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Galactoglucomannan from the secondary cell wall of *Picea abies* L. Karst

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Abstract

The fine structural features of alkali-extracted galactoglucomannan composed of D-galactose, D-glucose and D-mannose in a 1:8:33 mole proportion from the secondary cell walls of *Picea abies* L. Karst have been determined. Compositional and methylation analyses of the polymer, partial acid hydrolysis, as well as ^{1}H and ^{13}C NMR measurements of the polymer and products of partial acid hydrolysis confirmed a β-(1→4)-linked backbone of galactoglucomannan containing the segments of mannosyl residues (Man_2, Man_3, Man_4, etc.) interrupted with the segments having both mannose and glucose residues, as well as the segments in which D-Glc*p* units can be adjacent to each other (Glc_2). Further, the low content of branching points (~3%) at the positions of O-6, O-3 and O-2 of mannosyl and O-6 and O-3 of glucosyl residues, but preferably of mannosyl ones, indicates the presence of short side-chains terminated at position O-6 predominantly by D-galactose units as single stubs.

Keywords: *Picea abies* L. Karst; Secondary cell walls; Galactoglucomannan

1. Introduction

(Galacto)glucomannans are significant structural components of the secondary cell-walls of gymnosperms and angiosperms and were extensively studied in the 1960s [1–3]. Polysaccharides of this type have been found to lesser extent in other plant species, such as mosses, ferns, legumes [1], blackberry and tobacco and are also secreted into the culture medium by *Rubus fruticocus*, *Nicotiana tabacum* and *N. plumbaginifolia* suspension-cultured cells [4–7].

In general, two kinds of galactoglucomannans (GGMs), namely water-soluble and alkali-soluble, have been isolated from the secondary cell walls of angiosperms and gymnosperms [1]. Both consist of (1→4)-linked β-D-mannopyranosyl and β-D-glucopyranosyl residues, carrying single α-D-galactopyranosyl units at the O-6 position of D-mannose or D-glucose residues. In GGM from *Larix leptolepis*, the galactosyl stubs are attached to O-2 and O-3 of D-mannosyl units [8]. The side chains of GGMs from primary cell walls and extracellular polymers were shown to be composed of either single α-D-galactopyranosyl units or β-D-Gal*p*-(1→2)-D-α-Gal*p*-(1→ dimers. GGMs from the secondary cell walls differ mutually in the molar proportions of D-mannose and D-glucose residues, in D-galac-

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tose contents in the side chains and in molecular weights, as well as in the content and distribution of acetyl groups in the molecule. The proportions of D-glucose and D-mannose residues in gymnosperms varied between 1:3 and 1:4 [1], while GGMs from the primary cell walls and polysaccharides secreted into the extracellular space by suspension-cultured cells contained approximately equal proportions of these residues [7]. GGMs rich in D-galactose residues or carrying a higher content of these sugars in the side chains are water-soluble, while the GGMs with D-galactose units under 4–5% are less or insoluble. The solubility of these hemicelluloses depends on the content of *O*-acetyl substituents in the molecule. The content of *O*-acetyl groups in water-extractable polysaccharides (in alkali-extracted GGMs the *O*-acetyl groups are cleaved) varies widely. Hardwoods contain a higher proportion of acetyl groups (3–7%) than softwoods (1–2%) [9]. About 20–30% of the backbone units in GGM from softwood are substituted by acetyl groups [10]. In pinewood polysaccharide the *O*-acetyl groups were found on mannose residues equally distributed between the C-2 and C-3 positions [11]. Others reported [12] that 16% of mannose and 6% of glucose units in pine glucomannan carry acetyl groups, but only at the C-3 position. It seems that *O*-acetyl substitution of the backbone appears to be common on the GGMs from the secondary cell-walls of conifers [13].

In our previous work [14–16] we have found that GGM from poplar and spruce is not only the structural constituent of the secondary cell-wall of gymnosperms and angiosperms, but lower fragments of this polymer (oligosaccharides) showed biological activity in elongation growth induced by auxin, in some morphogenic processes and in regenerating protoplasts. In view of this, as well as the fact that most present knowledge of the structure of this complex macromolecule is based only on the results of methylation analysis and periodate oxidation [17], we were prompted to focus attention on reinvestigating the alkali-extracted GGM from the secondary cell walls of *Picea abies* L. Karst, using mass spectrometry and ^{13}C and ^{1}H NMR spectroscopy of the oligosaccharides released by partial acid depolymerization of the polysaccharide.

2. Experimental

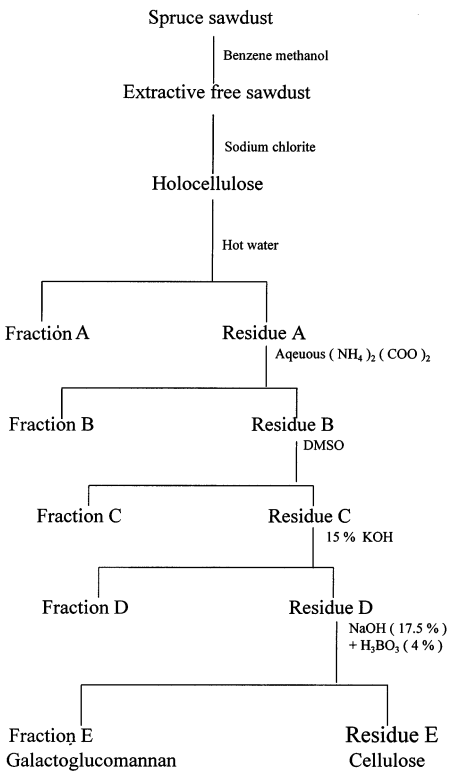
Plant material.—Sawdust was prepared from the trunk of the spruce (*P. abies* L. Karst) cultivated in Male Karpaty, Slovak Republic.

General methods.—Solutions were concentrated under diminished pressure below 40 °C. Free-boundary electrophoresis of a 1% solution of the polysaccharide was effected with a Zeis 35 apparatus, using 0.05 M sodium tetraborate buffer (pH 9.2) at 150 V/cm and 6 mA for 30 min. The number average molecular mass (M_n) was determined osmometrically at 30 °C, using a Knauer vapor-pressure osmometer. Infrared spectra of the methylated products were recorded with a Nicolet Magna 750 spectrometer. Polysaccharides were hydrolyzed with 2 M trifluoroacetic acid for 1 h at 120 °C.

Preparative paper chromatography was performed using the descending method on Whatman No. 3MM papers with the systems S1, 9:2:2 ethyl acetate–acetic acid–water and S2, 10:3:3 *n*-butanol–pyridin–water. The saccharides were detected with anilinium hydrogenphthalate. Thin-layer chromatography (TLC) was carried out on Kieselgel 60 (E. Merck, Germany) in the solvent system S3, 2:3:1 *n*-butanol–formic acid–water. The saccharides were visualized by spraying the plates with 20% aq $(NH_4)_2SO_4$ and heating at 200 °C. The uronic acid content was determined by potentiometric titration and spectrophotometrically with the 3-hydroxybiphenyl reagent [18]. Quantitative determination of the neutral sugars was carried out in the form of their trifluoroacetates [19] by gas chromatography on a Hewlett–Packard Model 5890 Series II chromatograph equipped with a PAS-1701 column (0.32 mm × 25 m) at the temperature program of 110–125 (2 °C/min) – 165 °C (20 °C/min) and flow rate of hydrogen 20 mL/min. Gas chromatography–mass spectrometry of partially methylated alditol acetates [20] was carried

out on a Finnigan MAT SSQ 710 spectrometer equipped with an SP 2330 column (0.25 mm × 30 m) at 80–240 °C (6 °C/min), 70 eV, 200 μA, and ion-source temperature 150 °C.

Isolation of the GGM.—The modified procedure of Mills and Timell [21] and Karacsonyi et al. [22] for isolation of the GGM was used. The air dried sawdust (500 g) prepared from the trunk of spruce (*P. abies* L. Karst) was exhaustively extracted with 2:1 (w/w) benzene–methanol and delignified by the sodium chlorite method [23]. The holocellulose was extracted twice with boiling water (5 L) for 3 h. The aq extracts were combined, concentrated, dialyzed and freeze-dried (Fr. A). The residue A was further extracted twice with 0.5% aq ammonium oxalate (5 L) for 3 h at 60 °C. The combined ammonium oxalate solutions were concentrated, dialyzed and freeze-dried (Fr. B). The depectinated residue B was washed with distilled water and extracted twice with Me_2SO (5 L) for 48 h at laboratory temperature. The Me_2SO extracts were combined, exhaustively dialyzed, concentrated and freeze-dried (Fr. C). The remaining residue C after washing with water was treated twice with 15% aq KOH (5 L) containing a 10 mM solution of $NaBH_4$ for 2 h at laboratory temperature. The alkaline extracts were cooled, neutralized with 4 M AcOH, concentrated, dialyzed and freeze-dried (Fr. D). The residue D remaining after extraction with 15% aq KOH was finally treated twice with 17.5% aq NaOH containing 4% boric acid (5 L) and 10 mM solution of $NaBH_4$ for 2 h at laboratory temperature. The extracts were recovered in the same way as above to give a crude GGM (Fr. E). The remaining material (residue E) after the final extraction afforded a cellulose (Scheme 1).



Scheme 1.

The crude GGM (Fr. E) was dissolved in NaOH solution and purified by precipitation with saturated aq $Ba(OH)_2$. The precipitate was centrifuged, suspended in water, neutralized with AcOH, dialyzed and after freeze-drying the GGM was subjected to structure analysis.

Partial acid hydrolysis of the GGM.—The GGM was partially hydrolyzed with 0.4 M trifluoroacetic acid for 70 min at 100 °C. Trifluoroacetic acid was evaporated and a mixture of mono- and oligo-saccharides was separated on a column (200 × 2.5 cm) of Bio-Gel P-2 by water elution. Fractions of 5 mL were collected and analyzed for the carbohydrate content by phenol–H_2SO_4 assay [24]. The elution profile of GGM-derived oligosaccharides (GGMOs) is shown in Fig. 1. Their degree of polymerization (dp) was identified by com-

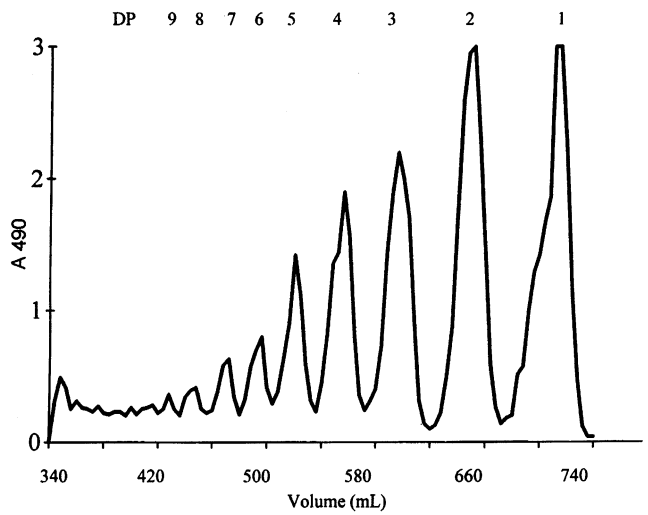


Fig. 1. Gel-filtration pattern (Bio-Gel P-2) of the GGM-derived oligosaccharides.

parison with the elution volumes of maltooligosaccharides (Serva, Germany) used as reference standard.

PPC and HPLC chromatography of the GGMOs.—The oligosaccharides with dp **2** and **3** from gel-filtration column (Bio-Gel P2) were dissolved in distilled water and separated by PPC in the systems S_1 (dp **2**) and S_2 (dp **3**). The individual fractions $\mathbf{2_1}$–$\mathbf{2_4}$ and $\mathbf{3_1}$–$\mathbf{3_4}$ were further purified on a Hewlett–Packard Series 1050 using a preparative column (2.15 × 30 cm) of TSK-GEL AMIDE-80 at a flow rate of 10 mL min^{-1}. Oligosaccharides were eluted with 65:35 (v/v) acetonitrile–water and monitored by an RI detector.

Monosaccharide analysis.—The constituent monosaccharides of the cell-wall polymers were identified after prehydrolysis by 13 M H_2SO_4 for 1 h followed by hydrolysis with 1 M H_2SO_4 at 100 °C for 3 h, or with 2 M trifluoroacetic acid at 120 °C for 1 h and reduction, in the form of their trifluoroacetates [19] by gas chromatography. The absolute configurations of the monosaccharides were established using the method of Gerwig et al. [25].

Methylation analysis.—The dry samples of oligo-(~ 1 mg) and polysaccharides (~ 5 mg) were solubilized in dry Me_2SO (1 mL) and methylated by the Hakomori method [26]. The methylated products were purified using a Sep-Pak C_{18} cartridge (Waters Assoc.) to give a fully methylated carbohydrates. The permethylated samples were hydrolyzed with 90% formic acid (1 h, 100 °C) and 2 M trifluoroacetic acid (1 h, 120 °C), reduced with sodium borodeuteride, acetylated and analyzed by GLC–MS. A mixture of disaccharides was reduced with borodeuteride, freeze-dried, and methylated as above. Fully methylated disaccharide alditols were analyzed by GLC–MS.

NMR spectroscopy.—The saccharides were dissolved in 0.5 ml D_2O (99.99 atom%) in 5-mm tubes. Spectra were recorded at 25 °C, on a Bruker DPX Avance 300 spectrometer equipped with a selective exitation unit and gradient-enhanced spectroscopy kit, for generation of *z*-gradients, operating at a 300 MHz for ^1H and 75.46 MHz for ^{13}C. The acetone was used as internal standard (δ 2.225 ppm for ^1H and 31.07 ppm for ^{13}C). The following pulse programs were used: 2D DQF COSY [27], TQF COSY [28] and a 1D TOCSY [29] sequence with pulse field gradients. In HSQC [30] experiment the signal of water was suppressed using pulse sequence with pulse field gradients. An HMBC [31] pulse program was applied with low- pass J-filter to suppress one bond correlations. The data matrices for the COSY experiment were processed with sine window functions in both dimensions. For HSQC and HMBC experiments a multiplication with squared sine function was applied. All processing was performed using Bruker software XWIN-NMR version 1.3 on a Silicon Graphics INDY computer system.

3. Results and discussion

Isolation of the GGM.—Sawdust prepared from the trunk of *P. abies* L. Karst was delipidized with benzene–methanol and delignified by the chlorite method [23]. Holocellulose was successively extracted with hot water (A), hot 0.5% aqueous ammonium oxalate (B), Me_2SO (C), aqueous 15% potassium hydroxide (D). A final step with aqueous 17.5% sodium hydroxide containing 4% boric acid (E) afforded a crude GGM and cell-wall residue (Scheme 1). The yields, protein content, and monosaccharide composition of the individual polymeric fractions released from spruce cell walls are shown in Table 1. The solubilized fractions (A–E) differed in the protein content, in the composition of the constituent neutral sugars, as well as in the uronic acid content. Fraction E was partly contaminated with xylan-related polymers, and therefore was purified in the next step with saturated aqueous barium hydroxide solution to give a GGM as the strongest bound hemicellulose polymer of angiosperms and gymnosperms. It represents about 9% of the delipidized and delignified spruce cell wall. GGM was proved to be homogeneous on free-boundary electrophoresis and HPLC, and had $M_n = 40{,}500$ (dp = 250). Monosaccharide analysis of GGM revealed the presence of D-galactose, D-glucose and D-mannose in 1:8:33 mol proportion. Water soluble GGM

Table 1
Extraction of polysaccharides from the secondary cell walls (CW) of *P. abies* L. Karst

Fractions	Extractant	Yield [a] (%)	Protein (%)	Monosaccharide composition (mole%)						
				Rha	Ara	Xyl	Man	Glc	Gal	UA
CW		100	nd [b]	tr [c]	4.3	1.6	41.5	49.4	3.2	nd
A	H_2O	6.3	–	tr	6.1	30.2	31.1	8.9	8.9	14.8
B	aq 0.5% $(NH_4)_2(COO)_2$	1.0	14.4	tr	5.9	24.0	34.9	8.7	7.5	19.0
C	DMSO	1.8	–	–	5.9	49.9	24.6	8.4	2.1	9.1
D	aq 15% KOH	14.8	1.2	–	11.0	56.9	8.3	2.1	4.1	17.6
E	aq 17.5% NaOH+4% H_3BO_3	10.7	1.2	–	1.1	10.9	67.1	18.7	2.1	tr
F	Cell wall residue	64.8	nd	–	–	1.4	3.4	93.6	1.6	nd

[a] Based on lipid and lignin free cell walls.
[b] Not determined.
[c] Traces.

isolated from fraction A contained the *O*-acetyl groups in its molecule. The yield of this polymer (~0.1–0.2%) was very low in comparison with alkali solubilized GGM (~9%) in fraction E. This polysaccharide may contain in its native state the *O*-acetyl groups as well; however, upon exposure to alkali these groups were cleaved.

Partial acid depolymerization of GGM.—In order to obtain oligomeric fragments from the polymer for its structural studies, the native GGM was partially hydrolyzed with TFA and the hydrolyzate mixture was further separated on a Bio-Gel P-2 column to nine distinct fractions. The elution profile of the GGMOs is shown in Fig. 1. It is evident that the major components of this hydrolyzate are oligomers of dp **2**, **3** and **4**, which represented 39, 28 and 23% of fractions **2**–**8**, respectively. Compositional analysis of the GGMOs of dp **2**–**8** revealed the presence of D-galactose, D-glucose and D-mannose in a different molar ratio but with dominant content of the last sugar. Increasing dp of oligosaccharides increases the content of D-galactose while the content of D-mannose decreases (Table 2).

PPC and HPLC chromatography showed that GGMOs of dp **2**, **3**, and **4** were mixtures of more oligosaccharide components. In order to identify the individual oligomers present in the fractions **2** and **3**, the oligosaccharides were separated by a combination of PPC and HPLC, and thus oligomers $\mathbf{2_1}$, $\mathbf{2_2}$, $\mathbf{2_3}$ and $\mathbf{2_4}$ and $\mathbf{3}_1$, $\mathbf{3_2}$, $\mathbf{3_3}$ and $\mathbf{3_4}$, respectively, were obtained. Compositional analysis of the purified oligomers revealed the presence of D-Man (51.7%), D-Glc (46.3%) and D-Gal (2.0%) in $\mathbf{2_1}$; D-Man (98.5%) and D-Glc (1.5%) in $\mathbf{2_2}$; D-Man (15.3%), D-Glc (72.0%) and D-Gal (12.8%) in $\mathbf{2_3}$; D-Man (48.6%), D-Glc (46.7%) and D-Gal (4.7%) in $\mathbf{2_4}$; D-Man (63.5%), D-Glc (34.7%) and D-Gal (1.8%) in $\mathbf{3_1}$; D-Man (70.9%) and D-Glc (29.1%) in $\mathbf{3_2}$; D-Man (92.1%), D-Glc (5.6%) and D-Gal (2.3%) in $\mathbf{3_3}$; and D-Man (60.6%), D-Glc (36.1%) and D-Gal (3.3%) in $\mathbf{3_4}$. The dimers $\mathbf{2_1}$, $\mathbf{2_2}$ and $\mathbf{2_4}$ were only partly contaminated, while compound $\mathbf{2_3}$ was composed of D-glucose, D-galactose and D-mannose residues confirming the presence of more dimers. D-glucose was found to be a dominant component of this fraction. The

Table 2
Degree of polymerixation (dp), yield (%) and sugar composition of GGMOs obtained by gel-filtration chromatography (Fig. 1)

Oligomer (dp)	Yield [a] (%)	Monosaccharide composition (mol%)		
		D-Man	D-Glc	D-Gal
2	39.4	76.6	22.0	1.4
3	28.2	77.2	21.6	1.2
4	22.5	75.7	22.5	1.8
5	5.7	73.5	23.9	2.7
6	2.7	66.6	30.7	2.7
7	1.2	72.4	24.4	3.2
8	0.3	68.5	29.0	2.5

[a] In percent of total amount of fractions **2**–**8**.

Table 3
Methylation analysis data of GGM and GGMOs of dp 2 ($\mathbf{2_1}$, $\mathbf{2_2}$, $\mathbf{2_3}$ and $\mathbf{2_4}$) and **3** ($\mathbf{3_1}$, $\mathbf{3_2}$, $\mathbf{3_3}$ and $\mathbf{3_4}$)

Sugar derivative	Mol%											Mode of linkage
	GGM	**2**	$\mathbf{2_1}$	$\mathbf{2_2}$	$\mathbf{2_3}$	$\mathbf{2_4}$	**3**	$\mathbf{3_1}$	$\mathbf{3_2}$	$\mathbf{3_3}$	$\mathbf{3_4}$	
2,3,4,6-Me_4-Man [a]	1.9	38.7	–	50.1	4.2	47.0	28.8	16.8	34.0	29.3	32.3	Man*p*-(1→
2,3,4,6-Me_4-Glc	–	10.3	50.9	–	39.4	–	0.6	18.1	–	–	–	Glc*p*-(1→
2,3,4,6-Me_4-Gal	2.2	1.5	–	–	5.7	3.2	0.9	tr.	–	1.4	1.7	Gal*p*-(1→
2,3,6-Me_3-Man	74.4	42.0	48.1	48.3	7.2	3.4	49.2	50.2	33.9	60.9	30.2	→4)-Man*p*-(1→
2,3,6-Me_3-Glc	18.3	6.2	0.5	1.6	37.8	45.3	19.2	14.9	31.8	4.7	33.5	→4)-Glc*p*-(1→
2,3,4-Me_3-Man	tr.	1.0	–	–	3.1	–	0.7	–	–	–	2.3	→6)-Man*p*-(1→
2,3,4-Me_3-Glc	tr.	0.3	–	–	–	–	tr.	–	–	–	tr.	→6)-Glc*p*-(1→
2,3,4-Me_3-Gal	tr.	tr.	0.5	–	2.6	1.1	–	–	–	1.0	–	→6)-Gal*p*-(1→
2,6-Me_2-Man	0.2	–	–	–	–	–	–	–	–	0.8	tr.	→3,4)-Man*p*-(1→
2,6-Me_2-Glc	0.1	–	–	–	–	–	–	–	–	–	–	→3,4)-Glc*p*-(1→
3,6-Me_2-Man	0.4	–	–	–	–	–	–	–	–	0.9	tr.	→2,4)-Man*p*-(1→
2,3-Me_2-Man	1.8	–	–	–	–	–	0.4	–	–	1.0	tr.	→4,6)-Man*p*-(1→
2,3-Me_2-Glc	0.6	–	–	–	–	–	tr.	–	0.3	–	–	→4,6)-Glc*p*-(1→
2,3-Me_2-Gal	tr.	–	–	–	–	–	–	–	–	–	–	→4,6)-Gal*p*-(1→
2,4–Me_2-Man	tr.	–	–	–	–	–	–	–	–	–	–	→3,6)-Man*p*-(1→

[a] 2,3,4,6-Me_4-Man = 1,5-di-*O*-acetyl- 2,3,4,6-tetra-*O*-methyl-mannitol, etc.

hydrolytic products of the borohydride-reduced dimer fractions proved the reducing end positions of mannose in $\mathbf{2_1}$ and $\mathbf{2_2}$, glucose and mannose in $\mathbf{2_3}$ and glucose in $\mathbf{2_4}$. In trisaccharide moieties the compound $\mathbf{3_2}$ was homogeneous, while other oligomers were only partly contaminated with other ones. The reducing units mannose in $\mathbf{3_1}$, $\mathbf{3_2}$ and $\mathbf{3_3}$ and glucose in $\mathbf{3_4}$ were determined.

Methylation analysis of GGM.—Linkage analysis of GGM showed two main sugar derivatives, namely 4-linked mannose and glucose units and pointed to a linear structure of the polymeric chains in GGM (Table 3). About 3% of the residues were involved in branched points. Low portions of 4,6-linked mannose (1.8%) and glucose (0.6%) residues indicate the prevalence of mannosyl units in branched points of the polymer. Besides, the minor proportions of 4-linked mannose residues were found to be branched points at position O-2 (0.4%) and O-3 (0.2%), while 4-linked glucose units were branched only at O-3 (0.1%). In addition to the above-mentioned 4-linked residues, trace amounts of 3,6-linked mannose and 6-linked glucose residues were also found. Galactose was found to occur predominantly at the nonreducing terminal (2.2%), 4,6-(traces), and 6-linked (traces) positions in the polymer. The detected derivatives in the table demonstrate that the ring form of all saccharide components was pyranose.

Methylation analysis of GGMOs.—The results of the linkage analysis of the mixture of GGM-derived oligosaccharides dp **2** and **3**, as well as their purified components are shown in Table 3. The prevalence of terminal and 4-linked mannose and glucose residues in these oligomers is evident. Linkage analysis of the purified oligomer fractions showed the terminal glucose and 4-linked mannose residues in $\mathbf{2_1}$, the terminal and 4-linked mannose units in $\mathbf{2_2}$, and the terminal mannose and 4-linked glucose units in $\mathbf{2_4}$. The terminal and 4-linked glucose as the dominant derivatives in $\mathbf{2_3}$ confirms the presence of cellobiose. A minor portion of the terminal galactose, 6-linked mannose and glucose residues with a slight preference for mannose indicates the presence of galactosylated dimers.

In order to determine the number and molar proportions of the individual compounds in **2** the oligomers in this fraction were reduced, methylated and analyzed by GLC–MS. Six permethylated disaccharide alditols were determined and their retention times were compared with the retention times of disaccharide alditols (Glc-(1→4)-Glc-ol, Man-(1→4)-Glc-ol, Glc-(1→4)-Man-ol, Man-(1→

4)-Man-ol, Gal-(1→6)-Man-ol and Gal-(1→6)-Glc-ol) used as reference standards. The mobilities of the individual compounds in **2** were identical with the retention times of cellobiitol ($\mathbf{2_3}$), Man-(1→4)-Glc-ol ($\mathbf{2_4}$), Glc-(1→4)-Man-ol ($\mathbf{2_1}$), mannobiitol ($\mathbf{2_2}$), Gal-(1→6)-Glc-ol and Gal-(1→6)-Man-ol (a minor portion of $\mathbf{2_3}$). Further, a small amount of (1→6)-linked hexose alditol was determined. From the intensities of peaks it was evident that mannobiose ($\mathbf{2_2}$) was the dominant compound (68.8%) of **2**. The other components as $\mathbf{2_1}$, $\mathbf{2_4}$, $\mathbf{2_3}$, Gal-(1→6)-Glc-ol and Gal-(1→6)-Man-ol (a minor portion of $\mathbf{2_3}$) and (1→6)-linked hexose alditol were recovered in 16.6, 8.6, 3.9, 1.4 and 0.7% yield, respectively.

Linkage analysis of the individual oligomeric components of dp **3** showed the prevalence of terminal and 4-linked mannose units and only in oligomer $\mathbf{3_1}$ was glucose identified in the terminal non-reducing position. The equal proportions of methylated derivatives in $\mathbf{3_2}$ (Table 3), and finding that mannose residue occupies reducing and non-reducing positions indicates the presence of a Man-(1→4)-Glc-(1→4)-Man sequence. High contents of 4-linked and terminal mannose residues suggest mannotriose [Man-(1→4)-Man-(1→4)-Man] as the dominant component in $\mathbf{3_3}$. Low portions of terminal galactose, 4-linked glucose, 6-linked mannose, as well as 4,6-linked mannose and glucose residues point to the presence of galactosylated trimers in $\mathbf{3_3}$. The equimolar portions of terminal mannose, 4-linked mannose and glucose residues, and the finding that glucose residue occupies only the reducing end confirmed the Man-(1→4)-Man-(1→4)-Glc sequence of the main component in $\mathbf{3_4}$. Minor portions of terminal galactose, 6-linked mannose and glucose residues point to the presence of galactosylated oligomers as well. The main components of trimers were compounds $\mathbf{3_3}$ (38%) and $\mathbf{3_1}$ (31%), and the other oligomers, i.e., $\mathbf{3_2}$ and $\mathbf{3_4}$ were recovered in 18 and 13% yields, respectively.

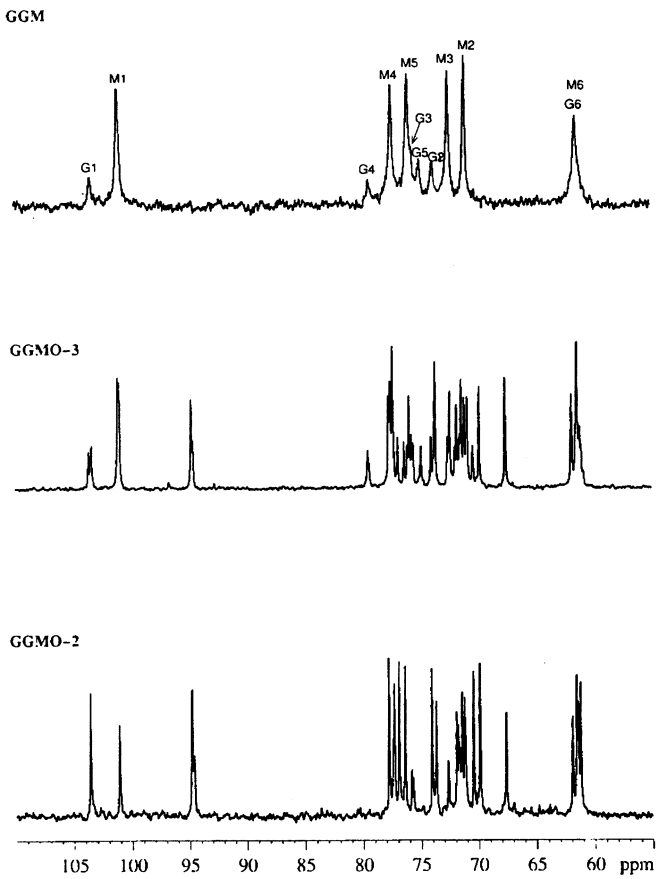


Fig. 2. ^{13}C NMR spectra of GGM and GGM-derived oligosaccharides of dp 2 and 3.

NMR spectroscopy of non-purified GGMOs.—In the studied oligomeric fractions **2** and **3**, the ^{1}H NMR anomeric signals showed the presence of mannopyranose and glucopyranose units. In fraction **2** the anomeric signals at 4.73, 5.17 and 4.90 ppm were assigned to the terminal D-Man*p*, and to the reducing ends of α and β D-Man*p* residues, respectively, and the main methylated derivative of this fraction 4-linked Man*p* (Table 3), confirmed the presence of the dominant dimer Man*p*-(1→4)-Man*p* in **2**. The relative intensity of ^{1}H signals of Man/Glc was 2.6:1. An anomeric signal at 4.49 ppm ($J_{1,2} = 8.2$ Hz) was attributed to a terminal D-Glc*p* in the β-configuration of glycosidic linkage [32] and indicates the presence of D-Glc*p*-β-(1→4)-Man*p* in this fraction. The above-mentioned anomeric signals were also found in the ^{1}H NMR spectrum of fraction **3** [7].

The ^{13}C NMR spectra of oligomeric fractions **2** and **3** (Fig. 2) showed signals in the anomeric region (105–95 ppm) showing only the presence of mannopyranose and glucopyranose units. The resonances at 79.50 and 77.55 ppm indicate 4-linked β-D-Glc*p* and D-Man*p* units, respectively [33–35]. The value of

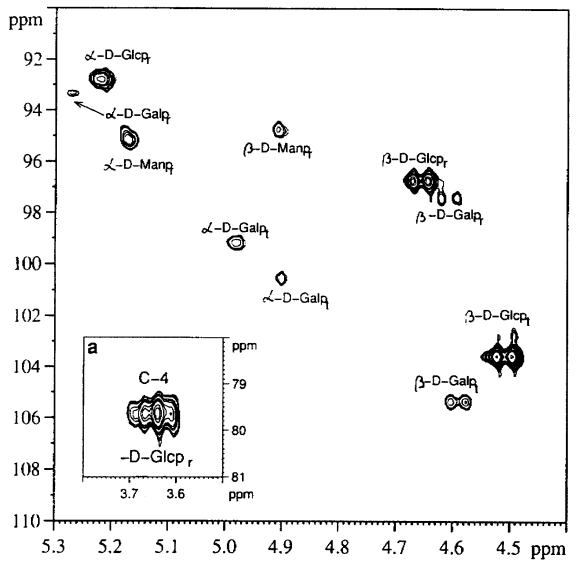


Fig. 3. The anomeric region of HSQC spectrum of the purified fraction 2_3. The inserted part (a) represents C-4 of the reducing Glc*p* residue.

$J_{C1H1} = 160.2$ Hz estimated from a ^{13}C-proton coupled 2D HSQC experiment gives evidence about β-linked D-Man*p* units in all GGMOs moieties [36]. In non-purified fractions **2** and **3** was possible to determine the presence of dominant components only.

NMR spectroscopy of purified GGMOs.— The mixture of oligomers of dp **2** was purified into four dimer components-$\mathbf{2_1}$, $\mathbf{2_2}$, $\mathbf{2_3}$ and $\mathbf{2_4}$. The ^{1}H and ^{13}C spectra of purified dimers were measured and signals of diagnostic value assigned using the pulse programs presented in Section 2.

Oligomer $\mathbf{2_1}$ yielded on hydrolysis D-glucose, D-mannose and a trace of D-galactose. The characteristic anomeric signals H-1/C-1 at 5.17/94.7 and 4.90/94.50 ppm were assigned to reducing α and β ends of D-Man*p* residues and doublets at 4.49 ppm ($J_{1,2} = 8.3$ Hz) and C-1 at 103.56 ppm were attributed to a terminal β-D-Glc*p* residue. The presence of 4-linked Man*p* derivative found in $\mathbf{2_1}$ (Table 3) and cross peaks (H-1/C-4) in the HMBC spectrum at 4.49/77.76 and 77.38 ppm confirmed the following structure of the dimer: D-Glc*p*-β-(1→4)-D-Man*p*.

Saccharide composition of $\mathbf{2_2}$ revealed on hydrolysis the presence of D-mannose and traces of D-glucose units. The anomeric region of $\mathbf{2_2}$ shows the chemical shifts (H-1/C-1) at 4.72/101.07 for terminal and the same characteristic shifts for reducing α and β ends of D-Man*p* residues as at oligomer $\mathbf{2_1}$. The cross peaks (H-1/C-4) in the HMBC spectrum at 4.49/77.76 and 77.38 ppm, and 4-linked Man*p* derivative from linkage analysis give evidence of β-(1→4) linkage between mannopyranose residues in D-Man*p*-β-(1→4)-D-Man*p*.

In purified fraction $\mathbf{2_3}$ D-glucose was found to be a dominant component, while D-galactose and D-mannose residues were present in small proportions. The relative intensities of anomeric proton signals in ^{1}H spectrum of $\mathbf{2_3}$ showed over 60% of D-Glc*p* moieties. The compositional and sugar linkage analyses (Table 3) showed that $\mathbf{2_3}$ was a mixture of more dimers with prevalence of D-glucose units. The anomeric signals in the HSQC spectrum (Fig. 3) of $\mathbf{2_3}$ observed at 4.50/103.5 ($J_{1,2} = 8.3$ Hz), 4.66/96.70 and 5.22/92.70 ppm were assigned to terminal and reducing α and β ends of D-Glc*p* residues, respectively. The signal at 79.50 ppm (C-4) in the spectrum (Fig. 3(a)) and a dominant component of linkage sugar analysis, 4-linked Glc*p* residue, proved the (1→4) linkage in D-Glc*p*-β-(1→4)-D-Glc*p*. The relatively high content of D-Gal*p* (5.7%) found as a terminal residue (linkage analysis) and the anomeric cross peak (H-1/C-1) in the HSQC spectrum (Fig. 3) at 4.95/99.14 ($J_{1,2} = 3.5$ Hz), as well as the presence of a 6-linked Man*p* (3.1%) derivative in linkage analysis of $\mathbf{2_3}$ suggests the α-configuration of glycosidic linkage of D-Gal*p* to →6)-D-Man*p* unit (chemical shifts of the anomeric atoms were in good agreement with the values of the model compound α-D-Gal*p*-(1→6)-D-Man*p* [7]). Very weak resonances in this spectrum (Fig. 3) at 4.92/100.50 ($J_{1,2} = 3.2$ Hz), 4.61/97.35 and 5.28/93.33 ppm ($J_{1,2} = 3.1$ Hz) and 6-linked Gal*p* derivative (2.6%) in linkage analysis indicate the trace amount of D-Gal*p*-(1→6)-D-Gal*p* dimer [7] in $\mathbf{2_3}$. Further, the signals at 4.59/105.20 ppm ($J_{1,2} = 8.0$ Hz) for terminal β-D-Gal*p*, 5.17/94.70 and 4.90/94.5 ppm for reducing α and β ends of D-Man residues, as well as the presence of terminal Gal*p* and 6-linked Man*p* derivatives in linkage analysis, suggest β-glycosidic linkage of D-Gal*p* unit in β-D-Gal*p*-(1→6)-D-Man*p*.

Oligosaccharide $\mathbf{2_4}$ formed on hydrolysis of D-mannose and D-glucose in equimolar pro-

portion and a small amount of D-galactose. The signals for anomeric atoms H-1/C-1 at 4.72/101.07 ppm (terminal β-D-Man*p* unit), the anomeric signals at 5.22/92.75 ($J_{1,2} = 3.5$ Hz) and 4.66/96.7 ppm ($J_{1,2} = 8.3$ Hz) for reducing α and β ends of D-Glcp residues, and the cross peak in the HMBC spectrum H-1/C-4 at 4.72 /77.7, gives the evidence of β-(1→4) linkage in dimer D-Man*p*-β-(1→4)-D-Glc*p*.

Purified fraction **3$_1$** was composed of D-mannose and D-glucose as the dominant sugars, and D-galactose was present only in a negligible amount. From the ^{1}H and ^{13}C NMR spectra of **3$_1$** two main oligomeric components were determined. The characteristic H-1/C-1 signals at 4.49/103.40 ($J_{1,2} = 8.1$ Hz), 4.74/101.11, 5.17/94.70 and 4.90/94.50 ppm were assigned to terminal β-D-Glc*p*, internal β-D-Man*p* and reducing α and β ends of D-Man*p* residues, respectively, in the (1→4) linkage sequence between D-Man*p* and D-Glc*p* residues (determined using an HMBC experiment) in D-Glc-β-(1→4)-D-Man*p*-β-(1→4)-D-Man*p*. It represents about 60% of **3$_1$**. The second component of **3$_1$** shows the signals in the anomeric region (H-1/C-1) at 4.72/101.00 ppm for terminal β-D-Man*p*, 4.52/103.60 ppm ($J_{1,2} = 8.2$ Hz) for internal β-D-Glc*p*, and 5.17/99.70 and 4.90/94.50 ppm for reducing α and β ends of D-Man*p* residues in the trisaccharide D-Man*p*-β-(1→4)-D-Glc*p*-β-(1→4)-D-Man*p*.

Sugar composition of compound **3$_2$** revealed on hydrolysis D-mannose and D-glucose. The ^{1}H and ^{13}C NMR spectra of **3$_2$** (the relative intensities of anomeric H-1 atoms of D-Man*p* and D-Glc*p* were in the ratio 2:1) showed the homogeneous character of this trisaccharide and its composition was identical with those of D-Man*p*-β-(1→4)-D-Glc*p*-β-(1→4)-D-Man*p*.

The fraction **3$_3$** afforded D-mannose (93.1%), D-glucose (5.6%), and small amounts of D-galactose (1.3%) on hydrolysis. The ^{1}H and ^{13}C NMR spectra of **3$_3$** revealed anomeric (H-1/C-1) signals at 4.72/101.10, 4.74/101.1, and 5.17/94.70 and 4.90/94.50 ppm attributed to terminal, internal and reducing α and β ends of D-Man*p* residues in the trisaccharide D-Man*p*-β-(1→4)-D-Man*p*-β-(1→4)-D-Man*p*. Besides, the signals of low intensities at 4.52/103.60 ppm indicate the presence of a low amount of an internal β-Glc*p* residue as a part of the trisaccharide **3$_2$**.

D-Mannose and D-glucose were the dominant sugars of the oligosaccharide fraction **3$_4$**, while D-galactose was present in a small amount (3.3%). In the ^{1}H and ^{13}C NMR spectra of **3$_4$** the anomeric signals at 4.72/101.10, 4.75/101.10, and 5.22/92.75 and 4.66/96.70 ppm were assigned to the terminal and internal D-Man*p*, and reducing α and β ends of D-Glc*p* units, respectively, in D-Man*p*-β-(1→4)-D-Man*p*-β-(1→4)-D-Glc*p*. Further, the resonances at 4.50/103.40 ppm ($J_{1,2} = 8.0$ Hz) and 4.53/103.63 ppm ($J_{1,2} = 8.1$ Hz) characteristic for terminal and internal β-D-Glc*p* residues, as well as the presence of the above-mentioned signals for terminal and internal β-D-Man*p* units, suggest two types of minor trimers with sequences of D-Man*p*-β-(1→4)-D-Glc*p*-β-(1→4)-D-Glc*p* and D-Glc*p*-β-(1→4)-D-Man*p*-β-(1→4)-D-Glc*p* in **3$_4$**. Besides, the signals of very low intensities at 5.10, 5.18 and 4.90 ppm characteristic for the anomeric atoms of terminal α-D-Gal*p* and reducing α and β ends of D-Man*p* residues, as well as the presence of terminal Gal*p* (1.7%) and 6-linked Man*p* (2.3%) derivatives in linkage analysis of **3$_4$** (Table 3) indicate the presence of galactosylated trisaccharide.

NMR spectroscopy of GGM.—The ^{13}C-NMR spectrum of the GGM is shown in Fig. 2. The signals at 101.2 (C-1), 70.9 (C-2), 72.4 (C-3), 77.5 (C-4), 75.9 (C-5) and 61.5 (C-6) ppm were assigned (based on ^{13}C NMR data of the purified oligomeric compounds described above) to 4-linked β-Man*p* units and the signals of lower intensities at 103.5 (C-1), 74.0 (C-2), 77.6 (C-3), 79.4 (C-4), 75.0 (C-5), and 61.50 (C-6) ppm to 4-linked β-D-Glc*p* residues. On the basis of the relative intensities of the anomeric C-1 signals of Man*p* and Glc*p* residues the ratio 4:1 was determined. This value is in good agreement with the results of compositional and methylation analyses. To detect the low content of D-Gal*p* residues (~ 2–3%) located in the side polysaccharide chains by ^{13}C NMR experiments was not possible.

The fine structural features of GGM from the secondary cell walls of *P. abies* L. Karst have been determined by NMR analyses of

the oligomeric fragments released by partial acid depolymerization. Sugar linkage analyses and NMR measurements of analyzed products after partial acid hydrolysis of the GGM indicated high number of oligosaccharides in individual fractions composed of D-Gal*p*, D-Glc*p* and D-Man*p* residues. Structural analyses confirmed the almost exclusively linear character of the polysaccharide chain with (1→4)-linked β-D-Glc*p* and β-D-Man*p* residues, containing segments of mannosyl residues (Man_2, Man_3, Man_4, etc.) interrupted with single glucose residues, as well as with segments containing the sequences in which D-Glc*p* units can be adjacent to each other (Glc_2). Relatively low content of branching points ($\sim 3\%$) at the positions O-2, O-3 and O-6 of mannosyl, and O-3 and O-6 glucosyl residues, but with preference to the mannosyl ones, indicates the presence of short side chains in the polymer.

The basic structural features of the alkali-extracted GGM from secondary cell walls of *P. abies* L. Karst are similar to those isolated from softwoods and hardwoods, such as *Tsuga canadensis*, *Picea Engelmannii*, *Abies amabilis*, *Populus tremuloides* (1), and *Populus monilifera* (37). Structural differences are evident in the molar proportions of D-mannose and D-glucose residues, in D-galactose contents in side chains, in degree of branching of mannosyl, glucosyl and galactosyl residues, in sequential distribution of glucosyl units, as well as in molecular weights. The occurrence of 6-linked D-Glc*p* residues, as well as 3,6-linked D-Man*p* and 4,6-linked D-Gal*p* units, although in trace amounts, indicates fine structural variabilities of GGM in various plant sources.

Developments over recent years made on the structure–activity relationship of cell-wall polysaccharides confirm their active participation in important physiological processes of plants. Their fragments as complex carbohydrates can activate the plant cell machinery and function as molecular signals involved in the regulation of growth, development and defense responses [37]. It seems that GGM, as the structural component of both primary and secondary plant cell wall may play an important role in the regulation of processes in plant cells by means of oligosaccharide fragments [14–16]. This results in the necessity to recognize the exact structural features of the biologically active molecule.

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