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Carbohydrate Research 328 (2000) 375–382

CARBOHYDRATE
RESEARCH

Application of nano-probe NMR for structure determination of low nanomole amounts of arabinoxylan oligosaccharides fractionated by analytical HPAEC-PAD

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Received 4 October 1999; accepted 11 April 2000

Abstract

A methodology for NMR analysis of low nanomole amounts of oligosaccharides fractionated by analytical HPAEC is presented. Arabinoxylan derived oligosaccharides purified by HPAEC-PAD on an analytical column, by single injections, were analyzed with nano-probe NMR and MALDI-TOF MS to provide full structural assignment. The NMR data were obtained with a 500 MHz NMR spectrometer equipped with a ^{1}H-observe nano-probe. Both one- and two-dimensional experiments on arabinoxylan samples in the low nanomole range were performed, including ^{1}H–^{1}H DQF-COSY, ^{1}H–^{1}H TOCSY and ^{1}H–^{1}H ROESY. These experiments allowed, in combination with MALDI-TOF MS and literature NMR data, a complete structural determination of several tetra-, penta-, hexa- and heptasaccharides. Two new structures: α-L-Ara*f*-(1 → 2)-β-D-Xyl*p*-(1 → 4)-β-D-Xyl*p*-(1 → 4)-β-D-Xyl*p*-(1 → 4)-D-Xyl*p* and α-L-Ara*f*-(1 → 2)[α-L-Ara*f*-(1 → 3)]-β-D-Xyl*p*-(1 → 4)-β-D-Xyl*p*-(1 → 4)-β-D-Xyl*p*-(1 → 4)-D-Xyl*p*) were characterized, as well as some previously published structures. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Barley; Arabinoxylan; Oligosaccharide; Structure; HPAEC-PAD; Nano-probe NMR; MALDI-TOF MS

1. Introduction

Arabinoxylan polysaccharides are found in the cell walls of a number of different plants, and have been studied extensively with NMR over the past 10 years, resulting in the elucidation of numerous oligosaccharide structures [1–8]. Barley arabinoxylan consists of a β-(1 → 4)-D-xylopyranoside backbone carrying O-2, O-3 or O-2,3 α-L-arabinofuranosyl substituents with an overall Ara/Xyl ratio of around 0.7 [9]. An unusual feature of arabinoxylan from barley is the high proportion of xylose residues monosubstituted at O-2 [10]. In studies of enzymes acting on arabinoxylan oligosaccharides, it is of paramount importance to have access to pure and well characterized substrates, i.e., arabinoxylan oligosaccharides, and also to be able to characterize the products of the enzymatic reactions. Since small amounts of these substances can be efficiently isolated by analytical HPAEC, a method for the analysis of such fractions based upon NMR spectroscopy and supplemented by MS, was highly desirable. Furthermore, since enzymatically degraded

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0008-6215/00/$ - see front matter
PII: S0008-6215(00)00108-7

arabinoxylan polysaccharides provide similar but diverse structures, which are not easily characterized by other methods than NMR, this group of carbohydrates serves as a good test sample for evaluating the methodology.

NMR is the most powerful tool for structural investigation of organic compounds. Quite often, however, the amount of sample available is limited, which makes NMR analysis time consuming or sometimes even impossible. To overcome such problems different NMR methodologies have been developed to handle small sample amounts [11]. One solution is to put the sample in an NMR probehead adapted to measurements on samples in small diameter NMR tubes [12]. This requires, however, that a large portion of the sample is kept outside the receiver coil of the NMR spectrometer to obtain ‘a infinite cylinder’ shape of the sample to minimize line-broadening effects from differences in magnetic susceptibility, just as in a conventional 5-mm probe head [12]. This reduces the sensitivity of the measurement, since the sample has to be diluted. Another approach is to reduce the line-broadening effects from differences in magnetic susceptibilities by magic-angle spinning technology. This enables all the sample to be kept inside the receiver coil, since line-broadening effects from the differences in magnetic susceptibilities are efficiently eliminated [13]. Such a methodology is used in the nano-probe employed in the present study. This NMR technique enables the structural analysis of different types of natural products at sample levels down to the nanomole range, using different one- (1D) and two-dimensional (2D) ^{1}H NMR techniques, e.g., [14–16].

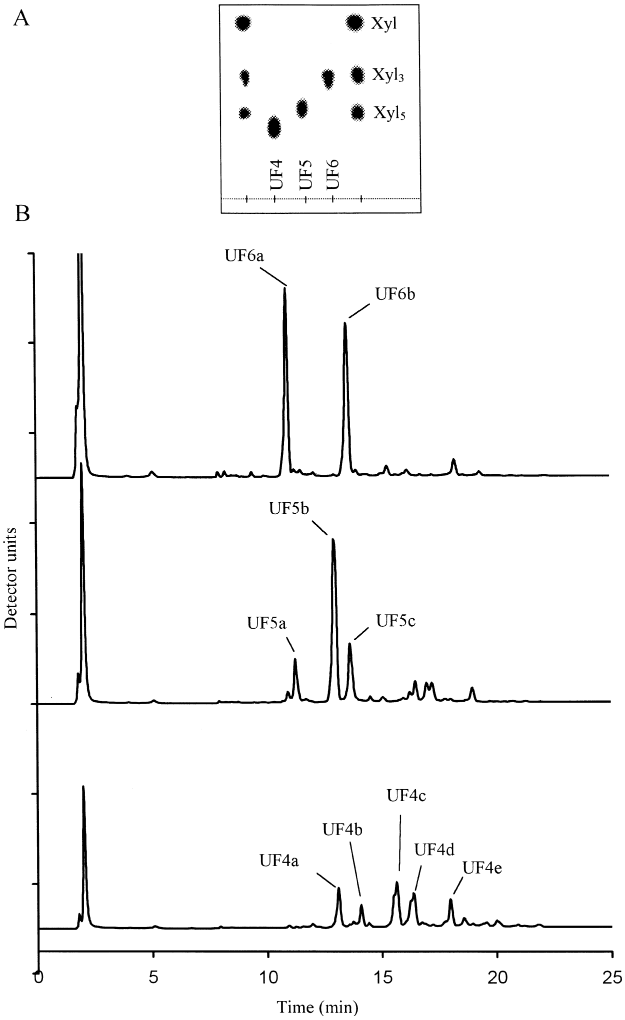


Fig. 1. Analysis of oligosaccharide fractions UF6, UF5 and UF4 with TLC (A) and HPAEC-PAD (B). Xyl, Xyl_3 and Xyl_5 refer to xylose, β-(1 → 4)-xylotriose and β-(1 → 4)-xylopentaose, respectively. The collected fractions from semi-preparative operation of the HPAEC system are indicated. The chromatographic conditions were as described in the experimental.

A combination of HPAEC-PAD separation with nano-probe NMR investigation supplemented by MALDI-TOF MS has been used for the structure elucidation of oligosaccharides originating from enzymatic digestion of barley malt arabinoxylans. This combination of techniques has proved to be a very powerful tool for the isolation and structure determination of minute amounts of oligosaccharides, as demonstrated below.

2. Results and discussion

General.—Barley malt arabinoxylan was digested with a commercially available enzyme mixture, and subsequently fractionated by size-exclusion chromatography. Three pools of fractions, UF6, UF5 and UF4, were collected and analyzed using thin-layer chromatography (TLC) and HPAEC-PAD (Fig. 1). Fractions UF6, UF5 and UF4 were further fractionated into subfractions UF6a-b, UF5a-c and UF4a-e by semi-preparative operation of the analytical HPAEC-PAD system (Fig. 1(B)). The sample amounts in the collected subfractions UF6a-b, UF5a-c and UF4a-e ranged from approximately 3 to 18 μg (3–26

Table 1
Molecular masses of main constituents of HPAEC-PAD subfractions isolated from size-exclusion chromatography fractions UF6, UF5 and UF4 as determined by MALDI-TOF MS [a]

Fraction	m/z		DP [b]
	Exp.	Theor.	
UF6a	569.10	569.17	4
UF6b	701.16	701.21	5
UF5a	701.29	701.21	5
UF5b	833.32	833.25	6
UF5c	833.29	833.25	6
UF4a	965.27	965.30	7
UF4b	965.29	965.30	7
UF4c	1097.28	1097.34	8
UF4d	1097.21	1097.34	8
UF4e	1229.21	1229.38	9

[a] The given values for all m/z correspond to Na^+ adducts.
[b] DP, degree of polymerization, i.e., total number of pentoses.

nmol, respectively) of the major arabinoxylan oligosaccharide, as determined by HPAEC-PAD using known amounts of reference compounds and assuming identical response factors. Previous studies have shown PAD response factors to be similar for neutral oligosaccharides of similar structures and sizes [17]. The sample amounts obtained were enough to produce 1D ^{1}H NMR spectra and in many cases 2D ^{1}H NMR spectra of reasonably good signal-to-noise ratio using nanoprobe NMR. In this study ^{1}H–^{1}H double quantum filtered correlation spectroscopy (DQF-COSY), ^{1}H–^{1}H total correlation spectroscopy (TOCSY) and ^{1}H–^{1}H rotating frame Overhauser enhancement spectroscopy (ROESY) experiments on sample amounts down to approximately 4 nmol (3 μg), have been measured, each requiring less than 24 h of instrument time.

Frequently, MS data provide valuable support to structural assignments from NMR analysis. MALDI-TOF MS requires very small sample amounts, in the range of 1 pmol of underivatized oligosaccharides, which makes the technique very suitable for analyzing small amounts of oligosaccharides fractionated on an analytical HPAEC-PAD system [18]. Thus, good quality mass spectra, with a mass accuracy within 0.01% from theoretical values, could be recorded on subfractions UF6a-b, UF5a-c and UF4a-e (Table 1).

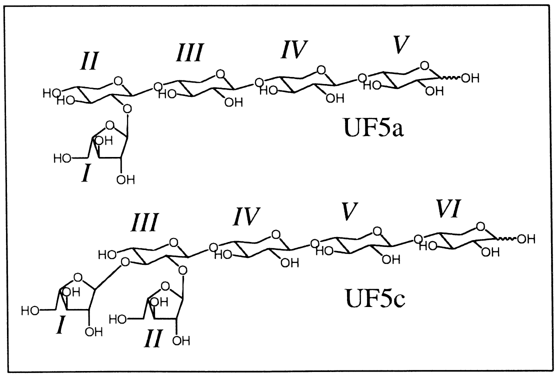
Details from the 1D ^{1}H NMR spectra of the ten subfractions isolated from UF6, UF5 and UF4 are shown in Fig. 2. The anomeric signals in these ^{1}H NMR spectra are found in two spectral regions: δ 5.15–5.3 and δ 4.4–4.65. The coupling constants ($^3J_{1,2}$) for these signals are approximately 1–1.4 and 7.2–8 Hz, respectively, which are in accordance with the presence of α-L-arabinofuranosyl and β-D-xylopyranosyl groups in the investigated oligosaccharides [1–3,5,19]. MALDI-TOF MS showed that the oligosaccharides in the analyzed subfractions ranged from tetra- to nonasaccharides in size (Table 1). These molecular sizes correspond well with the number of anomeric signals in the 1D ^{1}H NMR spectra of the HPAEC fractions, which contained mainly one component (i.e., UF6a-b, UF5a-c and UF4a-b).

Analysis of the recorded 1D and 2D NMR data resulted in complete structural elucidation of six oligosaccharide structures together with the partial characterization of one structure. Four of the fully characterized structures were previously published (UF6a: α-L-Ara*f*-(1→2)-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-D-Xyl*p* [2]; UF6b: α-L-Ara*f*-(1→2)-[α-L-Ara*f*-(1→3)]-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-D-Xyl*p* [1]; UF5b: β-D-Xyl*p*-(1→4)-[α-L-Ara*f*-(1→2)-[α-L-Ara*f*-(1→3)]]-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-D-Xyl*p* [3,5]; and UF4a: β-D-Xyl*p*-(1→4)-[α-L-Ara*f*-(1→2)-[α-L-Ara*f*-(1→3)]]-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-D-Xyl*p* [3]), and will not be discussed any further. The two novel compounds (UF5a and UF5c) will be discussed in more detail below, together with the partially solved structure UF4b. Since subfractions UF4c-e contained two or more components no serious attempts to make complete structural assignments were made.

Subfraction UF5a.—The dominating oligosaccharide in subfraction UF5a was found by a 1D ^{1}H NMR spectrum (Fig. 2) and MALDI-TOF MS-data (Table 1) to contain four D-xylopyranosyl groups and one α-L-arabinofuranosyl group. According to the chemical shift for the signal of the anomeric proton of the α-L-arabinofuranosyl group (δ

5.280), this should be (1 → 2)-linked to a β-D-xylopyranosyl group [2]. From the 2D ^{1}H–^{1}H TOCSY and 2D ^{1}H–^{1}H DQF-COSY spectra of UF5a (Fig. 3(A) and (B), recorded on approximately 3 μg (4 nmol) of oligosaccharide), it was possible to extract the chemical shifts for the signals from the ring protons of the β-D-xylopyranosyl and α-L-arabinofuranosyl groups (Table 2). The signal at δ 4.555 is from the anomeric proton of the α-L-arabinofuranosyl substituted β-D-xylopyranosyl group (residue II). The signal of H-2 of residue II is shifted downfield (δ 3.411) and the signal of H-4 is shifted upfield (δ 3.660) relative to the signals of H-2 and H-4 of internal xylopyranosyl groups in linear β-(1 → 4)-D-xylans (approximate chemical shifts δ 3.29 and δ 3.79, respectively) [3]. Thus, the NMR data are consistent with the following structure: α-L-Ara*f*-(1 → 2)-β-D-Xyl*p*-(1 → 4)-β-D-Xyl*p*-(1 → 4)-β-D-Xyl*p*-(1 → 4)-D-Xyl*p*. This conclusion was further verified by a 2D ^{1}H–^{1}H ROESY experiment on the same sample (Table 2 and Fig. 3(C)). The ROESY spectrum was recorded on approximately 3 μg (4 nmol) of oligosaccharide, which appears to be close to the limit for recording NMR data of this type on a reasonable time scale using this methodology (Fig. 3(C)).

Subfraction UF5c.—The initial subfraction UF5c contained approximately 9 nmol of the major oligosaccharide, but also a considerable amount of the hexasaccharide from subfraction UF5b. Thus, a second batch of UF5c was prepared from the same starting material, resulting in approximately 14 μg (17 nmol) of the oligosaccharide. The 1D ^{1}H NMR spectrum (Fig. 2) of subfraction UF5c contains signals from the anomeric protons of four D-xylopyranosyl groups and two α-L-arabinofuranosyl groups, which is in agreement with the MS data (Table 1). The arabinofuranosyl branching pattern can be determined directly from the 1D ^{1}H NMR spectrum, since the two α-L-arabinofuranosyl anomeric signals at δ



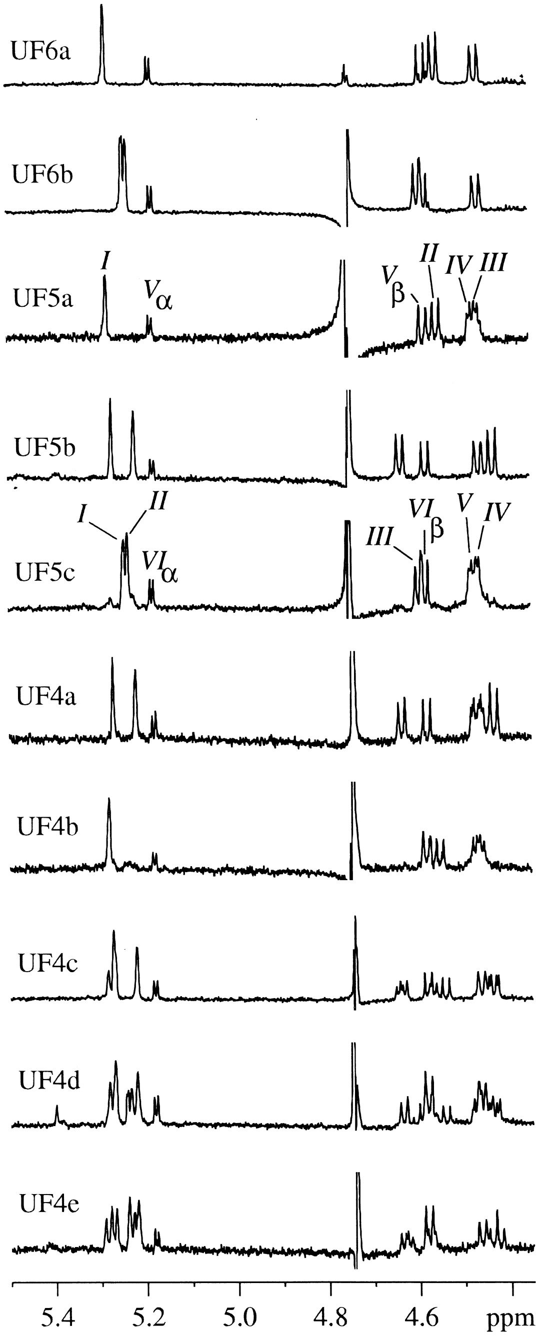


Fig. 2.

Fig. 2. The anomeric regions of 1D ^{1}H NMR spectra of subfractions UF6a-b, UF5a-c and UF4a-e recorded with 256 scans at 500 MHz with a 4-mm ^{1}H-observe Nano NMR probe on approximate sample amounts between 3 and 26 nmol (3 and 18 μg, respectively). The assignments of some characteristic signals of the novel oligosaccharides in subfractions UF5a and UF5c are indicated in the figure.

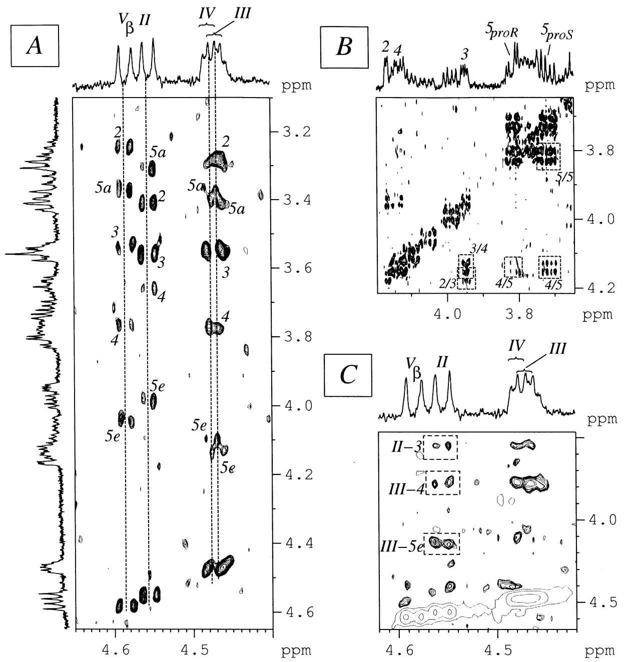


Fig. 3. Details from 2D ^{1}H–^{1}H TOCSY (A), ^{1}H–^{1}H DQF-COSY (B) and ^{1}H–^{1}H ROESY (C) spectra of subfraction UF5a recorded on approximately 4 nmol (3 μg) oligosaccharide at 500 MHz with a 4-mm ^{1}H-observe Nano NMR probe. The labels of the different monosaccharide residues refer to the labels in Fig. 2. (A) and (B) illustrates how the chemical shifts for the signals of the xylopyranosyl and arabinofuranosyl ring protons, respectively, can be extracted. (C) demonstrates diagnostic ROE cross-peaks for the determination of the sequence of xylopyranosyl group.

5.236 and δ 5.244 are diagnostic for two α-L-arabinofuranosyl groups (1 → 2)- and (1 → 3)-linked, respectively, to a β-D-xylopyranosyl group, which is not substituted at C-4 [1]. This suggests that the structure of the oligosaccharide in fraction UF5c is α-L-Ara*f*-(1 → 2)-[α-L-Ara*f*-(1 → 3)]-β-D-Xyl*p*-(1 → 4)-β-D-Xyl*p*-(1 → 4)-β-D-Xyl*p*-(1 → 4)-D-Xyl*p*). This structure and the NMR data have not previously been described. To corroborate this tentative structure and to assign all ^{1}H NMR signals 2D ^{1}H–^{1}H DQF-COSY, 2D ^{1}H–^{1}H TOCSY and 2D ^{1}H–^{1}H ROESY experiments were performed (data shown in Table 2).

Subfraction UF4b.—The major oligosaccharide in subfraction UF4b contained five D-xylopyranosyl groups and two α-L-arabinofuranosyl groups, according to the 1D ^{1}H NMR spectrum (Fig. 2) and the MS data (Table 1). According to the chemical shifts for the signals from the α-L-arabinofuranosyl anomeric protons (δ 5.282), both α-L-arabinofuranosyl groups should be (1 → 2)-linked. From the 2D ^{1}H–^{1}H TOCSY (data not shown) it was difficult to extract the chemical shifts for all ring-protons due to the overlap of the anomeric protons. However, the H-2 and H-4 signals from one of the β-D-xylopyranosyl groups display the characteristic downfield (δ 3.41) and upfield shifts (δ 3.65), respectively, as mentioned earlier for fraction UF5a (Table 2). This indicates that one of the α-L-arabinofuranosyl groups is (1 → 2)-linked to a β-D-xylopyranosyl group, which is not substituted at C-4. The position of the other (1 → 2)-linked α-L-arabinofuranosyl group could not be established with the recorded NMR data.

Concluding remarks.—The methodology and results described here clearly demonstrate that it is possible to solve the structures of oligosaccharides, at low nanomole sample level, in samples originating from complex mixtures fractionated by analytical HPAEC-PAD. The combination of these techniques opens the possibilities for more detailed descriptions of substrate preferences and action pattern of carbohydrate degrading enzymes.

These analyses can be performed quite rapidly. The fractionation on HPAEC-PAD takes less than 1 h, whereas the remainder of the sample work-up (neutralization, desalting and drying) takes a few hours. To record a 1D ^{1}H NMR spectrum on a sample of a few nanomoles of oligosaccharide, including insertion of the sample in the spectrometer, temperature equilibration and shimming, normally requires less than 0.5 h. It naturally takes more time to record 2D NMR data (usually overnight). Thus, interpretable NMR data can be available after some hours from the start of the fractionation procedure, in the case of 1D ^{1}H NMR data, or the next day in the case of 2D NMR data. The operation of an NMR spectrometer equipped with a nanoprobe is not very different from the operation with a conventional 5-mm probe head. The main difference is that the probe-head has to be removed and reinserted when the sample is inserted. Another difference is the shimming procedure, but this is only a question of using a different set of shim coils for routine shimming. The sample preparation can be significantly simplified by on-line neutralization and desalting using a Carbohydrate Membrane Desalter (Dionex), which makes the samples amenable to NMR or MS analysis directly after lyophilisation. During the preparation of this work we did not have access to such equipment. However, subsequent work in this laboratory indicates that nano-probe NMR analysis of on-line desalted HPAEC fractions containing a few micrograms of oligosaccharide can be performed directly after lyophilisation.

At present we are using HPAEC-isolated and nano-probe NMR characterized arabinoxylan oligosaccharides, at low microgram levels, to investigate the substrate preference and action pattern of an enzyme acting on arabinoxylans isolated from barley.

3. Experimental

Chemicals.—Maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose were all purchased from Sigma (St. Louis, MO, USA). β-(1→4)-D-Xylohexaose, 2,5-dihydroxybenzoic acid and D_2O were bought from Megazyme International (Bray, Ireland), Aldrich (Steinheim, Germany) and Cambridge Isotope Laboratories (Andover, MA, USA), respectively.

Extraction of arabinoxylan and preparation of oligosaccharides.—Finely ground barley malt (500 g; Danish Malting Group, Vordingborg, Denmark) was stirred with 5 L of 100 mM NaOAc buffer, pH 5.0 for 2 h at 4 °C. The extract was centrifuged at 17,500 × *g* for 20 min, the supernatant was concentrated to 250 mL and dialyzed against 20 mM NaOAc buffer (pH 5.0) using an Amicon CH 2A concentrator equipped with a spiral-wound membrane cartridge having a nominal molecular weight limit of 10 kDa. Protein was removed by passing the solution through an S-Sepharose column (2.6 × 40 cm; Amersham Pharmacia Biotech AB, Uppsala, Sweden) and after buffer exchange to 20 mM Tris–HCl (pH 8.0) through Q-Sepharose (2.6 × 20 cm; Amersham Pharmacia Biotech AB, Uppsala, Sweden). The preparation was then incubated for 16 h with 500 μL of Cereflo (NOVO-Nordisk, Bagsværd, Denmark) at 56 °C for degradation of high-molecular-weight (1→3),(1→4)-β-glucan and starch. Cereflo is a com-

Table 2
^{1}H NMR chemical shifts and important ROE cross-peaks for arabinoxylan subfractions UF5a and UF5c [a]

Fraction	Residue	δ						H-1 ROE [d]
		H-1	H-2	H-3	H-4	H-5eq [b]	H-5ax [c]	
UF5a	I	5.280	4.167	3.948	4.139	3.815	3.716	II-2
	II	4.555	3.411	3.559	3.660	3.988	3.307	III-4, 5e
	III	4.470	3.29	3.55	3.78	4.134	3.409	
	IV	4.478	3.29	3.55	3.78	4.102	3.375	
	V_β	4.584	3.246	3.545	3.77	4.053	3.375	
	V_α	5.183	3.56	3.74	nd	nd	nd	
UF5c	I	5.244	4.176	3.973	4.199	3.816	3.706	III-3
	II	5.236	4.151	3.957	4.134	3.819	3.720	III-2
	III	4.596	3.541	3.699	3.719	4.025	3.345	IV-4, 5e
	IV	4.471	3.29	3.56	3.791	4.142	3.417	V-5e
	V	4.477	3.29	3.56	3.78	4.106	3.378	VI-5e
	VI_β	4.582	3.250	3.549	3.78	4.057	3.378	
	VI_α	5.183	3.55	3.75	nd	nd	nd	

[a] The D-Xyl*p* and L-Ara*f* residues are labeled according to the structures in Fig. 2.

[b] Equatorial for D-Xyl*p* or proR for L-Ara*f*.

[c] Axial for D-Xyl*p* or proS for L-Ara*f*.

[d] Key ROE cross-peaks from the corresponding H-1 signal, II-2 means a cross-peak from the corresponding H-1 to H-2 of residue II; nd: not determined. Assignment of the signals from H-5 proR and proS of the α-L-arabinofuranosyl group was according to literature data [20].

mercial enzyme preparation without arabinoxylan degrading activities. The extract was diluted to 600 mL and high-molecular-weight arabinoxylan was precipitated from 75% EtOH at 4 °C over night. Precipitated material was collected by centrifugation at 17,500 × *g* for 20 min, dissolved and chromatographed in water on Sephacryl S-100 HR (2.6 × 100 cm; Amersham Pharmacia Biotech AB, Uppsala, Sweden) equilibrated with dextran standards. Material of molecular weight >10 kDa was used for preparation of oligosaccharides.

High-molecular-weight arabinoxylan was incubated with Ultraflo (NOVO-Nordisk) for 2 h at 56 °C using 0.2 mL of the commercial enzyme solution per gram of arabinoxylan. Following precipitation from 70% EtOH at 4 °C over night and centrifugation at 17,000 × *g* for 20 min, the oligosaccharide containing supernatant was evaporated to dryness on a rotatory evaporator. The oligosaccharides were fractionated by size-exclusion chromatography on Toyopearl HW-40S (TosoHaas) using two tandemly connected columns (1.6 × 95 cm) operated at 56 °C at a flow rate of 12 cm h^{-1}. Oligosaccharides were monitored by a refractive index detector (Gilson RI 132) and fractions of 5 mL were collected and analyzed by TLC.

TLC analysis of Toyopearl fractions.—Fractions of interest were freeze dried, redissolved in 500 μL of water and analyzed on TLC plates (Silica 60, E. Merck). The mobile phase was 2:2:1 EtOAc–HOAc–water. Following development and drying, the plates were flooded with 20% H_2SO_4 in EtOH, dried with a hair dryer and incubated at 125 °C for 10–15 min for visualization of carbohydrate spots. Toyopearl fractions containing apparently homogenous oligosaccharides with identical R_f values were pooled (i.e., UF4, UF5 and UF6) for further fractionation with HPAEC-PAD.

HPAEC-PAD fractionation of arabinoxylan oligosaccharides.—Fractionation of arabinoxylan oligosaccharides was performed on an HPAEC-PAD system (Dionex DX500 with a GP40 gradient pump coupled to a CarboPac PA-100 analytical column (4 × 250 mm) and an ED40 electrochemical detector operated in pulsed amperometric detection mode; Dionex Corporation, Sunnyvale, CA, USA). Aqueous NaOH (0.1 M) was used as eluent and an elution gradient was formed with aq NaOAc (0–0.5 M in 35 min at 0.8 mL min^{-1}). Samples (20 μL) from solutions of approximately 1 mg mL^{-1} of oligosaccharide mixtures (UF4, UF5 and UF6) were injected (1 injection/sample) on the analytical HPAEC column and fractions UF4a-e, UF5a-c and UF6a-b were manually collected. For analytical purposes 1 μL of the same samples were injected. The collected fractions were immediately neutralized with HOAc, desalted on columns (1 × 1 cm) of AG 50W-X8 (H$^+$) cation-exchanger (Bio-Rad Laboratories, Hercules, California, USA) and dried in a centrifugal evaporator.

Nano-probe NMR analysis.—NMR analyses were performed at 25 °C on a Varian Unity Inova 500 MHz NMR spectrometer (Varian Inc., Palo Alto, CA, USA) equipped with a 4-mm ^{1}H observe Nano NMR probe. Each arabinoxylan sample was dissolved in 40 μL D_2O and transferred to a nano-tube, which subsequently was positioned in the probe-head. During all NMR analyses the nano-tube spinning rates of around 2000 Hz were used. 1D ^{1}H NMR spectra (sweep width 8000 Hz, 256 scans, acquisition time 2.0 s) were recorded with presaturation of the residual HDO signal during the repetition delay (1.5 s; total time for recording 15 min). 2D homonuclear NMR experiments, i.e., ^{1}H–^{1}H DQF-COSY (64–96 scans, 256 t_1 increments, sweep width 4000 Hz, repetition delay 1.4 s), ^{1}H–^{1}H TOCSY (24–80 scans, 256 t_1 increments, sweep width 6000 Hz, repetition delay 1.55 s) and ^{1}H–^{1}H ROESY experiments (40–120 scans, 128–256 t_1 increments, sweep width 3600 Hz, repetition delay 1 s), were performed with presaturation of the residual HDO signal during the repetition delay. The ^{1}H–^{1}H TOCSY experiments were performed with a 90 ms adiabatic Wurst 8 mixing period [21] and the ^{1}H–^{1}H ROESY experiments with a 400 ms continuous spin-lock pulse of a power-level corresponding to a 90° pulse of 110 μs duration. The chemical shifts for the anomeric signals were determined directly from the 1D ^{1}H NMR spectra, whereas the chemical shifts for the other ring protons were extracted from 2D ^{1}H–^{1}H DQF-COSY and ^{1}H–^{1}H TOCSY experiments. All ^{1}H NMR chemical shifts were referenced to internal acetone (δ_H 2.225).

For quantification of the arabinoxylan oligosaccharide samples analyzed with nanoprobe NMR, a portion of each sample was injected on the HPAEC-PAD system (approximately 60–400 pmol of each oligosaccharide, in triplicate), using the same HPAEC-PAD conditions as mentioned above. β-(1 → 4)-Xylohexaose was used as internal standard (170 pmol was injected together with each sample). The approximate arabinoxylan oligosaccharide amount in each NMR sample was calculated from the area of the peak of the arabinoxylan oligosaccharide and the peak of internal xylohexaose.

MALDI-TOF MS analysis.—MS analyses were performed on a Bruker Reflex^{iii} MALDI-TOF MS (Bruker Daltonik GmbH, Bremen, Germany), operated in the delayed extraction mode with an accelerating voltage of 20 kV, reflectron voltage of 22.8 kV and detector voltage of 1.5 kV. 2,5-Dihydroxybenzoic acid was used as matrix (20 mg mL^{-1} in 3:2 water–MeCN with 0.1% CF_3CO_2H). Samples were dissolved in water and mixed 1:1 (v/v) with the matrix solution. Subsequently, 1 μL of the sample/matrix mixture was applied to the stainless steel target and dried under reduced pressure. Prior to MS analyses the sample spots were 'recrystallized' with a small volume of EtOH. After inserting the target in the ion source, the laser attenuation was set to a value slightly below the threshold value for the formation of observable ions and 100 shots were acquired and summed for each sample. A mixture of maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose was used as external standard for the mass assignments.

Acknowledgements

The authors thank Dr Charlotte H. Gotfredsen for valuable assistance and support during the nano-probe NMR analyses.

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