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Type I arabinogalactan contains β-D-Gal*p*-(1→3)-β-D-Gal*p* structural elements

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Abstract—Arabinogalactan type I from potato was partially degraded by *endo*-galactanase from *Aspergillus niger*. High-performance anion-exchange chromatography revealed that several of the oligomeric degradation products eluted as double peaks. To investigate the nature of these products, the digest was fractionated by Bio-Gel P2 chromatography. The pool that contained tetramers was treated with a β-D-Gal*p*-(1→4)-specific galactosidase from *Bifidobacterium adolescentis* to obtain a dimer with deviating linkage type, which was further purified by BioGel P2 chromatography. By obtaining all ^{1}H and ^{13}C chemical shifts and the presence of intra residual scalar coupling (HMBC) it could be concluded that the dimer contained a β-(1→3)-linkage instead of the expected β-(1→4)-linkage. Using the same NMR techniques as for the dimer, it was found that the pool of tetramers consisted of the following two galactose tetramers: β-Gal*p*-(1→4)-β-Gal*p*-(1→4)-β-Gal*p*-(1→4)-α/β-Gal*p*-OH and β-Gal*p*-(1→4)-β-Gal*p*-(1→4)-β-Gal*p*-(1→3)-α/β-Gal*p*-OH. The fact that the deviating β-(1→3)-linked galactose was found at the reducing end of the dimer showed that this deviating linkage is present within the backbone. The β-(1→3)-galactosyl interruption appeared to be a common structural feature of type I arabinogalactans with a frequency ranging from approximately 1 in 160 (potato, soy, citrus) to 1 in 250 (onion).

Keywords: Arabinogalactan; Potato; Structure elucidation; NMR analyses

1. Introduction

Galactans and arabinogalactans are plant cell wall polysaccharides.[1–4] Generally, two types of arabinogalactans are distinguished. Type I arabinogalactan contains a β-(1→4)-linked galactan backbone, which can be substituted with L-Ara*f* at C-3.[1,2] Type I arabinogalactans are found linked to rhamnogalacturonan I regions of pectic molecules,[5–7] for example, in potato, or non-covalently associated with other wall polysaccharides.[6] Linkage analysis by permethylation showed that 64% of the linkages in potato arabinogalactan are β-(1→4)-Gal*p*-linkages.[8]

In comparison to type I arabinogalactans, type II arabinogalactans are more widespread in plants.[1] They can also occur as arabinogalactan-proteins (AGP)[3] or as a polysaccharide present in exudate gums, not linked to other constituents.[1,2] The structural basis of this type of arabinogalactans is a backbone of β-galactopyranose residues that are predominantly (1→3)-linked. Type II arabinogalactans are most frequently branched with D-Gal*p*, L-Ara*f*, L-Rha*p*, D-Glc*p*A, and D-Gal*p*A, although other side chains such as D-Man*p*, D-Xyl*p*, and D-Glc*p* are also possible.[2] Only minor amounts of (1→3, 1→6)-β-linked galactan are found within potato galactans.[9]

Although the structure of arabinogalactans seems rather well established,[1,2,8,10] occasionally new structural elements are found in these polysaccharides. Huisman et al.[11] showed the presence of (1→5)-Ara*f* residues in the backbone, and terminal Ara*p* residues at the galactosyl side chains, of type I arabinogalactans from soy. This suggests that the structural variation within the arabinogalactans is large. In this paper, we add another example to arabinogalactan complexity and report a new structural element to type I arabinogalactan.

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2. Experimental

2.1. Materials

Potato, soybean, onion, and citrus arabinogalactan were obtained as described by Van de Vis.[8] The *endo*-galactanase from *Aspergillus niger* was purified by Van de Vis et al.[12] The β-D-Gal*p*-(1→4)-specific β-galactosidase from *Bifidobacterium adolescentis* DSM 20083 was cloned and purified by Hinz et al.[13] A β-(1→4)-galacto-disaccharide standard was purchased from Megazyme International Ireland Ltd. (Bray, Ireland).

2.2. Enzymatic degradation of potato arabinogalactan

A solution of 100 mg/mL potato arabinogalactan in 10 mM NaOAc buffer pH 5 was partially digested with an *endo*-galactanase from *A. niger* (26.4 μg, 26 U) for 384 h at 30 °C, while continuously being mixed. The incubation was stopped by heating for 10 min at 100 °C and insoluble material was removed by centrifugation (5 min at 10,000*g*). The extent of the depolymerization was followed by high-performance anion-exchange chromatography (HPAEC) and high-performance size-exclusion chromatography (HPSEC).

2.3. Fractionation of arabinogalacto-oligosaccharides

The arabinogalacto-oligosaccharides were separated from the polymeric material by fractionation on a Bio-Rad Bio-Gel P2 (200–400 mesh, Richmond, CA, USA) size-exclusion column (100 × 2.6 cm), using an Amersham Biotech Hiload system (Uppsala, Sweden). Components were eluted with Millipore water at 60 °C (flow rate was 0.5 mL/min) and monitored by refractive index detection using a Shodex RI-72 detector (Tokyo, Japan). Fractions (5 mL) were collected and appropriate fractions were pooled as indicated in Figure 2.

2.4. Analysis of sugar composition

The sugar composition of the various Bio-Gel P2 pools was determined using methanolysis according to De Ruiter et al.[14] For this, the different pools were treated with 2 M HCl in dry methanol for 16 h at 80 °C, followed by 1 h of 2 M TFA at 121 °C. The sugars released were quantified using HPAEC.

2.5. Enzymatic degradation of arabinogalacto-oligosaccharides

Bio-Gel P2 pools III, IV, and V were incubated with 0.015 U of the β-galactosidase β-Gal II from *B. adolescentis*. The incubations were carried out in 20 mM phosphate buffer, pH 6 at 37 °C for 60 min, and stopped by heating for 10 min at 100 °C. Insoluble material was removed by centrifuging (10 min at 10,000*g*). The reaction products were analyzed by HPAEC.

2.6. Complete enzymatic depolymerization of arabinogalactans

A solution of 1 mg/mL arabinogalactan in 50 mM NaOAc buffer pH 5 was incubated with the *endo*-galactanase from *A. niger* (0.14 μg, 0.26 U) for 24 h at 30 °C, under continuous stirring. The total volume of the incubation was 100 μL. The incubation was stopped by heating for 10 min at 100 °C, and insoluble material was removed by centrifugation (10 min at 10,000*g*). The incubation was monitored by HPAEC.

2.7. Estimation of the β-(1→3)/β-(1→4)-ratio in arabinogalactan from different sources

The products from the complete enzymatic depolymerization of arabinogalactans (50 μL) were incubated with the β-galactosidase β-Gal II from *B. adolescentis* (0.15 μg, 0.012 U). The volume of the reaction mixtures was adjusted to 100 μL with 20 mM phosphate buffer pH 6, and incubated at 37 °C for 60 min. The incubations were stopped by heating for 10 min at 100 °C, and insoluble material was removed by centrifuging (10 min at 10,000*g*). Reaction products were determined by HPAEC analysis.

2.8. Analytical methods

High-performance size-exclusion chromatography was performed on three TSKgel columns (7.8 mm ID × 30 cm per column) in series (G4000 PWXL, G3000 PWXL, G2500 PWXL; Tosohaas, Stuttgart, Germany), in combination with a PWX-guard column (Tosohaas, Stuttgart, Germany). Elution took place at 30 °C with 0.2 M sodium nitrate at 0.8 mL/min. The eluate was monitored by refractive index detection using a Shodex RI-72 detector. Calibration was performed using dextrans.

High-performance anion-exchange chromatography was performed on a Thermo-Quest HPLC system equipped with a Dionex CarboPac PA-1 (4 mm ID × 250 mm) column in combination with a Dionex CarboPac PA guard column (3 mm ID × 25 mm) and a Dionex ED40 PAD-detector (Dionex Co., Sunnyvale, USA). A flow rate of 1 mL/min was used.

Arabinogalacto-oligosaccharides were determined using a gradient of sodium acetate in 100 mM NaOH: 0–40 min, 0–400 mM NaOAc in 100 mM NaOH; 40–41 min, 400–1000 mM NaOAc in 100 mM NaOH; 41–46 min, 1000 mM NaOAc in 100 mM NaOH; 46–60 min, 100 mM NaOH.

The determination of the sugar composition was performed using the same HPAEC system equipped with a Dionex CarboPac PA20 (3 mm ID × 150 mm) in combination with CarboPac PA20 guard column (3 mm ID × 30 mm) and Borate trap (4 mm ID × 50 mm) and separated at 0.5 mL/min by isocratic elution with 3 mM NaOH for 20 min followed by applying a linear gradient from 0 to 700 mM NaOH in 20 min.

2.9. MALDI-TOF mass spectrometry

For MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) an Ultraflex workstation (Bruker Daltronics GmbH, Germany) was used. The mass spectrometer was calibrated with a mixture of malto-dextrins (mass range 365–2309). The samples were mixed with a matrix solution (1 μL each). The matrix solution was prepared by dissolving 9 mg of 2,5-dihydroxybenzoic acid and 3 mg 1-hydroxyisoquinoline in a 1 mL mixture of acetonitrile/water (300 μL:700 μL). Two microliters of the prepared sample and matrix solutions were put on a gold plate and dried with warm air.

2.10. ^{13}C and ^{1}H NMR

Prior to NMR analyses, the samples were exchanged in 99.96% D_2O (Cambridge Isotope Laboratories, USA) and after freeze-drying dissolved in 99.996% D_2O (Cambridge Isotope Laboratories, USA). NMR spectra were recorded at a probe temperature of 25 °C on a Bruker AMX-500 spectrometer located at the Wageningen NMR Centre. Chemical shifts were expressed in parts per million relative to internal acetone: $\delta = 2.225$ ppm for ^{1}H and $\delta = 31.55$ ppm for ^{13}C.

The 1D ^{1}H proton spectra were recorded at 500.13 MHz using eight scans of 8192 data points and a sweep width of 3000 Hz. The 1D ^{13}C proton decoupled carbon spectra were recorded at 125.77 Hz using 100,000 scans of 32,768 data points and a sweep width of 31,250 Hz.

The 2D COSY spectra were acquired using the double quantum filtered (DQF) method with a standard pulse sequence delivered by Bruker.

2D TOCSY spectra were acquired using a standard Bruker pulse sequences with 110 ms mixing time, respectively. For all homonuclear 2D spectra, 512 experiments of 2048 data points were recorded using 32 scans per increment.

For the 2D HMBC spectrum[15] a standard gradient enhanced 2D HMQC pulse sequence delivered Bruker was changed into a HMBC sequence by setting the delay between the first proton and carbon pulse to 50 ms. For the HMBC experiment, 1024 experiments of 2048 data points were recorded with 128 scans per increment.

3. Results

3.1. Partial degradation of potato arabinogalactan

Arabinogalactan from potato was partially digested by *endo*-galactanase from *A. niger* to obtain (arabino)galacto-oligosaccharides of various chain lengths. The degradation was monitored by HPSEC and HPAEC analysis and the reaction was stopped after the formation of a broad range of oligosaccharides. It is emphasized that these oligosaccharides in the digest are not limit-digest products.

HPSEC analysis showed a shift from a polymeric fraction to a mixture of oligomers. Part of the starting material was not degraded by the *endo*-galactanase, as shown in the HPSEC profile (Fig. 1A). Interestingly, several oligomers seemed to elute as double peaks upon HPAEC analysis (Fig. 1B). To assign the peak belonging to the β-(1→4)-galactobiose in the chromatogram of the digest, a commercially available β-(1→4)-galactodisaccharide derived from potato arabinogalactan was analyzed on HPAEC with the same gradient. Typically, this β-(1→4)-galactobiose showed the same double peak at 8.2 and 8.7 min as observed in the digest described above.

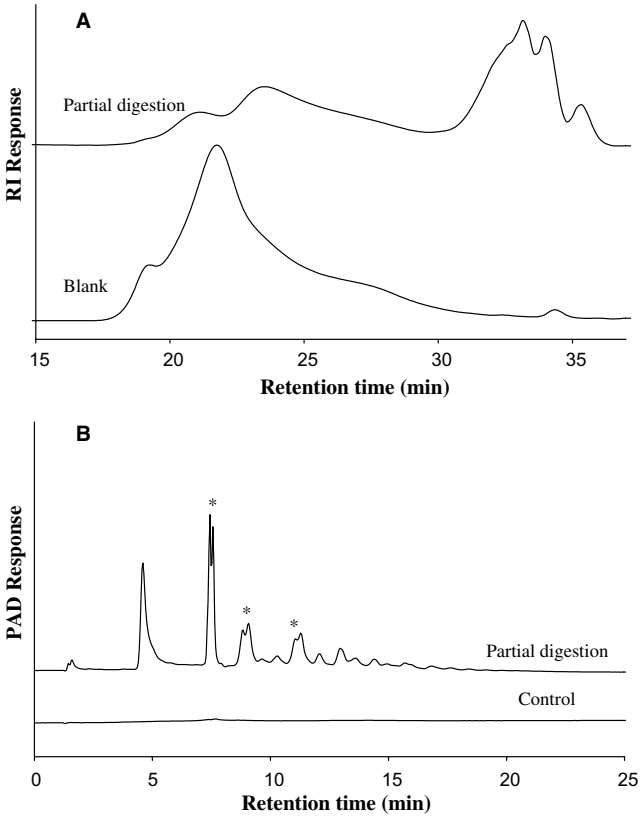

Figure 1. HPSEC (A) and HPAEC (B) profiles of partial degradation of potato arabinogalactan by an *endo*-galactanase from *A. niger*. *, double peak.

3.2. Fractionation of (arabino)galacto-oligosaccharides

To investigate the nature of the products eluting in these double peaks, the digest of potato arabinogalactan was fractionated by Bio-Gel P2 chromatography. Fractions were pooled as indicated and characterized for the presence of various (arabino)galacto-oligosaccharides (Fig. 2) by HPAEC and MALDI-TOF MS analysis. The monosaccharide compositions of the native potato arabinogalactan and the five pools were determined using HPAEC after methanolysis (Table 1). Because of the presence of galacturonic acid and rhamnose, it was concluded that the potato arabinogalactan contained some pectic material. Pool I also contained this pectic material, besides arabinogalactan material with a degree of polymerization of 10 and higher, as determined by MALDI-TOF MS (data not shown). In pools II, III, and IV mainly galactose and a small amount of arabinose was found. The Gal/Ara-ratio was calculated for the different pools (Table 1), and showed a shift from 1.1 in pool I to 39 in pool IV. This suggested that the arabinosyl-rich regions of the arabinogalactan are poorly degraded by the *endo*-galactanase. Pool II contained different types of arabinogalacto-oligosaccharides with a degree of polymerization of 5-9; examples of oligosaccharides present in this fraction are Gal_4Ara, Gal_5, Gal_4Ara_2, Gal_5Ara, Gal_6, and Gal_5Ara_2, as detected by MALDI-TOF MS (Fig. 3). Pools III, IV, and V contained tetrasaccharides, trisaccharides, and disaccharides, respectively. Pool V contained the disaccharides with 100% galactose, suggesting that the double peaks observed in the HPAEC elution profile could be derived from two galactosyl dimers with different linkage compositions.

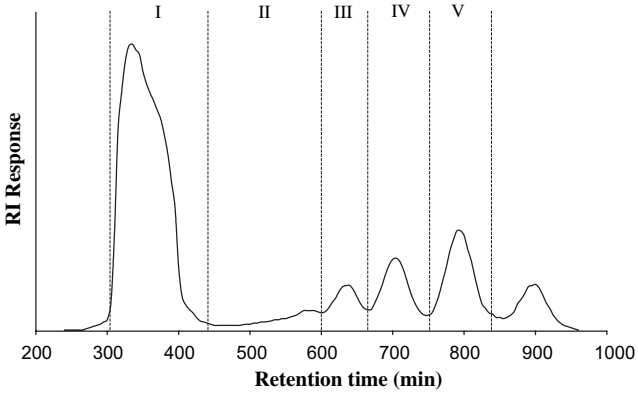

Figure 2. Bio-Gel P2 size-exclusion chromatography profile of arabinogalacto-oligosaccharides from the partial degradation of potato arabinogalactan by an *endo*-galactanase from *A. niger*. I: pool of larger galacto-oligomers and undigested material, II: pool of galacto-oligomers with a dp of 5–9, III: pool of galacto-oligomers with a dp of 4, IV: pool of galacto-oligomers with a dp of 3, V: pool of galacto-oligomers with a dp of 2.

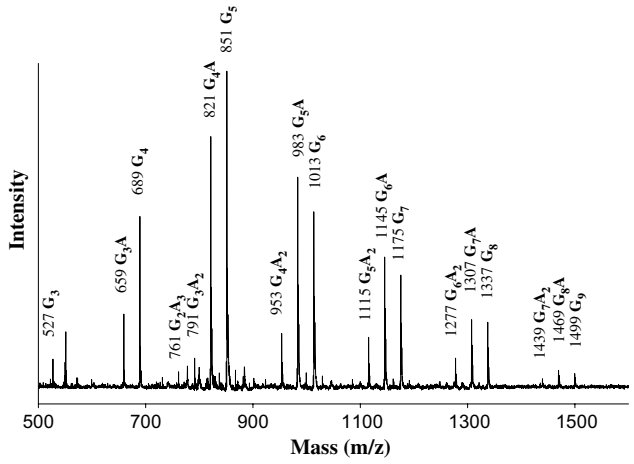

Figure 3. MALDI-TOF mass spectrum of Bio-Gel P2 pool II after fractionation of partially digested potato arabinogalactan. Masses of the sodium-adducted galacto-oligosaccharides are indicated (G = galactose; A = arabinose).

3.3. Characterization of the dimer, trimer, and tetramer fraction

To examine the presence of different linkage types in the galacto-oligosaccharides, the pools III, IV, and V were incubated with a β-D-Gal*p*-(1→4)-specific β-galactosidase from *B. adolescentis*.[13] We have shown previously that the enzyme was not able to degrade β-(1→6)-linkages, and only small amounts of β-(1→3)-linkages. The reaction products were analyzed by HPAEC. Only

Table 1. Sugar composition of potato arabinogalactan and fractions thereof

Fraction	Monosaccharide composition (mol %)						Carbohydrate content[a]	Gal/Ara-ratio
	Fuc	Rha	Ara	Gal	Glc	GalA		
Potato galactan	1.2 ± 0.2	6.5 ± 0.3	13.9 ± 0.2	65.8 ± 0.3	1.2 ± 0.0	11.3 ± 0.3	76.0 ± 2.6	4.7
I	0.8 ± 0.0	15.3 ± 0.8	27.1 ± 0.3	30.3 ± 0.3	1.2 ± 0.1	25.4 ± 0.8	57.9 ± 2.8	1.1
II	2.0 ± 0.4	—	9.0 ± 0.7	88.5 ± 0.3	0.6 ± 0.0	—	89.1 ± 5.3	9.8
III	—	—	5.9 ± 0.1	94.1 ± 0.1	—	—	97.6 ± 3.6	16
IV	—	—	2.5 ± 0.2	97.5 ± 0.2	—	—	94.5 ± 2.9	39
V	—	—	—	100.0 ± 0.0	—	—	96.5 ± 2.9	n.a.[b]

[a] Expressed as % w/w.
[b] n.a. = not applicable.

one of the two disaccharides in the dimer fraction (pool V) was converted to galactose (Fig. 4A). This suggested that the disappearing oligomer was the β-(1→4)-linked galacto-disaccharide, whereas the other dimer may contain a different linkage type. Digestion of the trimeric (Fig. 4B) and tetrameric (Fig. 4C) fractions yielded galactose and the same undigested galacto-disaccharide.

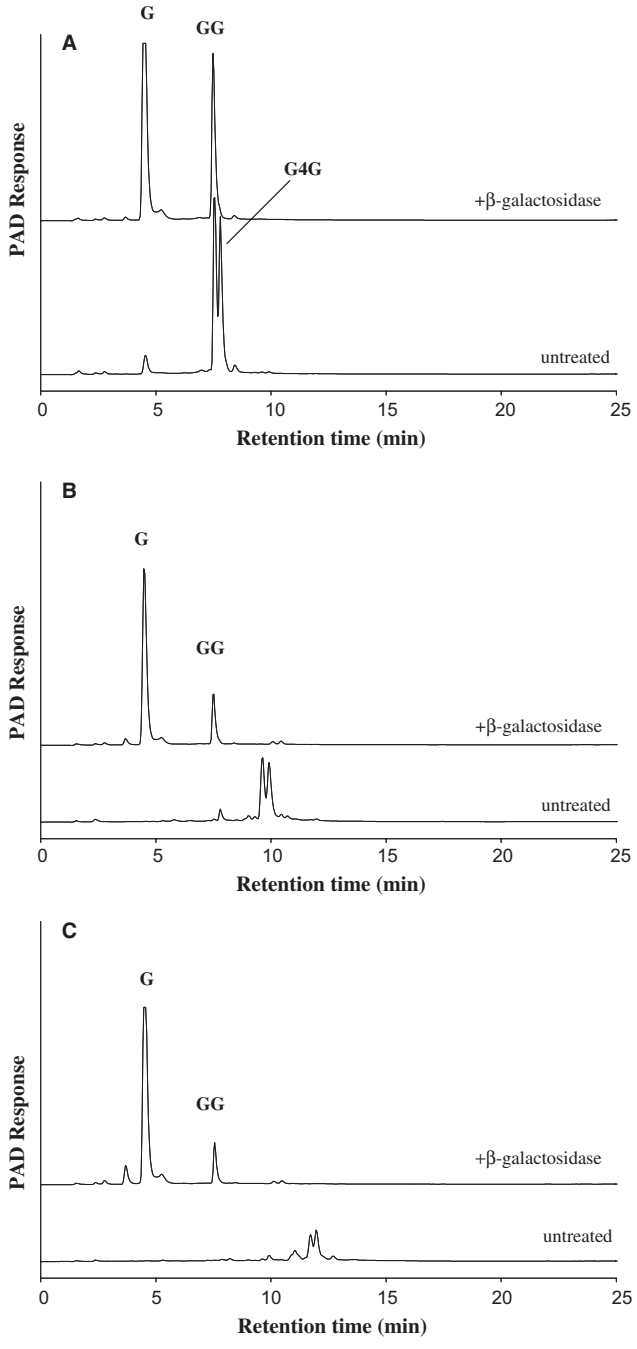

Figure 4. HPAEC profile for the Bio-Gel P2 pools V (A), IV (B), and III (C) after digestion with a β-D-Gal*p*-(1→4)-specific β-galactactosidase from *Bifidobacterium adolescentis*. **G**: galactose; **GG**: galacto-disaccharide with unknown linkage-type and **G4G**: β-D-Gal*p*-(1→4)-β-D-Gal*p*.

This result showed that the tri- and tetramers contained only one linkage-type differing from β-(1→4). Dimer pool V, digested by the β-galactosidase from *B. adolescentis*, was fractionated by Bio-Gel P2 size-exclusion chromatography to obtain the purified, non-digested galacto-disaccharide. To unambiguously prove the presence of two galactose dimers with different linkage types, the purified dimer was subjected to NMR analysis.

3.4. NMR analysis of the indigestible galacto-disaccharide

By recording a 2D homonuclear COSY and TOCSY spectrum it was possible to assign all the ^{1}H chemical shifts as shown in Table 2. From the differences with the values known for both α- and β-*O*-methyl-galactoside,[16] it was apparent that the oligomer consisted of two galactosyl residues connected by a (1→3)-linkage, because protons 2, 3, and 4 of the reducing end moieties (**A** and **B**) showed a significant downfield glycosylation shift. The fact that also proton 2 showed a significant downfield shift, which is far less within a 4-linked galactose,[17] indicated the presence of a (1→3)-linkage.

The presence of this linkage type was further substantiated by the assignment of the ^{13}C chemical shifts (Table 2) from the 2D heteronuclear HMBC spectrum recorded. The inter-residue coupling of C-1 and H-1 of the terminal galactosyl residue (**C**) with C-3 and H-3 of both the α and β reducing end galactose (**A** and **B**), which is indicated in the segment shown in Figure 5A, proves the presence of a β-(1→3)-linked galactose dimer. These findings already explain the double peak found in the HPAEC elution profiles shown in Figure 1B. It is suggested that the double peak in the HPAEC profile of the commercial preparation is also due to a β-(1→3)-linked galactose dimer. The presence of this new linkage type in type I potato arabinogalactans raised the question whether the β-(1→3)-linked galactosyl residue is the point of galactosyl side chain attachment or whether the new structural element is an integral part of the β-(1→4)-linked backbone.

To examine this, similar NMR spectra were recorded of the mixture of tetramers present in pool III. Figure 4C indicated that one of these tetramers contained only (1→4)-linkages whereas the other contained an atypical linkage. From the COSY, TOCSY, and HMBC spectra most of the different residues could be assigned up to proton 5 and carbon 5 (Table 3). Only two terminal residues could be found as indicated by residues **G** and **F** in Table 3. These residues could be separated from the other residues due to the fact that none of these proton and carbon resonances show a considerable downfield shift when compared to the terminal residue of the dimer shown in Table 2. The fact that only two terminal residues could be found already indicates that the

Table 2. ^{1}H and ^{13}C chemical shifts of β-(1→3) galactose dimer at 25 °C relative to internal acetone at 2.225 or 31.55 ppm, respectively

Residue	H-1 (C-1)	H-2 (C-2)	H-3 (C-3)	H-4 (C-4)	H-5 (C-5)	H-6 (C-6)	H-6′
A →3)-α-Gal*p*-OH	5.28	3.97	3.99	4.26	4.11	3.72	3.72
	0.06[b]	**0.19**	**0.18**	**0.31**	0.08	0.03	0.03
	(93.6)	(68.9)	(**81.0**)	(70.5)	(71.6)	(62.5)	—
B →3)-β-Gal*p*-OH	4.63	3.65	3.79	4.2	3.73	3.76[a]	3.76[a]
	0.1	**0.20**	**0.20**	**0.31**	0.08	0.12	0.041
	(97.6)	(72.3)	(**84.0**)	(69.9)	(76.2)	(62.4)	—
C β-Gal*p*-(1→	4.6	3.62	3.67	3.94	3.69	3.77[a]	3.77[a]
	0.07	0.17	0.08	0.05	0.04	0.13	0.05
	(105.8)	(72.7)	(73.9)	(70.0)	(76.4)	(62.2)	—

[a] Might be interchanged.
[b] Difference with values found for α- and β-*O*-methyl-galactoside.[16]

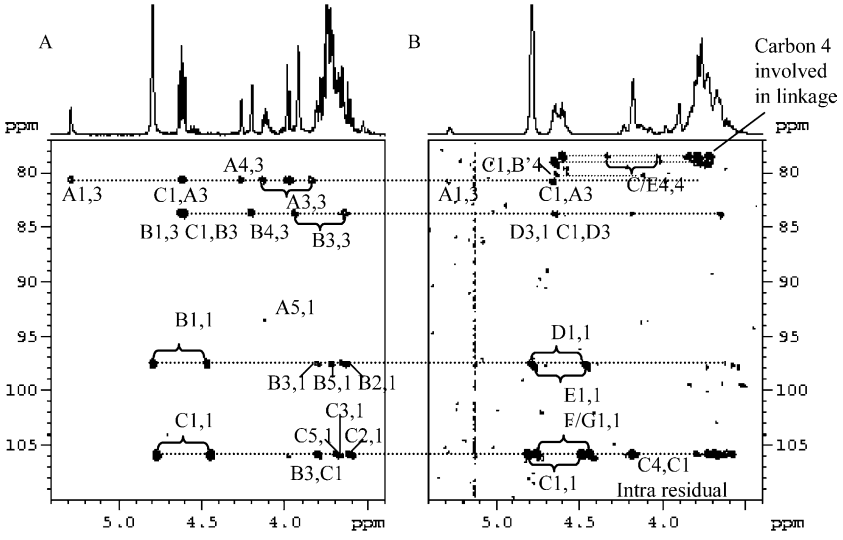

Figure 5. Segments of the 500-MHz 2D ^{1}H ^{13}C undecoupled HMBC spectrum of both the galactose dimer (A) and tetramer (B) recorded in D_2O at 70 °C. A: **A** = →3)α-Gal*p*-OH; **B** = →3)β-Gal*p*-OH; **C** = β-Gal*p*(1→. B: **A** = →3)α-Gal*p*-OH; **B′** = →4)α-Gal*p*-OH; **C** = →4)β-Gal*p*-(1→; **D** = →3)β-Gal*p*-OH; **E** = →4)β-Gal*p* **G/F** = β-Gal*p*(1→. The code **C**1,1 stands for the coupling between **C** H-1 and C-1, **C**2,1 stands for the coupling between **C** H-2 and C-1 and **B**3, **C**1 stands for the long range coupling between **B** H-3 and **C** C1, etc.

(1→3)-linked galactose is present in the backbone of the tetramer. The proton chemical shifts of both reducing galactosyl residues in their α anomeric configuration could be assigned completely as can be seen from Table 3 residues **A** and **B′**.

The proton chemical shifts of residue **A** were the same as the ones found for the α reducing end of the dimer measured before. This together with the fact that residue **B′** showed a significantly less downfield shift of proton 2, demonstrated that residue **A** belongs to a reducing end derived from a (1→3)-linked galactose and **B′** is the α anomer of the (1→4)-linked reducing galactose. In fact, if residue **A** is derived from a galactose linked both (1→3)- and (1→4)-galactose, a far larger downfield shift for protons 4, 3, and 2 would be expected.[17] The chemical shifts of the β-(1→3)-linked galactose (**D**) were also the same as the ones found for the same residue in the dimer (residue **B**, Table 2).

By recording 2D heteronuclear HMBC spectrum, see segment Figure 5B, further evidence for a (1→3)-linked galactose at the reducing end of the second tetramer could be obtained. In Figure 5B the C-3 signals of both the α-(1→3)- and β-(1→3)-linked galactose ($\delta = 80.8$ and 84.0 ppm) could be clearly separated from the C-4 signals of the (1→4)-linked galactosyl residues around $\delta = 79$ ppm. The ^{13}C chemical shifts of both residues **A** and **D** representing the α and β anomeric configuration of the (1→3)-linked galactose, respectively, were the same as the ones found for the dimer shown in Table 2. Therefore the β-(1→3)-linked galactose is at the reducing end of the tetramer. Taking into account the difference in calibration used, internal acetone 31.55 ppm or 1,4-dioxane 67.40 ppm relative to external Me_4Si, both the chemical shifts of the α-(1→4)- and β-(1→4)-linked galactose residue (**B′** and **E**) are similar to those reported.[16,18,19] It has to be noted that the C-6

Table 3. ^{1}H and ^{13}C chemical shifts of the mixture of 2 galactose tetramers at 25 °C relative to internal acetone at 2.225 or 31.55 ppm, respectively

Residue	H-1 (C-1)	H-2 (C-2)	H-3 (C-3)	H-4 (C-4)	H-5 (C-5)	H-6 (C-6)	H-6′
A →3)-α-Gal*p*-OH	5.28	3.99	3.99	4.23	4.12	3.72	3.72
	0.06[b]	**0.21**	**0.18**	**0.28**	0.09	0.03	0.03
	(93.7[a])	(68.7)	(**80.8**)	(70.5)	(71.4)	(61.8)	—
B′ →4)-α-Gal*p*-OH	5.27	3.89	3.954	4.22	4.12	3.82[a]	3.75
	0.05	**0.11**	**0.14**	**0.27**	0.09	0.13	0.06
	(93.7[a])	(nd)	(70.4)	(80.2)	(71.2)	(61.8)	—
C→4)-β-Gal*p*-(1→	4.66	3.68	3.78	4.19	3.73	nd	nd
	0.13	0.23	0.19	0.30	0.08	—	—
	(105.8)	(72.1)	(73.9)	(78.8, 78.5[c])	(75.8)	(62.0)	—
D →3)-β-Gal*p*-OH	4.63	3.65	3.81	4.18	3.73	nd	nd
	0.1	**0.20**	**0.22**	**0.29**	0.08		
	(97.5)	(72.3)	(**84.0**)	(70.1)	(75.6)	(62.0)	—
E →4)-β-Gal*p*-OH	4.61	3.57	3.74	4.18	3.73	nd	nd
	0.08	**0.12**	**0.15**	**0.29**	0.08		
	(97.8)	(73.5)	(74.0)	(79.1)	(75.6)	(62.0)	—
F β-Gal*p*-(1→	4.59	3.59	3.65	3.9	3.68	nd	nd
	0.06	0.14	0.06	0.01	0.03		
	(105.7)	(72.7)	(74.2)	(69.9)	(76.5)	(62.0)	—
G β-Gal*p*-(1→	4.58	3.62	3.67	3.91	3.68	nd	nd
	0.05	0.17	0.08	0.02	0.03		
	(105.7)	(72.7)	(74.2)	(69.9)	(76.5)	(62.0)	—

nd: not determined.
[a] Might be interchanged.
[b] Difference with values found for α- and β-*O*-methyl-galactoside.[16]
[c] C4 = 78.5 ppm for both residues neighboring the terminal residues (**G** and **F**).

chemical shift for the reducing β-(1→4)-linked galactose in Fransen et al.[19] determined at 69.44 ppm is probably a type error. All internal galactoses, residues **C**, show approximately the same shifts as β-(1→4)-linked galactose residue (**E**) except for their anomeric carbon and C-2. Due to extreme overlap, the different internal residues could not be distinguished. However the second and third galactose within both tetramers could be separated by their C-4 chemical shifts 78.5 ppm and 78.8 ppm, respectively. Both terminal residues (**G** and **F**) could be assigned due the up field signal of their carbon 4. From these results, we concluded that the mixture consisted of the following two tetramers.

1. β-Gal*p*-(1→4)-β-Gal*p*-(1→4)-β-Gal*p*-(1→4)-α/β-Gal*p*-OH

G/F **C** **C** **B'/E**

2. β-Gal*p*-(1→4)-β-Gal*p*-(1→4)-β-Gal*p*-(1→3)-α/β-Gal*p*-OH

G/F **C** **C** **A/D**

Tetramer **2**, together with the known mode of action of *endo*-galactanase, showed that potato galactan not only contained (1→4)-linkages in its backbone, but also (1→3)-linkages. This is corroborated by the mode of action of the β-galactosidase from *B. adolescentis* toward this tetramer. Previous research showed that the β-galactosidase was not able to cleave the β-(1→4)-linkage of α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Gal*p*, suggesting that the enzyme was only able to cleave β-(1→4)-linkages at the non-reducing end.[13] Figure 4C showed that the β-galactosidase degraded tetramer **2** to galactose and β-(1→3)-galactobiose, confirming that the β-(1→3)-linkage is located at the reducing end of the oligosaccharide.

3.5. Estimation of the β-(1→4)/β-(1→3)-ratio in arabino- galactan from different sources

To investigate if the new structural element is also present in other type I arabinogalactans and to estimate the β-(1→4)/β-(1→3)-ratio, arabinogalactans from different botanical sources, like potato (*Solanum tuberosum*), soybean (*Glycine max*), onion (*Allium cepa*), and citrus (*Citrus sinensis*) were incubated with the *endo*-galactanase from *A. niger* to an end-point. These incubations were analyzed by HPAEC (Fig. 6). It was found that all four arabinogalactans contained β-D-Gal*p*-(1→3)-β-D-Gal*p*, besides major products as arabinose, galactose, GalAra, and β-D-Gal*p*-(1→4)-β-D-Gal*p*. The products of complete enzymatic depolymerization of potato (Fig. 6A), soybean (Fig. 6B), and citrus arabinogalactans (Fig. 6D) gave a similar HPAEC profile. The HPAEC profile of onion arabinogalactan showed an extra peak (Fig. 6C*). The nature of this peak could not be revealed by digestion of the product with an arabinofuranosidase from *A. niger*, to liberate a possible arabinosyl residue,

or a β-galactosidase from *A. niger*, which was able to degrade different types of galactosyl linkages.

The end-point degradations were subsequently incubated with the β-D-Gal*p*-(1→4)-specific β-galactosidase from *B. adolescentis*.[13] The β-D-Gal*p*-(1→4)-β-D-Gal*p* was completely degraded to galactose, whereas the β-D-Gal*p*-(1→3)-β-D-Gal*p* was not. Based on these results, the amount of β-(1→3)- and β-(1→4)-linkages in the four different arabinogalactans was estimated, in which every liberated galactose represents a cleaved β-(1→4)-linkage (Table 4). The highest amount of β-(1→3)-linkages was found in potato arabinogalactan. The β-(1→4)/β-(1→3)-ratio was 163; the ratio for the other arabinogalactans was higher. The amount of β-(1→3)-linkages in arabinogalactan was rather low, but it is possible that this amount is underestimated. The *endo*-galactanase seemed to be able to degrade β-(1→3)-linkages (although at a slower rate), since the β-D-Gal*p*-(1→3)-β-D-Gal*p* peak in the HPAEC diagram of the partially degraded arabinogalactan (Fig. 1B) was approximately four times larger than that of the completely degraded arabinogalactan (Fig. 6A). The ability of an *endo*-galactanase to degrade β-(1→3)-linkages was also observed with an *endo*-galactanase from *Bifidobacterium longum* (unpublished results). Approximately 20% of the galactan backbone was substituted with arabinosyl side chains, which were only poorly degraded by the *endo*-galactanase. We did not take this part of the galactan into account when estimating the

Table 4. Estimation of the β-(1→4)/β-(1→3)-ratio in different arabinogalactans

	β-(1→4)[a]	β-(1→3)[a]	β-(1→4)/β-(1→3)-ratio
Potato galactan	97.6	0.6	163
Soy galactan	96.4	0.5	193
Onion galactan	99.2	0.4	248
Citrus galactan	98.1	0.5	196

[a] Expressed as mol %.

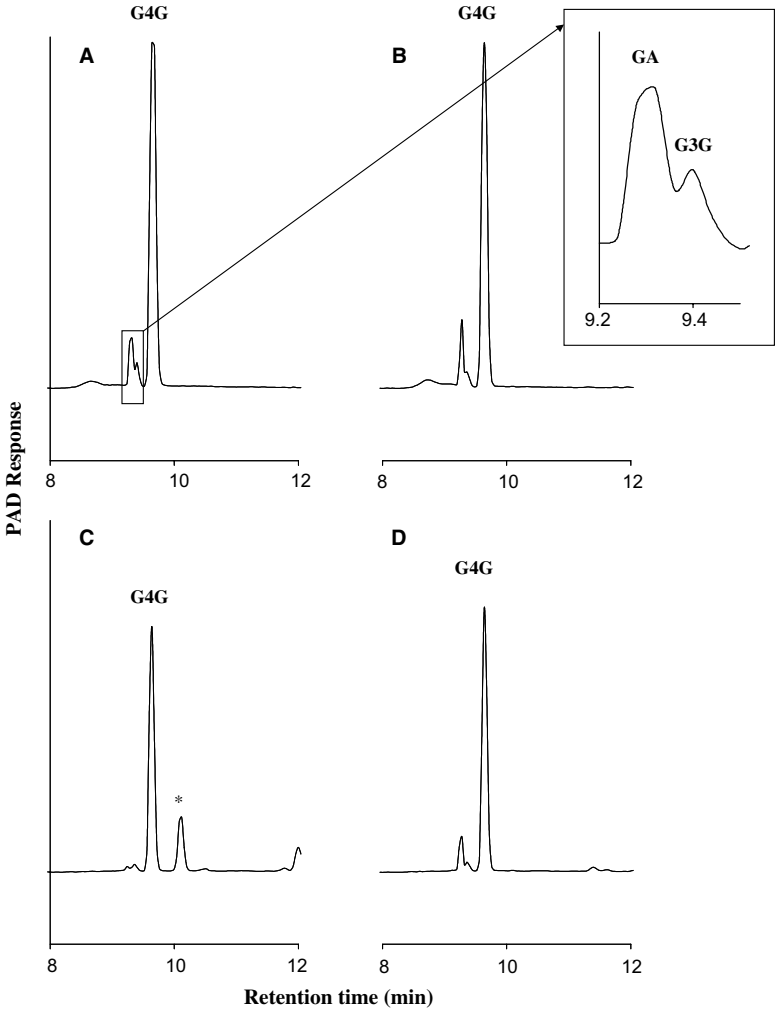

Figure 6. HPAEC profiles of the end-point degradation of arabinogalactans from different sources with *endo*-galactanase from *A. niger*. **A**: potato galactan; **B**: soybean galactan; **C**: onion galactan; **D**: citrus galactan. **G**: galactose; **A**: arabinose, **G4G**: β-D-Gal*p*-(1→4)-β-D-Gal*p*; **G3G**: β-D-Gal*p*-(1→3)-β-D-Gal*p*; *, unknown peak.

β-(1→4)/β-(1→3)-ratio. It is assumed that the β-(1→3)-linkages are evenly distributed in the backbone, and consequently this ratio will not be affected.

4. Discussion

It is generally accepted that the type I potato arabinogalactan contains β-(1→4)-linked galactosyl residues in the backbone, substituted with α-(1→5)-linked arabinosyl residues.[8,9] In this study, we showed that the potato arabinogalactan also contained β-(1→3)-linked galactosyl residues as an integral part of the backbone. The presence of peculiar structural elements in the arabinogalactan backbone was previously found for soybean arabinogalactans. For this type I arabinogalactan, it is known that the β-(1→4)-linked galactosyl backbone can be interspersed with (1→5)-linked arabinosyl residues.[11]

In this research, we also demonstrated that the presence of β-(1→3)-linked galactosyl residues is not restricted to the type I arabinogalactan from potato. Very different botanical sources such as soybean, onion, and citrus arabinogalactan also contained this structural element, which suggests that all type I arabinogalactans may contain β-(1→3)-linkages as an intrinsic part of the backbone. From the combined *A. niger endo*-galactanase and *B. adolescentis* β-galactosidase treatment the β-(1→4)/β-(1→3)-ratio was estimated as approximately 160–250.

The presence of β-(1→3)-linkages in a β-(1→4)-polymer is also found in other cell wall polysaccharides, like (1→3, 1→4)-β-D-glucans. These glucans in, for example, barley contain a β-(1→4)/β-(1→3)-ratio of approximately 2.5.[20] This ratio is much lower than that found in type I arabinogalactans (a ratio of 163 was found in potato galactan), demonstrating that the β-(1→3)-linkages occur at a much higher frequency in the β-glucans.

For β-glucan synthesis the insertion of β-(1→3)-glucosyl residues seems to be intentional; evidence is accumulating that the addition of these linkages is an intrinsic property of the β-glucan synthase (or synthesizing complex). Buckeridge et al.[21] found that the β-glucan synthesizing machinery inserts single β-(1→3)-linkages between cellotriose or cellobiose units in a linear backbone structure. The much lower frequency of β-(1→3)-linkages in type I arabinogalactans compared to β-glucans suggests that these elements are inserted incidently. We speculate that the β-(1→3)-linkages in type I arabinogalactan are 'errors' in the biosynthesis. Since both β-(1→4) and β-(1→3)-galactosyltransferases are present in the same compartment of the Golgi apparatus,[22] the β-(1→3)-galactosyltransferase might incidently use the growing β-(1→4)-galactan chain as an acceptor substrate and add a β-(1→3)-galactosyl unit. Subsequently, the β-(1→4)-galactosyltransferase continues the elongation of the nascent galactan. The variation in β-(1→4)/β-(1→3)-ratio observed for the galactans from different botanical sources might be related to the ratio of the two galactosyltransferases. However, it is also possible that differences in biochemical properties of the transferases (such as K_m for UDP-gal, processive or distributive mode of action) underlies this variation.

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