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Carbohydrate Research 341 (2006) 2449–2455
Carbohydrate RESEARCH

Note

Structural characterisation of novel lichen heteroglycans by NMR spectroscopy and methylation analysis

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Received 16 March 2006; received in revised form 8 June 2006; accepted 27 June 2006
Available online 1 August 2006

Abstract—Two galactofuranomannans, Ths-4 and Ths-5, were isolated from the lichen, *Thamnolia vermicularis* var. *subuliformis*, using ethanol fractionation and anion-exchange and size-exclusion chromatography. The average molecular weights of Ths-4 and Ths-5 were estimated to be 19 and 200 kDa, respectively. Structural characterisation of Ths-4, Ths-5 and their partially hydrolysed derivatives was performed by methanolysis and methylation analysis. The intact and partially hydrolysed Ths-4 was further analysed using NMR spectroscopy (1D, COSY, NOESY, TOCSY, HSQC and HMBC).

According to the data obtained, the heteroglycans Ths-4 and Ths-5 have similar structures, but have large differences in molecular weight. The structure is composed of 3-O-linked and 5-O-linked galactofuranosyl chains linked to a mannan core. The mannan core consists of a main chain of α-(1→6)-linked mannopyranosyl residues, substituted at O-2 with either a single α-mannopyranosyl unit or an α-Man*p*-(1→2)-α-Man*p*-(1→2)-α-Man*p* group in the ratio of approximately 1:3, respectively. The polysaccharides have idealised repeating blocks as is shown.

Keywords: Lichens; *Thamnolia*; Galactofuranomannan; NMR spectroscopy; Methylation analysis

Thamnolia vermicularis (Sw.) Schaer. var. *subuliformis* (Ehrh.) Schaer.[1] previously named *Thamnolia subuliformis* (Ehrh.) W. Culb.,[2] does not belong to a lichen family, but is classified in a group named Lichen imperfecti, which is a heterogeneous group of sterile lichen species.[3] *T. vermicularis* var. *subuliformis* grows throughout the world, but is more common in the Northern Hemisphere.[1] Polysaccharides isolated from lichens are primarily linear glucans and galactomannans; however, a few complex heteroglycans have been described.[4] Previously, two unusual lichen polysaccharides have been isolated from *T. vermicularis* var. *subuliformis*: a novel cold water-soluble heteroglycan named thamnolan, which consists of (1→3)- and (1→5)-β-linked galactofuranosyl chains and a rhamnan-rich core[5] and an alkali-extractable gel-forming β-glucan of lentinan-type, named Ths-2.[6] Both thamnolan and Ths-2 have been shown to be active in an in vitro classical anti-complementary assay, and thamnolan has also shown activity in a phagocytosis assay in vitro.[5,6]

The aim of the present work was to isolate and chromatographically purify two new water-soluble heteroglycans, Ths-4 and Ths-5, from the lichen, *T. vermicularis* var. *subuliformis*, and to characterise the structure using 1D proton, COSY, NOESY, TOCSY, HSQC and HMBC NMR experiments in combination with partial acid hydrolysis, methylation analysis and methanolysis.

Ths-4 and Ths-5 were obtained by anion-exchange chromatography, followed by preparative HPGPC (high-performance gel-permeation chromatography) using conditions described in Section 1. The mean M_r

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doi:10.1016/j.carres.2006.06.026

values of Ths-4 and Ths-5 were determined to be 19 and 200 kDa, respectively, by comparison with dextran standards. Ths-4 and Ths-5 were treated with oxalic acid giving partially hydrolysed Ths-4 (phThs-4) and Ths-5 (phThs-5). The results of the metanolysis of the heteroglycans, Ths-4 and Ths-5, as well as phThs-4 and phThs-5, are given in Table 1. These results show that Gal and Man are the dominant monosaccharide units in all four fractions; however, Ths-4 and Ths-5 contain relatively larger amounts of Gal compared to Man than that which is observed for the partially hydrolysed derivatives, phThs-4 and phThs-5, indicating that Ths-4 and Ths-5 contain labile Gal linkages (Table 1).

Partially methylated alditol acetates prepared from Ths-4 and Ths-5 were analysed by GC–MS and revealed unusual lichen-type galactomannans, mainly consisting of galactofuranosyl and mannopyranosyl units. Both polysaccharides contained 2-O-, 6-O-, 2,3-di-O- and 2,6-di-O-substituted mannopyranosyl units, in addition to nonreducing, 5-O- and 3-O-substituted galactofuranosyl chains. The results of the methylation analysis of both the intact and the partially hydrolysed polysaccharides are shown in Table 2. The number of terminal Gal*f* units is considerably overestimated in the methylation analysis as compared to the proton NMR analysis discussed below; this could be explained by the relatively labile glycosidic linkage of the (1→5)-galactofuranosyl chains.

The structures of Ths-4 and Ths-5 were further analysed by 1D NMR spectroscopy and showed similar spectra apart from broader signals observed for Ths-5 (Fig. 2). Thus, the structure of Ths-4 and phThs-4 was further analysed by NMR spectroscopy using a series of two-dimensional spectra (DQF-COSY, NOESY, TOCSY, HSQC and HMBC). Analysis of the anomeric region of Ths-4 in the 2D ^{1}H NMR COSY spectrum revealed two overlapping signals at δ 5.14 and 5.13. These signals were assigned to the anomeric protons of galactofuranose residues labelled K and L, respectively, from the chemical shifts[7,8] and the ^{1}H–^{1}H bond coupling constant ($J_{1,2} < 2$ Hz), suggesting the β-D-configuration.[9] This was supported by the chemical shifts of the anomeric carbons ($\delta > 107.6$) (Fig. 3 and Table 3).[10] Comparison of the observed values with those reported in the literature[8–10] led to the identification of residue K as 3-O-substituted Gal*f* and residue L as 5-O-substituted Gal*f*. A 2D NOESY experiment did not reveal any cross peaks between the K and L residues, as could be expected for alternate (1→3)- and (1→5)-galactofuranosyl units as described for cell-wall galactomannans from the fungi, *Aspergillus wentii* and *Chaetosartorya chrysella*.[7] Therefore, it is suggested that these residues elaborate (1→5)-β-Gal*f* and (1→3)-β-Gal*f* chains, respectively. Despite the small difference in chemical shifts, 5.14 and 5.13 ppm, it is possible to uniquely define that there

Table 1. Monosaccharide composition for the two heteroglycans[a]

Monosaccharides	Ths-4 (%)	Ths-4 hydrolysed (%)	Ths-5 (%)	Ths-5 hydrolysed (%)
Man	31	48	19	51
Gal	45	25	52	32
Glc	11	14	13	8
Ara	4	2	7	3
Rha	6	6	6	3
Xyl	3	5	3	3

[a] Ths-4 and Ths-5 and partially hydrolysed Ths-4 and Ths-5 were determined by methanolysis, followed by GC of their TMS-derivatives. The results are presented as percentages of total monosaccharide content.

Table 2. GC–MS analysis of partially methylated alditol acetates[a]

O–Me–alditol acetates	Molar ratio %				Linkage type
	Ths-4 intact	Ths-4 hydrolysed	Ths-5 intact	Ths-5 hydrolysed	
3,4,6-Me_3-Man*p*	19.3	25.7	9.0	23.4	2)-Man*p*-(1→
2,3,4,6-Me_4-Man*p*	2.1	5.9	1.5	6.9	Man*p*-(1→
2,3,4-Me_3-Man*p*	2.5	4.9	1.0	3.7	6)-Man*p*-(1→
4,6-Me_2-Man*p*	1.2	2.4	1.2	3.7	2,3)-Man*p*-(1→
3,4-Me_2-Man*p*	4.6	8.4	5.4	9.8	2,6)-Man*p*-(1→
4-Me-Man*p*	0.9	1.3	0.8	3.0	2,3,6)-Man*p*-(1→
2,3,5,6-Me_4-Gal*f*	15.0	7.7	18.3	12.5	Gal*f*-(1→
2,5,6- Me_3-Gal*f*	5.7	11.0	8.8	9.1	3)-Gal*f*-(1→
2,3,6-Me_3-Gal*f*	19.7	4.8	15.7	5.4	5)-Gal*f*-(1→
3,5,6-Me_3-Gal*f*	3.2		4.3		2)-Gal*f*-(1→
2,3,5-Me_3-Gal*f*			3.2	3.0	6)-Gal*f*-(1→
2,3,4,6-Me_4-Glc*p*	9.6	8.2	7.6	5.3	Glc*p*-(1→
2,4-Me_2-Glc*p*			5.4	2.3	3,6)-Glc*p*-(1→
3,4-Me_2-Rha*p*	6.2	6.2			2)-Rha*p*-(1→
2,3,4-Me_3-Xyl*p*	2.5	4.2	2.5	2.4	Xyl*p*-(1→
2,3,5-Me_3-Ara*f*	3.4		5.9		Ara*f*-(1→

[a] Obtained by hydrolysis of the methylated heteroglycans, Ths-4 and Ths-5 and partially hydrolysed Ths-4 and Ths-5. The molar ratios were determined by dividing the peak area with molar response factor according to Sweet et al.,[24] and then presented as percentages.

Table 3. ^{1}H (800 MHz) and ^{13}C (201 MHz) NMR chemical shifts (δ), NOE and HMBC correlations, for the galactofuranomannan, Ths-4 and its partially hydrolysed derivative phThs-4, isolated from the lichen *Thamnolia vermicularis* var. *subuliformis*

	Unit		1	2	3	4	5	6a	6b	NOE	HMBC
3)-β-Gal*f*-(1→3	K	H	5.14	4.23	4.13	3.97	3.84	3.65	3.61		82.5
		C	107.6	80.1	82.5	82.6	71.3	63.4			
5)-β-Gal*f*-(1→5	L	H	5.13	4.09	4.05	4.10	3.89	3.75	3.75	3.89	76.1
		C	107.6	81.8	77.1	82.1	76.1	61.2			
2,6)-α-Man*p*-(1→6	M21	H	5.06	3.98	3.88	3.78	3.76	3.96	3.65	3.96/3.65	66.0
		C	98.8	79.2	70.7	66.8	71.1	66.0			
α-Man*p*-(1→2	M22	H	4.99	4.03	3.76	3.62	3.71	3.84	3.70	3.98	79.2
		C	102.8	70.5	71.2	67.4	73.9	61.5			
2,6)-α-Man*p*-(1→6	M31	H	5.06	3.98	3.92	3.78	3.76	3.96	3.65	3.96/3.65	66.0
		C	98.8	79.2	70.7	66.8	71.1	66.0			
2)-α-Man*p*-(1→2	M32	H	5.18	4.06	3.91	3.63	3.72	3.84	3.70	3.98	79.2
		C	101.3	78.8	70.7	67.5	78.9	61.5			
2)-α-Man*p*-(1→2	M33	H	5.18	4.05	3.90	3.62	3.68	3.84	3.70	4.06	78.8
		C	101.3	78.8	70.7	67.5	78.9	61.5			
α-Man*p*-(1→2	M34	H	4.99	4.02	3.79	3.62	3.71	3.84	3.70	4.05	78.8
		C	102.8	70.5	71.2	67.4	73.9	61.5			

are no NOEs between the residues of the →3)-β-Gal*f*-(1→3) and →5)-β-Gal*f*-(1→5) galactofuranosyl units, which again indicates that these are two separate chains.

For further analysis of the core, the heteroglycan, Ths-4, was partially hydrolysed with oxalic acid, which partially removed the Gal*f* residues, and the phThs-4 was analysed by 2D NMR spectroscopy. The fact that the partly hydrolysed sample mainly contains (1→3)-β-Gal*f* and only a small amount of (1→5)-β-Gal*f* further illustrates the lability of the (1→5)-Gal*f* linkages. The NMR spectra of phThs-4 contained six anomeric signals. A series of homo- (COSY, TOCSY, NOESY) and hetero-(HSQC, HMBC) 2D-NMR experiments allowed partial assignment of six residues. The NMR data for the different residues are shown in Table 3.

By comparing the chemical shifts with those of known values,[11] as well as from the ^{1}H–^{1}H coupling constants and NOE correlations, it was concluded that residue M^{21} is 2,6)-α-Man*p*-(1→6)-, M^{22} is α-Man*p*-(1→2)-, M^{31} is 2,6)-α-Man*p*-(1→6)-, M^{32} and M^{33} are 2)-α-Man*p*-(1→2)- and M^{34} is α-Man*p*-(1→2)- (Table 3). The molar ratio for $M^{21}/M^{22}/M^{31}/M^{32}/M^{33}/M^{34}$ (Table 3 and Fig. 1) can be estimated to be approximately 1:1:3:3:3:3 based on the relative integrals for the anomeric signals in the ^{1}H NMR spectrum.

Analysis of the 2D-NOESY spectrum of the anomeric region of the core, showed cross-peaks from H-1 of residue M^{21} and M^{31} to the H-6s of residue M^{21} and M^{31} at δ 3.96 and 3.65, indicating that residues M^{21} and M^{31} are (1→6)-linked and substituted at the 6-position. Moreover, NOE correlations between the H-1 of residue M^{22} and H-2 of residue M^{21} (at δ 3.98), between the H-1 of residue M^{32} and H-2 of M^{31} (at δ 3.98), between the H-1 of residue M^{33} and H-2 of M^{32} (at δ 4.06) and between the H-1 of the residue M^{34} and H-2 of residue M^{33} (at δ 4.05) (Fig. 1), all demonstrate the (1→2) linkages. In addition, cross-peaks from the HMBC spectra were observed and showed correlations between H-1/C-6 of M^{21} and M^{31}, H-1/C-2 of M^{22} and M^{21}; and between H-1/C-2 of M^{32} and M^{31} as well as between H-1/C-2 of M^{33} and M^{32} and between H-1/C-2 of M^{34} and M^{33} (Table 3 and Fig. 1).

Considering the ratios of Man and Gal in Ths-4 and Ths-5 observed from the methanolysis (Table 1), together with their M_r of 19 and 200 kDa, respectively, and the structure of the mannan core described above (Fig. 1), it can be concluded that the Gal*f* chains must be quite long. However, it is not possible from the NMR data to determine the attachment sites of the (1→3)- and (1→5)-linked Gal*f* chains to the mannan core. The possibility that the (1→5)-linked Gal*f* chains of Ths-4 could be attached to the mannan core through a phosphorodiester bridges as described for phosphorogalactomannans of the fungus *Aspergillus versicolor*[12] was excluded by a phosphorus NMR experiment (data not shown).

In the literature, the proposed attachment sites for short (1→5)-linked Gal*f* chains and alternating (1→5)- and (1→3)-linked Gal*f* chains, on similar mannan core of galactomannans from *Aspergillus* sp. and *Chaetosar-*

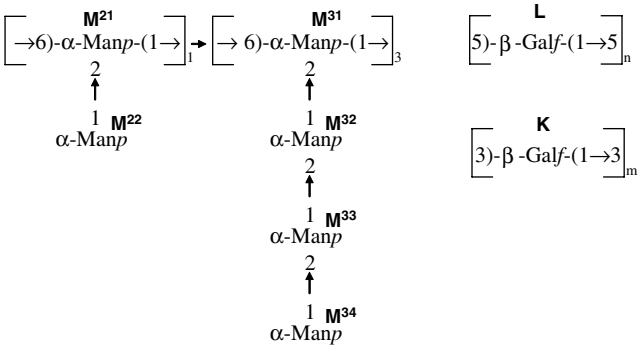

Figure 1. Repeating units of the galactofuranomannan, Ths-4.

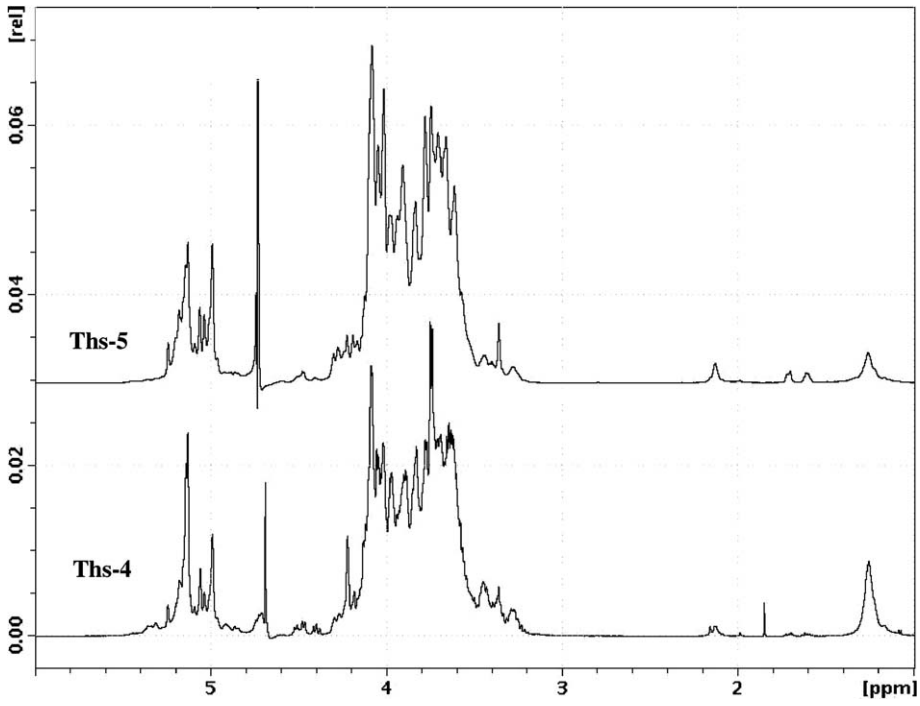

Figure 2. ^{1}H NMR spectra (D_2O, 25 °C, 800 MHz) of the water-soluble polysaccharides, Ths-4 and Ths-5.

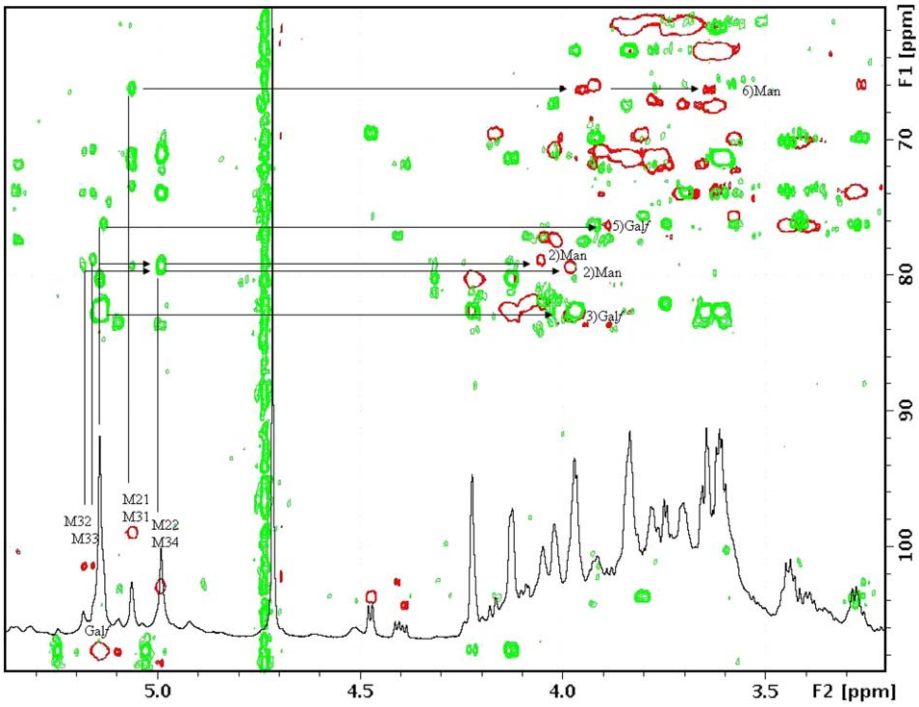

Figure 3. The ^{1}H–^{13}C–HSQC and HMBC spectra of the galactofuranomannan, Ths-4.

torya sp., are at the end of the (1→2)-linked Man*p* chains on the mannan core. The arguments were that the ratio of terminal Man*p* compared to the other Man*p* units, estimated by integration of the anomeric signals of the ^{1}H NMR spectra, is considerably larger in the core when all the Gal*f* units have been removed by hydrolysis.[7] Another study on galactofuranomannans from *Aspergillus fumigatus* suggests that the attachment side for (1→5)-linked Gal*f* chains of 4–5 units, could be at positions-3 and -6 of an α-(1→2)-linked Man*p* unit of

the core; however, the attachment sides are stated to be questionable.[13] It can be suggested that in Ths-4 and Ths-5, the (1→5)-linked Gal*f* chains are attached to the mannan core, probably to the terminal or the (1→2)-linked Man*p* units; however, the (1→3)-linked Gal*f* chains, which are more resistant to the weak oxalic acid hydrolysis used in this study, could either be side chains on the mannan core or compose a bridge between two mannan cores.

According to the GC–MS data, minor amounts of Glc, Xyl, Rha and Ara residues were present in the samples. In the NMR data the following types of glucose resides could be assigned: an α-glucan with (1→3) and (1→4) links; a β-glucan with (1→4) links; a terminal β-glucose and in addition a reducing end. For xylose, only (1→4)-linked and terminal xylose could be assigned. In the NMR data, rhamnose could only be partly assigned, that is the H-4/C-4, H-5/C-5 and H-6/C-6. No arabinose could be identified in the spectra. None of these minor components could be correlated with either the Gal*f* or the Man*p* residues, and these residues are not considered to be a part of the galactofuranomannan, Ths-4. It might be suggested that these residues are derived from algal polysaccharides present in small amounts, as complex heteroglycans containing similar components are known constituents of algae.

Galactofuranomannans, with a mannan core consisting of an α-Man*p*-(1→6)-linked main chain, substituted at O-2, O-4 and O-2,4 and with α-Man*p*-(1→2)-linked side chains and terminal β-Gal*f* residues, have been reported from lichens,[14–16] but glycans such as Ths-4 and Ths-5 with long (1→3)-linked and (1→5)-linked Gal*f* chains attached to a mannan core have not been previously described from lichens. In addition, fungal galactofuranomannans consisting of short (1→5)-linked Gal*f* chains attached to a mannan core similar to that of Ths-4, have been isolated from *Aspergillus* and *Chaetosartorya* species; however, these do not include (1→3) Gal*f* chains[7] as described above. Taken together, the results observed in this study, in addition to the two previous reports describing unusual lichen polysaccharide, that is, thamnolan[5] and Ths-2[6] from *Thamnolia vermicularis* var. *subuliformis*, further emphasise the unusual origin of this fascinating lichen species.

1. Experimental

1.1. Biological material

The lichen, *T. vermicularis* var. *subuliformis*, belonging to the group of Lichen imperfecti, was collected in 2002 in Ulfarsfell near Reykjavik in southwestern Iceland. The lichen was identified by S. Baldursdottir, lichenologist, Prokaria Ltd, Reykjavik, Iceland, and a voucher specimen is preserved at the Faculty of Pharmacy, University of Iceland, Reykjavik, Iceland.

1.2. Isolation and purification of the polysaccharide

The dried lichen material (108 g) was powdered and extracted with hot water as previously described.[5] The supernatant was further processed with ethanol fractionation according to a previously described fractionation process to give 300.6 mg of the crude fraction III.[5,17] Fraction III was purified with anion-exchange chromatography (DEAE Sepharose fast flow, Amersham Bioscience), giving 60.2 mg of fraction III-a using water as the eluent, and 101.8 mg of fraction III-b eluted with 0.25 M NaCl. Fractions III-a and III-b were further purified by HPGPC on a Superose 6 preparative column (16/50) (Amersham Biosciences, GE Healthcare), by elution with 0.05 M sodium phosphate buffer, pH 6.0, containing 0.15 M NaCl, with a flow rate of 1.0 mL/min, using refractive index detection (Hewlett–Packard, HP 1047A RI detector). The samples were applied in 1% solutions in the mobile phase, and the injected volume was 900 μL. Seven injections of fraction III-a gave 14.1 mg of thamnolan,[5] 30.6 mg (0.03%, yield) of Ths-4 and 11 injections of fraction III-b gave 70.2 mg (0.07%, yield) of Ths-5. The fractions were collected, dialysed (MWCO: 6–8 kDa) and lyophilised.

1.3. Determination of mean M_r and homogeneity

Homogeneity and mean M_r of Ths-4 and Ths-5 were determined by HPGPC on a Superose 6 HR 10/30 column (Amersham, GE Healthcare) eluted with 0.05 M sodium phosphate buffer, pH 6.0, containing 0.15 M NaCl, with a flow rate of 0.5 mL/min, using refractive index detection (HP 1047A RI detector). The samples were applied in 1% solutions in the mobile phase, and the injected volume was 20 μL. HPGPC elution profiles of the isolated Ths-4 and Ths-5 are shown in Figure 4. For the M_r-estimation, calibration was performed using dextrans of known M_r (T10, T40, T70, T250, T500 and T2000, Amersham, GE Healthcare).

1.4. Partial acidic hydrolysis

Ths-4 and Ths-5 (10.0 mg each) were dissolved in 3 mL of 0.2 M oxalic acid and heated at 60 °C for 2 h. The solution was neutralised by $NaHCO_3$ and applied to a PD-10 desalting column (Amersham Bioscience) with a 5000 Da cut-off.

1.5. Monosaccharide composition

The monosaccharide composition of the purified Ths-4 and Ths-5 and their hydrolysed derivatives was deter-

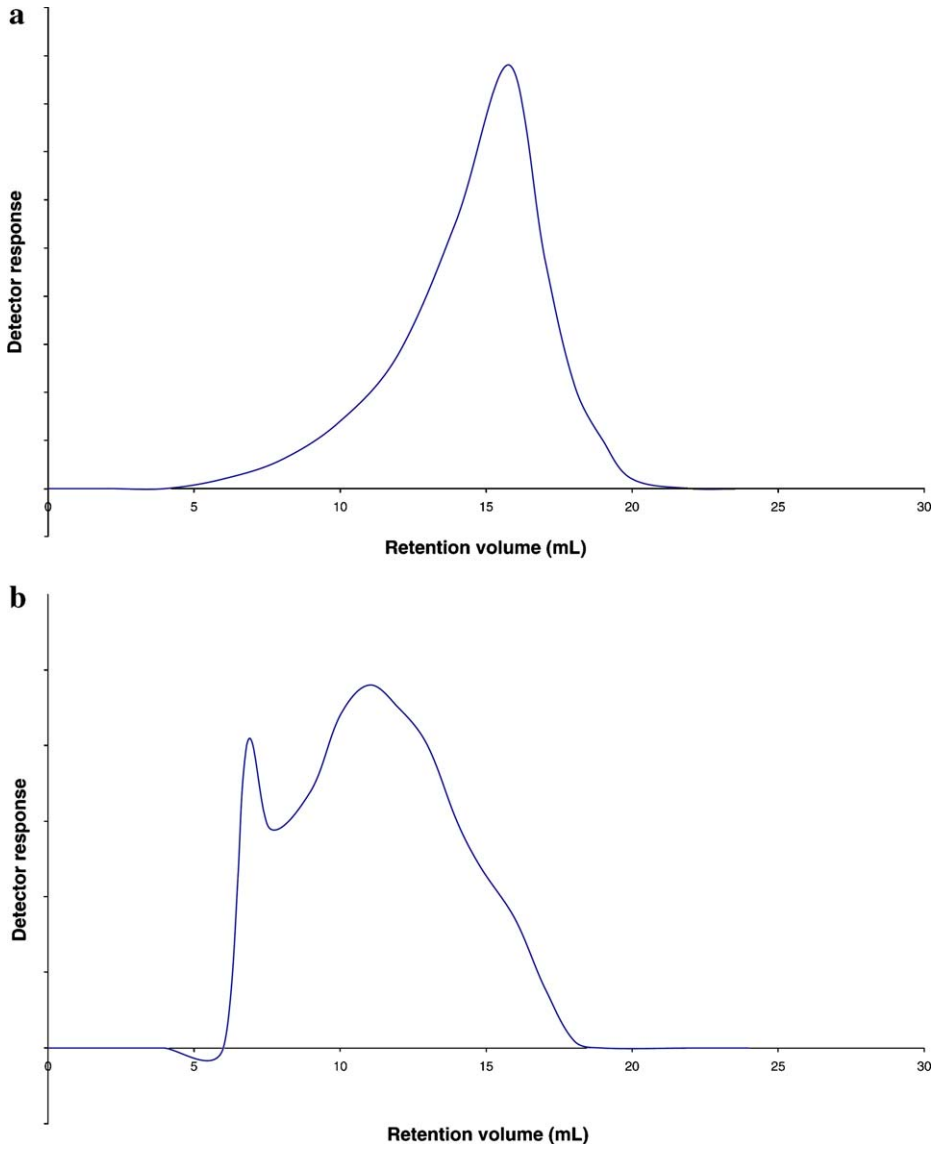

Figure 4. The elution profiles for the HPGPC of (a) Ths-4 and (b) Ths-5 on a Superose 6 10/30 HR gel-permeation column as recorded by refractive index detection.

mined by GC of the trimethylsilylated derivatives of the methyl glycosides obtained by methanolysis of 1 mg of polysaccharide in 1 mL of 4 M HCl in methanol at 80 °C for 24 h with mannitol as an internal standard. GC was performed on a DB-5 capillary column (30 m × 0.32 mm i.d.) with a film thickness of 0.25 μm, in a Carlo Erba 6000 Vega Series 2 gas chromatograph and a Shimadzu C-R6A integrator as described earlier.[18,19]

1.6. Methylation analysis

Ths-4 and Ths-5 (1 mg each) and their hydrolysed derivatives in dimethyl sulfoxide was methylated using NaOH and iodomethane according to the method of Ciucano and Kerek[20] as described by Kim and Carpita.[21] After hydrolysis with 2.5 M trifluoroacetic acid, the methylated sugar residues were converted to partially methylated alditol acetates by reduction with $NaBD_4$ in 2 M NH_4OH, followed by acetylation with acetic anhydride. The derivatised sugar residues were extracted into dichloromethane and evaporated to dryness, dissolved again in 100 μL methanol and analysed by GC–MS. GC–MS was performed with a split–splitless injector operated in the split mode and a Supelco fused silica capillary column (30 m × 0.20 mm i.d.) with film thickness 0.20 μm in the same way as described before. EI mass spectra were obtained using Fisons Instruments MD800 Mass Selective Detector 5970 with a Fisons Instruments GC8000 series (8065) gas chromatograph.[5,17,19,22]

1.7. Nuclear magnetic resonance spectroscopy

The NMR chemical shifts were assigned from 2D homo- and heteronuclear experiments at 799.96 MHz for ^{1}H and 201.12 MHz for ^{13}C, using acetone as reference for proton (δ 2.225) and 1,4-dioxane for carbon (δ 67.4). Ths-4, Ths-5 (1 mg each) and 0.5 mg of phThs-4 and phThs-5 were dissolved in 0.7 mL of D_2O, and spectra were recorded at 25 °C on a Varian UNITY INOVA 800 in a 5 mm tube. In addition to 1D proton spectra, a series of 2D spectra were obtained. The double quantum-filtered phase-sensitive COSY experiment was performed using the Varian standard program TNDQCOSY, with 0.37 s acquisition time and 4096 data points in the F2 dimension. The data matrix was zero-filled in the F1 dimension to give a matrix of 4096*2048 points and was resolution enhanced in both dimensions by a shifted sine-bell function before Fourier transformation. Similarly, the nuclear Overhauser experiment was performed using the Varian standard program TNNOESY, with a mixing time of 75 ms. The TOCSY experiment was performed using standard Varian program TNTOCSY with a spinlock time of 80 ms. A heteronuclear experiment was performed using the pulse field gradient program gHSQC and HMBC. The spectra were assigned using the computer program PRONTO,[23] which allows the simultaneous display of different two-dimensional spectra and the individual labelling of cross-peaks.

Acknowledgements

The NMR spectra at 800 MHz were obtained on the Varian Unity Inova spectrometer of the Danish Instrument Center for NMR Spectroscopy of Biological Macromolecules. Supporting grants from Icelandic Council of Science and University of Iceland Research Fund, The Icelandic Research Fund for Graduate Students, as well as from the Nordic Council of Ministers and The Bergthoru and Thorsteins Scheving Thorsteinsson fund are gratefully acknowledged. The contribution of Finn Tønnesen to the GC–MS analyses is also acknowledged. Adiaratou Togola was funded by the NUFU Project PRO22/2002.

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