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Structures of enzymically derived oligosaccharides from sorghum glucuronoarabinoxylan

Marian A. Verbruggen [a], Bertina A. Spronk [b], Henk A. Schols [a], Gerrit Beldman [a], Alphons G.J. Voragen [a,*], Jerry R. Thomas [b], Johannis P. Kamerling [b], Johannes F.G. Vliegenthart [b]

[a] *Wageningen Agricultural University, Department of Food Science, P.O. Box 8129, NL-6700 EV Wageningen, Netherlands*

[b] *Bijvoet Centre, Department of Bio-Organic Chemistry, Utrecht University, P.O. Box 80075, NL-3508 TB Utrecht, Netherlands*

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Abstract

Oligosaccharides derived from alkali-extracted sorghum glucuronoarabinoxylan by digestion with a combination of (1 → 4)-β-D-arabinoxylan arabinofuranohydrolase (AXH) and endo-(1 → 4)-β-D-xylanase (Xyl I), both from *Aspergillus awamori*, were purified by size-exclusion chromatography followed by preparative high-performance anion-exchange chromatography. Structural studies including monosaccharide analysis, methylation analysis, ^{1}H NMR spectroscopy, and mass spectrometry were carried out, resulting in the characterisation of four novel oligosaccharides, namely, α-D-Glc*p*A-(1 → 2)-β-D-Xyl *p*-(1 → 4)-β-D-Xyl *p*-(1 → 4)-β-D-Xyl *p*, α-D-Glc*p*A-(1 → 2)-β-D-Xyl *p*-(1 → 4)[α-L-Ara*f*-(1 → 3)]-β-D-Xyl *p*-(1 → 4)-β-D-Xyl *p*, α-D-Glc*p*A-(1 → 2)-β-D-Xyl *p*-(1 → 4)-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*, and α-D-Glc*p*A-(1 → 2)-β-D-Xyl *p*-(1 → 4)[α-L-Ara*f*-(1 → 3)]-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*. The various oligosaccharides identified provide additional insight into the structure of sorghum glucuronoarabinoxylan. Furthermore, novel data were generated with respect to the substrate specificity of AXH and Xyl I towards glucuronoarabinoxylans in general.

Keywords: Glucuronoarabinoxylan; Sorghum; Oligosaccharides; Structural analysis; Enzymic digestion

1. Introduction

Arabinoxylans are components widely found in cereal endosperm cell wall materials. These polysaccharides consist of a backbone of (1 → 4)-linked β-D-xylopyranose (Xyl) residues substituted primarily with single α-L-arabinofuranose (Ara) units at O-3 or at both O-2 and O-3 of certain Xyl units. Single substitution with Ara at O-2 occurs, but is quite rare. For the arabinoxylans of the kernel endosperms from wheat [1,2], barley [3], rye [4,5], and oat [6], it has been shown that they differ in degree and pattern of Ara-substitution and in molecular mass [7]. However,

* Corresponding author.

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the arabinoxylans from the grains of sorghum [8], maize [9], and some rice varieties [10] are far more complex. In the latter group of arabinoxylans, the molar ratio of Ara to Xyl is generally much higher ($\sim$ 0.9–1.1), indicating that they have a higher level of Ara-substitution. Moreover, they contain considerable amounts of uronic acid residues, predominantly D-glucuronic acid (GlcA) attached at O-2 of Xyl residues [10–12].

Several reports on the isolation, fractionation and partial characterisation of glucuronoarabinoxylans (GAX) have appeared [9,10,13–15], but so far detailed structural information is missing. However, partial characterisations are not sufficient to be interpreted in terms of functional properties and behaviour, and further structural knowledge is a prerequisite. Recently, structural studies on neutral arabinoxylo-oligosaccharides, enzymically generated from arabinoxylans of wheat [16–21], barley [22,23] and rye [24], have been published, and have resulted in detailed information about the chemical structure of the polysaccharides as well as about the substrate specificity of the applied enzymes [25]. Here, we focus on the identification of a number of acidic oligomeric structures derived from sorghum GAX by digestion with endo-(1 → 4)-β-D-xylanase (Xyl I) [26] in combination with (1 → 4)-β-D-arabinoxylan arabinofuranohydrolase (AXH) [27]. Both enzymes were isolated from *Aspergillus awamori* CMI 142717. It should be noted that the degradation of sorghum GAX by xylanases alone is limited, due to the high degree of Ara-substitution. However, to create more sites of attack for these xylanases, α-L-arabinofuranosidases can be used to selectively release O-3 linked arabinofuranosyl groups.

2. Experimental

Materials.—Glucuronoarabinoxylan (GAX) has been extracted by using saturated aqueous barium hydroxide [8] from the water-unextractable cell wall material from sorghum (*Sorghum vulgare* cv. Fara Fara) [28]. Endo-(1 → 4)-β-D-xylanase I (Xyl I, EC 3.2.1.8) [26] and (1 → 4)-β-D-arabinoxylan arabinofuranohydrolase (AXH, EC 3.2.1.55) [27] were purified from *A. awamori* CMI 142717.

Preparation of GAX oligosaccharides.—A solution of sorghum GAX (250 mg) in 50 mM NaOAc (100 mL, pH 5.0) was digested with a combination of Xyl I (0.65 μg protein/mL) and AXH (0.30 μg protein/mL) for 24 h at 50 °C, continuously mixed head-over-tail. The incubation was stopped by heating (15 min, 100 °C).

The molecular mass distribution of the generated mixture was determined by high-performance size-exclusion chromatography (HPSEC) as described elsewhere [8]. Analytical high-performance anion-exchange chromatography (HPAEC) was performed similarly as described elsewhere [8]. Elutions (1 mL/min, 20 °C) involved linear gradients of 0–0.2 M NaOAc in 0.1 M NaOH in 30 min, then increasing the NaOAc concentration to 0.6 M in 0.1 M NaOH in 15 min, followed by an increase to 1 M NaOAc in 0.1 M NaOH in 5 min. This step was followed by elution with 0.1 M NaOH for 15 min.

For preparative work-ups, digests were concentrated and fractionated by size exclusion chromatography on two columns (600 × 26 mm) in series, packed with Fractogel TSK HW-40(S) (25–40 μm, Merck, Darmstadt, Germany) and thermostated at 60 °C, using a Spectra Physics SP8800-ternary HPLC pump. Samples (1 mL) were injected (several repetitions) using a Spectra Physics SP8880 autosampler and eluted with 0.1 M NaOAc (pH 5, 2.5 mL/min). The effluent was recorded using a Shodex RI-SE-61 detector. Fractions (1.67 mL) were collected starting from 65 min, and assayed for total neutral sugar [29] and uronic acid [30] content using an autoanalyser (Skalar Analytical, Breda, The Netherlands), pooled accordingly and concentrated by reduced pressure. NaN_3 (0.01%) was added as a preservative.

Preparative HPAEC was performed using a Spectra Physics P4000 pump equipped with a CarboPac PA-100 column (250 × 22 mm). A Spectra Physics AS3000 autosampler was used to inject 900 μL samples (several repetitions). The flow rate was 25 mL/min, and the gradients were optimised for each sample using 0.2 M NaOH, 2 M NaOAc in 0.2 M NaOH, and water (Millipore) as eluents. A Dionex PED detector was used for detection. The effluent actually passing the detector was reduced to 1 mL by splitting the effluent post-column. Fractions of 10 mL were collected and immediately neutralised by on-line addition of 1 M HOAc, then pooled according to the recorded chromatogram. The pooled fractions were concentrated and desalted on a Sephadex G-10/column (600 × 50 mm). Samples up to 50 mL were applied and eluted with distilled water (5 mL/min) using a Pharmacia Hiload system equipped with a Pharmacia P50 pump. A Shodex RI-72 detector was used to monitor the refractive index. Due to the low amounts of oligomers present leading to low RI-responses, fractions of 7.5 mL were also checked for

their conductivity, pooled according free of, poor or rich in salt, and concentrated. The oligomers were present in the pools free of or poor in salt, as checked by analytical HPAEC. In the latter case, the desalting procedure had to be repeated. Although the desalting method, using Sephadex G-10, is quite impractical, it should be noted that the application of Dowex 50W X8 in conjunction with Dowex AG3 X4A or with HOAc evaporation [17,20,23] resulted in severe hydrolysis of the oligosaccharides. Finally, the purity of the oligosaccharides was always checked by analytical HPAEC, and the samples were stored frozen.

NMR spectroscopy.—Prior to ^{1}H NMR analysis, GAX oligomers were exchanged twice in deuterium oxide (99.9 at.% deuterium oxide, MSD Isotopes) with intermediate lyophilisation. Finally, samples were dissolved in 99.96 at.% deuterium oxide (MSD Isotopes). ^{1}H NMR spectra were recorded at 500 MHz on a Bruker AMX-500, or at 600 MHz on an AMX/2-600 spectrometer, at a probe temperature of 27 °C. Chemical shifts (δ) are expressed in ppm relative to the signal of internal acetone (δ 2.225). Typically, 1D spectra were recorded with a spectral width of 5000 Hz at 500 MHz, collecting 80–1000 free induction decays of 8 K or 16 K complex data points. Suppression of HOD was achieved by applying the WEFT pulse sequence as described [31]. The resolution of the 1D spectra was enhanced by Lorentzian-to-Gaussian transformation and the final spectra were baseline corrected with a polynomial function when necessary.

2D Homonuclear Hartmann–Hahn (HOHAHA) measurements were performed using a MLEV-17 mixing sequence of 100–120 ms, and 512 measurements of 2 K data points were recorded. The spectral width was 4032 Hz or 4500 Hz in each dimension. 2D Rotating frame nuclear Overhauser enhancement spectroscopy (ROESY) was carried out using a spin-lock mixing pulse of 250 ms at a field strength corresponding to a 90° pulse-width between 100 and 110 ms. The carrier frequency was placed at the left side of the spectrum at δ 5.9 in order to minimise HOHAHA-type magnetisation transfer. The spectral width was 5500 Hz in each dimension, and 512 experiments of 4 K data points were recorded. The HOHAHA and ROESY experiments were performed using the time-proportional phase-increment method to create t_1 amplitude modulation. The HOD signal was suppressed by presaturation for 1.0 s. 2D NMR data were processed on Silicon Graphics Iris Indigo or 4D/35 stations, using Triton software.

Methylation analysis.—Oligosaccharides were reduced with $NaBD_4$, permethylated [32,33], and then converted into mixtures of partially methylated alditol acetates by hydrolysis with 2 M CF_3CO_2H (1 h, 120 °C), reduction with $NaBD_4$, and acetylation with Ac_2O (3 h, 120 °C) [34]. The samples were analysed by GLC–MS [35] using a Fisons GC8060 gas chromatograph, equipped with a DB-1 capillary column (30 m × 0.32 mm; J&W Scientific), coupled to a Fisons MD800 mass spectrometer.

Monosaccharide analysis.—Oligosaccharides were converted into a mixture of (methyl ester) methyl glycosides by treatment with 1 M methanolic HCl (18 h, 85 °C). Methyl glycosides were trimethylsilylated in 10:2:1 pyridine–hexamethyldisilazane–trimethylchlorosilane [36], and analysed directly by GLC–MS as described for the methylation analysis.

Fast atom bombardment mass spectrometry.—The molecular masses of the underivatised oligosaccharides were determined using FABMS. Positive-ion mode FAB mass spectra were obtained using MS1 of a JEOL JMS-SX/SX102A tandem mass spectrometer operated at accelerating voltages of 10 kV (sample **F3.2**) or 9 kV (sample **F3.1**). The FAB gun was operated at 6 kV accelerating voltage with an emission current of 10 mA and using xenon as the bombarding gas. Spectra were scanned at a speed of 30 s for the full mass range specified by the accelerating voltage used, and were recorded and averaged on a Hewlett Packard HP9000 data system running Jeol Complement software.

Notation.—A coding system for the monosaccharide residues has been introduced earlier [17,18,20,21,23], but is now extended for the new oligomers. The Xyl residues are numbered (**1** to **4**), starting from the reducing side; **X**, **A**, and **GA** represent Xyl, Ara, and GlcA, respectively. Their type of linkage and position at the backbone are indicated in the superscripts. As an example, $\mathbf{A^{2A(3\times2)}}$ means that the Ara unit is linked to O-2 of another Ara residue, which in turn is linked to O-3 of the second Xyl residue in the xylan backbone counting from the reducing end.

3. Results and discussion

Degradation of a GAX population, obtained by barium hydroxide extraction of water-unextractable cell wall material from sorghum, by Xyl I and AXH resulted in a significant shift in molecular mass distribution on HPSEC (Fig. 1A). Analytical HPAEC of the later digest gave rise to a complex chromatogram

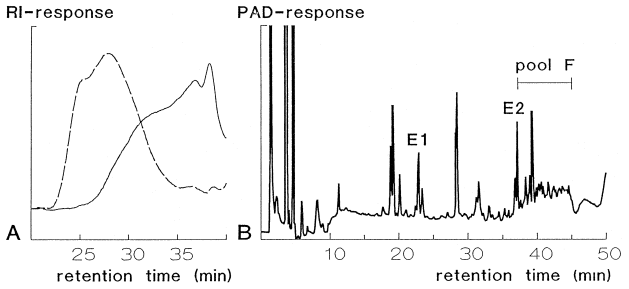


Fig. 1. Elution patterns of GAX digested with a combination of AXH and Xyl I on (A) HPSEC (--- blank; ———— digest) and (B) HPAEC.

(Fig. 1B). The digest turned out to be composed of both neutral and acidic oligosaccharides. The structural analysis of the acidic oligosaccharides is the subject of this study.

The first step in isolating the acidic oligosaccharides was a fractionation of the digest on Fractogel TSK (Fig. 2), yielding nine fractions denoted **A**–**I** (**A**, monomers; **I**, void volume). The polymeric degradation products in fraction **I** (dp > 10) were not seen by HPAEC. The compounds, which are observed by HPAEC, were present in the fractions **A**–**F**. Fraction **A** was mainly composed of Ara and Xyl. Oligomers in the fractions **B**–**F** were present in relatively low amounts when compared to the polymeric fraction **I** or the monomeric fraction **A**. Fractions **F**, **G** and **H** were not well separated, and contained oligomers with an estimated dp > 6. Fraction **E** contained acidic oligosaccharides, since the uronic acid assay nicely followed the pattern of the

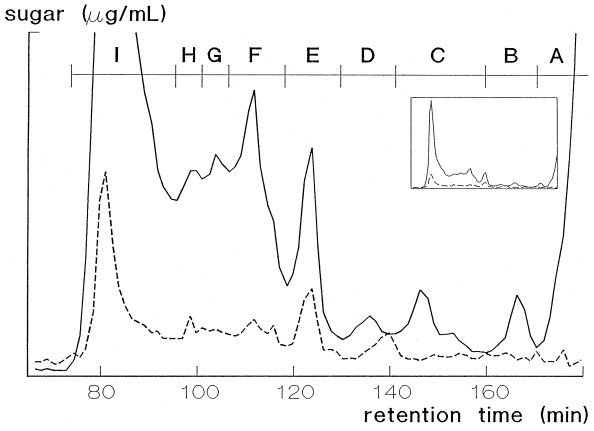


Fig. 2. Fractionation of a GAX digest on Fractogel TSK (———— total neutral sugar; --- uronic acid). The pools of interest are indicated by **E** and **F**. A complete picture of the SEC elution profile is shown in the upper right corner.

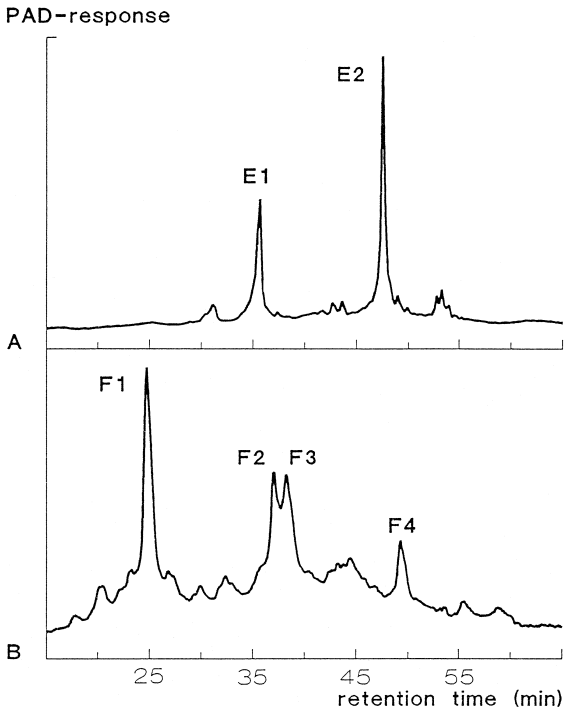


Fig. 3. HPAEC elution profile of (A) fraction **E** and (B) fraction **F** obtained after fractionation of a GAX digest on Fractogel TSK (see Fig. 2).

total neutral sugar assay in this fraction. Re-analysing the desalted fraction **E** on HPAEC showed that this fraction contained two compounds, **E1** and **E2** (Fig. 3A; adjusted gradient; see also Fig. 1B). Fraction **F** (see also Fig. 1B) contained a mixture of oligomers of an approximate dp 8, which could be subfractionated by HPAEC, yielding four major compounds, indicated by **F1** to **F4** (Fig. 3B). However, when desalted fraction **F3** was rechromatographed by HPAEC with a flatter NaOAc gradient, two peaks appeared (data not shown), and therefore **F3** was refractionated by preparative HPAEC, yielding compounds **F3.1** and **F3.2**. Fractions eluting earlier than fraction **F** contained materials that were too large to be fractionated on HPAEC with the gradients used. In Fig. 1B, a relatively large peak can be seen at ~39 min, but the component responsible for this peak was not one of the components isolated from fraction **F** in Fig. 2.

Fractions **E1**, **E2**, **F1**, and **F3.2** were subjected to ^{1}H NMR spectroscopy. In most cases also monosaccharide, methylation, and FABMS analyses were performed. The monosaccharide compositions and linkage patterns of **E1**, **F1**, and **F3.2** are summarised in Table 1. All compounds were composed of Xyl, Ara

Table 1
Molar ratios of constituent monosaccharides of isolated oligosaccharides (monosaccharide analysis), and relative amounts of substitution patterns of Ara*f* and Xyl *p* residues (methylation analysis, → 2)-Xyl *p*-(1 → = 1)

Residues	**E1**	**F1**	**F3.2**
Ara:Xyl:GlcA	0.6:3.0:1.2	2.4:4.0:0.8	3.6:4.0:0.8
Ara*f*-(1 →	−[a]	0.9	1.8
→ 2)-Ara*f*-(1 →	−	0.9	0.9
→ 5)-Ara*f*-(1 →	−	−	+[b]
→ 4)-Xyl *p*	1.0	1.1	1.0
→ 4)-Xyl *p*-(1 →	0.8	0.9	−
→ 2)-Xyl *p*-(1 →	1	1	1
→ 3,4)-Xyl *p*-(1 →	−	0.9	1.8

[a] −, Absent.
[b] +, Trace.

and GlcA, but in different molar ratios. The non-reducing terminal Xyl residue of the backbone was invariably substituted at O-2, whereas the reducing Xyl unit was unsubstituted. The Ara residues, if present, occur as terminal and internal units.

Compound **E1**.—The monosaccharide composition of **E1** (Table 1) indicated the presence of Ara, Xyl, and GlcA in the molar ratio 0.6:3.0:1.2. Methylation analysis showed the presence of 3 Xyl residues, (1 → 4)-linked forming a xylotriose, with a substituent at O-2 of the non-reducing terminal residue. Neither methylation analysis nor ^{1}H NMR spectroscopy (Fig. 4) of **E1** revealed the presence of Ara. Probably, the amount of Ara was overestimated in the monosaccharide analysis (vide supra).

On the H-1 tracks of the constituent monosaccharides in the 2D HOHAHA spectrum (not shown) the scalar coupled networks are observed, and the deduced chemical shift values are summarised in Table 2. The track at 5.323 ppm is typical for a Glc*p*A unit of which the H-5 doublet (δ 4.332) is shifted outside the bulk signal. The $J_{1,2}$ value of 3.9 Hz for the GlcA H-1 signal indicates α-configuration. The $J_{1,2}$ value of 7.8 Hz for the Xyl *p*-**2** and -**3** H-1 signals indicates β-configurations. The relative positions of

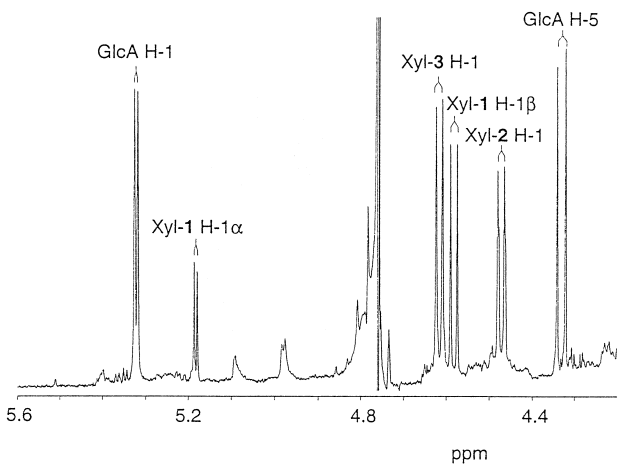


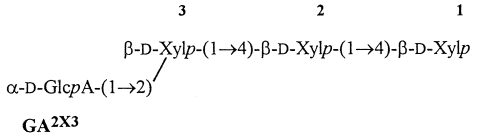
Fig. 4. Partial 500-MHz ^{1}H NMR spectrum (δ 4.2–5.6) of compound **E1**. The numbers in the spectrum refer to the corresponding residues in the structure. For notations, see Section 2.

Table 2
^{1}H NMR chemical shifts of GAX oligosaccharides, enzymically generated from water-unextractable sorghum cell wall material, recorded at 27 °C

Residue	Proton	Chemical shifts[a]			
		E1	**E2**	**F1**	**F3.2**
Xyl-**1**	H-1α	5.183	5.185	5.183	5.185
	H-1β	4.583	4.582	4.583	4.584
	H-2	3.24		3.24	
	H-3	3.54		3.53	
	H-4	3.78		3.76	
	H-5$_{eq}$	4.04		4.04	
	H-5$_{ax}$	3.37		3.36	
Xyl-**2**	H-1	4.473	4.504	4.494	4.484
	H-2	3.27		3.28	
	H-3	3.56		3.72	
	H-4	3.76		3.82	
	H-5$_{eq}$	4.14		4.11	
	H-5$_{ax}$	3.44		3.44	
Xyl-**3**	H-1	4.617	4.597	4.455	4.578
	H-2	3.46		3.24	
	H-3	3.46		3.55	
	H-4	3.65		3.75	
	H-5$_{eq}$	3.97		4.10	
	H-5$_{ax}$	3.30		3.40	
Xyl-**4**	H-1			4.603	4.609
	H-2			3.43	
	H-3			3.47	
	H-4			3.63	
	H-5$_{eq}$			3.97	
	H-5$_{ax}$			3.30	
GlcA-**GA**$^{2\times3(\times4)}$	H-1	5.323	5.366	5.314	5.356
	H-2	3.54		3.54	
	H-3	3.73		3.72	
	H-4	3.46		3.46	
	H-5	4.332	4.323	4.330	4.320
Ara-**A**$^{3\times2}$	H-1		5.401	5.509	5.509
	H-2		4.153	4.172	
	H-3			4.06	
	H-4		4.274	4.272	
Ara-**A**$^{2A(3\times2)}$	H-1			5.192	5.193
	H-2			4.10	
Ara-**A**$^{3\times3}$	H-1				5.401

[a] In ppm relative to the signal of internal acetone at δ 2.225. The chemical shift values determined from 2D spectra (**E1** and **F1**) are given in two digits.

the different Xyl residues in the xylotriose backbone are reflected by the interresidual ROESY cross-peaks between Xyl$_x$ H-1 and Xyl$_{x-1}$ H-4,5$_{eq}$ [17] (see Table 3). The ROESY cross-peak from GlcA H-1 to the overlapping terminal Xyl-**3** H-2 and H-3 resonances did not allow a definitive choice for the type of glycosidic linkage between GlcA and Xyl-**3**, but methylation analysis excluded a (1 → 3)-linkage (Table 1). Comparison of the ^{1}H NMR data of **E1** with those of β-D-Xyl*p*-(1 → 4)-β-D-Xyl*p*-(1 → 4)-β-D-Xyl*p* shows that in **E1** Xyl-**2** H-1 and Xyl-**1** H-1 α,β exhibit nearly identical chemical shift values, whereas Xyl-**3** H-1 was shifted downfield (Δδ +0.157) [17], thereby confirming the substitution on the terminal non-reducing Xyl residue. In conclusion, compound **E1** can be formulated as:

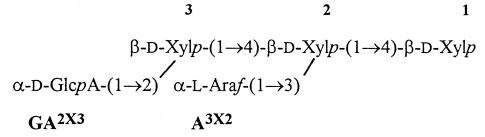


The 4-*O*-methylated GlcA analogue of **E1** has been described previously [37].

Compound **E2**.—The ^{1}H NMR spectrum of **E2** showed, in addition to the constituent monosaccharides of **E1**, the presence of an extra α-Ara residue (δ 5.401, $J_{1,2} \sim 1$ Hz), and the structural-reporter-group data are summarised in Table 2. When going from **E1** to **E2** a downfield shift was observed for Xyl-**2** H-1 (**E1**, δ 4.473; **E2**, δ 4.504; Δδ + 0.031) and an upfield shift for Xyl-**3** H-1 (**E1**, δ 4.617; **E2**, 4.597; Δδ −0.020). Similar Δδ values are calculated for the transition of β-D-Xyl*p*-(1 → 4)-β-D-Xyl*p*-(1 → 4)-β-D-Xyl*p* into β-D-Xyl*p*-(1 → 4)[α-L-Ara*f*-(1 → 3)]-β-D-Xyl*p*-(1 → 4)-β-D-Xyl*p*. The substitution of Xyl-**2** O-3 with Ara caused a downfield shift for Xyl-**2** H-1 from 4.479 to 4.509 ppm (Δδ +0.030) and an upfield shift for Xyl-**3** H-1 from 4.460 to 4.442 ppm (Δδ −0.018) [17,20]. In α-L-Ara*f*-(1 → 3)-β-D-Xyl*p*-(1 → 4)-β-D-Xyl*p* [20], the Ara H-1 signal is found at δ 5.395, and in **E2** at δ 5.401, supporting strongly the presence of an Ara residue at O-3 of Xyl-**2**. Going from **E1** to **E2** the GlcA H-1 signal shifts from δ 5.323 to 5.366, reflecting the close proximity of the neighbouring Ara residue.

Compound **E2** was shown [38] to be degradable by an α-glucuronidase, that specifically releases 2-linked GlcA from a non-reducing terminal Xyl residue [39]. The resulting substance co-eluted with β-D-Xyl*p*-(1 → 4)[α-L-Ara*f*-(1 → 3)]-β-D-Xyl*p*-(1 → 4)-β-D-Xyl*p* on HPAEC. Furthermore, the latter compound could be degraded by AXH, which typically removes Ara, (1 → 3)-linked to the xylan backbone, thereby forming xylotriose (data not shown). Apparently, the AXH action is restricted when Ara is linked at Xyl adjacent to a GlcA-substituted non-reducing terminal Xyl residue.

Summarising the various data, the structure of **E2** can be formulated as:



Compound **F1**.—As is evident from Table 1, monosaccharide and methylation analyses suggest that **F1** is built up from four Xyl, one GlcA, and two Ara

Table 3
Cross-peaks observed at the H-1 tracks in the 2D ROESY spectra, measured with a mixing time of 250 ms

Compound	Residue H-1	ROE connectivity
E1	Xyl-**2** H-1	Xyl-**1** H-5$_{eq}$, Xyl-**1** H-4, Xyl-**2** H-5$_{ax}$, Xyl-**2** H-3
	Xyl-**3** H-1	Xyl-**2** H-5eq, Xyl-**2** H-4, Xyl-**3** H-5$_{ax}$, Xyl-**3** H-3
	GlcA-**GA**$^{2\times3}$ H-1	Xyl-**3** H-2[a], Xyl-**3** H-3[a]
F1	Xyl-**2** H-1	Xyl-**1** H-4, Xyl-**2** H-5$_{ax}$, Xyl-**2** H-3
	Xyl-**3** H-1	Xyl-**2** H-5$_{eq}$, Xyl-**2** H-4, Xyl-**3** H-5$_{ax}$, Xyl-**3** H-3
	Xyl-**4** H-1	Xyl-**3** H-5$_{eq}$, Xyl-**3** H-4, Xyl-**4** H-5$_{ax}$, Xyl-**4** H-3
	GlcA-**GA**$^{2\times4}$ H-1	Xyl-**4** H-2, Xyl-**4** H-3 (weak), GlcA-**GA**$^{2\times4}$ H-2
	Ara-**A**$^{3\times2}$ H-1	Xyl-**2** H-3, Ara-**A**$^{3\times2}$ H-2, Ara-**A**$^{3\times2}$ H-3
	Ara-**A**$^{2A(3\times2)}$ H-1	Ara-**A**$^{3\times2}$ H-2, Ara-**A**$^{2A(3\times2)}$ H-2, Ara-**A**$^{2A(3\times2)}$ H-3

[a] Overlapping resonances.

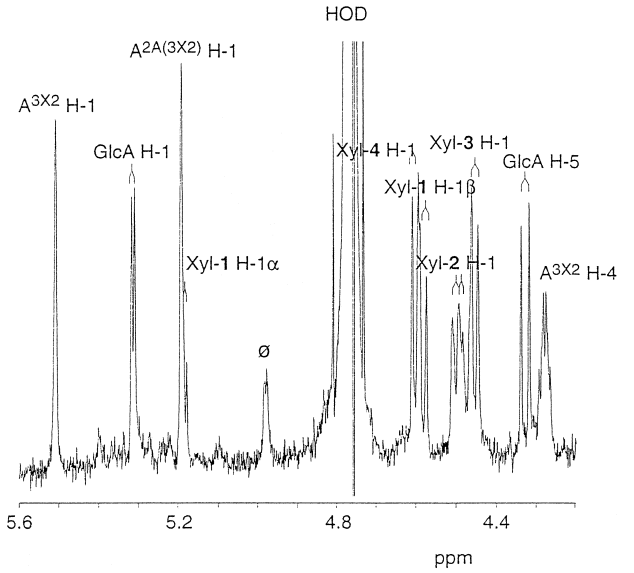
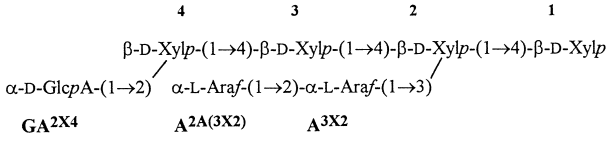


Fig. 5. Partial 500-MHz ^{1}H NMR spectrum (δ 4.2–5.6) of compound **F1**. The numbers in the spectrum refer to the corresponding residues in the structure. For notations, see Section 2.

residues, whereby the Ara content seems to be overestimated in the monosaccharide analysis. The Xyl residues form a (1 → 4)-linked xylotetraose backbone, of which the non-reducing terminal Xyl residue is substituted at O-2, and one of the two internal Xyl residues at O-3. Furthermore, a non-reducing terminal and a 2-linked Ara residue could be distinguished, suggesting that an arabinobiose side chain is present.

In the anomeric region of the ^{1}H NMR spectrum of **F1** (Fig. 5) four β-Xyl*p*, one α-Glc*p*A, and two α-Ara signals are detected. On the various H-1 tracks of the constituent monosaccharides in the 2D HOHAHA spectrum (Fig. 6) the scalar coupled networks could be characterised, and the chemical shift values are included in Table 2. Interresidual ROE connectivities are evident from Fig. 7 and are tabulated in Table 3. The Ara residues showed interresidual ROE connectivities between Ara-$\mathbf{A^{2A(3\times 2)}}$ H-1 and Ara-$\mathbf{A^{3\times 2}}$ H-2, and between Ara-$\mathbf{A^{3\times 2}}$ H-1 and Xyl-**2** H-3. These ROE connectivities together with the methylation analysis data established an Ara-(1 → 2)-Ara-(1 → 3)-Xyl element. The involved Xyl-**2** residue and the linkage between GlcA and Xyl-**4** are evident from additional interresidual ROE connectivities (Table 3). Taken together all results, **F1** is concluded to have the following structure:



Compound **F3.2**.—The positive-ion mode FAB mass spectrum of **F3.2** contained a $[M + H]^+$ ion at

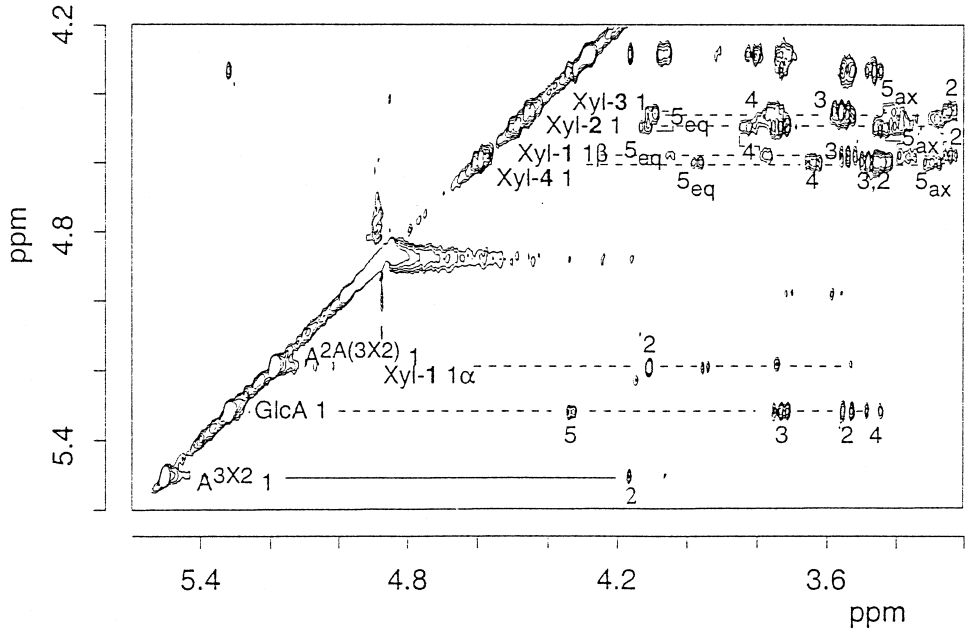


Fig. 6. Partial 500-MHz 2D HOHAHA spectrum (δ 3.2–5.6) of compound **F1**. In the spectrum, the diagonal peaks of the protons in the anomeric region are indicated. The numbers near cross-peaks in the spectrum refer to the protons of the scalar-coupling network to a diagonal peak. For notations, see Section 2.

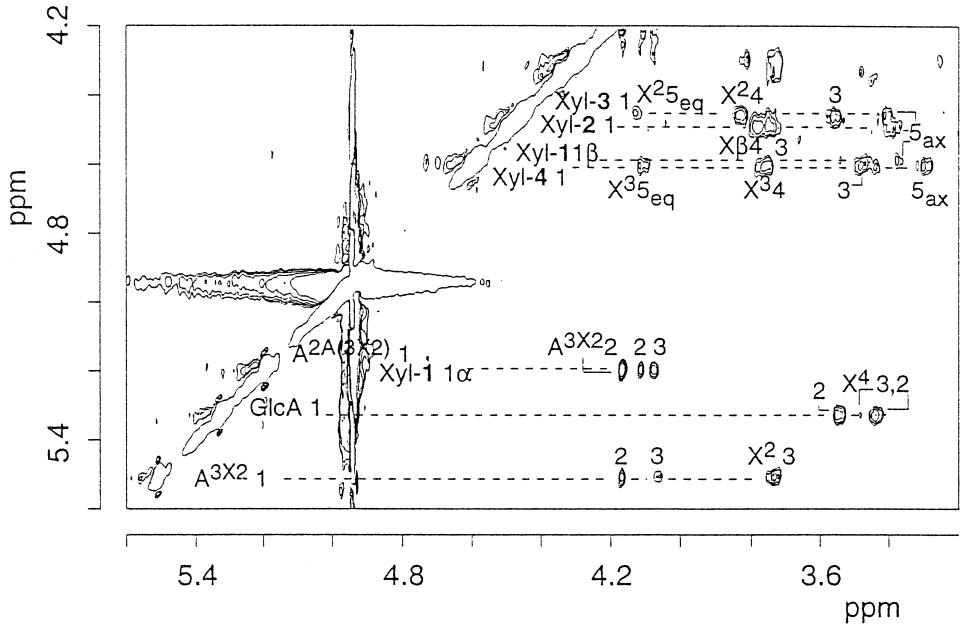
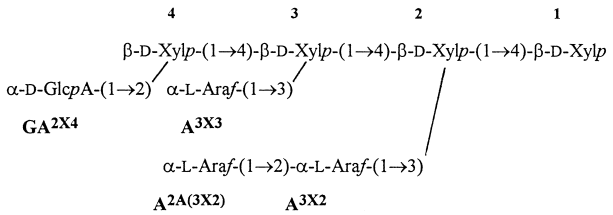


Fig. 7. Partial 500-MHz 2D ROESY spectrum (δ 3.2–5.6) of compound **F1**. The ROE connectivities along the H-1 tracks are denoted; $X^2$3 means Xyl-**2** H-3, etc. For further notations, see Section 2.

m/z 1163, corresponding to a composition of $Pent_7HexA$. This finding fits the methylation and monosaccharide analyses data (Table 1), except for the overestimated Ara content. The seven pentose residues are identified as four Xyl and three Ara. Furthermore, the methylation analysis showed the presence of two terminal and one 2-substituted Ara residues, and only O-3 substitution at the two internal Xyl residues of the xylotetraose backbone. The non-reducing terminal Xyl residue of this backbone is substituted at O-2.

The 1D ^{1}H NMR spectrum of this compound showed four β-Xyl*p*, one α-Glc*p*A and three α-Ara*f* H-1 signals. When comparing this spectrum to that of compound **F1** three major differences are observed (Table 2). First, besides the two Ara H-1 signals resonating at positions identical to those found for the α-L-Ara*f*-(1 → 2)-α-L-Ara*f*-(1 → 3) branch in **F1** (Ara-$\mathbf{A^{3\times2}}$, δ 5.509; Ara-$\mathbf{A^{2A(3\times2)}}$, δ 5.193), a third Ara H-1 signal is detected at δ 5.401, a value that correlates with that of the Ara-$\mathbf{A^{3\times2}}$ H-1 signal in **E2** (δ 5.401). Secondly, a downfield shift is observed for the GlcA H-1 signal (**F1**, δ 5.314; **F3.2**, δ 5.356; $\Delta\delta$ +0.042). A similar $\Delta\delta$ value (+0.043 ppm) was found for GlcA H-1 when going from **E1** (δ 5.323) to **E2** (δ 5.366), i.e., when going from an α-D-Glc*p*A-(1 → 2)-β-D-Xyl*p*-(1 → 4)-β-D-Xyl*p*-(1 → to an α-D-Glc*p*A-(1 → 2)-β-D-Xyl*p*-(1 → 4)[α-L-Ara*f*-(1 → 3)]-β-D-Xyl*p*-(1 → element. Thirdly, the chemical shift values of the Xyl-**2**, -**3**, and -**4** H-1 signals differ markedly from those in **F1**. Considering the ^{1}H NMR results and the methylation analysis data, the following structure can be proposed:



Concluding remarks.—In sorghum GAX single GlcA residues are attached at O-2 of Xyl residues in the xylan backbone and Ara residues, single or as chains, at O-3. However, methylation analysis of other compounds in pool **F** (Fig. 2) showed the presence of internal Xyl units substituted at O-2 (results not shown). The presence of Ara chains shows that the Ara to Xyl ratio, which is generally used to indicate the degree of substitution, is not suitable for this type of GAX. O-2,3-disubstituted Xyl residues in the xylan backbone were not found in the present study. These kind of structures, however, are present indeed in sorghum GAX, as could be previously demonstrated by methylation analysis [8].

Knowledge about the mode of action of Xyl I and

AXH has proven to be valuable for these kind of studies. In this study, the majority of the O-3 linked single Ara substituents has been removed from the polymeric GAX by AXH, prior to the generation of the oligosaccharides by Xyl I. Without this combination of enzymes, it would not have been possible to generate sufficient amounts of low-molecular-mass GAX oligomers. For this reason, the loss of some structural information with respect to the Ara substituents, due to the use of AXH, was accepted. However, apparently, AXH was not able to remove Ara residues linked to Xyl residues adjacent to GlcA-substituted Xyl units towards the reducing site. Also, the arabinobiose side chains, and the probably present O-2 linked single Ara residues, were not removed. The mode of action of Xyl I towards GAX was shown to be similar as towards neutral arabinoxylans. The enzyme 'treats' the GlcA substitution in the same way as a O-2,3-disubstituted Xyl residue [25]. It is clear that the AXH action is limited by the GlcA substitution.

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