Carbohydrate Polymers 91 (2013) 370–376

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Structural characterisation of a complex heteroglycan from the cyanobacterium *Nostoc commune*

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ARTICLE INFO

Article history:
Received 26 June 2012
Received in revised form 16 August 2012
Accepted 19 August 2012
Available online 25 August 2012

Keywords:
Nostoc commune
Cyanobacteria
Polysaccharides
High-field NMR
Structure elucidation

ABSTRACT

An alkali-extractable O-methylated ribofuranose-containing heteroglycan, Nc-5-s, was isolated from wild-growing field colonies of the cyanobacterium *Nostoc commune* collected in Iceland, using ethanol fractionation and anion-exchange chromatography. The average molecular weight was estimated to be 1500 kDa. Structural characterisation of the heteroglycan was performed by high-field NMR spectroscopy (1D proton, 2D-COSY, 2D-NOESY, 2D-TOCSY, ^{1}H ^{13}C-HSQC, HMBC, H2BC and HSQC-NOESY) as well as monosaccharide analysis after methanolysis by GC and supported by linkage analysis by GC–MS.

According to the data obtained, the structure of Nc-5-s is composed of repeating units of **1**, **1a**, **1b** and **2** and **2a** in approximate molar ratio of (10:25:50:5:10).

1: [4)2-OMe-β-Glc*p*A-(1-4)-β-Xyl*p*-(1-3)-β-Glc*p*-(1-4)-β-Ara*p*-(1-]$_n$
O K N D

1a: [4)2-OMe-β-Glc*p*A-(1-4)-β-Xyl*p*-(1-3)-β-Glc*p*-(1-4)-β-Ara*p*-(1-]$_n$
G
2-OMe-α-Ara*f*-(1-3)
A

1b: [4)2-OMe-β-Glc*p*A-(1-4)-β-Xyl*p*-(1-3)-β-Glc*p*-(1-4)-β-Ara*p*-(1-]$_n$
H
α-Rib*f*-(1-3)
B/C

2:
F P Q
α–Man*p*-(1-6)-β–Glc*p*-(1-6) β-Glc*p*A-(1-6)
[4)3-OMe-β-Man*p*-(1-4)-β–Xyl*p*-(1-4)-β–Glc*p*-(1-4)-α–Gal*p*-(1-]$_n$
I R M E

2a:
L
β–Man*p*-(1-6) β-Glc*p*A-(1-6)
[4)3-OMe-β-Man*p*-(1-4)-β–Xyl*p*-(1-4)-β–Glc*p*-(1-4)-α–Gal*p*-(1-]$_n$
J

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http://dx.doi.org/10.1016/j.carbpol.2012.08.063

1. Introduction

Cyanobacteria comprise a group of ancient photoautotrophic microorganisms that are widespread in nature. They produce exopolysaccharides (EPSs) that have been studied considerably in recent years for their viscousifying, suspending, emulsifying and biosorbent abilities that are of interest for biotechnological applications. However, knowledge about the structural details of these EPSs is scarce and is much needed in order to understand their chemical and physicochemical properties (De Philippis & Vincenzini, 1998; Pereira et al., 2009).

Nostoc is one of five genera of the nitrogen-fixing cyanobacteria family of Nostocaceae and has the ability to use atmospheric nitrogen when combined nitrogen is not available (Morsy, Kuzuha, Takani, & Sakamoto, 2008; Wright, Prickett, Helm, & Potts, 2001). The species *Nostoc commune* is a prominent component of microbial populations worldwide, distributed from the tropics to the polar regions of the Earth. It is a filamentous cyanobacterium adapted to the terrestrial environment, especially those associated with nutrient-poor soils. *N. commune* forms visible colonies of macroscopic proportions in which the entangled filaments are embedded in extracellular polysaccharides (Morsy et al., 2008; Pereira et al., 2009; Wright et al., 2001).

These colonies are in their natural environment subjected to frequent dehydration and rewetting cycles during which the cells produce large amounts of these protective polysaccharides. It is believed that the EPSs of *N. commune* play a role in the stabilisation of cells in the air-dried state, inhibiting the fusion of membrane vesicles during desiccation and acting as an immobilization matrix for secreted enzymes, which stay fully active after long-term air-dried storage. This contributes in many ways to the marked desiccation tolerance of this organism (Helm et al., 2000; Morsy et al., 2008; Pereira et al., 2009).

However, the main function attributed to the EPSs of cyanobacteria in general is to serve as a boundary between the bacterial cell wall and its immediate environment and for the cyanobacteria that live in association with higher plants or symbiosis with other organisms (e.g. lichens), they seem to act as an adhesive for cyanobacterial cells (De Philippis & Vincenzini, 1998; Morsy et al., 2008). It is thus clear that the structure and the physicochemical properties of the EPSs are adapted to the needs of the cyanobacteria in its particular environment.

According to the literature the extracellular matrix of cyanobacteria is mainly made up of high-molecular-weight heteropolysaccharides which are constituted of various sugar units such as glucose, galactose, xylose and uronic acids (Pereira et al., 2009). Cyanobacterial polysaccharides are characterised by a great variety in both number and type of constitutive monosaccharides, which varies with nutrient availability, growth phase and environmental conditions and previous reports suggest that their structures are not comparable to those of algae, other bacteria or fungi (De Philippis & Vincenzini, 1998; Helm et al., 2000; Morsy et al., 2008; Pereira et al., 2009).

The aim of the present study is to characterise the structure of the high molecular weight heteroglycan, Nc-5-s, from the wild-growing cyanobacterium *N. commune* in details, using high field (800 MHz for ^{1}H) 1D and 2D nuclear magnetic resonance spectroscopy; COSY, NOESY, TOCSY, HSQC, HSQC-NOESY, HSQC-TOCSY, HMBC and H2BC as well as monosaccharide and linkage analysis, and to provide useful reference data for future studies of complex cyanobacterial polysaccharides.

2. Experimental

2.1. Biological material

Wild growing colonies of *N. commune* Vauch. were collected in September 2004 in Botnsdalur, Hvalfjordur, in south-western Iceland (N64°23.18, W21°17.13). The field-collected samples were identified by Dr. Hordur Kristinsson, Icelandic Institute of Natural History, Akureyri, Iceland, and a voucher specimen is deposited at the Faculty of Pharmaceutical Sciences, University of Iceland.

2.2. Isolation and purification of the polysaccharide

The dried colonies (72.5 g) were powdered and extracted with organic solvents in a Soxhlet apparatus (light petroleum, acetone and methanol), followed by extraction with hot (95 °C) distilled water for 2 h and hot filtration. The sample residue from the water extraction was then extracted with 0.5 M aqueous NaOH according to Caldes, Prescott, Thomas, and Baker (1981) and kept at 4 °C overnight. The mixture was centrifuged and the supernatant processed further according to a previously described fractionation process (Caldes et al., 1981; Paulsen, Olafsdottir, & Ingolfsdottir, 2002) to give 566 mg of a crude fraction named Nc-5. 290 mg of this alkali-extractable, water-soluble fraction Nc-5, was then dissolved in 50 ml of water and purified further with anion-exchange chromatography on a 2.6 × 65 cm DEAE Sepharose Fast Flow column medium (Amersham, GE Healtcare), using 0–1.0 M stepwise NaCl gradient, giving one fraction Nc-5-s eluted in 0.25 M NaCl. The fraction Nc-5-s was collected, dialysed (MWCO: 6–8 kDa) and lyophilized giving 37 mg (a yield of 0.1% from dry bacteria colonies). The isolation process is outlined in Fig. 1.

2.3. Estimation of mean M_r and homogeneity

Homogeneity and mean M_r of Nc-5-s was estimated by HP-GPC 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. For the M_r-estimation, calibration was performed using dextrans of known M_r (T10, T40, T70, T250, T500 and T2000, Amersham, GE Healthcare).

2.4. Monosaccharide composition

The monosaccharide composition of Nc-5-s was determined 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 Carlo Elba 6000 Vega Series 2 gas chromatograph and a Shimadzu C-R6A integrator as described earlier (Barsett & Paulsen, 1992; Reinhold, 1972).

2.5. Linkage analysis

Carboxyl reduction of Nc-5-s (1 mg) was conducted as previously described (Kim & Carpita, 1992). Nc-5-s in dimethyl sulphoxide was then methylated or ethylated using NaOH and methyl- or ethyl iodide according to Ciucanu and Kerek (1984) as described by Kim and Carpita (1992). After hydrolysis with 2.5 M trifluoroacetic acid, the methylated/ethylated sugar residues were converted to partially methylated/ethylated alditol acetates by reduction with $NaBD_4$ in 2 M NH_4OH followed by acetylation with

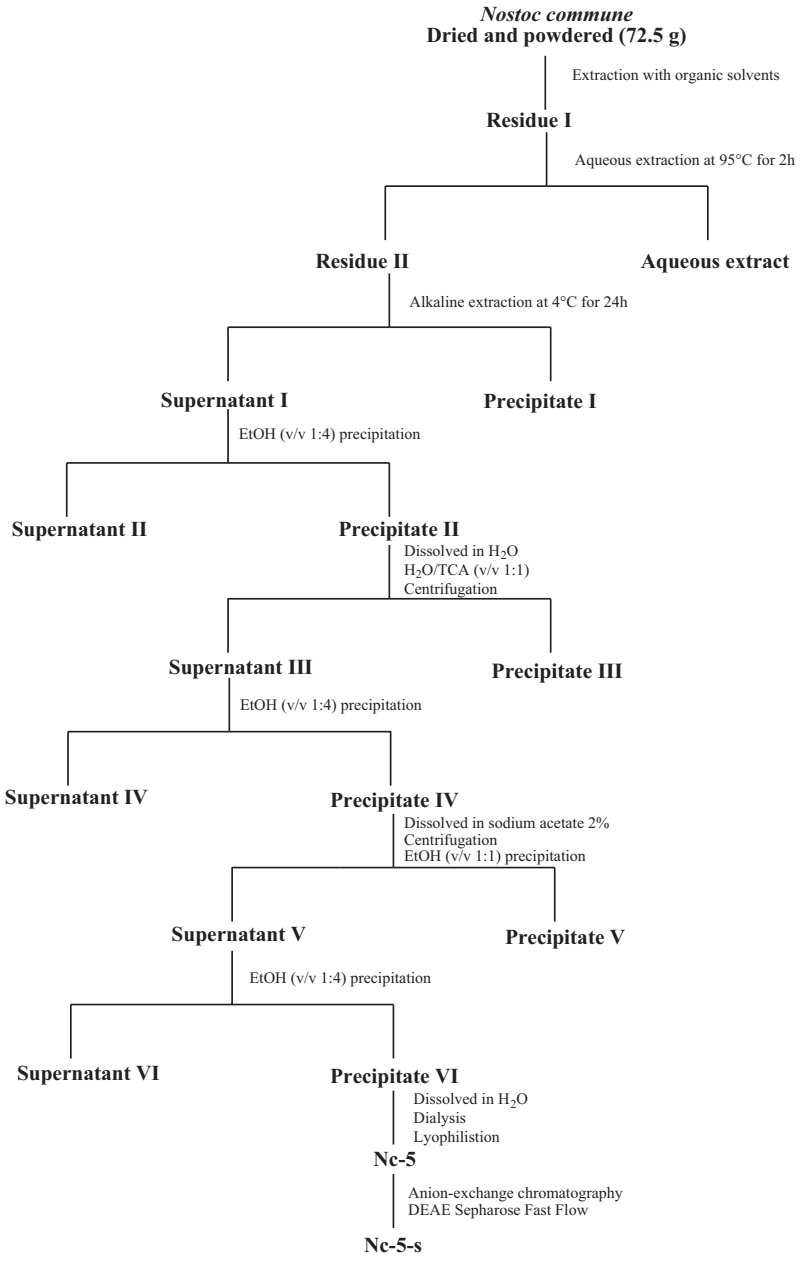

Fig. 1. Fractionation of the alkali extract of *Nostoc commune* by precipitation with ethanol, followed by anion-exchange chromatography.

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, used 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. E.I. mass spectra were obtained using Fisons Instruments MD800 Mass Selective Detector 5970 with a Fisons Instruments GC8000 series (8065) gas chromatograph (Barsett & Paulsen, 1992; Olafsdottir, Omarsdottir, Paulsen, Jurcic, & Wagner, 1999; Paulsen et al., 2002).

2.6. Partial acidic hydrolysis

Nc-5 (300 mg) was dispersed in deionized water (184 ml) by stirring at room temperature for 30 min. 16 ml of trifluoroacetic acid was added, and the mixture heated on a 80 °C water bath for 4 h. The mixture was evaporated to dryness and further evaporated three times with isopropanol (100 ml). The oligosaccharides were dissolved in water (8 ml) and centrifuged. The supernatant was lyophilized, re-dissolved in deionized water (3 ml) an separated by gel filtration on a Bio-Gel P2 column (1.6 cm × 50 cm) eluted with deionized water at a flow rate of 0.4 ml/min, using refractive index detection (HP 1047A RI detector) (Helm et al., 2000). Four fractions were obtained and the major fraction was subjected to NMR analysis and supported the assignments of signals of the intact polymer.

2.7. Nuclear magnetic resonance spectroscopy

The NMR chemical shifts were assigned from 2D homo- and heteronuclear experiments at 799.4 MHz for proton and 201.12 MHz for carbon, using acetone as reference for proton (δ 2.22 ppm) and for carbon (δ 30.9 ppm).

Less than 1.0 mg of both intact and partially hydrolysed Nc-5-s were dissolved in 0.6 ml of D_2O and spectra were recorded on a BRUKER AVANCE 800 equipped with cryo-probe in a 5 mm tube. All spectra were obtained with the temperature set to 33.5 °C.

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 Bruker standard program dqfcosy, with 0.3 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. Similar, the nuclear Overhauser experiment was performed using the Bruker standard noesyphpr, with a mixing time of 300 ms. With the same amount of data points the TOCSY experiment was performed using standard Bruker program mlevphpr with a spinlock time of 80 ms.

Furthermore, a 1D carbon spectrum and a series of heteronuclear experiments were performed using pulse field gradient programs HSQC, HMBC, HSQC-TOCSY, HSQC-NOESY and H2BC. All heteronuclear experiments were recorded with 2048 points in F2 and 1024 in F1 and zero filled in both dimension.

The spectra were assigned using the computer program Topspin®, which allows the simultaneous display of different two-dimensional spectra and the individual labelling of cross peaks.

3. Results and discussion

Nc-5-s was obtained from an alkali extract of a wild growing specimen of the colonized cyanobacterium *N. commune* followed by ethanol precipitation and anion-exchange chromatography (Fig. 1). Nc-5-s was eluted as a single peak in HP-GPC and the mean M_r was estimated to be about 1500 kDa by comparison to dextran standards. Monosaccharide composition was determined to be Glc/GlcA/Xyl/Man/Ara/Rib/Gal in approximate ratios of 24:24:15:13:13:7:4 by GC after methanolysis. The monosaccharide units and linkage patterns were studied further using methylation and ethylation analysis and GC–MS, where all of the linkage types later established by NMR, could be observed. However, determination of molar ratio was difficult due to overlapping of several signals in the GC chromatogram (data not shown). The fact that Nc-5-s is a complex heteroglycan involving eleven different monosaccharides, including both acidic and native monomethylated components as well as the unusual Rib*f*, made the analysis of the degradation products particularly complicated. However, by combining the preliminary results from the monosaccharide and linkage analysis with high field NMR spectroscopic analysis described below, a detailed structure elucidation of Nc-5-s backbones was performed.

The detailed structure was established by ^1H and ^{13}C NMR spectroscopy and a series of 2D experiments of the intact heteroglycan, Nc-5-s. The complete assignment of all connectivities from the spectra of the intact polymer alone, was somewhat difficult because of the high M_r and the resulting line-broadening, and was supported by the sharper NMR spectra of the partially hydrolysed fragments (data not shown).

The different carbohydrate residues (labelled **A**–**R**) were established and the chemical shifts were assigned as shown in Table 1. The anomeric region of the COSY spectrum of Nc-5-s can be divided into several regions characterised by well separated anomeric signals (Fig. 2). The signal at δ 5.69 ppm was assigned to the anomeric proton of a terminal 2-OMe-α-Ara*f* residue (**A**) from the chemical shifts shown in Table 1, the 1H–1H three bond coupling constants ($J_{1,2}$ ~ 1 to 1.7 Hz; $J_{2,3}$ ~ 3.5 Hz; $J_{3,4}$ ~ 6 Hz and $J_{4,5a}$ ~ 2.0–3.5 Hz and $J_{4,5b}$ ~ 5–6 Hz) (Ishii, Ono, & Maeda, 2005; Serianni & Barker, 1984). The signal at δ 5.39 ppm was assigned to β-Ara*p* residue (**D**) according to the chemical shifts and the coupling constants ($J_{1,2}$ ~ 3.5 Hz; $J_{2,3}$ ~ 9.5 Hz; $J_{3,4}$ ~ 10 Hz) and characteristically small couplings (~1–3 Hz) between H-4 and H-5 (Bock, Pedersen, & Pedersen, 1984; Bock & Thøgersen, 1982).

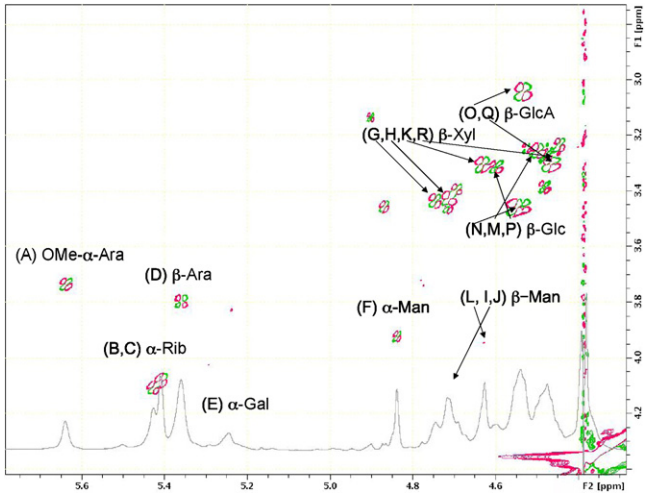

Fig. 2. 1D proton spectrum of the anomeric region of Nc-5-s with labelling of different anomeric signals overlaid with the DQF-COSY spectrum. The spectra shown represent the intact polysaccharide.

Two signals at δ 5.43 and 5.41 correlating to H-2 at δ 4.11 and 4.08, respectively, were assigned to anomeric proton of terminal α-Rib*f* residues (**B**, **C**), from the chemical shifts shown in Table 1 and the 1H–1H three bond coupling constants ($J_{1,2}$ ~ 4 Hz; $J_{2,3}$ ~ 6 Hz; $J_{3,4}$ ~ 3.5 Hz; $J_{4,5a}$ ~ 3 Hz and $J_{4,5b}$ ~ 5 Hz) (Serianni & Barker, 1984). Two anomeric α-Rib*f* signals (**B**, **C**) were observed probably due to slightly different environment of this unit dependent on the order of the linking of different variants of the polymer (Bock et al., 1984; Bock & Thøgersen, 1982; Duus, Gotfredsen, & Bock, 2000). The signal at δ 5.24 ppm was assigned to the anomeric proton of α-Gal*p* residue (**E**) of backbone 2 according to chemical shifts and the 1H–1H three bond coupling constants ($J_{1,2}$ ~ 3–4 Hz; $J_{2,3}$ ~ 10 Hz; $J_{3,4}$ ~ 4 Hz; $J_{4,5}$ ~ 1 Hz) (Bock & Thøgersen, 1982; Duus et al., 2000). Four signals at δ 4.84, 4.69, 4.69 and 4.63 (Table 1) were assigned to the anomeric protons of a α-Man*p* (**F**) and three β-Man*p* (**I**, **J**, **L**) residues, respectively, according to standard chemical shifts, the 1H–1H three bond coupling constants ($J_{1,2}$ ~ 1–2 Hz; $J_{2,3}$ ~ 4 Hz; $J_{3,4}$ ~ 10 Hz, $J_{4,5}$ ~ 10 Hz). The small coupling constants $J_{1,2}$ and $J_{2,3}$ give rise to the characteristically very weak cross-peak in the phase-sensitive COSY (Bock et al., 1984; Bock & Thøgersen, 1982; Duus et al., 2000).

The remaining anomeric signals in the region δ 4.75–4.45 ppm were assigned to β-Xyl*p* (**G**, **H**, **K**, **R**), β-Glc*p*A (**O**, **Q**) and β-Glc*p* (**M**, **N**, **P**) residues. The anomeric peaks at δ 4.75, 4.71, 4.63 and 4.45 ppm were assigned to β-Xyl*p* residues (**G**, **H**, **K**, **R**) from the chemical shifts (Table 1) and 1H–1H three bond coupling constants ($J_{1,2}$ ~ 8 Hz; $J_{2,3}$ ~ 9 Hz; $J_{3,4}$ ~ 9 Hz) and the COSY correlations within their spin systems ended at H-5 with a well indicated cross-peak at δ 4.07–4.05/3.38–3.35 ppm (Table 1) (Bock et al., 1984; Bock & Thøgersen, 1982; Duus et al., 2000).

The anomeric signals at δ 4.53 and 4.47 ppm were assigned to β-Glc*p*A residues (**O**, **Q**) from the chemical shifts (Table 1), the 1H–1H three bond coupling constants ($J_{1,2}$ ~ 8 Hz; $J_{2,3}$ ~ 10 Hz; $J_{3,4}$ ~ 10 Hz), strong COSY and TOCSY cross-peaks between H-1 and H-2,3,4, and 5 and NOE correlations between H-1–H-3 and H-1–H-5, and a long range proton/carbon correlation between H-5 and C-6 carbonyl at δ 174.2 and 174.0 ppm. The anomeric signals at δ 4.60, 4.55 and 4.50 ppm were assigned to β-Glc*p* (**M**, **N**, **P**) residues from the chemical shifts (Table 1), the 1H–1H three bond coupling constants ($J_{1,2}$ ~ 8 Hz; $J_{2,3}$ ~ 10 Hz; $J_{3,4}$ ~ 10 Hz), strong

Table 1
^{1}H (800 MHz) and ^{13}C (201 MHz) NMR chemical shifts (δ), NOE and HMBC correlations for the heteroglycan Nc-5-s, isolated from the cyanobacterium *Nostoc commune*.

		H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5a/C-5a	H-5b/6a C-5b/6a	H-6b/C-6b	O-Me	HMBC[a]	NOESY HMBC[b]
A	T-α-Ara*f*(1 → 3	5.69	3.74	4.07	4.14	3.65	3.59		3.39		3.82
	2-OMe	100.6	80.3	69.5	85.5	61.9			58.6	80.3	76.8
B	T-α-Rib*f*(1 → 3	5.43	4.11	3.97	4.12	3.68	3.60				3.71
		103.1	71.6	70.4	84.7	61.9					79.9
C	T-α-Rib*f*(1 → 3	5.41	4.08	3.96	4.11	3.66	3.58				3.74
		103.1	71.6	70.4	84.7	61.9					80.0
D	4)β-Ara*p*(1 → 4	5.39	3.80	3.82	4.02	3.81	3.78				3.81
		99.56	69.2	69.0	79.5	62.9					77.3
E	4)α-Gal*p*(1 → 4	5.24	3.81	3.84	4.16	3.91	3.75	3.75			3.68
		100.2	69.2	69.0	78.4	71.5	61.2				
F	α-Man*p*(1 → 6	4.84	3.92	3.76	3.61	3.60	3.81	3.70			3.87, 3.71
		100.1	70.4	71.1	67.1	73.1	61.3				65.9
G	3,4)β-Xyl*p*(1 → 3	4.75	3.44	3.82	3.92	4.06	3.37				3.63
		103.1	71.6	76.8	77.3	63.2					84.3
H	3,4)β-Xyl*p*(1 → 3	4.71	3.43	3.74	3.91	4.05	3.35				3.63
		103.3	72.3	80.0	76.7	63.2					84.3
I	4,6)β-Man*p*(1 → 4	4.69	4.14	3.53	3.68	3.66	4.10	3.87	3.36		
	3-OMe	98.4	66.5	82.7	74.0	74.0	69.7		55.8	82.7	
J	4,6)β-Man*p*(1 → 4	4.69	4.14	3.53	3.68	3.56	4.11	3.72	3.36		
	3-OMe	98.4	66.5	82.7	74.0	74.2	69.1		55.8	82.7	
K	4)β-Xyl*p*(1 → 3	4.63	3.30	3.56	3.74	4.06	3.36				3.63
		103.6	73.6	74.2	77.4	63.2					84.3
L	T-β-Man*p*(1 → 6	4.63	3.95	3.59	3.52	3.30	3.85	3.67			4.11, 3.72
		101.0	70.8	73.3	67.3	76.7	61.5				69.1
M	4,6)β-Glc*p*(1 → 4	4.60	3.31	3.55	3.60	3.68	4.20	3.87			4.16
		103.9	73.6	74.2	78.7	74.0	68.9				78.4
N	3)β-Glc*p*(1 → 4	4.55	3.46	3.63	3.41	3.38	3.83	3.65			4.02
		103.8	73.6	84.3	68.3	76.0	61.1				79.5
O	4)β-Glc*p*A(1 → 4	4.53	3.05	3.70	3.81	3.81			3.49		3.74
	2-OMe	101.3	82.8	75.8	77.3	75.9	174.0		60.6	82.8	77.4
P	6)β-Glc*p*(1 → 6	4.50	3.25	3.44	3.42	3.54	3.87	3.71			
		103.6	73.5	76.1	69.9	74.5	65.9				69.7
Q	T-β-Glc*p*A(1 → 6	4.47	3.30	3.47	3.51	3.77					4.20 3.87
		103.2	73.2	75.8	71.8	75.7	174.2				68.9
R	4)β-Xyl*p*(1 → 4	4.45	3.28	3.55	3.82	4.07	3.38				
		103.2	73.3	74.2	76.9	62.7					

[a] Intra-residual HMBC correlations from protons of the OMe groups to ^{13}C chemical shift.
[b] Inter-residual NOE correlation from H1- to ^{1}H chemical shift and HMBC correlations from H1 to ^{13}C chemical shift.

COSY and TOCSY cross-peaks between H-1–H-2,3,4,5, and H-6a/6b and NOE correlations between H-1–H-3 and H-1–H-5 (Table 1) (Bock et al., 1984; Bock & Thøgersen, 1982; Duus et al., 2000).

After establishing the monosaccharide residues composing the complete polysaccharide the connectivities were established using a combination of inter-residue NOE and HMBC, where possible. This is confirmed by comparing the observed glycosylations shifts with literature values. Two methoxy resonances (δ 3.39/58.6, 3.49/60.6 ppm) (Bock & Thøgersen, 1982) correlated to signals from H-2 of residue α-Ara*p* (**A**) and β-Glc*p*A (**O**) and intra-residual HMBC correlations between H-1 and C-2 (δ 5.69/80.3, 4.53/82.8 ppm) establishing the 2-OMe-linkages. Another methoxy resonance (δ 3.36/55.8 ppm) correlated to signals from H-3 of residues β-Man*p* (**I**, **J**), and intra-residual HMBC correlations between H-1 and C-2 (δ 4.69/82.7 ppm) establishing the 3-OMe-linkages (Bock & Thøgersen, 1982).

Furthermore, inter-residual NOE and HMBC correlations of backbone 1 was assigned between H-1 of 2-OMe-β-Glc*p*A (**O**) and H-4/C-4 of β-Xyl*p* (**K**) (δ 4.53/3.74; 77.4 ppm); between H-1 β-Xyl*p* (**K**) and H-3/C-3 of β-Glc*p* (**N**) (δ 4.63/3.63; 84.3 ppm) and between H-1 β-Glc*p* (**N**) and H-4 of β-Ara*p* (**D**) (δ 4.55/4.02; 79.5 ppm). In variations **1a** and **1b**, inter-residual NOE and HMBC correlations were assigned between H-1 of 2-OMe-α-Ara*f* (**A**) and H-3/C-3 of β-Xyl*p* (**G**) (δ 5.69/3.82; 76.8 ppm) and between H-1 of α-Rib*f* (**B**) and H-3 of β-Xyl*p* (**H**) (δ 5.43/3.71; 79.9 ppm) and between H-1 of α-Rib*f* (**C**) and H-3 of β-Xyl*p* (**H**) (δ 5.41/3.74; 80.0 ppm), respectively.

In addition, inter-residual correlation between H-1 of β-Glc*p* (**P**) and C-6 of 3-OMe-β-Man*p* (**I**) (δ 4.50; 69.7 ppm) in backbone 2 were assigned by HMBC. Moreover correlations (δ 4.47/4.20, 3.87; 68.9 ppm) between H-1 of β-Glc*p*A (**Q**) and H-6 and C-6 of β-Glc*p* (**M**) revealed that these residues are correspondingly (1 → 6)-linked and correlations (δ 4.60/4.16; 78.4 ppm) between H-1 of **M** and H-4 and C-4 of α-Gal*p* (**E**) shows that these residues are (1 → 4)-linked. Furthermore, correlation of units **F** and **P** (δ 4.84/3.87 and 3.71; 65.9 ppm) were assigned between H-1 of α-Man*p* and H-6/C-6 of β-Glc*p*, thus establishing a β-Man*p*-(1 → 6)-β-Glc*p* linkages in a side-chain of the repeating unit 2. In the repeating unit 2a, correlations in units β-Man*p* (**L**) and 3-OMe-β-Man*p* (**J**) were found between H-1 and H-6; C-6 at (δ 4.63/4.11, 3.72; 69.1 ppm).

NOE and inter-residual HMBC correlations were not detected between residues **I**/**J** and **R** and between **R** and **M** in the intact polymer. However, these correlations were detected in the partially hydrolysed fragments and the same chemical shifts were found both in the intact and hydrolysed polymers. In addition, the downfield shifts for β-Xyl*p* at H-4 and C-4 of **R** from δ 3.51 to 3.82 and from 70.2 to 76.9 ppm and the downfield shifts of β-Glc*p* at H-4 and C-4 of **M** from δ 3.27 to 3.60 and from 70.6 to 78.7 ppm were used to establish the linkage position for these residues (Bock & Thøgersen, 1982).

The remaining ^{1}H and ^{13}C signals (Table 1) of the heteroglycan Nc-5-s were established through a combination of COSY, TOCSY, NOESY, HMBC, HSQC, H2BC, HSQC-TOCSY and HSQC-NOESY experiments.

The anomeric carbon signals were identified in the region of the HSQC spectrum with resonances between δ 98.4 and 103.9 ppm (Table 1) (Fig. 3). All other carbon signals were assigned from proton/carbon correlations in the region between δ 55.8 and 85.5 ppm. Downfield shifts for β-Glc*p*A (**O**) C-4 at δ 77.3 ppm, β-Xyl*p* (**K**, **R**)

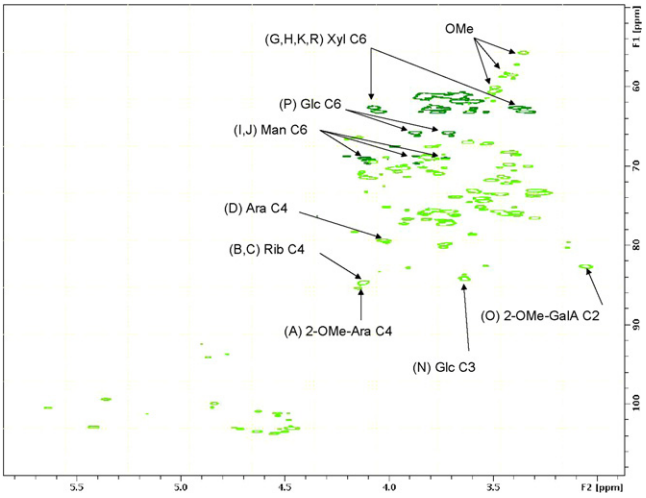

Fig. 3. HSQC spectrum of Nc-5-s (light green: CH, CH_3; dark green: CH_2). The assignments of selected proton–carbon correlations are shown. The spectrum represents the intact polysaccharide. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

C-4 at δ 76.9–77.4 ppm, β-Xyl*p* (**G**, **H**) C-3 at δ 76.8, 80.0 and C-4 at δ 77.3, 76.7 ppm, respectively, confirmed the linkage position for these residues. The difference between the chemical shifts for C-3 of units **G** (δ 76.8 ppm) and **H** (δ 80.0 ppm) is probably due to the 2-OMe group of α-Ara*f* (**A**) linked to unit **G**. Furthermore, the downfield shifts for β-Glc*p* (**N**) C-3 at δ 84.3 ppm, β-Glc*p* (**M**) C-4 and C-6 at δ 78.7 and 68.9, respectively, β-Glc*p* (**P**) C-6 at δ 65.9 ppm, β-Man*p* (**I** and **J**) C-6 at δ 69.7 and 69.1 ppm, respectively, and α-Gal*p* (**E**) C-4 at δ 78.4, confirmed the linkage positions for these residues (Table 1) (Bock et al., 1984). HSQC in combination with HMBC and HSQC-TOCSY were used to assign C-2, C-3, C-4, C-5 and C-6 of the residues.

Assignment of all ^{1}H and ^{13}C NMR signals for monosaccharide units of the polysaccharide Nc-5-s were accomplished and the complete analysis allowed revealing of the primary structure with approximate ratio of units **1**:**1a**:**1b**:**2**:**2a**, 10:25:50:5:10. Based on the relative integrals of the anomeric signals in the ^{1}H-spectrum the approximate monosaccharide composition of Nc-5-s is Glc/GlcA/Xyl/Man/Ara/Rib/Gal (20:20:20:5:22:10:3). This is similar to the results obtained from the methanolysis, where the ratios were determined to be Glc/GlcA/Xyl/Man/Ara/Rib/Gal (24:24:15:13:13:7:4). However, some differences between the molar ratios obtained by these two methods were found, especially for Man and Ara. Although molar ratios of the units could not be estimated by linkage analysis due to overlapping of signals, the results were very useful as a support for the NMR-analysis.

As stated in Section 1 cyanobacteria are known to produce complex EPSs of various compositions. Monosaccharide analysis show that they are usually composed of six to ten different monosaccharide residues including Glc, Gal, Xyl and uronic acids (De Philippis, Sili, Paperi, & Vincenzini, 2001; Otero & Vincenzini, 2003; Pereira et al., 2009). In addition some residues have been shown to contain sulphated or amino sugars, and in rare cases methylated sugar units have been described such as OMe-Rha (Hu, Liu, Paulsen, Petersen, & Klaveness, 2003), 2-OMe-Glc*p* (Brull et al., 2000), 3-OMe-Ara*f* (Volk, Venzke, & Blaschek, 2007); as well as 2-OMe-Man*p* and 2-OMe-Ara*p* in the case of colleman, which was isolated from a cyanolichen (Jensen et al., 2010). Previously, EPSs of three species of field growing *Nostoc* cyanobacteria; *N. commune*, *Nostoc flagelliforme*, *Nostoc sphaeroides*, are reported to consist of (1 → 4)-linked Glc, Xyl and Gal residues, Man and Ara were also present in *N. flagelliforme*. Ara was however detected in considerable amounts (up to 25%) in EPSs from *N. commune* cultured in nitrogen-free medium (Huang, Liu, Paulsen, & Klaveness, 1998). Further structural investigation of the *N. commune* polysaccharides revealed the presence of substantial amounts of Ara*f*, 2-OMe-Glc*p* and Glc*p*A in cultured samples and the amount of Ara was higher in the nitrogen-free cultivation (Brull et al., 2000). Structural investigation of an EPS released by *Nostoc insulare* during photoautotrophic cultivation revealed the constituents: GlcA, Glc and Ara, partly in the form of 3-OMe-Ara*f* (Volk et al., 2007). Ribose as a monosaccharide unit of cyanobacterial heteroglycans has been described before in samples of *Oscillatoria* and *Cyanothece* sp. (Parikh & Madamwar, 2006) as well as in samples of cultured *Nostoc* sp. (De Philippis, Ena, Paperi, Sili, & Vincenzini, 2000; De Philippis et al., 2001; Helm et al., 2000). The study of Helm et al. (2000) on an acidic heteroglycan DRH-1 from cultured *N. commune* cyanobacteria, describes structural fragments with a backbone of (1 → 4)-linked Glc*p*, Xyl*p* and Gal*p* with terminal α-D-Rib*f* unit linked at O3 of Xyl*p* and a NosA (nosturonic acid) linked at O6 of Glc*p*. The only previous study on detailed structure of acidic heteroglycan from wild *N. commune* colonies does describe structural fragments with a backbone of GlcA and (1 → 4)-linked Glc*p*, Gal*p* and Xyl*p*, also containing small amounts of 2-OMe-Glc*p* and (1 → 3)-linked Ara*f* (Brull et al., 2000).

Taken together, the structure of Nc-5-s does have some similarities to structures described earlier for polysaccharides from *N. commune* and other *Nostoc* sp., e.g. a backbone with (1 → 4) linked Gal*p*, Xyl*p* and Glc*p*A. However, the Glc*p*A is 2-O-methylated in the present study and the Glc*p* is (1 → 3)-linked instead of (1 → 4)-linked as previously described. Additional new structural features for Nc-5-s are the terminal 2-OMe-Ara*f* and the Man*p*-(1 → 6)-Glc*p* branches. Terminal α-Rib*f* linked to O3 of the Xyl*p* in the backbone has been observed before by Helm et al. (2000). Although Rib*f* has rarely been described in cyanobacterial EPS studies the lability of the Rib*f* glycosidic linkage might have resulted in under-estimation of this monosaccharide unit. It has been suggested that Rib*f* could be involved in the protection of neighbouring glycosidic bonds in cyanobacterial EPSs and to be partially responsible for the gelatinous consistency of the native material (De Philippis & Vincenzini, 1998; Helm et al., 2000).

Cyanobacteria are nitrogen fixing and most of the previous studies have been devoted to assaying the effects of nitrogen deficiency on the monosaccharide compositions of cultured samples. The results have shown that different types of extracellular polysaccharides are produced by field samples and cyanobacteria grown in media with or without nitrogen. This suggests that variation of nitrogen metabolism influences the carbohydrate metabolism and even stimulates the polysaccharide synthesis, since the polymers could act as a product overflow metabolism, and are excreted to allow cells to get rid of excess carbon (Brull et al., 2000; De Philippis & Vincenzini, 1998; Huang et al., 1998). Cultivation of *N. commune* in nitrogen free medium elevates the amount of Ara substantially (Brull et al., 2000; Huang et al., 1998) and since the Icelandic soil is generally considered to be nitrogen poor (Arnalds & Kimble, 2001), this could explain the relatively high levels of Ara in the wild growing Icelandic *N. commune*.

Growth of free living colonies of *N. commune* under harsh conditions and for longer periods of time compared to cultured samples would therefore be expected to provide different composition of the heteroglycans, modified in accordance to the diverse environmental conditions. This flexibility in the cyanobacterial polysaccharide assembly process certainly guarantees the viability of the organism, but can make the structural analysis of cyanobacterial polysaccharides quite challenging (Brull et al., 2000; Helm et al., 2000). On the other hand variability in structural details with environmental factors, gives us the opportunity to influence the production of heteroglycans by varying conditions upon cultivation.

Studies revealing repeating blocks and showing detailed connective patterns of the monosaccharide units of cyanobacterial polysaccharide structures are rare in the literature. However, a recently published paper by the present authors (Jensen et al., 2010) describes the detailed structure elucidation by high field NMR spectroscopy, of a heteroglycan named colleman, from the cyanolichen *Collema flaccidum* that has a *Nostoc* cyanobacterium photobiont. Colleman was extracted and isolated in a similar manner as described for Nc-5-s in this study. Colleman was suggested to be of cyanobacterial origin as the structure was a complex acidic heteroglycan and had some similarities to *Nostoc* EPSs described earlier (Brull et al., 2000; De Philippis et al., 2000; Helm et al., 2000; Huang et al., 1998; Jensen et al., 2010; Parikh & Madamwar, 2006; Pereira et al., 2009).

However, the structural differences revealed by this detailed analysis of these comparable heteroglycan fractions, colleman from a lichenized *Nostoc* sp. on one hand, and Nc-5-s from a free living cyanobacteria *N. commune* on the other hand, reflects the structural variations that seem to be universal for heteroglycans of the *Nostoc* genus, and the same probably applies for the cyanobacteria in general, depending on various environmental factors. It has been shown that *Nostoc* cyanobacteria can be cultivated in bioreactors (Yu, Jia, & Dai, 2010) and, by manipulating with the growing conditions, it could be possible to produce EPSs with favourable chemical and physical properties of potential interest to the food-, cosmetic-, medical- and other industries.

In conclusion, the thorough structural analysis of the *N. commune* polysaccharide Nc-5-s, presented in this study revealed its primary structure. The detailed NMR assignments provide useful NMR reference data for future studies on cyanobacterial heteroglycans. Nc-5-s has some structural features in common with those polysaccharides previously isolated from *Nostoc* cyanobacteria, however, the terminal 2-OMe-Ara*f* and the Man*p*-(1 → 6)-Glc*p* branches are new features, as well as the relatively high Ara content, the methylation pattern and some differences in the linkage pattern.

The solving of the structure of Nc-5-s provides crucial data for future studies of *N. commune* EPSs. Knowledge about complete structures of cyanobacterial heteroglycans is important and essential to promote deeper understanding of the different factors that eventually could enable manipulation of the chemical and physical properties of these potentially valuable industrial products in the future.

Acknowledgements

The spectra at 800 MHz were obtained on the Bruker Avance spectrometer of the Danish Instrument Center for NMR Spectroscopy of Biological Macromolecules, Carlsberg Laboratory, Copenhagen, Denmark. Supporting grants from Icelandic Council of Science, University of Iceland Research Fund, Bergthora and Thorsteinn Scheving Thorsteinsson Fund and Selma and Kay Langvad Fund are gratefully acknowledged. Tanja Veselinovic's contribution to collecting and preparing the *Nostoc* material is gratefully acknowledged as well.

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