of dry unextracted spruce wood. 4-*O*-methylglucuronic acid was not analyzed. A screening of appropriate heat fractionation conditions of the spruce chips was performed. The heat fractionation conditions used are shown in Table 1. The filtrates can be expected to consist of carbohydrates (mainly hemicellulose) and lignin (Puls & Schuseil, 1993). It is well established that carbohydrates can be detected with a refractometer, but do not absorb in the ultra violet wavelength range where lignin has its absorbance maxima (Morohoshi, 1991). SEC was used to analyze the filtrates, obtained from heat fractionation of the spruce chips. In the light of the fact that galactoglucomannan is the major hemicellulose in spruce it can be expected that the major RI-detected material free from UV- absorbing material consists of galactoglucomannan or products obtained thereof. The heat fractionation was evaluated on the basis of the molecular weight for RI-detected material free from UV-absorbing material in the filtrates and on the basis of yield of mannose-containing soluble poly- and oligosaccharides in the filtrates.

3.1.1. Molecular weight distribution

Filtrates from the heat fractionations were collected. Initially, filtrates obtained from impregnation with alkali (0.05% NaOH) and water were analyzed with SEC to determine the M_w distribution. Figs. 2 and 3 show the influence of impregnation, with 0.05% NaOH and water respectively, on the M_w distribution. After 0.05% NaOH impregnation and heat fractionation at 200°C for 2, 5 and 10 min the peaks of the highest M_w were detected between 15–30 ml (approx. 8000–80 000 Da) in the RI chromatograms (Fig. 2a). For roughly the same volumes at which these peaks were detected, peaks were also found in the UV chromatogram (Fig. 2b). A low M_w peak (<500–5000 Da) was detected between 32.5 and 42.5 ml (Fig 2a) with no corresponding UV absorbing peak.

The M_w distribution after heat fractionation in water was lower (RI-detection, Fig. 3a) in comparison to filtrates from alkali impregnation (Fig. 2a). However, the fractions had less UV-absorbing material co-eluting (Fig. 3b). The filtrates from heat fractionation of water impregnation at 200°C for 2, 5 and 10 min displayed chromatograms with similar M_w distribution (Fig. 3). In the RI chromatograms major peaks eluting between 33 and 43 ml with maxima at approx. 37–38 and 41–42 ml were obtained. For the heat treatment with the shortest residence time (2 min), higher M_w material was eluted at 28–33 ml. This material was gradually lost with longer residence times, yielding a shift toward lower M_w distribution. No or very little UV-absorbance (<0.030 AU) was detected between elution volumes of 30–49 ml (Fig. 3b). The RI-chromatograms for the filtrates from treatment for 2–10 min showed small peaks eluting between 19 and 27.5 ml with corresponding peaks in the UV-chromatograms (Fig. 3).

3.1.2. Analysis of the filtrates

Several conditions for the heat fractionation were further tested (Table 1). Filtrates from the heat fractionation were separated on the SEC columns in the same way as described for samples from impregnation with water and 0.05% NaOH (previous section). With increased NaOH concentration (0.1–2%) the M_w for RI-detected material with no UV-absorbing material was decreased. Lower content of carbohydrates in the filtrate and a higher content of lignin were found estimated from SEC chromatograms with the higher pH (data not shown). A reasonable explanation for this is that when the pH is increased, more lignin is dissolved and also the mannan are likely to be deacetylated at alkaline conditions (Malinen & Sjöström, 1975; Rydholm, 1967) and thus precipitated.

Impregnation with KOH and H_2SO_4 was also investigated (Table 1). After heat fractionation the filtrates were analyzed with SEC. With KOH no further improvements were obtained. A slight difference was observed compared with impregnation with NaOH. The possible lignin–carbohydrate complexes were of somewhat lower M_w after impregnation with KOH. With H_2SO_4 apparently acid hydrolysis occurred and the results of these experiments showed mostly monomeric sugars in the filtrate, analyzed with HPLC (data not shown).

Further investigations on 0.05% NaOH and water impregnated spruce chips were carried out as described in Section 2.3.2. Figs. 4 and 5 show the yield of mannan obtained after heat fractionation and impregnation with

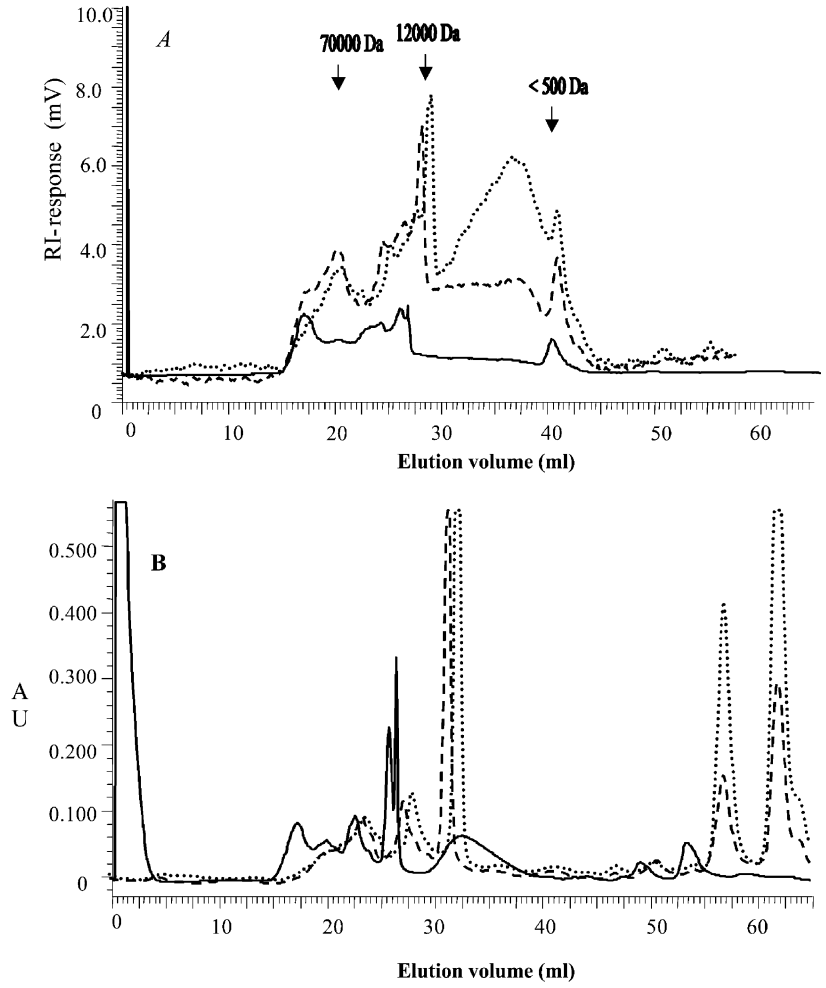

Fig. 2. Elution profile of the water-soluble material from microwave oven treated spruce chips after SEC using Superdex 75 and 200 columns. The spruce chips have been impregnated with 0.05% NaOH (100 μl of the filtrate was applied). The chromatograms are for filtrates from heat fractionation at 200°C/2 min (solid line), 200°C/5 min (hatched line) and 200°/10 min (dotted line). (a) RI-detection; (b) UV-absorption at 280 nm (AU: absorbance units). The applied sample volume was 100 μl. The arrows mark the elution volumes of dextran standards of 70 000, 12 000 Da, and mannotriose (504 Da) which coeluted with pentose and hexose monomers close to the total column volume.

0.05% NaOH and water respectively. The sugar residues in the filtrates after heat fractionation were mainly mannose (70–80%). The yield of mannan was increased with increased temperature and residence time for both conditions (Figs. 4 and 5). The yield of mannan in the filtrates from 0.05% NaOH impregnated spruce chips heat fractionated at 200°C (2, 5 and 10 min) were 8, 30 and 41% respectively (Fig. 4). For the water impregnated spruce chips heat fractionated at 200°C (2, 5 and 10 min) the yield were 35, 75 and 50% respectively (Fig. 5). Thus, the highest yield of oligomeric and polymeric mannan for water impregnated spruce chips filtrate was reached at 200°C (5 min) (75%, Fig. 5), which was higher compared to the highest yield obtained with 0.05% NaOH (about 55%, Fig. 4). The yield of xylan (oligo- and polysaccharides) in the filtrates showed similar trends as the yield of mannan, i.e. increased yield with increased temperature, except for 10 min and higher where a decrease of the yield occurred when the temperature was 200°C and above (data not shown).

In order to select a fraction of galactoglucomannan to be analyzed further, several criteria were considered. In the optimal case, the DP should be as high as possible without lignin contamination, and the yield of the selected fraction should be as high as possible. At low temperatures and high pH, the lignin content was higher than for higher temperatures and lower pH. The yield was considered to be an important factor in further evaluation. For water impregnation and heat fractionation at 200°C it is striking that the mannan yield increased from ~35 to close to 75% with an increased of residence time in the heat treatment from 2 to 5 min (Fig. 5). Furthermore, a similar overall shape of the chromatograms (with UV and RI-detection) for 2, 5 and 10 min were obtained, but a shift toward lower M_w RI-detected material was seen with increase in residence time (Fig. 3). As a first step to characterize the obtained carbohydrate from heat fractionation with water at 200°C, the filtrate from the 2 min heat fractionation was analyzed further.

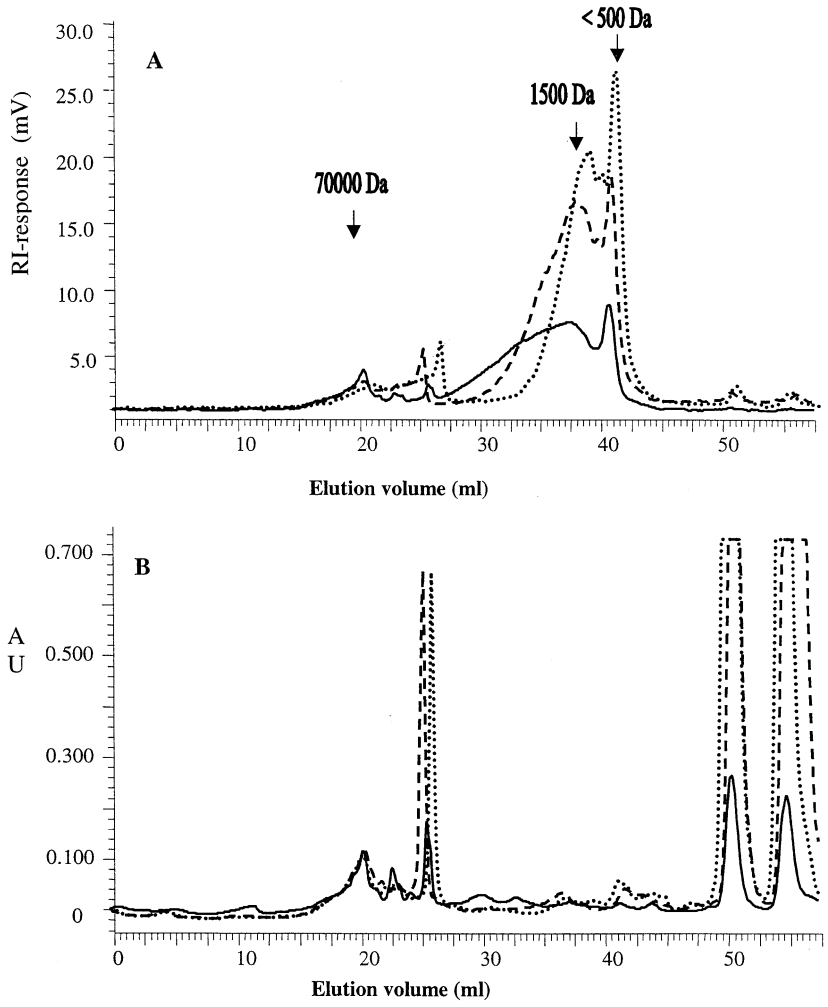

Fig. 3. Elution profile of the water-soluble material from microwave oven treated spruce chips after SEC using Superdex 75 and 200 columns. The spruce chips have been impregnated with water (100 μl of the filtrate was applied). The chromatograms are for filtrates from heat fractionation at 200°C/2 min (solid line), 200°C/5 min (hatched line) and 200°/10 min (dotted line). (a) RI-detection; (b) UV-absorption at 280 nm (AU: absorbance units). The applied sample volume was 100 μl. The arrows mark the elution volumes of dextran standards of 70 000, 1500 Da, and mannotriose (504 Da) coeluted with pentose and hexose monomers close to the total column volume.

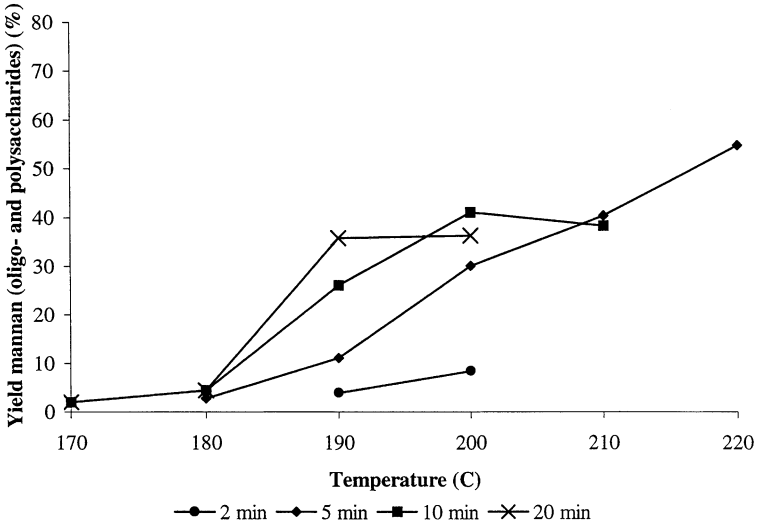

Fig. 4. Effects of heat fractionation temperature and time on the yield of mannan (oligo- and polysaccharides) for spruce impregnated with 0.05% NaOH. For calculations of yield see Section 2.

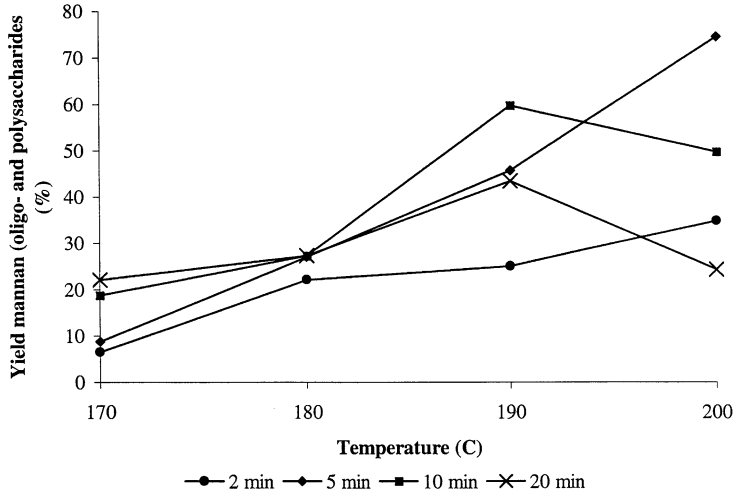

Fig. 5. Effects of heat fractionation temperature and time on the yield of mannan (oligo- and polysaccharides) for spruce impregnated with water. For calculations of yield see Section 2.

3.2. Isolation of galactoglucomannan

A preparative SEC-separation with the filtrate obtained from heat fractionation with water at 200°C for 2 min (500 μl) was done. Similar to the analytical run (Fig. 3), the refractometrically detected material showed a M_w range obtained by SEC between approx. 15–45 ml (peak maxima at elution volume 24, 37 and 41 ml) (Fig. 6). Distinct peaks with UV-absorbance were detected at elution volumes 17, 24, 50 and 55 ml. Fractions were collected and two fractions were selected containing a substantial amount of assumed carbohydrate polymers but no UV-absorbing material. These fractions (**8** and **9**, Fig. 6) with an estimated M_w from the SEC run of 5000 and 1600 Da respectively were subjected to acid hydrolysis and subsequent HPLC analysis which showed an approximate molar ratio for galactose:glucose:mannose of 0.3:1:3 (fraction **8**) and 0.5:1:4 (fraction **9**), roughly what is expected for *O*-acetyl-galactoglucomannan. The acid hydrolysis also showed that the saccharides recovered in fractions **1**–**5** (14.5–27 ml) consisted of both mannan and xylan polysaccharides.

3.3. Structures of the isolated saccharides

The fractions thus obtained denoted **8** and **9** (Fig. 6) were subsequently studied by NMR spectroscopy. The two samples could easily be dissolved in D_2O at neutral pD. NMR spectra were then obtained at 70°C. The 1D proton NMR spectra demonstrated that the saccharides recovered in fractions **8** and **9** are almost identical (Fig. 7). Fraction **9** was selected for more detailed structural analysis.

The proton NMR resonances in the fingerprint region between 4.4 and 5.5 ppm were assigned on the basis of phase-sensitive COSY and TOCSY experiments (Table 2). An anomeric proton chemical shift of 4.70–4.75 ppm and a $^3J_{1,2}$ value of 1 Hz are characteristic for β(1 → 4) linked mannopyranosyl residues (Harjunpää, Teleman, Siika-aho & Drakenberg, 1995; Tenkanen, Makkonen, Perttula, Viikari & Teleman, 1997). The signals at 2.1–2.2 ppm indicate that the saccharides in these samples are acetylated (Fig. 7). Two kinds of *O*-acetylated residues can be identified, both having $^3J_{1,2}$ and $^3J_{2,3}$ values of 1 and 3.4 Hz, respectively, which are characteristic for β-(1 → 4)-linked mannopyranosyl residues (Table 2). The *O*-acetyl group induces a large shift of the proton at the position of *O*-acetylation to higher chemical shift, in accordance with observations for *O*-acetylated glucopyranosyl and xylopyranosyl residues (Laignel, Bliard, Massiot & Nuzillard, 1997; Teleman, Lundqvist, Tjerneld, Stålbrand & Dahlman, 2000). The presence of more than one signal each for both the 2 *O*-acetylated and 3 *O*-acetylated mannopyranosyl residues indicates that various possible combinations of these sugar residues, resulting from the random distribution along the backbone (Kenne, Rosell & Svensson, 1975), probably are present. A detailed assignment of these combinations has not been made here.

The $^3J_{1,2}$ value of 7.7 Hz and the through bond connectivities network from the anomeric proton at 4.53 ppm (Table 2) is consistent with the presence of β(1 → 4)linked glucopyranosyl residues in galactoglucomannan (Tenkanen et al., 1997). A minor cross-peak is observed in the COSY spectrum originating from α-D-galactopyranosyl groups (1 → 6)-linked to mannose (Table 2) (Davis, Hoffman, Russel & Debet, 1995; McCleary, Taravel & Cheetham, 1982; Sims, Craik & Bacic, 1997; Tenkanen et al., 1997). On the basis of this NMR analysis, it can be concluded that fractions **8** and **9** consist of a linear β-(1 → 4)-linked glucomannan, *O*-acetylated at some of the C–2 and C–3 positions of mannose. The data suggest the presence of α-(1 → 6)-linked D-galactosyl side-groups. Both the ^{1}H NMR spectra and chemical shifts reported here are in good agreement

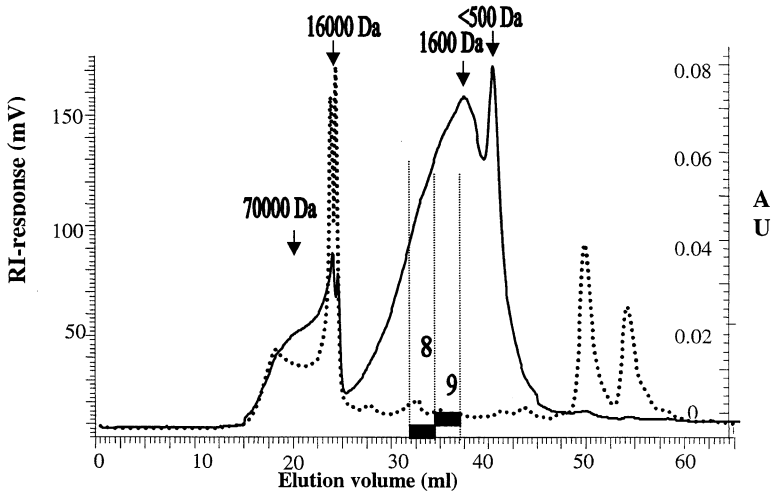

Fig. 6. RI- (filled) and UV (dotted) chromatograms for material after heat fractionation (200°C for 2 min) of spruce impregnated with water (AU: Absorbance units). SEC analysis with Superdex 75 and Superdex 200 columns as described in materials and methods. The applied sample volume was 500 μl. The arrows mark the elution volumes of dextran standards of 70 000, 16 000 Da, 1600 and mannotriose (504 Da) coeluted with pentose and hexose monomers close to the total column volume. Fractions **8** and **9** were collected at the volumes indicated by the vertical dotted lines (approx.0.70 and 0.75 column volumes, respectively).

with corresponding spectra and values published earlier for acetylated glucomannan from fibre flax (van Hazendonk, Reinerink, de Waard & van Dam, 1996).

Two doublets, with narrower linewidths than the signals originating from *O*-acetyl-galactoglucomannan, were also observed in the fingerprint region (Fig. 8). Two structural elements can be identified from the cross-peaks in the COSY and TOCSY spectra: β-xylose (H-1,2,35: δ 4.49, 3.32, 3.40, 3.58, 3.79, 4.12) and unknown (H-1,2,34: δ 4.64, 3.69, 3.77, 4.16). These peaks probably originate from a minor contamination of other saccharides.

3.4. Enzymatic hydrolysis

The action of *Aspergillus niger* endo-(1 → 4)-β-mannanase on the isolated *O*-acetyl-galactoglucomannans was studied by ^{1}H NMR spectroscopy by analyzing the whole hydrolysate obtained after extensive enzyme treatment. As we previously have shown for mannopentaose hydrolysis (Ademark et al., 1998), an increase of the mannose unit reducing end α and β signals was observed in the proton NMR spectra (data not shown). The relative amount of the β-(1 → 4)-linked mannose signal was concomitantly decreased. This hydrolysis pattern is expected for endo-(1 → 4)-β-mannanases on mannan substrates (Ademark et al., 1998; Tenkanen et al., 1997), and thus convincingly shows that the *O*-acetyl-galactoglucomannan polysaccharides are partially degraded by endo-(1 → 4)-β-mannanase, and thus have β-(1 → 4)-mannosyl bonds accessible to the enzymes.

The results from the hydrolysis of the acetyl-galactoglucomannan (fraction **8**) with the *Aspergillus niger* (1 → 6)-α-galactosidase AglB showed a release of galactose (analyzed with HPAEC-PAD), further demonstrating the presence of α-(1 → 6)-linked galactosyl side groups. Furthermore, the released galactose (0.05 mg produced galactose from 4.3 mg acetyl-galactoglucomannan) correspond to a release of roughly 100% (calculated value 110%) of the galactose groups calculated on the basis of molar ratio in Table 3.

3.5. Relative amounts of the different moieties

The relative amounts of acetyl and sugar residues were determined by integration of the signals in the fingerprint region between 4.4 and 5.5 ppm (Fig. 8 and Table 3) and the degree of acetyl substitution (DS) subsequently calculated (Table 3). Similar values for the DS are obtained by integration of the signals assigned to acetyl groups at

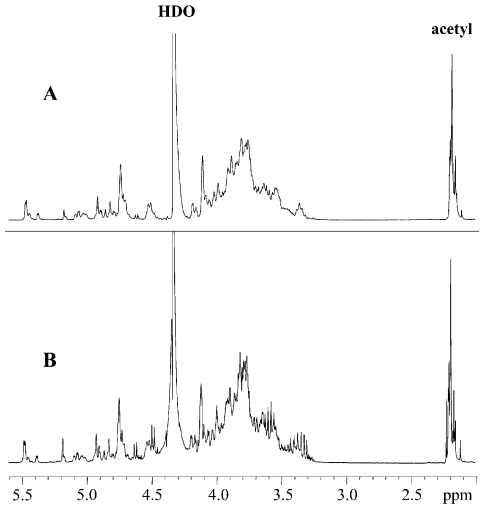

Fig. 7. ^{1}H NMR spectra of *O*-acetyl-galactoglucomannans from spruce. (a) Fraction **8**; and (b) fraction **9**.

Table 2
^{1}H NMR data on the constituent monosaccharide residues of the oligo- and polysaccharides present in Fraction **9**

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′
M red α	5.184 (1.6)	3.99	[3.76, 3.90, n.d.[c]][d]		
M red α	5.167	3.99	n.d.		
M red β	4.908	4.00	n.d.		
M	4.753 (~1)	4.12	[3.804, n.d.]		
M	4.727 (~1)	4.06	[3.653, 3.77, n.d.]		
M	4.720 (~1)	4.09	n.d.		
M	4.704 (~1)	4.03	[3.78, n.d.]		
M2	4.932 (~1)	5.477 (3.3)	4.01	[3.62, 3.78, 3.85, 3.92]	
M2	4.902 (~1)	5.449 (3.3)	3.96	[3.53, 3.75, 3.83, 3.88]	
M2	4.873 (~1)	5.383 (3.4)	3.97	[3.54, 3.79, 3.82, 3.86]	
M3	4.834 (~1)	4.19 (2.9)	5.084 (9.8)	4.06	[3.63, 3.78, 3.93]
M3	4.806	4.17	5.022	4.04	[3.75, 3.94, 3.91]
G red α	5.222 (3.6)	3.58	n.d.		
G red β	4.584	3.27	n.d.		
G	4.526 (7.7)	3.36	3.67	[3.63, 3.74, 3.86, 3.95]	
Gal	5.025	3.83	n.d.		

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

[b] Relative to an internal acetone standard at 2.225 ppm (D_2O, 70°C, pD 6.5) and acquired at 400 MHz. The following ^{1}H shifts were observed for the CH_3 groups ^{1}H chemical shift in ppm): 2.154, 2.156, 2.164, 2.171, 2.189, 2.203 and 2.209.

[c] n.d. = not detected.

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

2.2 ppm and of all carbohydrate signals. The observed ratio of galactosyl:glucosyl:mannosyl units of approximately 0.1:1:4 (Table 3) is about the same as for a typical galactoglucomannan with a low galactosyl content found in softwoods (Timell, 1967). This galactoglucomannan fraction is often referred to as glucomannan.

About one-third of the D-mannosyl units are substituted by *O*-acetyl groups almost equally distributed between the C-2 and C-3 positions. It is unclear whether this reflects the distribution present in the wood itself or if this is due to *O*-acetyl migration (Garegg, 1965) occurring during the isolation procedure.

The DS values obtained in this study (Table 3) are somewhat lower than earlier reported values of DS 0.36 for pine glucomannan (Lindberg, Rosell & Svensson, 1973) and DS 0.32 for spruce galactoglucomannan (Tenkanen, Puls, Rättö & Viikari, 1993).

3.6. Approximate degree of polymerization

In the proton NMR spectra, resonances originating from reducing end residues are seen (Fig. 8). Comparison of the integration of the anomeric proton of the reducing end residues with those of the non-reducing residues allows to calculate approximate DP of ~20 and ~11 for the polysaccharides present in fractions **8** and **9**, respectively. The fact that the polysaccharides present in fraction **8** are larger than those in fraction **9** agrees well with their order of elution in connection with size-exclusion chromatography. The obtained DP values correspond to molecular masses somewhat lower than those found in the SEC analysis (Fig. 6). However, the molecular masses (Fig. 6) are estimations since they are determined by calibration with linear dextrans, probably not possessing the same hydrodynamic volume as the hemicellulose polysaccharides.

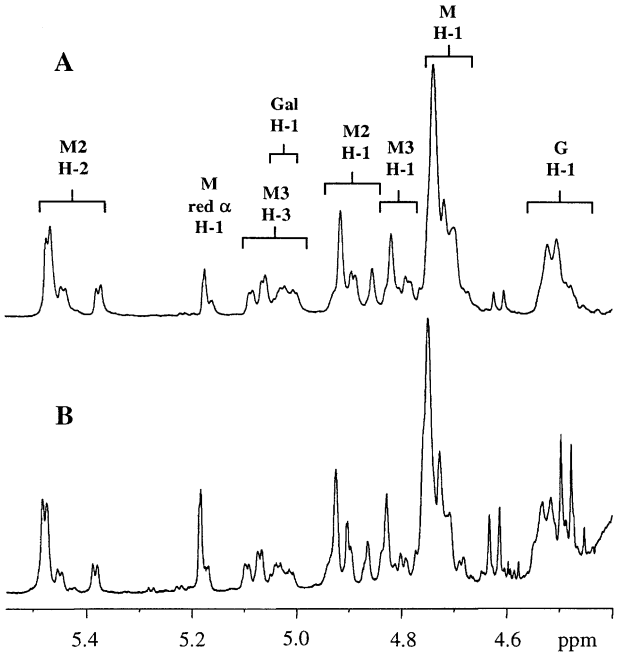

Fig. 8. The fingerprint region of ^{1}H NMR spectra of *O*-acetyl-galactoglucomannans from spruce. (a) Fraction **8**; and (b) fraction **9**. See footnote a in Table 2 for an explanation of the peak designations.

Table 3
Relative amounts[a] of acetyl groups and monosaccharide residues in galactoglucomannans extracted from spruce

Fraction	Structural element (mol%)				Degree of substitution
	Man[b]	Glc	Gal[c]	Acetyl	
8	62	15	1	22	0.28
9	64	16	1	20	0.25

[a] Determined by integration of quantitative ^{1}D NMR spectra.
[b] The mannose substituted by *O*-acetyl groups has about 55 and 45% of the acetyl groups at the C-2 and C-3 positions, respectively.
[c] Approximate value, due to overlap with the mannose resonances.

4. Conclusions

The same trends, for the dissolved carbohydrates obtained from heat fractionation were found for alkali and water impregnation of the spruce chips: increasing temperature and residence time increases the yield of mannan (oligo- and polysaccharides) and decreases the M_w of the saccharides. The major difference found is that for alkali impregnated spruce with concentrations of 0.1–2%, the yield of dissolved mannan is lower.

The polysaccharides isolated here (heat fractionation at 200°C for 2 min) from water impregnated spruce are galactoglucomannans. The relative sugar and acetyl composition of these polysaccharides is the same as for a typical *O*-acetyl-galactoglucomannan with low galactose substitution (Shimizu, 1991; Timell, 1967). It thus appears that few acetyl groups are removed by the microwave oven treatment and subsequent size fractionation by SEC. However, the degree of polymerization obtained in this study is lower than for a natural softwood galactoglucomannan (Shimizu, 1991; Timell, 1967), indicating fragmentation of the polysaccharide probably during the heat fractionation. The heat fractionation using water impregnation should be a feasible method for further optimization in order to obtain higher yield and higher molecular weight *O*-acetyl-galactoglucomannan in order to be able to further study the structure and the properties of this polysaccharide.

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