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Structures of small oligomers liberated from barley arabinoxylans by endoxylanase from *Aspergillus awamori*

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ABSTRACT

Arabinoxylans consisting of more than 90% of arabinose and xylose were extracted from water-insoluble cell wall material of dehusked barley grain, and degraded with a purified endo-(1 → 4)-β-D-xylanase from *Aspergillus awamori*. Twelve of the oligosaccharide fragments released were isolated by a combination of size-exclusion and anion-exchange chromatography, and their structures were determined by ^{1}H NMR spectroscopy. The oligosaccharides identified consisted of (1 → 4)-linked β-D-xylopyranosyl residues, some of which were substituted at O-2, at O-3, or at both O-2 and O-3, with α-L-arabinofuranosyl groups. Two new structures, a tetrasaccharide and a pentasaccharide, both containing terminal 2-*O*-arabinofuranosylxylopyranose as a structural element are described.

INTRODUCTION

Barley arabinoxylans consist of a backbone of (1 → 4)-linked β-D-xylopyranosyl residues. Some of these residues are substituted at O-2, at O-3, or at both O-2 and O-3 with mainly single α-L-arabinofuranosyl groups[1,2]. 4-*O*-Methylglucuronic acid[3], 3-*O*-xylopyranosylarabinofuranose[4], and 5-*O*-feruloylarabinofuranose[5] have also been found in small amounts in barley arabinoxylans, depending on the tissues from which the arabinoxylans were extracted.

Enzymes with different specificities are required for complete degradation of these arabinoxylans. Major enzymes in this respect are endo- and exo-(1 → 4)-β-D-xylanase, xylosidase, and arabinofuranosidase. The nature and distribution of the

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substituents along the xylan backbone determine the extent to which these enzymes can degrade the arabinoxylan.

In a previous report, we described the isolation and linkage composition of arabinoxylan fractions extraction with a $Ba(OH)_2$–$NaBH_4$ solution from water-insoluble cell wall material from barley[2]. These arabinoxylan fractions consisted of more than 90% of arabinose and xylose, even without any purification after extraction. A feature unique to arabinoxylans from barley and malt was the presence of large amounts of (1 → 4)-linked xylopyranosyl residues bearing a single arabinofuranosyl substituent at O-2 (ref 2). Those results, however, gave no information about the distribution of the arabinose residues over the xylan backbone. As a first step in resolving the distribution of arabinosyl substituents over the xylan backbone, we studied the structures of small oligomeric fragments liberated by enzymic degradation of arabinoxylans isolated from water-insoluble cell wall material from barley[2]. The structures of such oligosaccharides can give information on the structure of the polysaccharide as well as about the xylan structures recognised by the endoxylanase used.

EXPERIMENTAL

General methods.—An arabinoxylan fraction was extracted from barley cell wall material with satd aq $Ba(OH)_2$ containing $NaBH_4$ (0.13 M). Isolation and chemical composition of this extract (BE fraction) have been described elsewhere[2]. The material consisted of 38 mol% of arabinose, 53 mol% of xylose, and 8 mol% of glucose, with trace amounts of mannose and galactose. Methylation analysis showed arabinose to be present mainly as terminal arabinofuranose; xylose was present as xylopyranose, 56% of which was 1,4-linked, 10% 1,2,4-linked, 14% 1,3,4-linked, and 19% 1,2,3,4-linked, with a trace of terminal xylopyranose[2].

Purified endoxylanase I from *Aspergillus awamori* CMI 142717 (ref 6) was a gift from F. Kormelink (Agricultural University, Department of Food Science). Preliminary tests had shown that this enzyme was the most active of the endoxylanase preparations available that did not release arabinose from the substrate (data not shown).

Total sugar content in column eluent fractions was determined with an orcinol–H_2SO_4 reagent, using a Skalar autoanalyzer. Methylation analysis[2] of oligosaccharides, using partially methylated alditol acetates, was carried out after reduction of the oligosaccharide with $NaBD_4$ (ref 7).

Enzyme incubations.—For enzymic degradation of arabinoxylan fractions, 100 mg of substrate and 20 μg of endoxylanase I in 100 mL of buffer (50 mM NaOAc, pH 5.0, containing 1.5 mM NaN_3) were incubated for 16 h at 40°C. Enzyme activity was destroyed by heating the mixture for 10 min in a boiling water bath.

HPAEC analysis.—Analytical and preparative high performance anion-exchange chromatography (HPAEC) were performed on CarboPac PA-1 columns (Dionex) (250 × 4.5 mm and 250 × 9 mm, respectively, and a corresponding guard

column was used with the analytical column), using electrochemical detection (PED-detector, Dionex). For analytical runs, a linear gradient of 0–150 mM NaOAc in 100 mM NaOH in 10 min, followed by 150–500 mM NaOAc in 100 mM NaOH in 35 min, was used at a flow rate of 1 mL/min. For preparative work, the flow rate was 5 mL/min and the gradients were optimised for each sample. The eluent was immediately neutralised by on-line addition of 1 M AcOH and fractions of 1.25 mL were collected. Total sugars in the collected fractions were determined and fractions were pooled according to sugar distribution. The pooled fractions were desalted using columns filled with Dowex 50W-X8 (H^+) (100–200 mesh, Bio-Rad) and AG-3 (HO^-) (200–400 mesh, Bio-Rad) resins, consecutively[7].

Monosaccharide composition.—Oligosaccharides were hydrolysed in 2 M CF_3CO_2H for 1 h at 121°C. The hydrolysates were dried in a stream of air and dissolved in water. The monosaccharide solutions obtained were analysed by HPAEC on a CarboPac PA-1 column (see above), using 100 mM NaOH as eluent. Analysis time was 10 min. Calibration curves were used for quantification of arabinose and xylose contents.

Purification of degradation products.—Arabinoxylan oligosaccharides were fractionated according to size on a column (100 × 1.6 cm) of Bio-Gel P-2 (200–400 mesh, Bio-Rad) and eluted at 65°C with water containing 0.15 mM NaN_3. The eluent was collected in fractions of 1.3 mL and analysed for total sugar. Fractions were pooled and, if necessary, further fractionated by HPAEC.

^1H NMR spectroscopy.—Samples were repeatedly treated with D_2O (99.9 atom% D, MSD Isotopes), finally using 99.96 atom% D at pD ≥ 7. Resolution-enhanced 600-MHz ^1H NMR spectra were recorded using a Bruker AM-600 spectrometer (SON-hf-NMR facility, Department of Biophysical Chemistry, Nijmegen University), operating at a probe temperature of 27°C. Chemical shifts (δ) are expressed in ppm and were measured by reference to internal acetone (δ 2.225 in D_2O at 27°C)[8].

Full details of the HOHAHA spin-lock experiments and ROESY spectroscopy have been reported in a previous paper[7].

RESULTS AND DISCUSSION

Isolation of arabinoxylan oligosaccharides.—The mixture of oligosaccharides, obtained by degradation of barley arabinoxylan BE-fraction with endoxylanase I, was fractionated on Bio-Gel P-2 to yield 7 fractions (fractions **1–6** and **void** fraction, see Fig. 1). Incubation of the **void** fraction with fresh endoxylanase I did not result in any further degradation, showing that degradation had proceeded as far as possible with the enzyme used (data not shown). Investigation of **1** and **2** by HPAEC in combination with monosaccharide analysis showed that these fractions consisted of one component each, xylose and xylobiose, respectively. Fractions **3–6** contained 2 or 3 major components, consisting of arabinose and xylose (Fig. 2 and Table I). These components were isolated by preparative HPAEC.

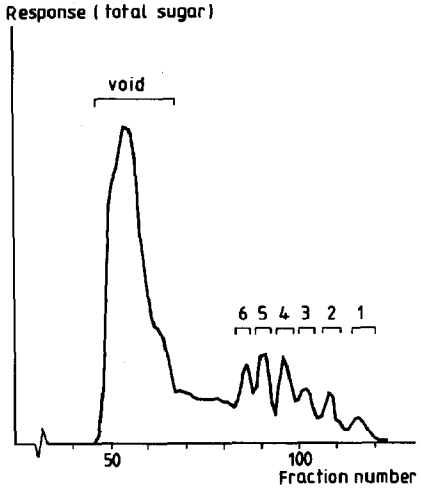


Fig. 1. Separation on Bio-Gel P-2 of fragments in the digest obtained from barley arabinoxylans with endoxylanase I.

^{1}H NMR analysis of oligosaccharides.—Primary structures of the major oligosaccharides present in the isolated HPAEC fractions were further elucidated by ^{1}H NMR spectroscopy. All fractions isolated, except **4.2** and **5.3**, contained compounds having structures identical to recently published compounds[7,9,10], and the H-1 chemical shifts and structures are given in Table II and Fig. 3, respectively.

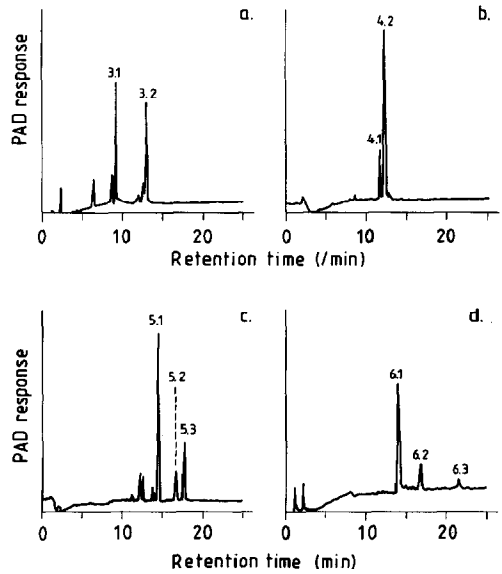


Fig. 2. HPAEC elution patterns of Bio-Gel P-2 fractions **3–6** in Fig. 1 (a–d, respectively).

TABLE I

Distribution and monosaccharide composition of fractions from arabinoxylans digested with endo-xylanase I from *Aspergillus awamori*

Fractions from Bio-Gel P-2										
Fraction	**1**		**2**		**3**		**4**		**5**	**6**
% of total	1.8		4.0		4.5		5.7		4.5	3.9
Ara : Xyl ratio[a]	0.00		0.00		0.17		0.37		0.46	0.52
Fractions from HPAEC										
Fraction	**3.1**	**3.2**	**4.1**	**4.2**	**5.1**	**5.2**	**5.3**	**6.1**	**6.2**	**6.3**
% of pool	59	41	60	40	57	30	12	69	21	10
Ara : Xyl ratio	0.00	0.50	0.36	0.39	0.69	0.66	0.74	0.51	0.49	0.74

[a] Ara : Xyl ratio = ratio of arabinose-to-xylose; other monosaccharides were not found in any of the fractions. Fractions **1** and **2** consisted of xylose only.

The monosaccharide composition of these oligosaccharides was in agreement with the structures assigned (Table I). The ^{1}H NMR spectra of fractions **4.2** and **5.3** did not occur in the reference library, and will be discussed below.

Fraction **4.2**.—The intensities of the signals for anomeric protons in the ^{1}H NMR spectrum of **4.2** (Fig. 4) indicated the presence of an arabinosylxylotriose as the major compound with the xylopyranosyl (Xyl*p*) units in the β configuration ($J_{1,2}$ 7–8 Hz) and the arabinofuranosyl (Ara*f*) unit in the α configuration ($J_{1,2} \approx 1$ Hz)[11]. On the various H-1 tracks of the constituent monosaccharides in the 2D HOHAHA spectrum (Fig. 5), the total scalar-coupled networks of each residue were observed, and the data obtained are summarised in Table III. Specific assignment of the α-Ara*f* H-5*proR*,5*proS* signals is based on their relative

TABLE II

^{1}H NMR chemical shifts of the anomeric protons of the constituent monosaccharides for the oligosaccharides **3.1–6.3**, derived by enzymic degradation of barley endosperm arabinoxylans

Proton[a]	Compound									
	3.1	**3.2**	**4.1**	**4.2**	**5.1**	**5.2**	**5.3**[b]	**6.1**	**6.2**	**6.3**
α-Ara*f*-$\mathbf{A^{3X2\alpha}}$		5.335	5.401			5.396	5.426		5.391	5.427
α-Ara*f*-$\mathbf{A^{3X2\beta}}$		5.331	5.396			5.391	5.421		5.387	5.422
α-Ara*f*-$\mathbf{A^{2X3}}$				5.280	5.237		5.293	5.226		5.244
α-Ara*f*-$\mathbf{A^{3X3}}$					5.246	5.329		5.274	5.398	5.244
α-Xyl*p*-**1**	5.184	5.185	5.185	5.183	5.183	5.185	5.183	5.183	5.186	5.185
β-Xyl*p*-**1**	4.584	4.584	4.584	4.583	4.584	4.584	4.583	4.584	4.584	4.584
β-Xyl*p*-$\mathbf{2_\alpha}$	4.478	4.487	4.508	4.462	4.465	4.510	4.494	4.466	4.510	4.495
β-Xyl*p*-$\mathbf{2_\beta}$	4.475	4.490	4.510	4.466	4.468	4.510	4.494	4.468	4.510	4.495
β-Xyl*p*-**3**	4.461		4.443	4.555	4.597	4.475	4.527	4.640	4.489	4.562
β-Xyl*p*-**4**								4.437	4.432	

[a] The Xyl*p* residue in the reducing position is denoted **1**, etc; $\mathbf{2_{\alpha/\beta}}$ means that the reducing Xyl*p*-**1** residue is α/β (anomerisation effect). Ara*f*-$\mathbf{A^{2X3}}$ means arabinofuranose linked to O-2 of Xyl*p*-**3**, etc.
[b] Assignments for this fragment are tentative (see text).

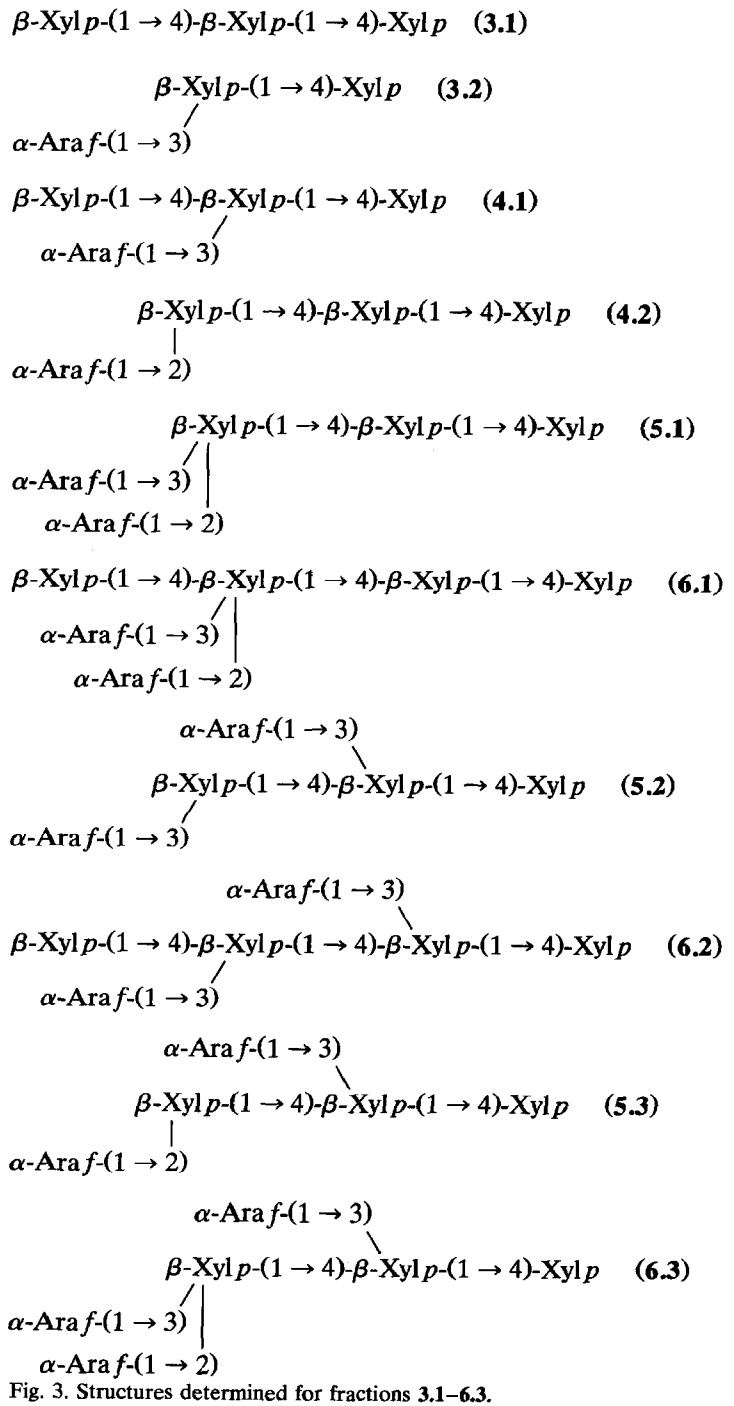


Fig. 3. Structures determined for fractions **3.1–6.3.**

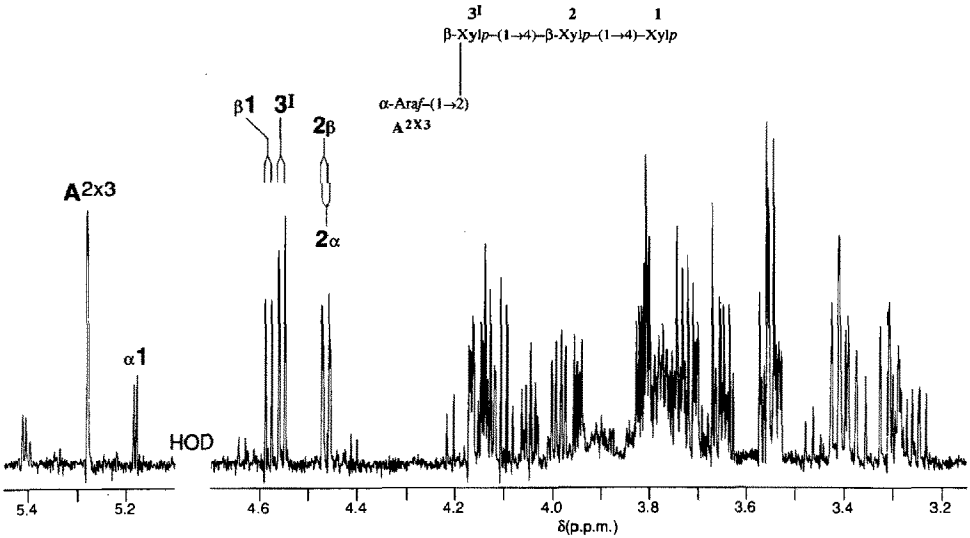


Fig. 4. Resolution-enhanced 600-MHz ^{1}H NMR spectrum of fraction **4.2**. The numbers and letters in the spectrum refer to the corresponding residues in the structure.

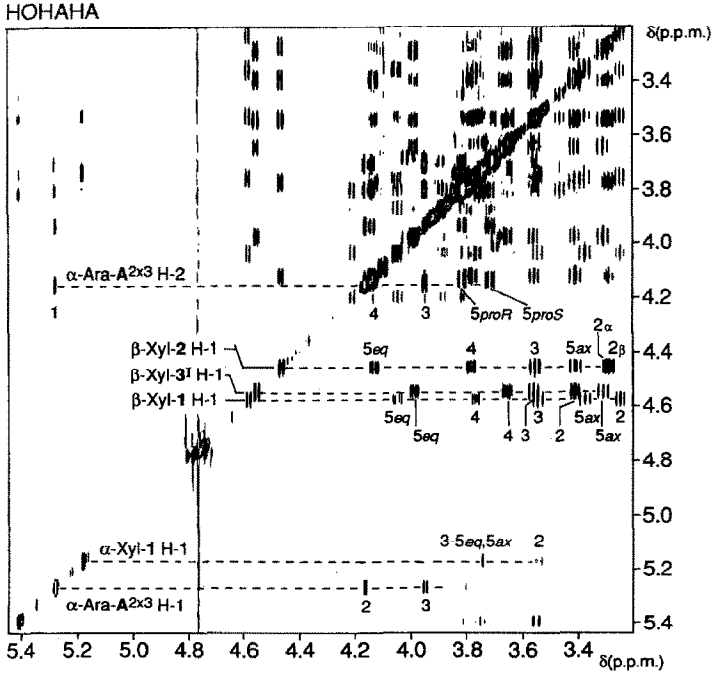


Fig. 5. 600-MHz HOHAHA spectrum of fraction **4.2**. Diagonal peaks of the anomeric protons are indicated. The numbers near cross-peaks refer to the protons of the scalar-coupling network belonging to a diagonal peak.

TABLE III

^{1}H NMR data for the arabinoxylan oligosaccharide **4.2**, together with those of reference compounds **3.2** and **5.1**, derived from barley and wheat[10] arabinoxylans

Compound	Residue [a]	Chemical shift [b]					
		H-1	H-2	H-3	H-4	H-5*eq*(H-5*proR*)	H-5*ax*(H-5*proS*)
	α-Xyl*p*-**1**	5.185	3.545	———	———	3.73–3.82	———
	β-Xyl*p*-**1**	4.584	3.250	3.545	3.781	4.055	3.377
	β-Xyl*p*-2^{II}_{α}	4.487	3.413	3.594	3.692		
						4.004	3.340
3.2	β-Xyl*p*-2^{II}_{β}	4.490	3.403	3.591	3.690		
	α-Ara*f*-$A^{3X2\alpha}$	5.335					
			4.175	3.959	4.185	3.817	3.705
	α-Ara*f*-$A^{3X2\beta}$	5.332					
	α-Xyl*p*-**1**	5.183	3.543	———	———	3.73–3.82	———
	β-Xyl*p*-**1**	4.583	3.247	3.544	3.770	4.050	3.375
	β-Xyl*p*-2_{α}	4.462	3.295				
				3.556	3.785	4.134	3.409
	β-Xyl*p*-2_{β}	4.466	3.287				
4.2	β-Xyl*p*-3^{I}	4.555	3.411	3.560	3.660	3.988	3.308
	α-Ara*f*-A^{2X3}	5.280	4.166	3.948	4.140	3.814	3.716
	α-Xyl*p*-**1**	5.183	3.546	———	———	3.73–3.82	———
	β-Xyl*p*-**1**	4.584	3.249	3.546	3.771	4.050	3.376
	β-Xyl*p*-2_{α}	4.465	3.298				
				3.558	3.792	4.140	3.416
	β-Xyl*p*-2_{β}	4.467	3.290				
5.1	β-Xyl*p*-3^{III}	4.596	3.539	3.689	3.724	4.023	3.344
	α-Ara*f*-A^{2X3}	5.238	4.151	3.956	4.132	3.816	3.720
	α-Ara*f*-A^{3X3}	5.246	4.175	3.973	4.198	3.813	3.706

[a] The Xyl*p* residue in the reducing position is denoted **1**, etc.; $2_{\alpha/\beta}$ means that the reducing Xyl*p*-**1** residue is α/β (anomerisation effect). Ara*f*-A^{2X3} means arabinofuranose linked to O-2 of Xyl*p*-**3**, etc.; Xyl*p*-3^{I} means Xyl*p*-**3** branched at O-2; Xyl*p*-3^{II} means Xyl*p*-**3** branched at O-3; Xyl*p*-3^{III} means Xyl*p*-**3** branched at both O-2 and O-3. [b] In ppm relative to the signal of internal acetone at δ 2.225 in D_2O at 27°C, acquired at 600 MHz.

chemical shifts ($\delta_{5proR} > \delta_{5proS}$), supported by the $J_{4,5}$ values ($J_{4,5proR} < J_{4,5proS}$)[12]. Part of the ROESY spectrum is presented in Fig. 6. The observed ROEs between H-1 of β-Xyl*p*-(**n**) and H-4,5*eq* of β-Xyl*p*-(**n** − **1**), together with the α-Ara*f*-A^{2X3} H-1,β-Xyl*p*-3^{I} H-2 connectivity, established the structure of **4.2**.

$$\begin{array}{l} \qquad\qquad\quad 3^{I} \qquad\quad 2 \qquad\quad 1 \\ \beta\text{-Xyl}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-Xyl}p\text{-}(1\rightarrow 4)\text{-Xyl}p \\ \quad\diagup \\ \mathbf{A^{2X3}}\quad \alpha\text{-Ara}f\text{-}(1\rightarrow 2) \end{array} \qquad \textbf{(4.2)}$$

Comparison of the ^{1}H NMR data of **4.2** with those of reference compounds **3.2** and **5.1** (ref 10) shows an intermediate chemical shift position of the β-Xyl*p*-3^{I} H-1 signal of [α-Ara*f*-(1 → 2)-β-Xyl*p*-(1 →] relative to that of the β-Xyl*p*-2^{II} H-1 signal of [α-Ara*f*-(1 → 3)-β-Xyl*p*-(1 →] in **3.2** and the β-Xyl*p*-3^{III} H-1 signal of {α-Ara*f*-(1 → 2)-[α-Ara*f*-(1 → 3)]-β-Xyl*p*-(1 →} in **5.1**. A similar observation holds

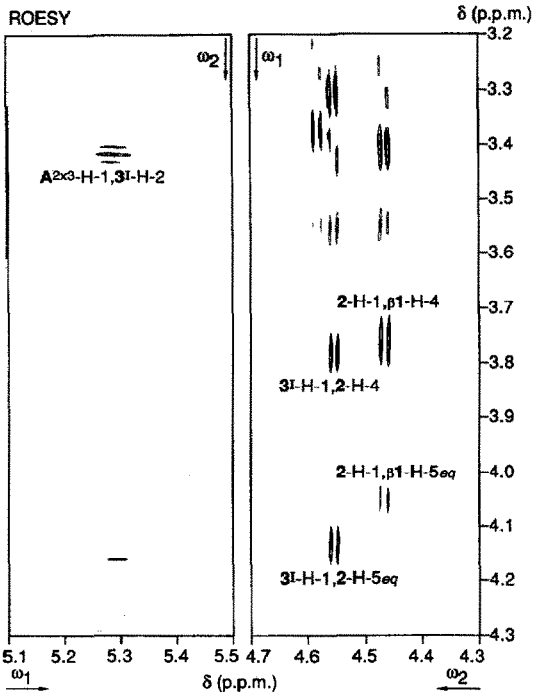


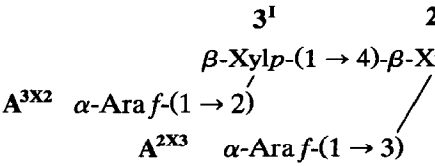
Fig. 6. 600-MHz ROESY spectrum of fraction **4.2**. ROE connectivities are given along the H-1 tracks in the ω_1-dimension for the β-Xyl*p* residues, and in the ω_2-dimension for the α-Ara*f* residue. Only the inter-residue ROE connectivities are denoted and the negative levels given. A^{2X3}-H-1,3^{I}-H-2 means the cross-peak between H-1 of α-Ara*f*-A^{2X3} and H-2 of β-Xyl*p*-3^{I}, etc.

also for the α-Ara*f*-A^{2X3} H-1 signal, relative to the α-Ara*f*-A^{3X2} H-1 signal of **3.2** and the α-Ara*f*-A^{2X3} and α-Ara*f*-A^{3X3} H-1 signals of **5.1**, respectively. Owing to the presence of a reducing residue, an anomerisation effect occurs, doubling the H-1,2 signals of β-Xyl*p*-**2** (refs 7 and 13).

The structure for **4.2** was confirmed by methylation analysis of the borodeuteride-reduced oligosaccharide, showing the presence of terminal arabinofuranose, 4-linked xylopyranose, and 2-linked xylopyranose in molar ratios of 1 : 0.9 : 0.9. The 4-linked xylitol could not be detected by methylation analysis, due to the high volatility of its permethylated derivative.

Fraction **5.3**.—Based on Bio-Gel P-2 chromatography, fraction **5.3** contain fragments consisting of five pentose residues. This was confirmed by the arabino to-xylose ratio (0.74, Table I) and by the 1D ^{1}H NMR spectrum, showing 5 n signals in the anomeric region (Table II). Too little material of fraction **5.3** available for a complete 2D NMR analysis. The H-1 signals of Xyl*p*-1, β-Xyl and α-Ara*f*-A^{3X2} of the major component of **5.3** (Table II) resonate at the chemical shift positions as those of the corresponding residues in **6.3**. The t arabinosylxylose unit was characterised by the β-Xyl*p*-3^{I} and α-Ara*f*-A signals at δ 4.527 and 5.293, respectively. Both H-1 signals resona

intermediate chemical shift position relative to the β-Xyl*p* and α-Ara*f* H-1 signals of the terminal arabinosylxylose unit of **5.2** and the diarabinosylxylose unit of **6.3**, respectively. Based on these data, the structure of the major component of fraction **5.3** is:



(5.3)

CONCLUSIONS

The structures of all the major oligosaccharides with dp ≤ 6 could be determined. Quantities of larger oligosaccharides were insufficient for structural analysis.

The presence of 2-*O*-Ara*f*-Xyl*p* in barley arabinoxylans[2] was confirmed by the presence of fractions **4.2** and **5.3**. Since this structural element appears to block the endoxylanase I to the same extent as 2,3-di-*O*-Ara*f*-Xyl*p*, its presence can explain the lower degradability of barley arabinoxylans by endoxylanase I when compared to wheat arabinoxylans[10], although the distribution of substituted and unsubstituted regions over the xylan backbone could also be of importance. Further work is in progress to gain information on the distribution of substituents over the xylan backbone.

ACKNOWLEDGMENTS

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