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Short communication

A water soluble glucomannan isolated from an immunomodulatory active polysaccharide of *Salvia officinalis* L.

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ABSTRACT

A water soluble glucomannan has been isolated from the decoloured and defatted aerial parts of sage (*Salvia officinalis* L.) by water extraction, followed by ion-exchange chromatography, and precipitation with Fehling reagent. It was composed of D-glucose and D-mannose residues in the mole ratio of 1.0:1.3 and had $M_w \sim 8000$. Chemical and spectroscopic analyses revealed a linear structure of the polymer with a backbone composed of β-1,4-linked glucopyranosyl and mannopyranosyl units slightly branched at C-6 by side chains, mainly as single α-glucosyl and mannosyl stubs.

1. Introduction

The medicinal plant *Salvia officinalis* L. (*Lamiaceae*) has been known for a long time as a remedy in the traditional medicine. The ethanolic tinctures and decoctions from its aerial parts have been used for treatment of inflammations of oral cavity, digestive and intestinal tract, gastritis and tonsillitis. Phytochemical investigation of drug revealed a great number of bioactive compounds possessing a variety of biological activities (Lu & Foo, 2002). In our previous papers we found that crude polysaccharides extracted from sage were shown to exhibit immunomodulatory and antitussive activities (Capek & Hříbalová, 2004; Capek et al., 2003; Nosáľová, Capek, Šutovská, Fraňová, & Matulová, 2006; Nosáľová et al., 2005). This finding prompted us to characterize more detail the structural features of the polymers occurring in the water extractable fraction. Lately, we described a highly branched α-L-arabino-β-3,6-D-galactan which was shown to be a dominant component of a neutral part of water extractable fraction (Capek, 2008). Now, we report on isolation and structural characterization of a further neutral polymer of sage, a glucomannan.

2. Material and methods

2.1. Plant material and general methods

Dry aerial parts of sage (*Salvia officinalis* L.) were purchased from Slovakofarma, Malacky, Slovakia. Polysaccharides were hydrolyzed with 2 M trifluoroacetic acid for 1 h at 120 °C. All the solutions were concentrated under reduced pressure. The uronic acid content was determined with the 3-hydroxybiphenyl reagent (Blumenkrantz & Asboe-Hansen, 1973).

The quantitative determination of the neutral sugars was carried out in the form of their trifluoroacetates by gas chromatography on a Hewlett-Packard Model 5890 Series II chromatograph equipped with a PAS-1701 column (0.32 mm × 25 m), the temperature program of 110–125 (2 °C/min) −165 °C (20 °C/min) and flow rate of hydrogen 20 cm^3/min (Shapira, 1969). The absolute configuration of monosaccharides was established by the method of Gerwig, Kamerling, and Vliegenthart (1979).

Gas chromatography-mass spectrometry of partially methylated alditol acetates was effected on a FINNIGAN MAT SSQ 710 spectrometer equipped with a SP 2330 column (0.25 mm × 30 m) at 80–240 °C (6 °C/min), 70 eV, 200 μA and ion-source temperature of 150 °C (Jansson, Kenne, Liedgren, Lindberg, & Lönngren, 1976).

HPLC measurement was performed with a Shimadzu apparatus (Japan) equipped with a differential refractometer RID-6A and a UV-vis detector SPD-10AV using a HEMA-BIO 100 column (8 mm × 250 mm) of particle size 10 μm. As a mobil phase 0.02 M phosphate buffer pH 7.2 containing 0.1 M NaCl was used at a flow rate 0.8 ml/min. A set of pullulan standards was used for calibration of the column (Gearing Scientific, Polymer Lab. Ltd., UK).

Infrared spectra were obtained on a NICOLET Magna 750 spectrometer with DTGS detector and OMNIC 3.2 software. 128 scans were recorded with 4 cm^{-1} resolution. The samples were measured as polymer films on NaCl discs.

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Elemental analysis was performed with EA 1108 apparatus (FISONS Instruments, UK). Protein was calculated from the nitrogen content (% N × 6.25). Optical rotation was measured with automatic polarimeter Perkin-Elmer Model 241.

NMR spectra of polysaccharides were recorded on a Bruker DPX AVANCE 300 spectrometer operating at 300 MHz for ^{1}H and 75.46 MHz for ^{13}C. The acetone was used as the internal standard (δ 2.225 ppm for ^{1}H and 31.07 ppm for ^{13}C). 2D HSQC spectrum was measurement with optimalization on one bond $^{1}J_{CH}$ coupling constant on 165 Hz.

2.2. Isolation and purification of sage glucomannan

The air dried and crushed aerial parts of sage (*Salvia officinalis* L.) were exhaustively extracted with methanol–chloroform (10:1 v/v). The residues were extracted twice in distilled water at laboratory temperature in the presence of 0.02% solution of natriumazid. Both extracts were combined, concentrated and polysaccharides were precipitated from 96% ethanol. The precipitate was collected by centrifugation, dissolved in water, dialyzed and recovered by freeze-drying (A).

Crude polysaccharide (A; 1 g) was dissolved in distilled water (50 mL) and applied to a column (8 × 20 cm) of DEAE-Sephacel and eluted successively with water (A_1), 0.1 M (A_2), 0.25 M (A_3), 0.5 M (A_4) and 1 M (A_5) NaCl solutions, and finally with 1 M NaOH (A_6). Fractions of 10 mL were collected and analyzed for the carbohydrate content by the phenol–sulphuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Pooled fractions were dialyzed and polysaccharide fractions were recovered by freeze-drying.

The non-retained fraction on DEAE-Sephacel (A_1) was dissolved in water and treated with the Fehling reagent to forms a suspension, which on centrifugation gives the precipitated glucomannan-copper complex and the supernatant (Jones & Stoodley, 1965). The precipitate was washed with cold water, collected by centrifugation, dissolved in distilled water, acidified with 1 M HCl solution, dialyzed and freeze-dried. The whole precipitation process with Fehling reagent was repeated twice to obtain a glucomannan as a white material, which was subjected to structural analyses.

2.3. Methylation analysis

The dry glucomannan (~2–3 mg) was dissolved in dry dimethyl sulfoxide (1 mL) and methylated with methyl iodide in the presence of methylsulfinyl carbanion (Hakomori, 1964). The methylated sample was purified using the Sep-Pak C_{18} cartridge (Waters Assoc.) and concentrated to dryness. The permethylated glucomannan was hydrolyzed with 2 M TFA at 120 °C for 1 h, reduced with sodium borodeuteride, and the partially methylated derivatives of glucose and mannose residues were acetylated and analyzed by GLCMS (Jansson et al., 1976).

3. Results and discussion

From the extracted free aerial parts of sage a crude mixture of polysaccharides (A) was obtained by water extraction, followed by ethanol precipitation, dialysis and freeze-drying. The water extractable polysaccharide complex (A) showed a broad molecular-mass distribution pattern and indicated its heterogenous character. It was obtained in 3.8% yield and contained 9.4% of protein. Sugar analysis revealed the dominance of arabinose, galactose and glucose, and smaller amounts of mannose, xylose, rhamnose, fucose and uronic acids (Table 1). Ion-exchange chromatography of A afforded six fractions by the step-wise elution with water (A_1), sodium chloride solutions (A_2A_5) and sodium hydroxide (A_6) solutions (Capek & Hříbalová, 2004).

Table 1
Monosaccharide composition of sage polysaccharides

Monosaccharide composition (%)	A	A_1	GM
Arabinose	30.4	35.0	tr
Galactose	17.9	22.1	tr
Glucose	15.5	13.0	48.1
Mannose	8.3	10.1	51.9
Uronic acid	8.0	4.7	
Xylose	7.6	2.9	
Rhamnose	6.7	1.2	
3-*O*-Methyl-galactose	3.0	10.6	tr
Fucose	2.6	0.4	

A, crude polysaccharide complex; A_1, ion-exchange fraction of A; GM, glucomannan; tr, traces.

The non-retained polysaccharide fraction (A_1) showed a single peak of lower molecular mass on HPLC. It was recovered in 7.5% yield and on hydrolysis showed the dominance of arabinose, galactose, glucose, 3-*O*-methyl-galactose and mannose, and smaller amounts of uronic acid, xylose, fucose, and rhamnose, suggesting heterogeneity of A_1. Therefore, A_1 was further purified by Fehling reagent (Jones & Stoodley, 1965) to give the glucomannan as pellet and the arabinogalactan as supernatant, already described (Capek, 2008). GM had a similar molecular-mass pattern as that of A_1 (~8000), an optical rotation of −3° (c 1.0, water, λ = 589 nm) and on hydrolysis afforded D-glucose and D-mannose in the mole ratio of 1.0:1.3 and trace amounts of accompanying sugars originated from arabinogalactan, which is supposed to be a contaminant (Table 1).

Methylation analysis data of GM revealed two main sugar derivatives, i.e. 2,3,6-tri-*O*-methylmannose and 2,3,6-tri-*O*-methylglucose and confirmed the 1,4-linked glucomannopyranosyl backbone. The low content of 2,3-di-*O*-methylmannose (~6%) and 2,3-di-*O*-methylglucose (~2%), derived from 1,4,6-linked residues, demonstrated the slight branching of the polymer at C-6. Besides, ~4% of 2,3,4-tri-*O*-methylglucose suggested the presence of 1,6 linkages, ~3% of 2,3,4,6-tetra-*O*-methylmannose and ~5% of 2,3,4,6-tetra-*O*-methylglucose indicated their terminal positions in the polymer Besides, small amounts of terminal and 3,6-linked galactose were determined (Table 2).

Chemical shifts of the glucomannan residues were assigned according 1D (^{1}H and ^{13}C) and 2D (HSQC) NMR spectroscopy and reference data (Capek, Alföldi, & Lišková, 2002; Hazendonk, Reinerink, de Waard, & Dam, 1996; Sims, Craik, & Bacic, 1997). The NMR spectral data were in a good agreement with conclusions derived from sugar linkage analysis. The ^{13}C NMR spectrum showed characteristic anomeric signals at δ 103.6 with a slight shoulder at δ 103.4 due to C-1 resonances of β-D-glucose residues, at δ 101.0 with a clear shoulder occurring at δ 100.6 due to C-1 resonances of D-mannose residues, and a low intensity signal at δ 99.7 due to anomeric resonances of galactose residues originated from the

Table 2
Sugar linkage analysis data of the glucomannan

Sugar derivative	mol%	Linkage indicated
2,3,4,6-Me_4-Man[a]	2.6	Man*p*-(1→
2,3,4,6-Me_4-Glc	4.6	Glc*p*-(1→
2,3,4,6-Me_4-Gal	tr	Gal*p*-(1→
2,3,6-Me_3-Man	44.1	→ 4)-Man*p*-(1→
2,3,6-Me_3-Glc	36.5	→ 4)-Glc*p*-(1→
2,3,4-Me_3-Glc	4.4	→ 6)-Glc*p*-(1→
2,3-Me_2-Man	5.6	→ 4,6)-Man*p*-(1→
2,3-Me_2-Glc	2.2	→ 4,6)-Glc*p*-(1→
2,4-Me_2-Gal	tr	→ 3,6)-Gal*p*-(1→

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

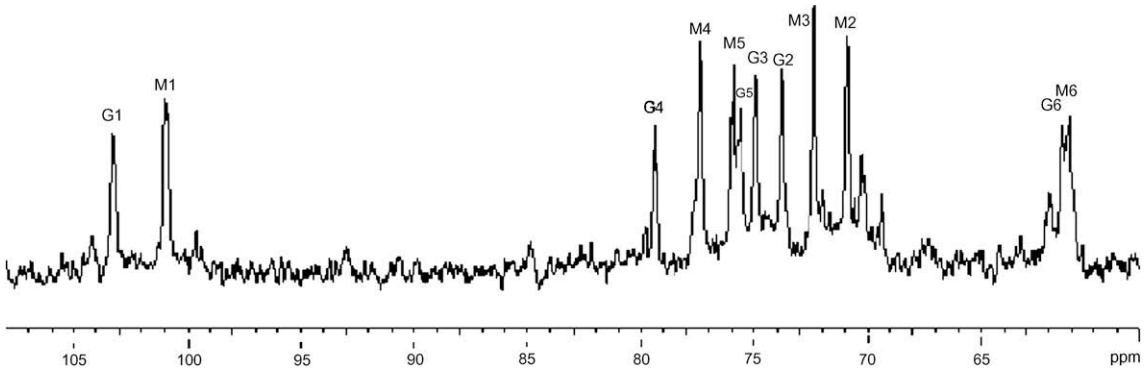

Fig. 1. ^{13}C NMR spectrum of the sage glucomannan. G1-6: C1-C6 of glucose, M1-6: C1-C6 of mannose residues.

arabinogalactan. The C-4 chemical shifts of the glucosyl and mannosyl units involved in glycosidic linkages appeared at δ 79.4 and 77.4, respectively. The signals at δ 75.9, 72.4 and 70.1 were assigned to C-5, C-3 and C-2 of mannose residues, respectively. The characteristic resonances of C-5, C-3 and C-2 of 1,4-linked glucose residues were observed at δ 75.6, 74.9 and 73.7, respectively. The signals in the high magnetic field at δ 61.4-61.0 were generated by the resonances of non-substituted C-6 of glucosyl and mannosyl residues (Fig. 1). Besides, in the 1D DEPT spectrum (not shown), a low intensity signal at δ 67.6 could be assigned to substituted C-6 of glucosyl or mannosyl residues.

The ^{1}H-^{13}C heterocorrelated HSQC NMR spectrum revealed the presence of β glucosyl and mannosyl residues (β-linkage of manosyl residue was evidenced by the coupling constant $^{1}J_{CH}$ = 161.5 Hz) in the polymer. Two dominant H1/C1 cross peaks could be identified at δ 4.50/103.6 and 4.74/101.0 due to β-1,4-linked glucose and mannose residues, respectively. Smaller intensity signals at δ 5.00/99.7 and 4.48/104.2 originate from arabinogalactan, which is supposed to be a contaminant (Fig. 2). Cross-peaks at δ 3.67/79.4 and 3.79/77.4 are due to H4/C4 signals of substituted glucosyl and mannosyl residues, respectively. Cross peaks at δ 3.56/75.9 (H5/C5), 3.79/72.3 (H3/C3) and 4.11/ 70.9 (H2/C2) are characteristic of mannosyl residues involved in 1,4-linkage, and those at δ 3.58/75.7 (H5/C5), 3.67/74.8 (H3/C3) and 3.41/73.7 (H2/C2) of 1,4-linked glucosyl ones. Signals H6 and C6 appeared at δ 3.89–3.74/61.4–61.0 due to non-substituted glucosyl and mannosyl residues.

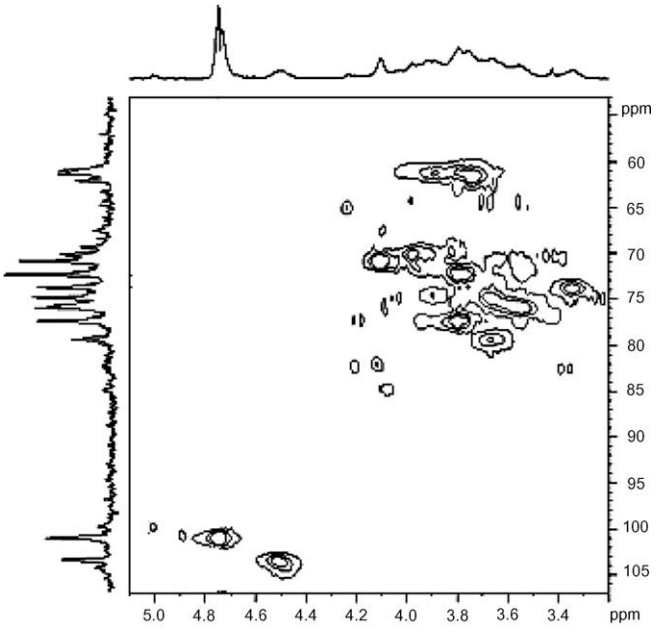

Fig. 2. HSQC spectrum of the sage glucomannan.

In conclusion, the results of compositional and linkage analyses as well as NMR measurements confirmed a linear structure of sage glucomannan composed of 1,4 linked D-glucosyl and D-mannosyl units in the mole ratio of 1:1.3, respectively, the β configuration of glycosidic bond in the main chain, and the presence of short side chains at C-6, mainly as single stubs. Moreover, D-mannosyl residues showed a higher degree of branching compare to D-glucosyl ones. However, D-glucosyl residues were shown to have a higher substitution in the side chains. Splitting of the anomeric signal of D-glucosyl unit to δ 103.6 and 103.4, and D-mannosyl one to δ 101.0 and 100.6 in the ^{13}C NMR spectrum of the polymer is due to different mutual arrangement of D-glucosyl (e.g. MGM, MGG, etc.) and D-mannosyl units (e.g. MMM, MMG, etc.) in the main chain (Capek et al., 2002; Hazendonk et al., 1996; Sims et al., 1997). Structural studies (chemical or enzymatic) on glucomannan backbone revealed many possible sequences and as well the presence of smaller mannosyl blocks up to d.p. 4 and glucosyl ones up to 3, however, did not confirm the occurrence of higher mannosyl- or glucosyl-blocks in the polymeric chain (Capek, Kubačková, Alföldi, Bilisics, & Lišková, 2000; Cescutti, Campa, Delben, & Rizzo, 2002). Although sage polysaccharide has the similar composition of the backbone as hemicellulose (galacto) glucomannans extracted from the cell walls of angiosperms and gymnosperms, legumes, moss and ferns and seeds of some plant species by alkaline solutions (Matheson, 1990; Stephen, 1983), differs in molecular size and content of side short chains. About 8% of branched points resulted in the complete solubility of sage glucomannan in water.

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