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Structural studies of the pectic polysaccharide from duckweed *Lemna minor* L.

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Abstract

The pectic polysaccharide of duckweed *Lemna minor* L. termed lemnan (LM) was shown to contain the ramified, ''hairy'' region. Using partial acid hydrolysis and Smith degradation followed by NMR spectroscopy of the fragments obtained, some structural features of the hairy region of LM were elucidated. Partial acid hydrolysis of LM afforded the crude polysaccharide fraction LMH that was separated into two polysaccharide fractions: LMH-1 and LMH-2. In addition, the oligosaccharide fraction LMH-3 contained 97% D-apiose was obtained from the supernatant. A further more rigorous acidic hydrolysis of LMH led to the crude polysaccharide fraction LMHR which was separated in to two fractions: LMHR-1 and LMHR-2. Smith degradation of LMH afforded the polysaccharide fragment LMHS differed in low contents of apiose residues. Unfortunately, NMR-spectroscopy failed to provide significant evidence concerning the structure of LMH-1 due to the complexity of the macromolecule. The structure of the $^{1}H/^{13}C$-NMR spectroscopy including the correlation 2D NMR spectroscopy. As a result, α-1,4-D-galactopyranosyluronan was confirmed to be the main constituent of the LM backbone. In addition, the ramified, ''hairy'' region of the macromolecule appeared to contain segments consisting of residues of terminal and β-1,5-linked apiofuranose, terminal and α-1,5-linked arabinofuranose, terminal and β-1,3- and β-1,4- linked galactopyranose, the terminal and β-1,4-linked xylopyranose, and β-1,4-linked 2-mono-*O*-methyl xylopyranose. Analytical and NMR-spectral data of LMHS confirmed the presence of considerable amounts of the non-oxidized of 1,4-linked D-galactopyranosyl uronic acid residues. Thus, some side chains of the ramified region of lemnan appeared to attach to D-galactopyranosyl uronic acid residues of the backbone.

Keywords: Lemnaceae; *Lemna minor* L.; Plant polysaccharides; Pectin structure; Lemnan; Pectin fragmentation; NMR-spectroscopy of pectins

1. Introduction

Apiogalacturonans have been previously isolated from duckweed *Lemna minor* L. and preliminary characterized (Hart and Kindel, 1970; Kindel et al., 1996; Cheng and Kindel, 1997). The apiogalacturonan fragment appeared to be part of a more complicated pectin, which we have isolated from fresh duckweed *L. minor* L. and termed lemnan (LM) (Ovodova et al., 2000). The saccharide chains of LM has been shown to consist of D-galacturonic acid (64%) and D-apiose (25%), as the main constituents, and galactose, arabinose, rhamnose, and xylose. LM was reported to have α-1,4-D-galacturonan as the backbone of the macromolecule. The branched apiogalacturonan appeared to be the main component of the ramified, ''hairy'' region of LM in addition to the side chains contained the residues of galactose, arabinose, and xylose. Some D-apiose residues appeared to occupy the terminal positions of the macromolecule (Ovodova et al., 2000).

LM has been shown to possess immunomodulatory activity namely to enhance phagocytosis (Popov et al., 2000). The present paper reveals the further structural studies of the ramified region of LM macromolecule.

2. Results and discussion

A previous isolation of apiogalacturonan from duckweed *L. minor* L. (Hart and Kindel, 1970) appeared to demonstrate the presence of linkages between galacturonan

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Table 1
Sugar compositions of LM and its fragments

LM and its fragments	Yield of the LM (%)	Contents of the sugar residues (%)						
		Gal A	Api	Ara	Gal	Rha	Xyl	Xyl-2-OMe
LM	100	64.0	23.3	3.4	4.5	2.3	4.4	Trace
LMH	78.9	72.2	16.7	3.7	4.8	2.0	5.4	Trace
LMH-1	60.9	70.7	12.2	3.0	4.9	1.2	5.2	Trace
LMH-2	1.1	24.7	18.2	15.0	22.7	2.7	4.1	0.7
LMH-3	18.0	–	97.0	–	–	–	–	Trace
LMHR-1	2.8	84.1	1.3	3.3	6.4	Trace	3.1	Trace
LMHR-2	4.0	60.8	0.9	2.4	12.9	1.0	11.8	0.4
LMHS	36.8	31.2	1.0	14.4	19.5	1.5	19.3	Trace

backbone and side chains composed of D-apiose residues. This, in connection with our own studies, suggested that the LM merited further studies: the "hairy", ramified region.

A complete acidic hydrolysis of LM with 2 M aqueous trifluoroacetic acid (TFA) at 100 °C for 3 h led to the following monosaccharides in the hydrolysate: galacturonic acid, apiose, rhamnose, arabinose, galactose, xylose, and trace of 2-mono-*O*-methyl xylose (Table 1) as the constituents of the sugar chains of the macromolecule.

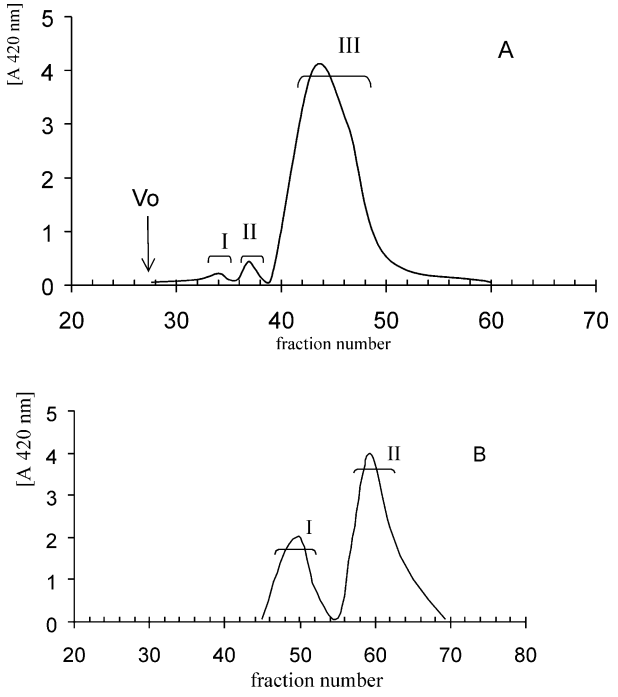

Fig. 1. Gel chromatography on Sephacryl S-500 (column 2.4×60 cm, void volume, $V_0 = 96$ ml, a rate of water as eluent is 32 ml/h). (A) Separation of the polysaccharide fraction LMH. Peaks: I and II—minor concomitant fractions ($K_{av} = 0.02$ and 0.05; yields 4.0 and 9.6% the parent LMH, respectively); III—fragment LMH-1 ($K_{av} = 0.10$; yield 77.2% the parent LMH). (B) Separation of the crude fraction LMHR. Peaks: I—fraction LMHR-1 ($K_{av} = 0.15$; yield 30% the parent LMHR); II—fraction LMHR-2 ($K_{av} = 0.24$; yield 43% the parent LMHR).

A partial acid hydrolysis of LM with 0.01 M TFA at 80 °C for 3 h released D-apiose as the only monosaccharide thus indicating the terminal positions of some D-apiose residues and afforded the crude polysaccharide fraction LMH which was precipitated from the hydrolysate by ethanol and was purified by gel chromatography on Sephacryl S-500 column to furnish the purified polysaccharide fraction LMH-1 (Fig. 1A). The second polysaccharide fraction LMH-2 was isolated from the supernatant after precipitation of LMH-1 and was purified by gel chromatography on Sephacryl-500 column. LMH-2 showed to the single peak only.

The sugar compositions of polysaccharide fractions obtained are given in Table 1. In addition, the purified apio-oligosaccharide fraction LMH-3 was isolated using evaporation of the residual supernatant with methanol followed by gel chromatography on Bio Gel P-2 column (Fig. 2). It can be seen from Table 1, that the D-apiose content of LMH-1 was only half that of the parent LM. This supports the suggestion of terminal positions of some D-apiose residues in the macromolecule. In addition, D-GalA increased whilst the contents of the other sugars were not changed significantly. The purified polysaccharide fraction LMH-2, $[\alpha]_D^{20} + 78.1°$ (*c* 0.1;

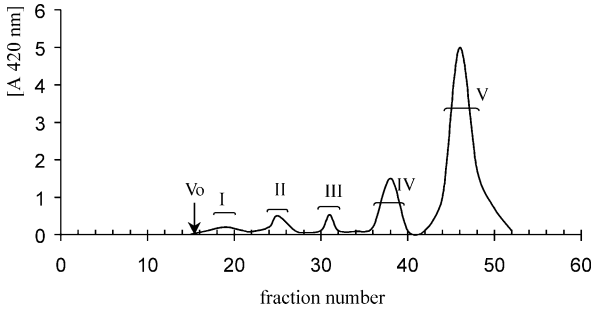

Fig. 2. Fractionation of D-apiose and apiooligosaccharides mixture on Bio Gel P-2; column (1.6×76 cm, void volume, $V_0 = 26$ ml, a rate of water as eluent is 9 ml/h). Peaks: I–III unidentified oligosaccharides fractions ($K_{av} = 0.08$; $K_{av} = 0.27$; $K_{av} = 0.47$; yield 1.1; 2.3 and 2.0% the parent mixture, respectively); IV—apio-oligosaccharide fraction LMH-3 ($K_{av} = 0.70$; yield 8.9% the crude mixture); V—apiose ($K_{av} = 0.96$; yield 60.0% the parent mixture).

H_2O), contained less amounts of D-galacturonic acid (24.7%) and may represent the apiogalacturonan fragment connected with rhamnogalacturonan I as main constituent of LMH-2, bearing the arabinogalactan hairy region.

A further acidic hydrolysis of the polysaccharide fragment LMH with 0.05 M TFA at 100 °C for 2 h led to the crude polysaccharide fraction LMHR which was separated using gel chromatography on Sephacryl S-500 column into two fractions as follows: LMHR-1 (30%), $[\alpha]_D^{20} + 141.4°$ (*c* 1.0; H_2O) and LMHR-2 (43%), $[\alpha]_D^{20} + 102.1°$ (*c* 1.0; H_2O) (Fig. 1B). The sugar compositions of both fragments are listed in the Table 1. LMHR-1 appeared to represent mainly the homogalacturonan part of the backbone. Apiose and arabinose were detected in the ethanolic supernatant after precipitation of polysaccharide LMHR from the hydrolysate. The arabinose residues are suggested to occupy the terminal positions of the hairy region in addition to the terminal residues of apiose in the apiogalacturonan fragment of LM.

Smith degradation of LMH was found to afford the polysaccharide fraction LMHS. Analytical data for LMHS are listed in Table 1. A substantial amount of the galacturonic acid residues was resistant to periodate oxidation, which suggested that they participated in glycosidic bonds between the galacturonan backbone and ramified regions of LM.

Unfortunately, NMR-spectroscopy failed to provide the significant evidence concerning the structural features of LMH-1 due to the complexity of the macromolecule. 2D NMR spectra of LMH-2 (Fig. 3) indicated the presence of β-1,5- linkage between the some D-apiose residues. The closed spin systems of CH_2-protons with cross-peaks at 4.12/3.95, 4.18/3.92, and 3.83/3.68 ppm are detected in the COSY and TOCSY spectra. The ^{13}C-resonances from the CH_2-groups were found according to HSQC data at 75.0, 74.5 and 72.3 ppm, respectively. The former two chemical shifts at 75.0 and 74.5 ppm are known to be typical for the resonance from C4 of D-apiose residues (Ovodova et al., 2000). The resonance at 73.3 ppm is most likely from a substituted CH_2-group. The correlation peak at 5.12/3.68 ppm is present in the ROESY spectrum.(data not show) These data demonstrated that the C-atom of CH_2-group which resonated at 73.3 ppm (^{13}C) and at 3.83 and 3.68 ppm (^1H) appeared to represent C5-atom of the apiose residue substituted by the second apiose residue showing the resonances at 5.12 and 110.3 ppm from H1 and C1 atoms, respectively (HSQC data).

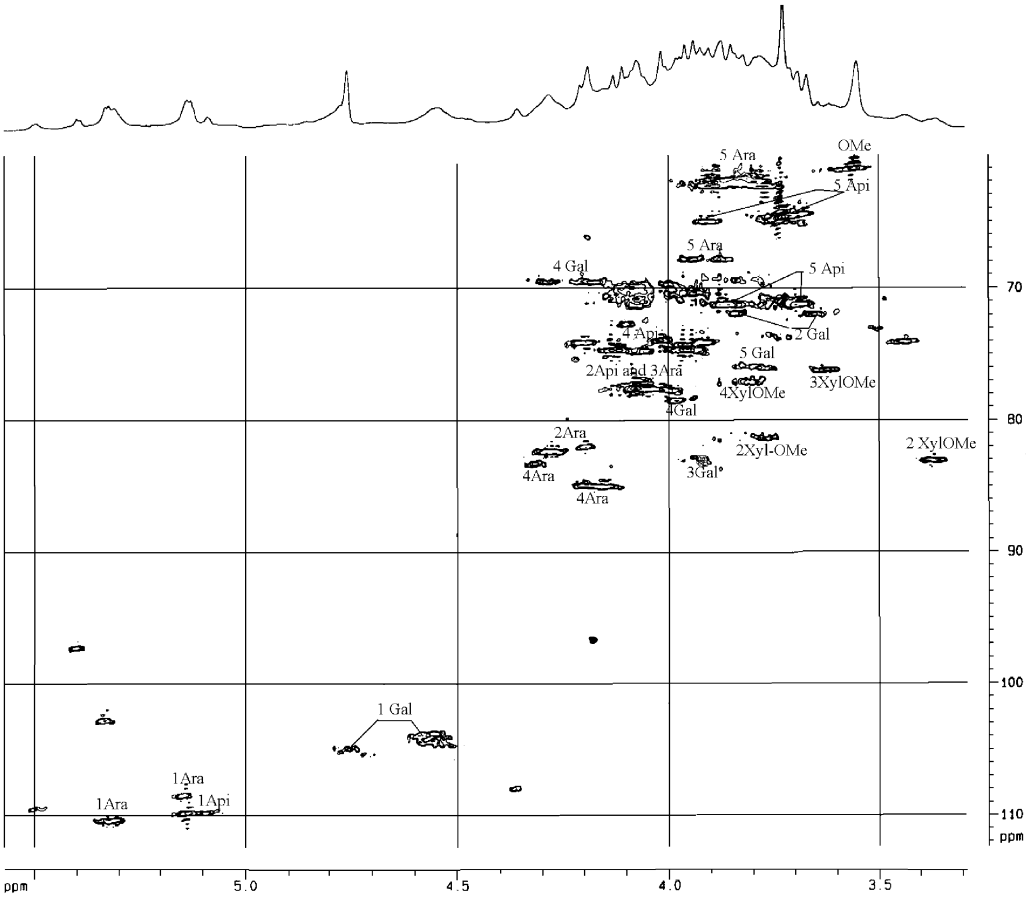

Fig. 3. $^1H/^{13}C$ HSQC spectrum of the polysaccharide fraction LMH-2.

Analysis of the two-dimensional spectra COSY, TOCSY, ROESY and HSQC demonstrated the presence of the terminal and 1,5-linked α-arabinofuranose residues which appeared to substitute the neighboring α-arabinofuranose residues in 3- and 5-positions (Table 2). In addition, the polysaccharide LMH-2 contained terminal β-galactopyranose residues. A downfield shift of the galactopyranose derived resonance C3-(83.2 ppm) and C4-(78.1 ppm) atoms suggest the presence of β-1,3 and β-1,4-linked galactopyranose residues. The signals of the other C-atoms were interpreted also (Table 2 and Fig. 3). A resonance of the anomeric atom at 104.2 ppm and a downfield shift of the C2-atom resonance (83.3 ppm) demonstrated a presence of the terminal residues of β-galactopyranose and 1,4-linked residues of 2-mono-*O*-methyl xylopyranose (Table 2 and Fig. 3).

Thus, all the above data indicated that the following sugar residues are involved in the ramified region of the LM macromolecule: terminal and β-1,5-linked apiose, terminal, β-1,3- and β-1,4-linked galactose, terminal and α-1,5-linked arabinose, and β-1,4-linked 2- mono-*O*-methyl xylose.

The NMR-spectral data for the purified apio-oligosaccharide fraction LMH-3 showed the presence of 1,5-linked D-apiose residues. The resonances of the anomeric atoms of the D-apiose residues are observed at 109.95 and 109.1 ppm in the ^{13}C-NMR spectrum (Table 3). The signals in the field of resonance from anomeric protons (4.7–5.3 ppm) are observed in the ^{1}H NMR spectrum with coupling constant 4 Hz which are characteristic of the D-apiose residues having β-configuration of the anomeric centre. Such residues showed the main resonances in the two-dimensional spectra. The most intensive signals are assigned to terminal and 1,5-linked D-apiose residues (Table 3). In addition, the most intensive correlation peak at 5.07/3.62–3.64 ppm as well as the correlation peak with low intensity at 5.07/3.80 and 5.07/4.03 ppm which demonstrated that the spatial interactions of the anomeric protons with H5, H5′ and H4 atoms of the apiose residues are observed in the ROESY spectrum. The HMBC spectrum showed the intra-residual correlation peak at 5.07/74.8 ppm (H1/C4, strong) and the inter-residual peak at 5.07/71.2 ppm (H1/C5, weak) thus confirming an occurrence of the 1,5-linked apiose residues as a disaccharide fragment of the apiooligosaccharide fraction LMH-3 (Fig. 4).

These data confirmed the presence of the β-1,5-linked apiose residues in the LM macromolecule.

The NMR spectra of the fragment LMHR-1 failed to be sufficiently informative due to high content of D-galacturonic acid residues. As ones of it, only the NMR spectral data for LMHR-2 were interpreted.

Table 2
Chemical shifts of resonances in the ^{13}C and ^{1}H NMR spectra of LMH-2

Residues	Chemical shifts (δ, ppm)						
	C1/H1	C2/H2	C3/H3	C4/H4;H4′	C5/H5;H5′	C6/H6;H6′	OMe
α-Ara*f*-(1→5A*	108.9/5.14	82.3/4.20	78.0/4.03	85.4/4.08	62.8/3.89;3.78		
α-Ara*f*-(1→3A*	110.0/5.48	82.8/4.26	78.0/4.00	85.4/4.14	62.8/3.88;3.77		
→5)-α-Ara*f*-(1→	110.6/5.32	82.8/4.28	78.0/4.08	84.7/4.32	68.2/3.94;3.86		
β-Gal*p*-(1→	104.8/4.55	72.0/3.63	73.9/3.76	69.8/4.16	76.6/3.77	62.0/3.83	
→3)-β-Gal*p*-(1→	105.2/4.71	71.8/3.85	83.2/3.93	69.8/4.27	76.6/3.77	62.5/3.85	
→4)-β-Gal*p*-(1→	105.2/4.69	72.4/3.67	73.8/3.72	78.1/4.00	76.0/3.79	62.2/3.88;3.75	
→4)-β-Xyl*p*-2-OMe-(1→	104.2/4.56	83.4/3.33	76.4/3.62	77.3/3.80	n.d.		61.08/3.55
β-Api-(1→	110.3/5.12	78.0/4.08	80.4	75.0/4.12;3.95	65.0/3.73;3.73		
→5)-β-Api-(1	110.3/5.12	78.0/4.08	80.4	74.5/4.18;3.92	72.3/3.83;3.68		

A*—is appeared to be Ara*f*.
n.d.—Not determined.

Table 3
Chemical shifts of resonances in ^{1}H/^{13}C NMR spectra of the apiooligosaccharide LMH-3

Residues	Chemical shifts (δ, ppm)				
	C1/H1	C2/H2	C3	C4/H4; H4′	C5/H5; H5′
β-Api-(1→A*	109.95/5.07	77.70/4.00	80.55	74.80/4.05;3.88	64.85/3.67;3.67
	109.10/5.07	77.70/3.95	80.55	74.80/4.05;3.88	64.65/3.64;3.64
→5-β-Api-(1→A*	n.d.	n.d.	80.55	74.8/4.03;3.90	71.60/4.02;3.64
					71.20/3.80;3.62
					70.90/4.01;3.53

n.d.—Not determined.
A*—Fragment of the residues of α-Gal A or 5-substituted Api.

The heteronuclear correlation $^{1}H/^{13}C$-HSQC spectrum of LMHR-2 showed the resonance of OCH_3-group in the field ca. 61.0 ppm. All the signals of the resonances from C-atoms of the terminal β-galactopyranose and 1,4-linked 2-mono-*O*-methyl xylopyranose residues are observed (Table 4 and Fig. 5). The signals, which are characteristic of the terminal, β-1,3- and β-1,4-linked galactopyranose, are also observed in the HSQC spectrum. These data coincided with those for LMH-2 (Table 2).

The homonuclear ROESY spectrum of LMHR-2 contained *trans*-glycosidic correlation peak of anomeric proton of the terminal β-galactopyranose residue with H3-atom of β-1,3-linked galactopyranose (H1/H3 4.50/3.92). In addition, *trans*-glycosidic correlation peak of anomeric proton of β-1,3-linked galactopyranose with H4 atom of β-1,4-linked galactopyranose (H1/H4 4.75/4.01) is observed. These data demonstrated the presence of the following fragment in the hairy region of LM:

β-Gal*p*-(1 → 3)-β-Gal*p*-(1 → 4)-β-Gal*p*(1 → ...

The $^{1}H/^{13}C$ HSQC spectrum of LMHR-2 indicated the occurrence of the D-galactopyranosyl uronic acid residues containing the non-methoxylated carboxyl groups due to an absence of the resonance of methoxyl group at 54.1 ppm and the signal at 172.4 ppm corresponding to C6-atom of methoxylated galacturonic acid

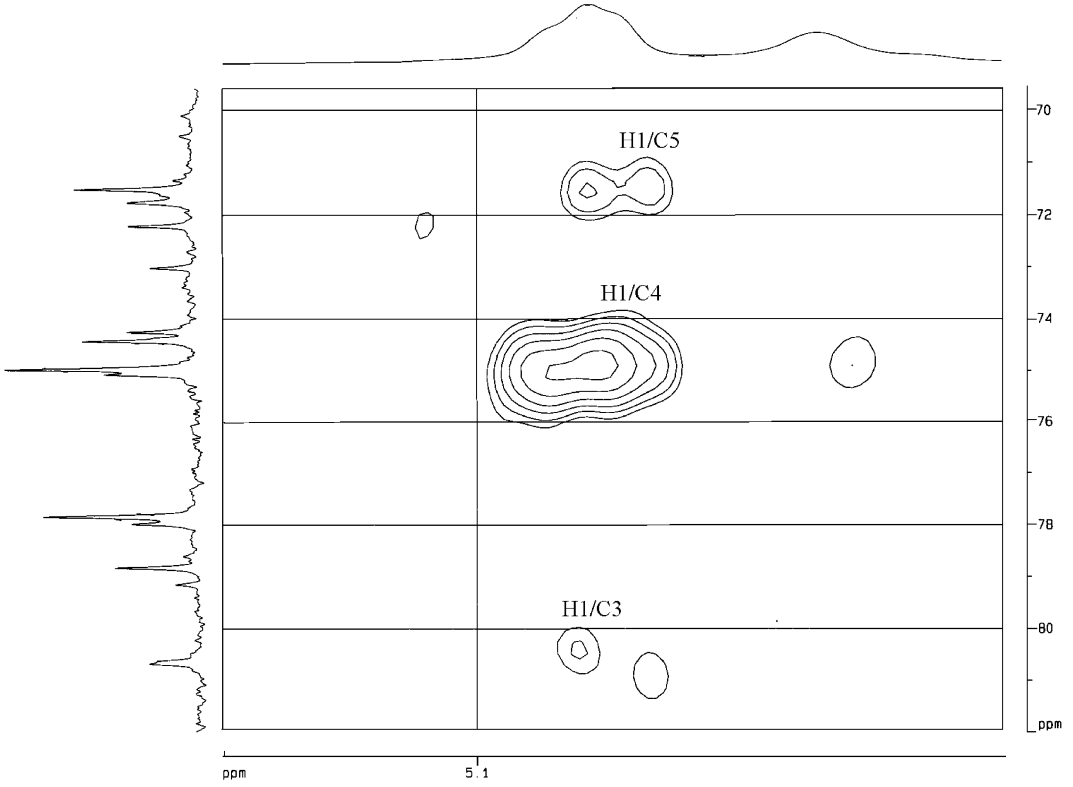

Fig. 4. $^{1}H/^{13}C$ HMBC spectrum of the polysaccharide fraction LMH-3.

Table 4
Chemical shifts of resonances in the ^{13}C and ^{1}H NMR spectra of LMHR-2

Residues	Chemical shifts (δ, ppm)						
	C1/H1	C2/H2	C3/H3	C4/H4;H4′	C5/H5;H5′	C6/H6;H6′	OMe
→4)-α-Gal*p*A-(1→	100.7/5.17	70.0/3.83	70.7/4.05	79.6/4.52	73.1/4.76	176.9	
α-Ara*f*-(1→	110.4/5.33	82.5/4.27	78.2/4.08	85.5/4.17	62.8/3.88;3.77		
β-Gal*p*-(1→	104.9/4.50	72.3/3.61	74.2/3.71	70.0/4.18	76.6/3.75	62.5/3.85	
→3)-β-Gal*p*-(1→	105.2/4.75	71.8/3.85	83.2/3.92	69.8/4.25	76.6/3.75	62.5/3.85	
→4)-β-Gal*p*-(1→	104.2/4.49	72.4/3.67	73.8/3.73	78.1/4.01	76.0/3.78	62.2/3.93;3.80	
β-Xyl*p*-(1→	103.1/4.54	74.2/3.37	76.6/3.61	70.5/3.70	66.5/4.18;3.46		
→4)-β-Xyl*p*-2-OMe-(1→	104.2/4.55	83.4/3.38	76.4/3.61	77.3/3.81	64.5/4.18;3.46		61.08/3.55
→4)-β-Xyl*p*-(1→	103.3/4.56	74.1/3.37	75.0/3.63	77.9/3.79	64.5/4.18;3.46		

residues (Odonmažig et al., 1992; Catoire et al., 1998) (Fig. 5). This statement was confirmed by an analytical estimation (Wood and Siddiqui, 1971) of low contents (0.98%) of methoxyl groups in LMHR-2 containing 60.8% galacturonic acid residues.

A combined analysis of the correlation spectra (COSY, TOCSY, ROESY, HSQC) demonstrated an occurrence of the terminal α-arabinofuranose residues in LMHR-2 (Table 4).

In addition, the $^{1}H/^{13}C$ NMR spectra indicated that the terminal and β-1,4- linked xylopyranose residues were involved in the side sugar chains of LMHR-2 (Table 4).

The NMR spectral data for the fragment LMHS confirmed the presence of β-1,4-xylopyranose residues, α-1,4-linked D-galactopyranosyl uronic acid residues and the terminal α-arabinofuranose residues (Table 5 and Fig. 6).

These data are in agreement with the above suggestion that the side sugar chains consisted of terminal and β-1,3- and β-1,4-linked galactose and terminal and α-1,5-linked arabinose residues. In addition, the β-1,4-linked xylose residues also implicated as present in the side sugar chains.

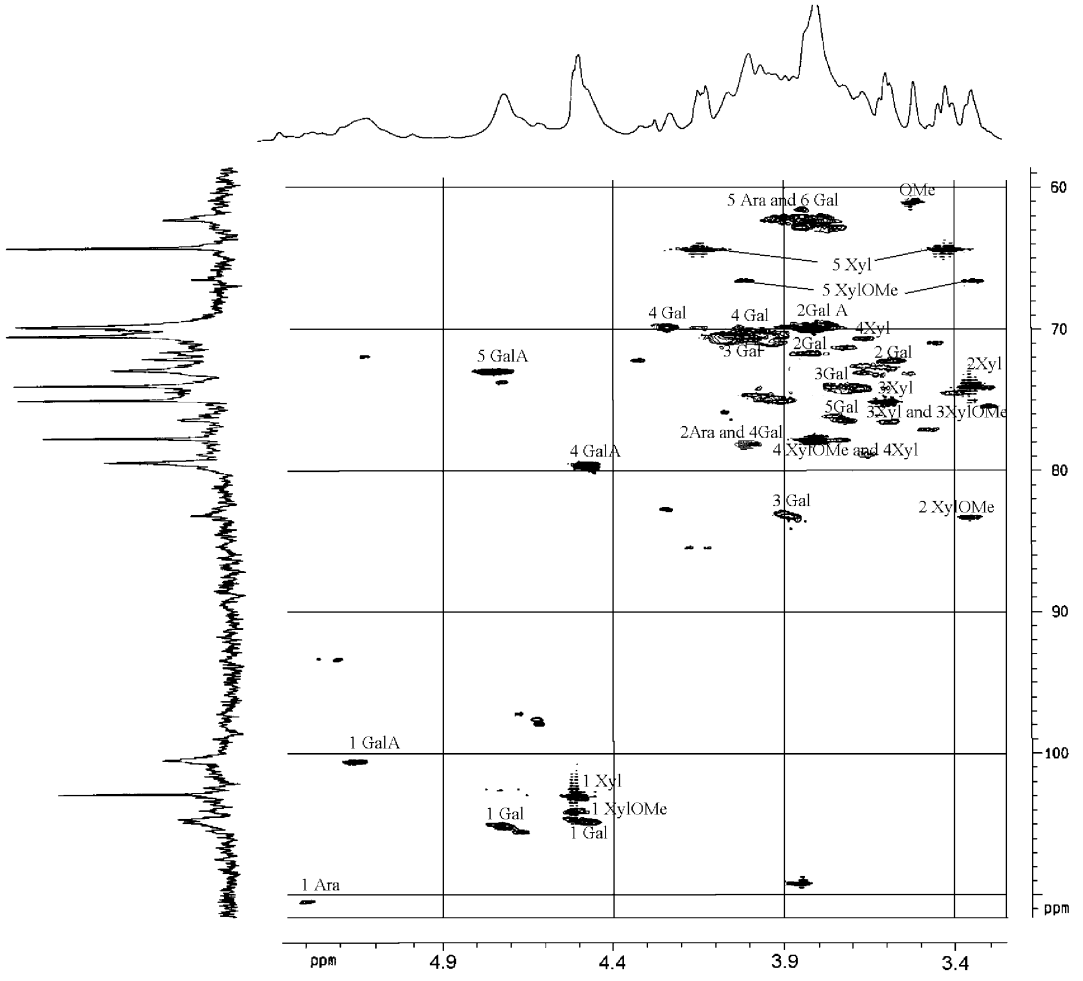

Fig. 5. $^{1}H/^{13}C$ HSQC spectrum of the polysaccharide fraction LMHR-2.

Table 5
Chemical shifts of resonances in the ^{13}C and ^{1}H NMR spectra of LMHS

Residues	Chemical shifts (δ, ppm)					
	C1/H1	C2/H2	C3/H3	C4/H4;H4′	C5/H5;H5′	C6/H6;H6′
→4)-α-Gal*p* A-(1→	100.0/5.18	69.4/3.82	70.0/3.90	79.1/4.52	73.1/4.76	176.9
α-Ara*f*-(1→	110.2/5.38	82.3/4.30	78.0/4.05	85.0/4.20	62.5/3.90;3.80	
→4)-β-Xyl*p*-(1→	102.7/4.60	73.7/3.39	74.8/3.65	77.3/3.79	64.5/4.18;3.46	64.0/4.18;3.48

The NMR spectral data for the fragment LMHS indicated the presence of the β-1,4-xylopyranose, α-1,4-linked galactopyranosyl uronic acid, and terminal α-arabinofuranose residues. (Table 5 and Fig. 6).

Thus, the following fragment proved to be the main constituent of the LM backbone:

$\ldots \rightarrow$ 4-α-D-Gal*p*A-$\left(1[\rightarrow 4)$-α-D-Gal*p*A-$(1 \rightarrow]_n 4\right)$

-α-D-Gal*p*A $\rightarrow \ldots$

In addition, the ramified region of the macromolecule appeared to contain the side chain segments as follows:

β-Api-(1 → 5)-β-Api-(1 → ...

α-Ara*f*-(1 → 5)-α-Ara*f*-(1 → ...

β-Gal*p*-(1 → 3)-β-Gal*p*-(1 → 4)-β-Gal*p*(1 → ...

β-Xyl*p*-(1 → 4)-β-Xyl*p*-(1 → ...

... → 4)-β-Xyl*p*-2 – OMe-(1 → ...

3. Experimental

3.1. Plant material

Duckweed *L. minor* L. was harvested at the period since September till October from the lake surface near Syktyvkar, Komi Republic, Russia.

3.2. Isolation of lemnan LM

LM was isolated from the fresh duckweed and purified as described earlier in details (Ovodova et al., 2000).

3.3. General experimental procedures

The galacturonic acid contents were determined using an interaction with 3,5-dimethyl phenol in the presence of conc. sulphuric acid and calibration against the authentic sample of D-galacturonic acid (Usov et al., 1995).

Quantitation of methyl ester groups was carried out as described earlier (Wood and Siddiqui, 1971) using a calibration curve for methanol. The sodium periodate

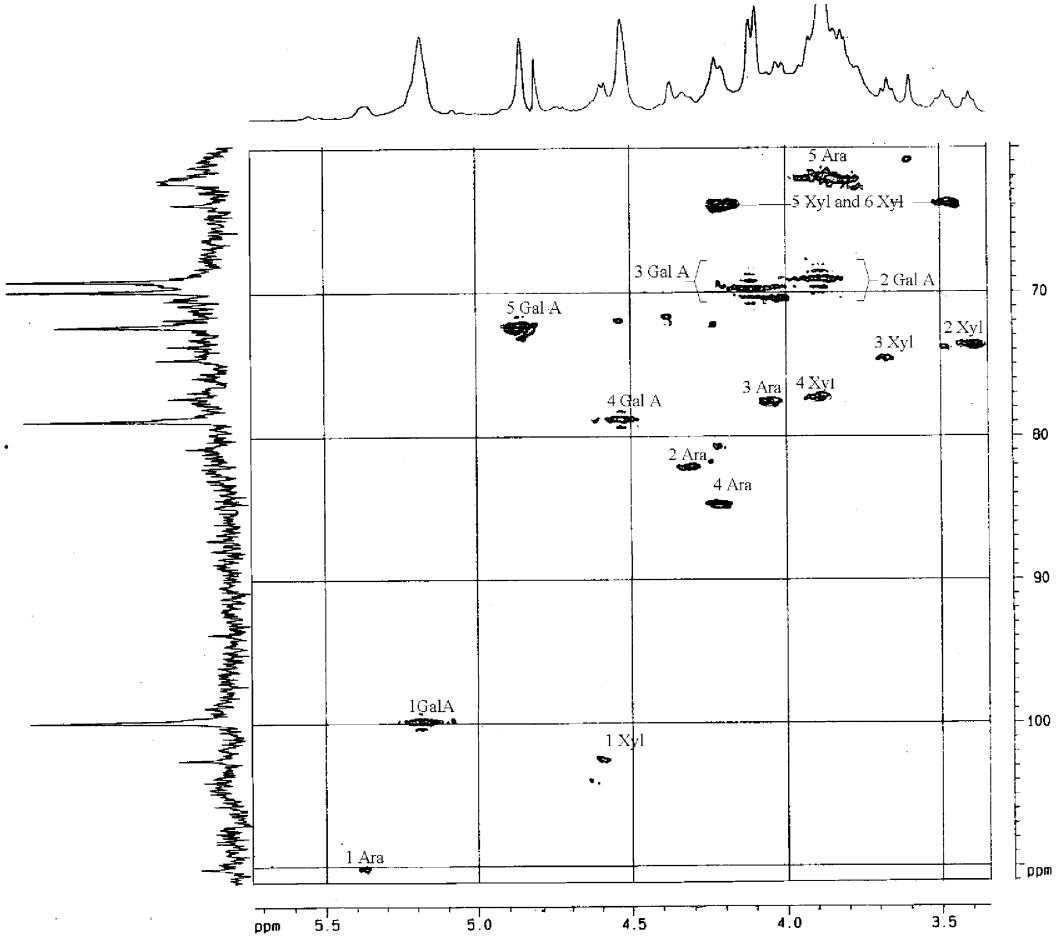

Fig. 6. 7 $^{1}H/^{13}C$ HSQC spectrum of the polysaccharide fraction LMHS.

consumption was estimated by spectrophotometry at 223 nm using Ultrospec 3000 spectrophotometer (England). Gel chromatography of polysaccharide fragments was carried out on Sephacryl S-500 (2.4×60 cm) column (void volume, $V_o = 96$ ml) using dist.water as eluent with a rate of 32 ml/h. Fractions (3.2 ml each) were collected and combined in the accord with the results of analysis by phenol-sulfuric acid procedure (Dubois et al., 1956).

Descending paper chromatography (p.c.) was run on Filtrak FN-12 and FN-13 papers in the solvent system of *n*-butanol-pyridine-water (6:4:3, v/v/v) followed by indication of sugars with anilin hydrogen phtalate at 105 °C.

Monosaccharides were determined and quantitated as the corresponding alditol acetates (York et al., 1985) by gas-liquid chromatography (g.l.c.) on Hewlett-Packard 4890 instrument (USA) equipped with a RTX-1 (0.25×30 m) column in the temperature range of 175–250 °C (Δ 3 °C/min). The monosaccharide quantities were calculated from the peak areas using molar coefficients and *myo*-inositol as the internal standard.

NMR-spectra were performed on DRX-500 Bruker instrument (Germany) for 3–5% solutions of saccharides in D_2O at 30 °C (the internal standard—aceton, δ_H 2.225 ppm, δ_C 31.45 ppm).

The two-dimensional spectra were run using the standard Bruker procedures.

Optical rotation values were measured in tubes of the 1 ml at 20 °C on Polatronic MHZ polarimeter (Germany).

The solutions were evaporated in vacuum at 40 °C.

3.4. Complete acidic hydrolysis

Saccharides (2–3 mg) were treated with 2 M trifluoroacetic acid (TFA, 1 ml) in the presence of *myo*-inositol (0.5 mg/ml) as the internal standard for 3 h at 100 °C. The excess of TFA was removed by repeated evaporation with methanol up to dryness. The sugars were identified by p.c. and g.l.c.

3.5. Partial acidic hydrolysis

(a) LM (2 g) was heated with 0.01 M TFA (160 ml) at 80 °C for 3 h, the mixture obtained was poured in 96% ethanol (160 ml), the precipitate was separated by centrifugation, dissolved in H_2O and lyophilised to afford the polysaccharide fraction LMH, yield 78.9%. The material obtained (50 mg) was dissolved in dist. water (5 ml) and subjected to gelchromatography on Sephacryl S-500 column (2.4×60 cm, void volume 96 ml, a rate of water as eluent is 32 ml/h) to furnish the purified polysaccharide LMH-1 (K_{av} 0.10; yield 38.6 mg) corresponding to the main peak on the elution curve (Fig. 1A).

The supernatant obtained after removing LMH-1 was evaporated up to 2 ml, and 96% ethanol (100 ml) was added to the residual material. The precipitate obtained was subjected to gel chromatography on Sephacryl S-500 column to give the purified polysaccharide fraction LMH-2 (14.7 mg) which showed the single peak with K_{av} 0.06.

The combined filtrates were evaporated with methanol to yield the mixture of D-Apiose and apiooligosaccharides (360 mg). D-Apiose, $[\alpha]_D^{20} + 5.4°$ (*c* 1.0; H_2O) was isolated as described earlier (Ovodova et al., 2000). The mixture of apio-oligosaccharides (300 mg) was separated using gel chromatography on Bio Gel P-2 column (1.6×76 cm, void volume 26 ml, a rate of water as eluent is 9 ml/h). Five oligosaccharide fractions were obtained and their sugar compositions were determined. (Fig. 2) The oligosaccharide fraction LMH-3 consisted of D-apiose (97%) was collected, yield ca. 9% (26.8 mg), and subjected to further investigations.

(a) The polysaccharide fraction LMH (1 g) was treated with 0.05 M TFA (100 ml) for 3 h at 100 °C, the mixture obtained was centrifugated and the supernatant was poured into ethanol (500 ml). The precipitate obtained was separated by centrifugation, the residual material was washed thrice with ethanol, dissolved in water and lyophilised to furnish a crude polysaccharide fraction (92 mg) which was subjected to gel chromatography on Sephacryl S-500 column to yeild two purified polysaccharide fractions as follows: LMHR-1, yield 27.6 mg, K_{av} 0.15, and LMHR-2, yield 39.7 mg, K_{av} 0.22. The elution curve is given in Fig. 1.

3.6. Smith degradation of LMH

LMH (40 mg) was dissolved in dist. water (3 ml), an aqueous solution of sodium metaperiodate (25 mg in 1 ml of water) was added and the mixture obtained was kept in the darkness at 5 °C for 24 h up to a complete consumption of sodium metaperiodate. 10% aqueous ethylene glycol (1.0 ml) was added to the solution and the mixture obtained was kept in the darkness at 5 °C for 1 h, sodium borohydride (100 mg) was added and the solution obtained was dialysed and lyophilised to afford the polyalcohol fraction (20 mg) which was subjected to partial hydrolysis with 1% acetic acid (5 ml) for 2 h at 100 °C. The material obtained was concentrated up to 1 ml and the residue was chromatographed on Sephadex G-25 column to afford the polysaccharide fraction LMHS (14.7 mg). Fraction corresponding to the single peak with K_{av} 0 were combined and the solution obtained was lyophilized to furnish the polysaccharide fraction.

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