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Structural characteristics of water-soluble polysaccharides from *Heracleum sosnowskyi* Manden

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ABSTRACT

Fractions containing arabinogalactan proteins (AGPs) and pectic polysaccharides were isolated from above-ground parts of *Heracleum sosnowskyi*. Major units of their structure were elucidated using ion-exchange chromatography, gel chromatography, and NMR spectroscopy. The carbohydrate backbone of the polysaccharides consisted of 1,3-β-D-galactopyranosyl residues, whereas side chains of the branched region consisted of the residues of 1,6-β-galactopyranose, 1,5-α-L-arabinofuranose, 1,4-β-D-glucuronic acid, and 1,6-β-D-glucopyranose. The branching points were identified as 1,3,6-β-D-galactopyranose residues. Side chains were terminated with β-D-galactopyranose, α-L-arabinofuranose and α-L-rhamnopyranose. A significant part of the side-chain β-1,6-galactan was substituted at C6 by 4-OMe-β-D-glucuronic acid. A minor part of glucuronic acid was included in the α-Rhap-(1→4)-β-GlcA-(→ fragment. All the studied fractions contained 1,4-β-D-galacturonic acid as well.

1. Introduction

The major cell wall components involved in organ development in plants are pectins and arabinogalactan proteins (AGPs) (Rafińska & Bednarska, 2011). AGP is a wide class of highly glycosylated proteins with complex structure. The bulk of AGP (up to 95%) is comprised by a carbohydrate part of the molecule which consists of arabinogalactan type II (AG-II) and 2–10% of a polypeptide core usually rich in hydroxyproline (Hyp), alanine (Ala), serine (Ser), or threonine (Thr) (Ellis, Egelund, Schultz, & Bacic, 2010; Tan et al., 2012).

Hydroxyproline of the protein core is the site of attachment for the branched β-1,3-galactans substituted at C6 with 1,6-β-D-linked galactopyranose-containing side chains. The side chains are often capped by a single α-L-arabinofuranose residue or other sugars, such as Fuc*p*, Rha*p*, Glc*p*A or 4-O-methyl-Glc*p*A (Ellis et al., 2010; Gaspar, Johnson, McKenna, Bacic, & Schultz, 2001; Gorshkova, 2007; Seifert & Roberts, 2007; Tan et al., 2012; Tryfona et al., 2010).

AG-II has been reported both as free polysaccharides and as side chains of rhamnogalacturonan I (RG-I) (Caffall & Mohnen, 2009; Ridley, O'Neill, & Mohnen, 2001). It was found in purified pectic fractions; however, its linkage to the pectin RG-I domains as covalently attached side-chains is still unclear, though RG-I and AG-II often seem to co-extract and are difficult to separate from one another. Structurally, AG-II is not significantly different from the glycan moiety of arabinogalactan proteins, which are frequently found in the vicinity of pectic RG-I (Yapo, 2011).

Heracleum (*Umbelliferae*) includes about 60–70 species; it is characterised by vast biomass, reproduces rapidly, and is easy to maintain. Several species of cow-parsnip are cultivated as ornamental plants, others are used as silage, and some are edible for human (Bulletin OEPP/EPPO Bulletin, 2009; Jahodová1, Trybush1, Pysek, Wade, & Karp, 2007; Pysek, Cock, Nentwig, & Ravn, 2007).

Heracleum nepalense (family *Apiaceae*) was used as breath rate and blood pressure stimulator in veterinary medicine, its dried root extract and isolated quercetin glycoside exhibiting a dose-dependent immunostimulant effect (Kumar, Gupta, Sharma, & Kumar, 2011). Dichloromethane extract from *Heracleum sphondylium* L. (*Apiaceae*) was shown to possess vasorelaxant properties, allowing its usage as antihypertensive therapy in traditional medicine (Senejoux et al., 2013).

Study of the monosaccharide composition and content of pectic polysaccharides, and glucan, and glucomannan hemicellulose in the stems and roots of *Heracleum moellendorffii* Hance were reported. The water-extractable fraction from stems of *H. moellendorffii* contained mainly glucose (45%) and mannose (35%). It was reported that polysaccharides from *H. moellendorffii* roots

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possessed high antiviral activity against the antigen of mink Aleutian disease virus (Tomshich et al., 1997).

A number of hemicellulose polysaccharides from laminas and leafstalks of *Heracleum sosnowskyi* Manden has been studied. They are characterised by the presence of xylan and xyloglucan and the absence of mannan. The water-soluble fraction of hemicellulose extracted from the *H. sosnowskyi* leaves contains galactoarabinoxyloglucan comprised by galactose, glucose, arabinose, and xylose in the molar ratio of 4:60:7:42 (Dudkin, Parfent'eva, & Cherno, 1984).

The structural studies of polysaccharides from *H. sosnowskyi* have been limited to the elucidation of their sugar composition (Dudkin et al., 1984). Investigation of structural and chemical characteristics of components from *H. sosnowskyi* may be useful for development of a scientific base for its processing, which, in turn, may assist to solve the problem of limitation of spreading and harmfulness of cow-parsnip as an aggressive invasive species (Jahodová1 et al., 2007; Pysek et al., 2007; Wrzesińska, 2004). In the meantime, one of the major spread control methods is mowing to prevent flowering and ripening of cow-parsnip seeds. This procedure can be combined with a simultaneous stocking of its above-ground parts to obtain valuable products.

This work is dedicated to characterisation of chemical structure of polysaccharides isolated from above-ground parts of *H. sosnowskyi* Manden by water extraction.

2. Materials and methods

2.1. Processing of raw plant material and isolation of polysaccharides

Above-ground parts of *Heracleum* (whole plants with leaves, flowers, and stems) were collected in July in the blooming period near Syktyvkar, Komi Republic, Russia. Isolation was performed as described earlier (Makarova, Patova, Shakhmatov, Kuznetsov, & Ovodov, 2013). Fresh plant material was homogenised and extracted with 90% ethanol (at ~55 °C, twice for 2 h) to remove low molecular weight contaminants. The residual material was air-dried, with the yield ~9% of the original fresh material. Then the dried material (25 g) was treated with distilled water at 70 °C for 2 h. The fivefold extraction was applied. An extract from each step was collected separately, centrifuged and concentrated. The supernatant was precipitated with four volumes of 96% ethanol. The precipitate was separated by centrifugation and re-dissolved in water, with subsequent dialysis and lyophilisation. Therefore, five polysaccharide fractions were obtained, HS-I, HS-II, HS-III, HS-VI, and HS-V, with yields of 138.5, 52.6, 54.7, 22.3, and 35.8 mg, respectively.

Water–alcohol supernatants obtained at precipitation of these five fractions with four volumes of 96% ethanol, were combined, concentrated and dialysed against distilled water (3 kDa membranes). The obtained solution was centrifuged, concentrated and lyophilised. The carbohydrate fraction (46 mg) was dissolved in water (1 ml) and subjected to further purification via gel filtration chromatography on a Sephacryl S-300 column. A fraction corresponding to the major peak was collected and lyophilised to obtain the homogeneous polysaccharide fraction HS-S (26 mg).

2.2. General analytical methods

The amount of protein and uronic acid moieties was determined as described (Makarova et al., 2013). Absorption spectra were measured on the Shimadzu UV-1700 (PharmaSpec) spectrophotometer.

Solutions were concentrated on a rotary evaporator under reduced pressure at 40–45 °C, centrifuged at 5000–6000 rpm for 10–20 min, and lyophilised.

Molecular weights and polydispersities of the polysaccharide fractions were determined by high pressure liquid chromatography (HPLC) as described (Makarova et al., 2013). Gel filtration chromatography was carried out as described earlier (Makarova et al., 2013). The fractions corresponding to separate peaks were combined, concentrated and lyophilised.

NMR spectra were recorded on Bruker Avance II 300 MHz (Bruker, Germany) using 3–5% solutions of polysaccharides in D_2O (99.9% D) containing DSS (Sigma–Aldrich), at 303 K and 328 K. Samples were freeze-dried from 95% D_2O and subsequently dissolved in 99.9% D_2O. Chemical shifts were referenced to internal DSS (^1H and ^{13}C at 0.00 ppm). NMR signals were assigned using two-dimensional (2D) COSY, TOCSY, ROESY, $\{^1H,^{13}C\}$ HSQC, and $\{^1H,^{13}C\}$ HMBC experiments. 2D TOCSY spectra were recorded using the MLEV-17 mixing sequence with spin-lock time of 90 ms. 2D ROESY experiments were performed with a mixing time of 200 ms.

During the spectra assignment, the reference ^{13}C NMR data for the unsubstituted residues and glycosylation effects were taken from a CSDB Carbohydrate NMR simulation tool (Toukach & Ananikov, 2013). Literature and database ^{13}C NMR spectra referenced to TMS were normalised using a correction of +1.56 ppm.

2.3. Monosaccharide analysis

Samples (3–5 mg) were hydrolysed with 2 M TFA (1 ml) containing *myo*-inositol (1 mg/ml) at 100 °C for 5 h. The mixture of neutral monosaccharides was converted to alditol acetates (York, Darvill, McNeil, Stevenson, & Albersheim, 1985) and identified by gas–liquid chromatography (GLC) on the Shimadzu GC-2010AF chromatograph equipped with a flame ionisation detector, using a capillary column HP-1 (Agilent, 30 m × 0.25 mm × 0.25 mcm). Helium was used as a carrier gas. GLC of alditol acetates was carried out at temperature programmed from 175 °C (1 min) to 250 °C (2 min) at the rate of 3 °C/min. The content of monosaccharides as a percent of the total preparation mass was calculated from the peak areas using the coefficients of detector response. Myo-inositol was used as an internal standard.

2.4. Ion-exchange chromatography of polysaccharide HS-I on DEAE-cellulose

Polysaccharide HS-I (100 mg) was dissolved in 0.01 M NaCl (3 ml) and fractionated on a DEAE-cellulose OH^--form column (34.5 cm × 2.2 cm). The fractions were eluted consecutively with 0.01, 0.1, 0.2, 0.3, and 0.4 M NaCl at flow rate of 1 ml/min, then collected and analysed by Smith's procedure (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The fractions corresponding to separate peaks were combined, concentrated, dialysed and lyophilised. Four polysaccharide fractions were as follows: HS-I_1 (eluted with 0.01 M NaCl, 19.1 mg), HS-I_2 (eluted with 0.1 M NaCl, 35.9 mg), HS-I_3 (eluted with 0.2 M NaCl, 5.8 mg), and HS-I_4 (eluted with 0.3 M NaCl, 4.7 mg).

3. Results and discussion

3.1. Characterisation of water-soluble polysaccharides from above-ground parts of H. sosnowskyi M.

Monosaccharide analysis of the obtained polymers HS-I, HS-II, HS-III, HS-VI, and HS-V revealed high content of D-uronic acids, D-galactose, and L-arabinose, characteristic components of arabinogalactan type II, which often occurred within arabinogalactan proteins (AGPs) and pectic polysaccharides (Table 1). The results

Table 1
Yields and composition (% w/w) of *Heracleum* fractions.

Fraction	Yield[a] (%)	Uronic acids	Neutral monosaccharides						Protein
			Gal	Ara	Rha	Xyl	Glc	Man	
HS-I	0.55	23.0	36.6	5.6	2.1	0.2	2.3	1.3	8.5
HS-II	0.21	35.0	16.3	3.2	3.7	1.5	4.0	1.4	9.1
HS-III	0.22	40.0	17.0	4.6	3.8	0.7	5.1	1.4	8.5
HS-VI	0.09	58.7	16.6	5.1	3.7	1.7	5.2	0.9	8.1
HS-V	0.14	57.0	17.4	4.9	3.6	1.7	8.2	0.6	6.6
HS-I_1	19.1[b]	17.2	65.0	8.0	2.0	0.5	2.0	0.7	2.1
HS-I_2	35.9[b]	20.0	43.6	8.0	2.0	0.5	1.9	1.3	2.0
HS-I_3	5.8[b]	19.9	15.9	6.4	12.4	0.7	8.0	2.4	12.3
HS-I_4	4.7[b]	18.9	8.8	3.3	5.7	0.5	6.5	2.3	14.1
HS-S	–	8.5	3.7	5.2	0.6	0.8	19.7	16.8	4.2

[a] Polysaccharide yields are calculated from the air-dried plant material.
[b] Yield related to the quantity applied to the DEAE-cellulose column.

showed that a sequential extraction increased the content of glucuronic acids from 23 to 58% (Table 1).

3.2. Ion-exchange chromatography of polysaccharide HS-I on DEAE-cellulose

By using ion-exchange chromatography on DEAE-cellulose (OH^--form), it was demonstrated that fraction HS-I consisted mainly of polysaccharides HS-I_1 and HS-I_2, which were eluted with 0.01 and 0.1 M NaCl, respectively, and were characterised by high content of galactose (44–65%), arabinose (8%), and uronic acids (17–20%) (Table 1). Minor fractions HS-I_3 and HS-I_4 were characterised by high content of protein (12–14%).

Major fractions eluted by 0.01 M and 0.1 M NaCl were separated by SEC on Sephacryl S-300 to give polysaccharides HS-I_1 (M_w 8.5 kDa) and HS-I_2 (M_w 9.5 kDa), corresponding to two biggest peaks. In fractions HS-I_1 and HS-I_2, an average ratio of Gal to Ara was 8:1 and 5:1, respectively. Similar Gal to Ara ratio has been reported for AGP from New Zealand kanuka honey (5.3:1), for AG-II of spruce heartwood was about 4.3:1, pine heartwood about 4.5:1, and larch heartwood about 6.7:1 (Steinhorn, Sims, Carnachan, Carr, & Schlothauer, 2011; Willfor & Holmbom, 2004).

3.3. Isolation of polysaccharide HS-S

From the combined water–alcohol supernatant obtained upon precipitation of fractions HS-I, HS-II, HS-III, HS-VI, and HS-V, a polysaccharide fraction was isolated as a minor component; the subsequent fractionation by gel filtration gave polysaccharide fraction HS-S that corresponded to the main peak on the elution curve, with low average-weight molecular mass (11.5 kDa) and low dispersion degree (1.4) (Table 1). The carbohydrate chain from fraction HS-S contained mainly D-glucose (19.7%) and D-mannose (16.8%) characteristic for the hemicellulose class of glucans and glucomannans. Fraction HS-S also contained minor amounts of uronic acids, Rha, Ara, Gal, and Xyl.

3.4. NMR spectroscopy of polysaccharides

Fractions HS-I_1, HS-I_2, HS-V and HS-S were studied by ^1H and ^{13}C NMR spectroscopy, using double-quantum filtered homonuclear COSY, TOCSY, Rotating frame Overhauser effect spectroscopy (ROESY), {^1H,^{13}C} HSQC, and {^1H,^{13}C} HMBC.

3.4.1. NMR studies of polysaccharides HS-I_1, HS-I_2

One- and two-dimensional ^1H NMR spectra of fraction HS-I_1 contained signals of a protein part of the molecule in the region of 0.8–3.0 ppm (Breitmaier & Voelter, 1990, chap. 5; Capek, Matulová, Navarini, & Suggi-Liverani, 2010; Matulová, Capek, Kaneko, Navarinic, & Liveranic, 2011).

The HSQC spectrum of fraction HS-I_1 (Fig. 1) showed C3/H3 correlation at 85.0/3.83 ppm, implying a characteristic glycosylation effect of β1→3 substitution of D-Gal. Accordingly, a ROESY spectrum did not contain other correlations of Gal, than expected H1/H3 and H1/H5 at 4.68/3.83 ppm and 4.68/3.76 ppm, respectively. These data allow identification of a …→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→… fragment. 1,3-Linked β-D-Gal*p* residues were reported as a core component of AGP carbohydate moiety (Capek et al., 2010; Matulová et al., 2011; Redgwell et al., 2011; Tan et al., 2010).

An HMBC spectrum of fraction HS-I_1 (Fig. 2) demonstrated the following cross-peaks from 1,6-linked β-D-Gal*p*, implying the presence of the fragment …→6)-β-Gal*p*-(1→6)-β-Gal*p*-(1→…: C6/H1 at 72.4/4.47 ppm, C4/H3 at 71.7/3.68 ppm, C4/H5 at 71.7/3.91 ppm, C5/H6 at 76.7/3.92 ppm, C3/H2 at 75.6/3.55 ppm, C1/H2 at 106.3/3.57 ppm, and C2/H5 at 73.9/3.91 ppm. A ROESY spectrum of fraction HS-I_1 confirmed the presence of …→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→… fragment by an *inter*-residue cross-peak H1/H6,6′ at 4.47/3.92;4.04 ppm (together with *intra*-residue cross-peaks H1/H3 at 4.47/3.68 ppm and H1/H5 at 4.47/3.91 ppm).

β-1,6-Galactans can be of various length and are presented by short chains of one or two residues (Gaspar et al., 2001), as well as long chains of more than 20 residues long (Haque, Kotake, & Tsumuraya, 2005; Tryfona et al., 2010).

The HMBC spectrum of fraction HS-I_1 demonstrated the following cross-peaks of the terminal β-D-Gal*p*: C1/H2 at 106.5/3.52 ppm; C1/H5 at 106.5/3.70; C3/H2 at 75.7/3.52 ppm; C4/H5 at 71.5/3.70 ppm; C6/H5 at 64.0/3.70 ppm; C5/H6 at 78.1/3.76 ppm; and C5/H4 at 78.1/3.92 ppm. In addition, the HMBC spectrum contained cross-peaks C1/H6,6′ at 106.5/4.04;3.91 ppm and C6/H1 at 72.4/4.45 ppm implying that 1,6-linked β-D-Gal*p* is capped by terminal galactose: β-Gal*p*(1→6)-β-Gal*p*-(1→…

A COSY spectrum of HS-I_1 contained cross-peaks H1/H2, H2/H3, and H3/H4 from the following residues: terminal β-D-Gal*p*, 1,3-linked β-D-Gal*p*, 1,6-linked β-D-Gal*p*, 1,3,6-linked β-D-Gal*p*, and 4-O-Me-β-Glc*p*A. Signals of 1,3,6-linked galactose (H1/H2 at 4.49/3.68 ppm and H1/H2 4.55/3.70 ppm) indicated athe presence of this residue in different surrounding: β-D-Gal*p*$^{(I)}$ and β-D-Gal*p*$^{(II)}$ (Table 2).

The anomeric region of the HSQC spectrum of fraction HS-I_1 (Fig. 1) contained intense anomeric signals from three different terminal α-L-Ara*f* residues at 111.4/5.45 ppm, 111.4/5.41 ppm and 112.6/5.25 ppm, and a weak signal at 110.7/5.09 ppm from 1,5-linked α-L-Ara*f*. Terminal Ara*f* residues were distinguished by its C4 chemical shift of 86.7–86.9 ppm indicating the absence of substitution at C3 and C5, with account to DSS to TMS difference (Pastell, Tuomainen, Virkki, & Tenkanen, 2008). α-L-Ara*f* C4 was

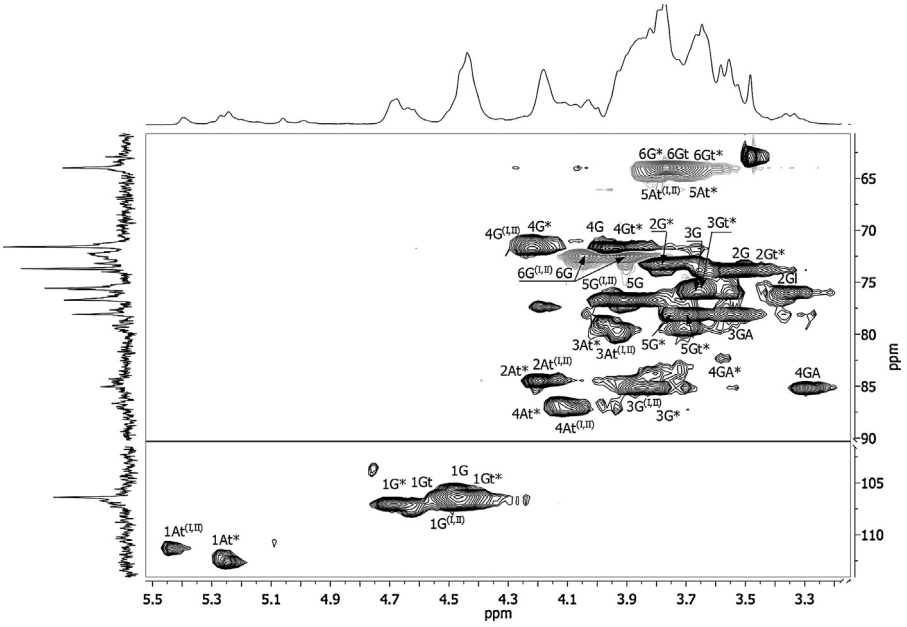

Fig. 1. {^{1}H,^{13}C} HSQC spectrum of polysaccharide fragment HS-I_1.

assigned using and HMBC cross-peaks H1/C4 at 5.45/86.8 ppm, 5.25/86.7 ppm. Ara*f* residues exhibited transglycosidic cross-peaks H1/H5;5′ in ROESY (at 5.25/3.81;3.72 ppm for terminal, and at 5.09/3.87;3.81 for 1,5-linked α-Ara*f*), indicating the presence of . . .→5)-α-Ara*f*-(1→5)-α-Ara*f*-(1→. . . fragment.

1,6-Linked β-D-Gal*p* residues in the side chain of AG-II were reported as 3-substituted by terminal arabinose having the anomeric proton signal at 5.25 ppm (Haque et al., 2005; Redgwell, Curti, Fischer, Nicolas, & Fay, 2002; Tryfona et al., 2012). This allows a suggestion of the presence of →6)[α-L-Ara*f*(1→3)]β-D-Gal*p*(1→ fragment in fraction HS-I_1. According to published data, the signal at 4.48 ppm was due to 1,3,6-β-Gal of the backbone substituted with a single β-Gal or 1,6-liked β-Gal oligosaccharide chains, whereas the one at 4.52 was due to 1,3,6-β-Gal substituted with α-Ara*f* in the side chains (Matulová et al., 2011).

1,3,6-β-Gal anomeric protons at 4.49 ppm and 4.55 ppm of fraction HS-I_1 indicate that 1,3,6-β-Gal residues may have two different substituents at C1. Suggestedly H1 at 4.49 ppm belongs to the backbone 1,3,6-β-Gal glycosylating 1,3-linked β-Gal, while H1 at 4.55 ppm belongs to side chain 1,3,6-β-Gal substituted by terminal Ara*f* at C3 and, probably, glycosylating 1,6- or 1,3,6-β-Gal residues.

In the ^{1}H NMR spectrum, intensity of H1 signals of terminal Ara*f* residues (5.09, 5.25, 5.41, 5.45 ppm) was ca. three times lower than that one of H1 signals of terminal β-D-Gal*p* (4.45 ppm), indicating that side chains were terminated mainly with β-D-Gal*p*.

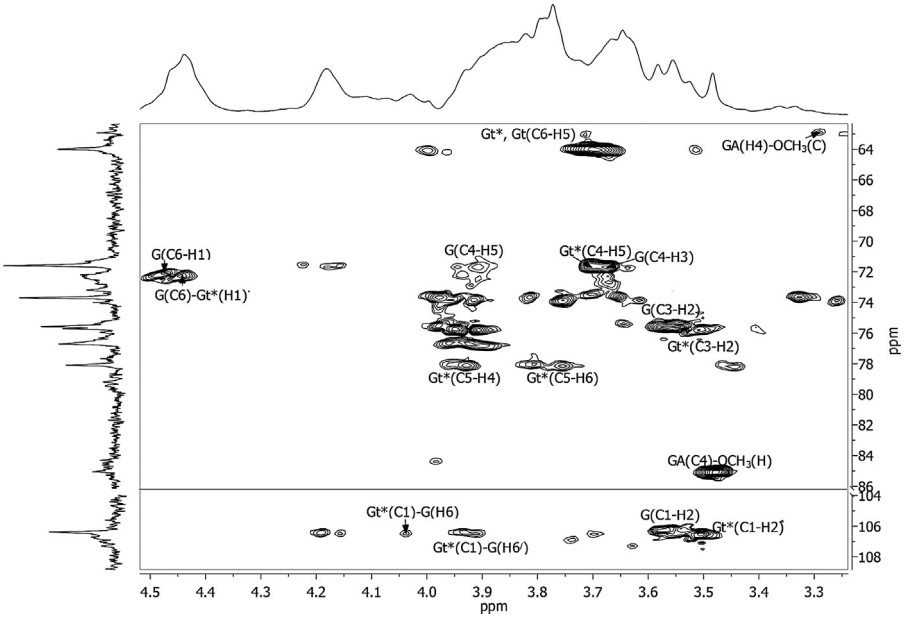

Fig. 2. Partial {^{1}H,^{13}C} HMBC spectrum of polysaccharide fragment HS-I_1.

Table 2
^{13}C and ^{1}H NMR data of polysaccharide fragment HS-I_1, referenced to DSS.

Residue		C-1 *H-1*	C-2 *H-2*	C-3 *H-3*	C-4 *H-4*	C-5 *H-5;5′*	C-6 *H-6;6′*
→4)-α-Gal*p*A-(1→		n.d. *5.00*	n.d. *3.82*	n.d. *3.97*	n.d.	n.d.	n.d.
α-Rha*p*-(1→		n.d.	n.d. *3.95*	n.d. *3.76*	74.7 *3.39*	71.7 *4.01*	19.3 *1.24*
β-Gal*p*-(1→3	Gt	107.2 *4.61*	73.7 *3.61*	75.4 *3.67*	71.7 *3.93*	78.1 *3.69*	64.0 *3.75*
→3,6)-β-Gal*p*-(1→$^{(I)}$	$G^{(I)}$	106.5 *4.49*	73.2 *3.68*	85.0 *3.85*	71.3 *4.20*	76.7 *3.91*	72.4 *4.04;3.92*
→3,6)-β-Gal*p*-(1→$^{(II)}$	$G^{(II)}$	106.5 *4.55*	73.2 *3.70*	85.0 *3.85*	71.3 *4.22*	76.7 *3.91*	72.4 *4.04;3.92*
→3)-β-Gal*p*-(1→	G*	107.2 *4.68*	73.2 *3.77*	85.0 *3.83*	71.3 *4.18*	78.0 *3.76*	64.0 *3.77*
β-Gal*p*-(1→6	Gt*	106.5 *4.45*	73.7 *3.52*	75.7 *3.66*	71.5 *3.92*	78.1 *3.70*	64.0 *3.76*
→6)-β-Gal*p*-(1→	G	106.3 *4.47*	73.9 *3.57*	75.6 *3.68*	71.7 *3.95*	76.7 *3.91*	72.4 *4.04;3.92*
→6)-β-Glc*p*-(1→	Gl	105.9 *4.48*	76.0 *3.35*	73.7 *3.55*	73.9 *3.65*	75.5 *3.54*	66.1 *3.98;3.71*
4-O-Me-β-Glc*p*A-(1→	GA	106.4 *4.62*	76.0 *3.29*	78.1 *3.54*	85.2 *3.28*	78.2 *3.74*	n.d.
→4)-β-Glc*p*A-(1→	GA*	106.4 *4.62*	76.0 *3.31*	78.1 *3.56*	82.4 *3.58*	78.2 *3.76*	n.d.
→5)-α-Ara*f*-(1→		110.7 *5.09*	84.2 *4.13*	n.d.	n.d. *4.20*	69.4 *3.87;3.81*	
α-Ara*f*-(1→$^{(I)}$	$At^{(I)}$	111.4 *5.41*	84.5 *4.19*	79.6 *3.93*	86.8 *4.13*	64.0 *3.81;3.73*	
α-Ara*f*-(1→$^{(II)}$	$At^{(II)}$	111.4 *5.45*	84.5 *4.20*	79.6 *3.94*	86.8 *4.13*	64.0 *3.81;3.72*	
α-Ara*f*-(1→3	At*	112.6 *5.25*	84.5 *4.20*	78.9 *3.98*	86.7 *4.11*	64.0 *3.81;3.71*	

Methyl group signals in the HSQC spectrum (Fig. 1) at 62.8/3.48 ppm and the chemical shift of C4/H4 at 85.2/3.28 ppm indicated the presence of 4-O-Me-GlcA. An intense cross-peak at 85.2/3.48 ppm and a minor cross-peak at 62.8/3.28 ppm in the HMBC spectrum confirmed an O-methyl substituent at position 4 of β-D-glucuronic acid (Perrone et al., 2002; Redgwell et al., 2011). According to published data, residues of 4-O-Me-GlcA can occur at the non-reducing termini of side chains of β-(1→6)-galactan: 4-Me-β-GlcA-(1→6)-β-Gal-(1→... (Redgwell et al., 2002; Tryfona et al., 2012).

Some AGP, such as those found in roots of *Echinacea pallida* (Nutt.), roots of radish (*Raphanus sativus*), maize (*Zea mays*), Chinese wolfberry (*Lycium barbarum*), and gum arabic, have been reported to contain 4-O-methylated and non-methylated Glc*p*A residues (Ellis et al., 2010; Kieliszewski, Kamyab, Leykam, & Lamport, 1992; Konishi et al., 2008; Redgwell et al., 2011; Thude & Classen, 2005).

Additionally, the COSY spectrum of fraction HS-I_1 demonstrated cross-peaks H1/H2 at 4.62/3.31 ppm from the 4-substituted residues of β-D-glucopyranosyluronic acid (Table 2). In the HSQC spectrum (Fig. 1), there was a cross-peak C4/H4 at 82.4/3.58 ppm, its chemical shift confirming the substitution of β-D-Glc*p*A at position 4 (Redgwell et al., 2011). Residues of 4-substituted β-D-Glc*p*A have also been found in the side chain of AGP from Chinese wolfberry (*L. barbarum*) (Redgwell et al., 2011).

The HSQC spectrum of fraction HS-I_1 contained a weak signal of α-rhamnopyranose C6/H6 at 19.3/1.24 ppm. The methyl group of terminal α-L-Rha*p* was assigned upon the cross-peaks C4/C6 at 74.7/1.24 ppm and C5/H6 at 71.7/1.24 ppm in the HMBC spectrum (Table 2). According to literature data, terminal α-L-Rha*p* can be present at the non-reducing termini of side chains and is 1,4-linked to Glc*p*A: α-Rha*p*-(1→4)-β-Glc*p*A-(1→... (Pellerin, Vidal, Williams, & Brillouet, 1995; Perrone et al., 2002).

The presence of 1,6-linked β-Glc*p* in a polysaccharide HS-I_1 was confirmed by the HSQC (Fig. 1) correlation C6/H6,6′ at 66.1/3.98;3.71 and other carbon chemical shifts typical for this substitution pattern. The spin system of 1,6-linked β-Glc*p* was assigned using HMBC correlations C3/H4 at 73.7/3.65 ppm, C3/H6 at 73.7/3.98 ppm, C5/H6 at 75.5/3.98 ppm.

Close NMR chemical shifts of C1 and C2, considering the difference between used standards (C1/H1 at 103.23/4.52 ppm and C2/H2 at 73.68/3.33 ppm), were observed for 1,6-linked β-D-Gal*p* in arabinogalactan from *Coffea arabica* instant coffee powder (Matulová et al., 2011).

According to published data, the residues of 4-O-Me-Glc*p*A can occur at the non-reducing termini of side chains of β-(1→6)-glucan: 4-Me-β-Glc*p*A-(1→6)-β-Glc*p*-(1→ (Tomoda & Kitamura, 1967).

NMR spectra of fraction HS-I_1 also demonstrated minor signals from the residues of 1,4-α-Gal*p*A (Petersen, Meier, Duus, & Clausen, 2008; Taboada et al., 2010). According to their integral intensities, the residues of glucuronic and galacturonic acids were present in the ratio of 4:1. Thus, in fraction HS-I_1, uronic acids were presented mainly by the residues of 4-O-Me-D-glucuronic and 1,4-β-D-glucuronic acids.

The upfield regions of the COSY spectrum contained cross-peaks H6/H3 at 1.32/4.08, H6/H2 at 1.32/4.12, and H6/H3 at 1.30/3.90, indicating the presence of 1,2-linked and 1,2,4-linked residues α-L-Rha*p*. The occurrence of the aforesaid residues of α-L-Rha*p* and 1,4-α-Gal*p*A implies the presence of rhamnogalacturonan I.

Detailed analysis of the NMR spectra of fraction HS-I_2 showed that it possessed structural elements similar to that of fraction HS-I_1. However, integral signal intensities indicated that ratio of GlcA to GalA in fraction HS-I_2 was 17:1.

3.4.2. NMR studies of polysaccharide HS-V

As followed from the detailed analysis of the NMR spectra, fraction HS-V possessed similar structural elements, as fractions HS-I_1 and HS-I_2. The fractions differed mainly in the ratio of 4-OMe-β-Glc*p*A to 1,4-α-Gal*p*A. The $\{^{1}H,^{13}C\}$ HSQC spectrum of fraction HS-V contained an intense signal from methoxy groups at

Table 3
^{13}C and ^{1}H NMR data of polysaccharide fragment HS-S, referenced to DSS.

Residue		C-1 *H-1*	C-2 *H-2*	C-3 *H-3*	C-4 *H-4*	C-5 *H-5;5'*	C-6 *H-6;6'*
→4)-β-Man*p*-(1→	M	102.8 *4.76*	72.9 *4.11*	74.4 *3.78*	79.2 *3.79*	77.9 *3.57*	63.1 *4.00;3.83*
β-Man*p*-(1→	Mt	102.6 *4.67*	72.8 *3.99*	n.d.	n.d.	n.d.	n.d.
→4)-β-Glc*p*-(1→	G	105.3 *4.54*	75.6 *3.38*	76.8 *3.68*	81.4 *3.69*	77.0 *3.65*	63.1 *3.97;3.89*
→4)-β-Glc*p*-(1→	G*	105.3 *4.52*	75.6 *3.36*	76.8 *3.67*	81.4 *3.69*	77.0 *3.65*	63.1 *3.97;3.89*
β-Glc*p*-(1→	Gt	105.3 *4.51*	75.6 *3.31*	77.5 *3.52*	72.4 *3.41*	77.8 *3.52*	63.1 *3.91;3.73*
α-Ara*f*-(1→	At	109.9 *5.16*	83.6 *4.15*	79.3 *3.97*	86.7 *4.06*	63.1 *3.85;3.76*	
→5)-α-Ara*f*-(1→	A	110.4 *5.10*	83.6 *4.14*	79.3 *3.97*	84.5 *4.19*	69.4 *3.87;3.81*	

55.8/3.81 ppm, suggesting the presence of regions with partially methyl-esterifyed D-Gal*p*A. On the ^{1}H NMR spectrum, the signals of the anomeric protons of 1,4-linked GalA (5.04 ppm) and 1,4-linked GalA 6-methyl ester residues (4.95 ppm) were significantly more intensive than those of 4-OMe-β-GlcA (4.62 ppm), which implied the prevalence of D-Gal*p*A residues.

The monosaccharide composition of fraction HS-IV was close to that of HS-V. Thus, the two last acidic fractions contained mainly the residues of 1,4-β-D-galacturonic acid, as well as of 4-OMe-D-glucuronic acid. These data suggested the presence of pectic polysaccharides.

Arabinogalactan proteins are thought to interact with other components of the cell wall, e.g. pectins; therefore, their isolation presents a difficult problem (Gorshkova, 2007; Oosterveld, Voragen, & Schols, 2002; Rumyantseva, 2005). It has been demonstrated that the fraction of arabinogalactan proteins contained pectic polysaccharides (Immerzeel, Eppink, de Vries, Schols, & Voragen, 2006; Oosterveld et al., 2002; Pellerin et al., 1995; Redgwell et al., 2002). Immerzeel et al. (2006) found that at least a part of AGP from the extracellular medium and cell walls of carrot could be covalently linked to pectin-containing homogalacturonan structural element. Oosterveld et al. (2002) suggested that the arabinogalactan protein present in the pectic extract from hop (*Humulus lupulus*) was also linked to pectin. AGP from red wine contained both glucuronic and galacturonic acids in association with 2- and 2,4-linked rhamnose, indicating the presence of AG rhamnogalacturonan fragments (Pellerin et al., 1995). Redgwell et al. (2002) speculated that AGP from *C. arabica* could be linked to pectin fraction.

Increase of uronic acid content from 23 to 58% in fractions HS-I–HS-V can be explained by decrease of the AGP part and increase of the pectic polysaccharide part.

3.4.3. NMR spectroscopy of polysaccharide HS-S

The COSY spectrum of fraction HS-S allowed the full assignment of protons from H1 to H6 of terminal β-D-Glc*p*, 1,4-linked β-D-Glc*p*, and 1,4-linked β-D-Man*p* (Table 3). An $\{^{1}H,^{13}C\}$ HSQC spectrum of fraction HS-S (Fig. 3) confirmed the presence of terminal and 1,4-linked residues of β-D-Man*p* and β-D-Glc*p*.

A ROESY spectrum of fraction HS-S indicated the presence of the following intra-residue cross-peaks of β-mannose: H1 with H2, H3, H5 at 4.76/4.11, 4.76/3.78, and 4.76/3.57 ppm, respectively, as well as transglycosidic cross-peak H1/H4 at 4.76/3.79 ppm implying the presence of the fragment . . .→4)-β-Man*p*-(1→4)-β-Man*p*-(1→. . . This spectrum showed the following inter-residue cross-peaks as well: H1/H4 at 4.54/3.69 and H1/H6 4.54/3.97 ppm, implying the presence of the fragment . . .→4)-β-Glc*p*-(1→. . . (Ghotra, Vasanthan, & Temelli, 2008; Hannuksela & Penhoat, 2004; Teleman, Nordstrom, Tenkanen, Jacobs, & Dahlman, 2003).

In addition, the ROESY spectrum of fraction HS-S contained the following transglycosidic cross-peaks: H1 of 1,4-linked β-D-Man*p* with H4 and H6 of 1,4-linked β-D-Glc*p* at 4.76/3.69 ppm and 4.76/3.89 ppm, respectively, implying the presence of the fragment . . .→4)-β-Man*p*-(1→4)-β-Glc*p*-(1→. . .; H1 of 1,4-linked β-D-Glc*p* with H4 and H6 of 1,4-linked β-D-Man*p* at 4.54/3.79 ppm and 4.54/3.83 ppm, respectively, implying the presence of the fragment . . .→4)-β-Glc*p*-(1→4)-β-Man*p*-(1→. . . Thus, NMR spectroscopic data allowed us to establish that the carbohydrate backbone of fraction HS-S consisted of statistically alternating residues of 1,4-β-D-mannopyranose and 1,4-β-D-glucopyranose. In the ^{1}H NMR spectrum, the ratio of integral intensities of H1 of 1,4-β-D-mannopyranose (4.76 ppm) and of 1,4-β-D-glucopyranose (4.54 ppm) was ∼ 1/0.35; therefore the part of 1,4-β-D-glucopyranose comprised ∼35%, which was characteristic of the hemicellulose class of glucomannans. It was reported that the typical part of 1,4-β-D-glucopyranose in glucomannans is 25–35% (Gorshkova, 2007).

In the COSY spectrum, the cross-peak H1/H2 at 4.52/3.36 ppm from 1,4-linked β-D-Glc*p* apparently suggested the presence of the hemicellulose class of glucans.

The monosaccharide composition and results of NMR spectroscopic analysis of fraction HS-S revealed the presence of several classes of polysaccharides. According to the obtained data, in addition to mannose and glucose, fraction HS-S also contained protein (4.2%), glucuronic acid (8.5%), arabinose (5.2%), and galactose (0.7%).

The COSY and HSQC spectra of fraction HS-S contained signals from the residues of terminal and 1,5-linked α-L-Ara*f* (Fig. 3). The residues of 1,5-linked α-L-Ara*f* were also found in AGP from roots of *E. pallida*, *Baptisia tinctoria*, exudate gum from *Meryta sinclairii* and gum arabic (Classen, Thude, Blaschek, Wack, & Bodinet, 2006; Sims & Furneaux, 2003).

In the polysaccharide HS-S, the protein content was higher than in HS-I_1 and HS-I_2, and signals characteristic for a protein moiety (Breitmaier & Voelter, 1990) were significantly more intense. Particularly, fraction HS-S demonstrated intense signals in the region of 3.0–0.8 ppm in the ^{1}H NMR spectrum; cross-peaks at 176.6/2.14, 180.1/2.01 and 184.3/1.91 ppm in the HMBC spectrum, cross-peaks at 23.1/2.19, 23.0/2.14, 24.0/2.01 and 25.9/1.91 ppm in the HSQC spectrum, as well as large group of CH signals in the upfield region 17–25/0.8–1.4 ppm.

Thus, fraction HS-S contains hemicellulose of glucan and glucomannan classes, and, probably, AGP fragments structurally similar to fraction HS-I_1.

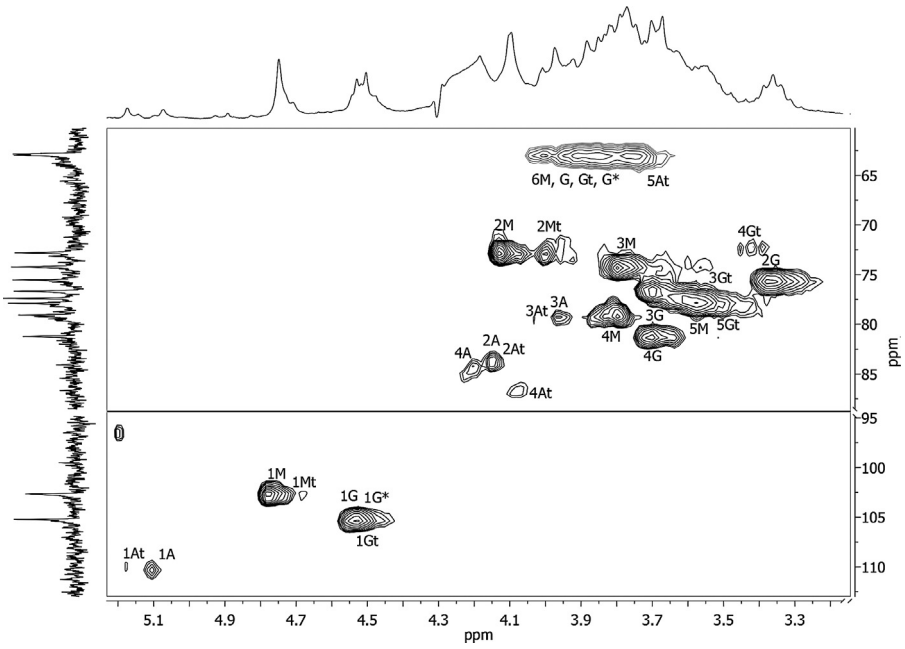

Fig. 3. {^{1}H,^{13}C} HSQC spectrum of polysaccharide fragment HS-S.

4. Conclusions

Using sequential water extraction, five polysaccharide fractions containing 23–58% of D-glucuronic acid residues and 6.6–9.1% of protein were obtained from above-ground parts of *H. sosnowskyi*.

Sugar analysis and NMR spectroscopy revealed that the backbone of the polysaccharide mainly consisted of 1,3-linked and 1,3,6-linked residues of Gal*p*. The side chains were composed of 1,3,6-β-linked and 1,6-β-linked Gal*p*, 1,5-linked Ara*f*, 1,4-linked β-Glc*p*A, 1,6-linked β-Glc*p*, and terminal 4-OMe-β-D-Glc*p*A, Ara*f*, Gal*p* and Rha*p*. A major part of the side-chain β-1,6-galactan was substituted with a single residue of 4-O-Me-β-Glc*p*A via the β-(1→6) linkage. A minor part of glucuronic acid was found in the fragment α-Rha*p*-(1→4)-β-Glc*p*A-(→.

Characteristic structural elements of carbohydrate moiety of AGP isolated from above-ground parts of *H. sosnowskyi* M. were elucidated as following:

…→3)-β-D-Gal*p*-(1→… …→3)-β-D-Gal*p*-(1→…

6

↑

…

major side chain fragments:

4-OMe-β-D-Glc*p*A-(1→6)-β-D-Gal*p*-(1→… β-D-Gal*p*(1→6)-β-D-Gal*p*-(1→…

…→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→… …→6)β-D-Gal*p*(1→…

3

↑

α-L-Ara*f*(1

minor side chain fragments:

α-L-Rha*p*-(1→4)-β-D-Glc*p*A-(1→… 4-Me-β-D-Glc*p*A-(1→6)-β-D-Glc*p*-(1→…

Absolute configurations were assumed basing on common distribution of monomer enantiomers in plant saccharides.

The obtained results suggested that fractions HS-I–HS-V were mixtures of arabinogalactan proteins and pectic polysaccharides, which differ in content of 4-O-Me-D-glucuronic, 1,4-β-D-glucuronic and 1,4-β-D-galacturonic acids. Since the obtained fractions also contained components of pectic polysaccharides, it is possible that the isolated arabinogalactan proteins might be linked to pectin.

This study reports the first structural evidence of the presence of an arabinogalactan protein in the cell walls of *H. sosnowskyi*. Investigation of the structure of polysaccharides from cow-parsnip will allow expansion of knowledge of structural characteristics of plant polysaccharides. In the future, while studying their physiological and biological activity, this investigation will promote establishment of the correlation between the structure of sugar macromolecules and their useful properties.

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