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Branched arabino-oligosaccharides isolated from sugar beet arabinan

Yvonne Westphal^a, Stefan Kühnel^a, Pieter de Waard^b, Sandra W. A. Hinz^c, Henk A. Schols^{a,*}, Alphons G. J. Voragen^a, Harry Gruppen^a

^a Wageningen University, Laboratory of Food Chemistry, PO Box 8129, 6700 EV Wageningen, The Netherlands

^b Wageningen NMR Centre, PO Box 8128, 6700 ET Wageningen, The Netherlands

^c Dyadic Netherlands, Nieuwe Kanaal 75, 6709 PA Wageningen, The Netherlands

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ABSTRACT

Sugar beet arabinan consists of an α -(1,5)-linked backbone of L-arabinosyl residues, which can be either single or double substituted with α -(1,2)- and/or α -(1,3)-linked L-arabinosyl residues. Neutral branched arabino-oligosaccharides were isolated from sugar beet arabinan by enzymatic degradation with mixtures of pure and well-defined arabinohydrolases from *Chrysosporium lucknowense* followed by fractionation based on size and analysis by MALDI-TOF MS and HPAEC. Using NMR analysis, two main series of branched arabino-oligosaccharides have been identified, both having an α -(1,5)-linked backbone of L-arabinosyl residues. One series carries single substituted α -(1,3)-linked L-arabinosyl residues at the backbone, whereas the other series consists of a double substituted α -(1,2,3,5)-linked arabinan structure within the molecule. The structures of eight such branched arabino-oligosaccharides were established.

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1. Introduction

Pectins belong to the main polysaccharides within the primary plant cell wall of dicotyls.¹ Four main pectic components have been identified: homogalacturonan, rhamnogalacturonan-I (RG-I), rhamnogalacturonan-II, and xylogalacturonan, which have been described extensively.^{2–4} RG-I consists of a backbone of repeating units of alternating α -(1,2)-linked rhamnose and α -(1,4)-linked galacturonic acids. Depending on the type of cell wall and tissue, 20–80% of the rhamnose residues are substituted with side chains composed of galactose and/or arabinose attached to O-3 and/or O-4. The length of these side chains can vary from one single sugar residue up to chains of 50 residues.⁴ The RG-I arabinans are branched molecules with a linear α -(1,5)-linked arabinose backbone, which can be single or double substituted with α -(1,2)-linked and/or α -(1,3)-linked arabinose side chains, which again may be further branched.^{5,6} In addition, the arabinan of, for example, sugar beet cell walls can be feruloylated at the O-2 and/or O-5 position of one of the arabinose moieties.^{7,8}

Enzymes able to degrade arabinans of various sources have been reviewed previously⁵ and include *endo*-arabinanases and *exo*-arabinanases to degrade the linear α -(1,5)-linked arabinan. For the degradation of branched arabinan, arabinofuranosidases (Abf A and B) are necessary to debranch the arabinan and to enable the degradation of the resulting linear α -(1,5)-linked arabinan backbone by the

endo- and *exo*-arabinanases.⁵ Complete insight of the linkage specificity of the arabinofuranosidases has not yet been achieved due to a lack of well-characterized branched arabino-oligosaccharides (AOS). Recently, novel arabinohydrolases from *Chrysosporium lucknowense*, Abn1 (*endo*-arabinanase), Abn2 (*exo*-arabinanase), and Abn4 (arabinofuranosidase), have been described.⁹ Together these enzymes were able to degrade 60% of the sugar beet arabinan resulting in the main degradation products arabinose and arabinobiose in the presence of significant amounts of AOS (DP3–6). Some of these oligosaccharides were hypothesized to be branched AOS.⁹ The isolation and identification of these unknown oligomeric arabinan structures would provide essential information for the detailed elucidation of the polymeric arabinan structure. In addition, the further characterization of arabinan-specific enzymes and possible exploration of these AOS for their prebiotic potential, as has been described for linear α -(1,5)-linked AOS,¹⁰ are of great interest. Therefore, this research deals with the isolation of these novel oligomeric branched arabinan structures derived from sugar beet arabinan by enzymatic degradation with a mixture of arabinohydrolases from *C. lucknowense* (Abn1, Abn2, and Abn4) and the characterization of purified branched AOS by NMR.

2. Results and discussion

2.1. Enzymatic preparation of AOS from sugar beet arabinan

Enzymatic degradation of sugar beet arabinan with a mixture of the arabinohydrolases Abn1, Abn2, and Abn4 (*C. lucknowense*)

* Corresponding author. Tel.: +31 317 482239; fax: +31 317 484893.
E-mail address: henk.schols@wur.nl (H.A. Schols).

releases the main degradation products arabinose and arabinobiose, but as well produces various unknown AOS, which elute differently in high performance anion exchange chromatography with pulsed amperometric detection (HPAEC–PAD) compared to linear α -(1,5)-linked AOS.⁹ To explore the precise structure of various unknown AOS, sugar beet arabinan was digested with two different treatments of Abn1, Abn2, and Abn4. Although sugar beet arabinan only contains 66% (w/w) arabinose in addition to significant amounts of residual rhamnogalacturonan-I, the use of pure and well-defined arabinohydrolases ensured specific degradation of the arabinan segments for this experiment. To the first digest (D-30) the arabinofuranosidase Abn4 has been added in a concentration that should ensure partial degradation of the side chains of sugar beet arabinan resulting in partly debranched backbone. HPAEC results showed that 30% of the maximal degradation by Abn4 took place. This equals the release of 6% of all arabinose present (data not shown). To the second digest (D-100) Abn4 has been added in an overdose, thereby allowing Abn4 to cleave all possible linkages by releasing about 18% of all arabinose present. This results in a heavily debranched arabinan backbone. Both digests were subsequently treated with a mixture of *endo*-arabinanase Abn1 and *exo*-arabinanase Abn2 to ensure degradation of the linear part of the arabinan present toward mono-, di-, and oligosaccharides. The HPAEC chromatograms of both enzyme digests, D-30 and D-100, are presented in Figure 1A and B, respectively. In both digests, the main degradation products were arabinose and arabinobiose. Their levels increased with an increase of Abn4 conversion level (D-30 to D-100). This is in good agreement with previous results.⁹ In addition to the monomer and dimer, several oligomeric structures were obtained as well. Most of these oligomeric structures do not co-elute with the α -(1,5)-linked AOS standards (Fig. 1), suggesting that these peaks are branched AOS as hypothesized already earlier by Kühnel et al.⁹ The peak at 19.1 min, which is present in the D-30 digest, and the peak at 22.6 min, which appears in the D-100 digest are the dominant peaks not co-eluting with linear α -(1,5)-linked AOS. Many more unknown AOS are present in minor quantities in both digests. As HPAEC analysis of both digests indicated the presence of various unknown AOS, both digests were subjected to further analysis.

2.2. Fractionation of the AOS derived from sugar beet arabinan after enzyme digestion

For detailed structural characterization of the AOS, a preparative fractionation based on size of both digests was performed. In Figures 2A and 3A the refractive index (RI) patterns of preparative Biogel P2 separations of both digests are given, including the DP as established using MALDI-TOF MS analysis (Figs. 2 and 3, inset tables). The total sugar analysis of all fractions taken (3.5 mL each) confirmed the RI patterns of both digests. Significant amounts of uronic acid were only detected in the first 15 fractions of the Biogel P2 separations. Sugar composition of these pools (fractions 1–15) of both digests is given in Table 1. It supports the assumption that the main peak in the beginning of the RI patterns in both digests consists of the remaining rhamnogalacturonan-I (RG-I) core structure ('RG-I remnants' in Figs. 2A and 3A) due to the presence of significant amounts of rhamnose, galacturonic acid, arabinose, and galactose. The arabinose levels (35 and 21 w/w% for D-30 and D-100, respectively) represent 34 and 11 w/w% of the arabinose present in the starting material (crude sugar beet arabinan), whereas the rest is present as small molecular weight material.

Fractions 18–71 from the Biogel P2 separations (3.5 mL each) have been analyzed by HPAEC and MALDI-TOF MS. The fractions have been pooled based on HPAEC analysis aiming at pools with high purity of the compounds present. The pool numbers are indicated as $I_{30-VII_{30}}$ and $I_{100-VIII_{100}}$ in Figures 2A and 3A for samples D-30 and D-100, respectively.

HPAEC showed that pools I_{30} and I_{100} consisted of arabinose monomers. In the following part HPAEC, MALDI-TOF MS, and NMR results of the various pools will be discussed in more detail. Concerning the NMR results of all resolved structures it can be stated that full assignment of both proton and carbon spectra was possible combining the data of the various 2D experiments (Table 2). All linkages could be confirmed with HMBC cross-peaks.

2.2.1. Dimers

NMR analysis of the pools II_{30} and II_{100} resulted in identical NMR data (Table 2). The component present was identified as an α -(1,5)-arabinobiose, which confirms the HPAEC results (data not shown). The NMR data are in agreement with data for α -(1,5)-arabinobiose.¹¹

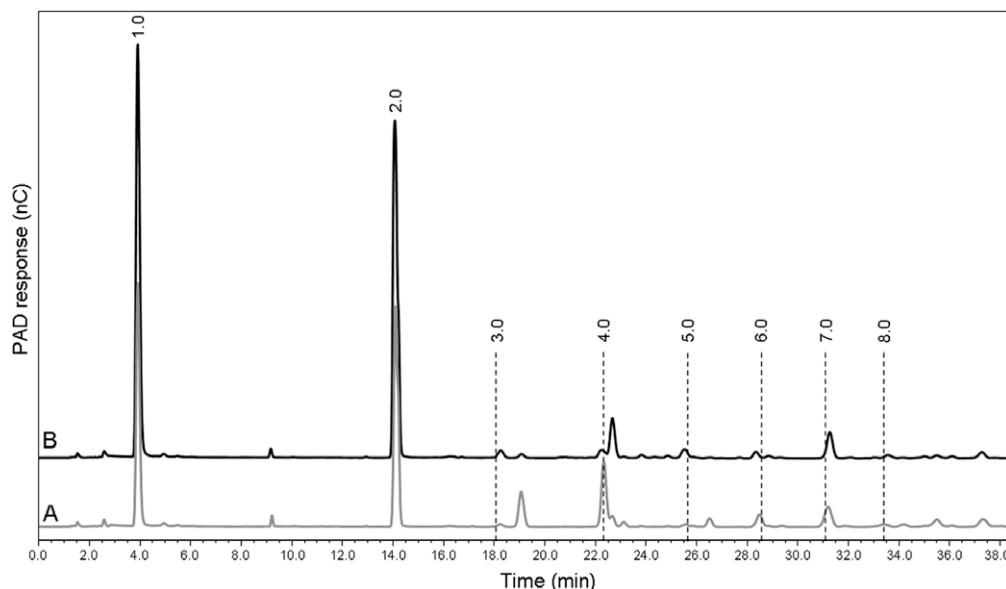


Figure 1. HPAEC elution pattern of AOS after degradation of sugar beet arabinan with different amounts of Abn4 followed by end-point-incubation with Abn1 and Abn2: D-30 (A), D-100 (B); indication of linear α -(1,5)-linked AOS (DP1–8; denoted 1.0–8.0).

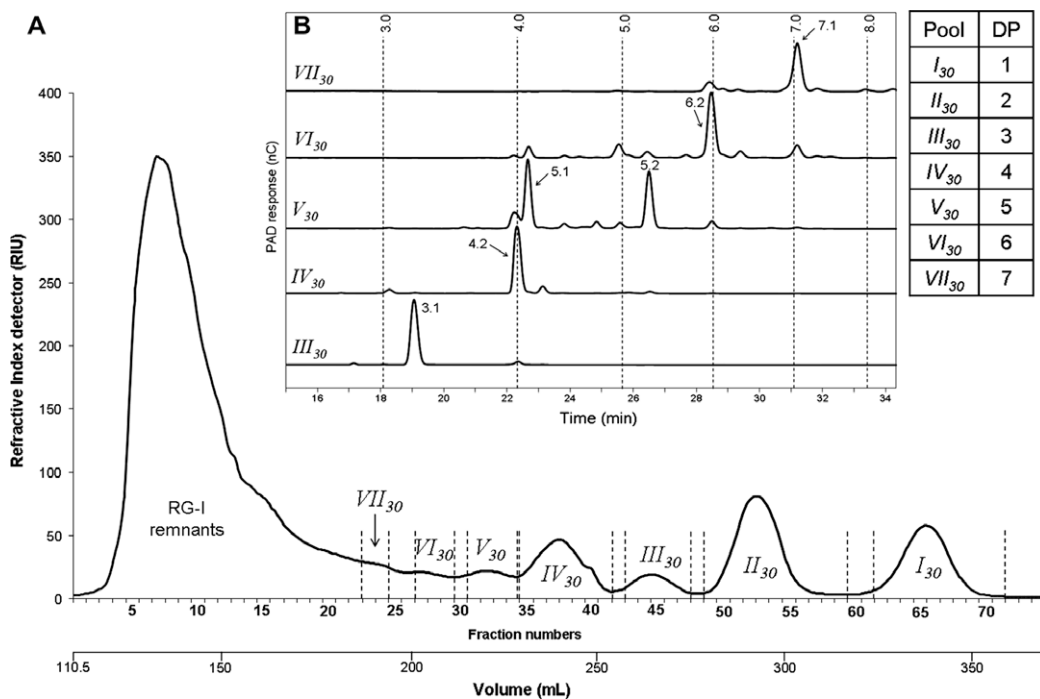


Figure 2. Biogel P2 elution pattern of the D-30 digest with indication of the pooled fractions (A); HPAEC elution pattern of pooled fractions (B; zoom) with indication of linear α -(1,5)-linked AOS (DP3–8); inset table represents the DP of the pools as analyzed with MALDI-TOF MS.

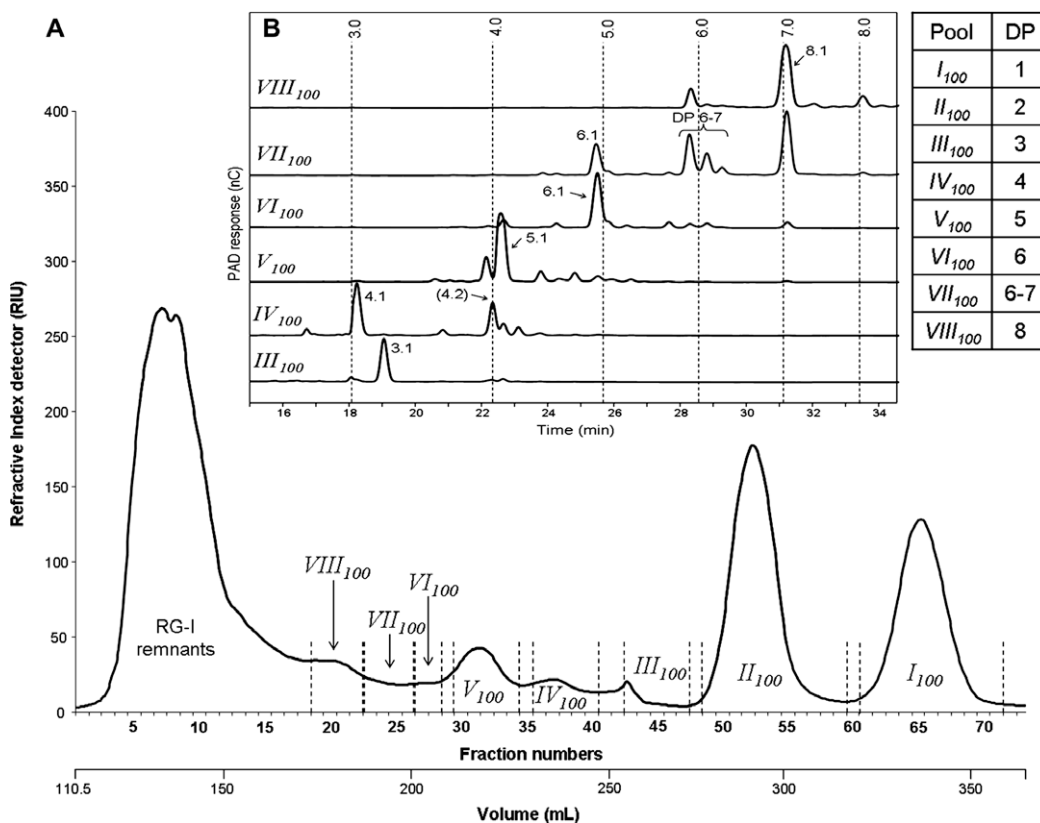


Figure 3. Biogel P2 elution pattern of the D-100 digest with indication of the pooled fractions (A); HPAEC elution pattern of pooled fractions (B; zoom) with indication of linear α -(1,5)-linked AOS (DP3–8); inset table represents the DP of the pools as analyzed with MALDI-TOF MS.

2.2.2. Trimers

HPAEC analysis of pools III₃₀ and III₁₀₀ reveals a major peak in the HPAEC chromatogram at 19.1 min for both samples, not

co-eluting with any linear α -(1,5)-linked AOS standard (Figs. 2B and 3B). MALDI-TOF MS indicates the presence of an oligomer with a degree of polymerization (DP) of 3 for both pools.

Table 1
Sugar composition (w/w%) of the 'RG-I remnants' (Biogel P2 fractions 1–15) of the D-30 and D-100 sugar beet arabinan digests

w/w%	Rha	Ara	Gal	Glc	GalA	Total sugar
D-30	4	35	17	4	10	70
D-100	5	21	21	6	11	64

Table 2
¹H and ¹³C NMR data of arabino-oligosaccharides identified from sugar beet arabinan

Pools <i>II</i> ₃₀ and <i>II</i> ₁₀₀	H-1	H-2	H-3	H-4	H-5R	H-5S	C-1	C-2	C-3	C-4	C-5
R α	5.265	4.04	4.04	4.24	3.769	3.87	104.01	84.18	78.71	84.23	69.69
R β	5.306	4.10	4.10	3.95	3.769	3.86	98.15	78.83	77.19	82.21	71.03
T	5.085	4.132	3.956	4.10	3.72	3.834	110.26	83.7 [†]	79.36	86.8 [†]	64.04
Pool <i>III</i> ₃₀	H-1	H-2	H-3	H-4	H-5R	H-5S	C-1	C-2	C-3	C-4	C-5
R α	5.257	4.04	4.06	4.236	3.77	3.86	103.98	84.22	78.55	84.10	69.13
R β	5.312	4.10	4.10	3.95	3.78	3.85	98.18	78.90	77.16	82.21	70.69
A	5.116	4.293	4.04	4.197	3.768	3.877	110.26	82.03	84.94	86.0 [†]	63.9
T	5.17	4.144	3.948	4.04	3.717	3.842	109.89	84.05	79.38	86.73	63.99
Pool <i>IV</i> ₃₀	H-1	H-2	H-3	H-4	H-5R	H-5S	C-1	C-2	C-3	C-4	C-5
R α	5.256	4.04	4.06	4.241	3.77	3.86	103.99	84.2	78.55	84.10	69.21
R β	5.308	4.10	4.10	3.95	3.78	3.85	98.19	78.88	77.15	82.16	70.73
A	5.122	4.294	4.10	4.319	3.856	3.95	110.29	81.92	85.2	84.52	69.29
T ₃	5.165	4.14	3.96	4.05	3.713	3.839	109.97	84.05	79.39	86.77	63.99
T ₅	5.097	4.14	3.96	4.115	3.734	3.839	110.18	83.78	79.39	86.83	63.99
Pool <i>IV</i> ₁₀₀	H-1	H-2	H-3	H-4	H-5R	H-5S	C-1	C-2	C-3	C-4	C-5
R α	5.26	4.04	4.06	4.242	3.78	3.86	103.99	84.17	78.58	84.10	69.21
R β	5.307	4.10	4.10	3.95	3.78	3.85	98.19	78.86	77.22	82.18	70.73
A	5.253	4.31	4.19	4.19	3.77	3.89	109.14	88.0 [†]	82.9	85.4 [†]	63.54
T ₂	5.189	4.13	3.97	4.08	3.727	3.84	109.86	84.1	79.31	86.98	63.93
T ₃	5.165	4.15	3.96	4.05	3.718	3.84	109.7	84.02	79.37	86.83	63.97
Pool <i>V</i> ₃₀	H-1	H-2	H-3	H-4	H-5R	H-5S	C-1	C-2	C-3	C-4	C-5
R α	5.26	4.04	4.05	4.24	3.77	3.86	103.99	84.21	78.56	84.10	69.22
R β	5.3	4.10	4.10	3.96	3.78	3.85	98.17	78.89	77.20	82.20	70.75
A	5.12 [†]	4.29 [†]	4.11	4.32	3.85	3.95	110.2	82.0 [†]	85.18	84.5 [†]	68.71
T ₃	5.16	4.14	3.96	4.05	3.725	3.84	110.04	84.07	79.40	86.77 ^a	63.97
B	5.125	4.3	4.04	4.221	3.77	3.88	110.2	82.04	84.95	86.08	63.89
T ₃	5.164	4.14	3.96	4.05	3.725	3.84	109.86	84.07	79.4	86.85 ^a	63.97
Pool <i>V</i> ₁₀₀	H-1	H-2	H-3	H-4	H-5R	H-5S	C-1	C-2	C-3	C-4	C-5
R α	5.26	4.04	4.05	4.24	3.782	3.86	104.03	84.2	78.62	84.12	69.41
R β	5.30	4.10	4.10	3.96	3.782	3.85	98.2	78.9	77.20	82.14	70.96
A	5.260	4.315	4.256	4.32	3.860	3.95	109.2	87.8 [†]	83.12	84.0	68.88
T ₂	5.189	4.14	3.97	4.08	3.725	3.84	109.8	84.2	79.40	86.92	63.96
T ₃	5.165	4.15	3.96	4.05	3.725	3.84	109.8	84.0	79.40	86.9	63.96
T ₅	5.092	4.14	3.96	4.11	3.725	3.84	110.19	83.93	79.44	86.69	63.96
Pool <i>VI</i> ₃₀	H-1	H-2	H-3	H-4	H-5R	H-5S	C-1	C-2	C-3	C-4	C-5
R α	5.26	4.04	4.06	4.24	3.77	3.85	103.98	84.21	78.56	84.1	69.22
R β	5.31	4.10	4.10	3.95	3.77	3.85	98.20	78.88	77.20	82.21	70.80
A	5.12 [†]	4.290	4.12	4.32	3.85	3.96	110.3 [†]	82.09	85.13	84.66	68.72
T ₃	5.162	4.15	3.96	4.05	3.72	3.84	109.94	84.09	79.40	86.74	63.95 ^b
B	5.133	4.303	4.10	4.346	3.77	3.85	110.21	81.9	85.21	84.66	69.27
T ₃	5.162	4.15	3.96	4.05	3.72	3.84	109.94	84.09	79.40	86.74	63.95 ^b
T ₅	5.098	4.14	3.96	4.12	3.73	3.84	110.23	83.8	79.44	86.83	64.00 ^b
Pool <i>VII</i> ₃₀	H-1	H-2	H-3	H-4	H-5R	H-5S	C-1	C-2	C-3	C-4	C-5
R α	5.26	4.04	4.06	4.24	3.773	3.86	104	84.22	78.55	84.10	69.2
R β	5.31	4.1	4.1	3.96	3.773	3.86	98.2	78.87	77.20	82.10	70.69
A	5.12 [†]	4.3	4.1	4.32	3.85	3.96	110.3 ^c	81.94 ^d	85.17	84.51	69.27 ^g
T ₃	5.164	4.15	3.96	4.05	3.73	3.84	109.96	84.08	79.40	86.74 ^f	63.9 ^h
B	5.09	4.14	4.05	4.23	3.807	3.901	110.3 ^c	83.72 ^e	79.37	85.08	69.07
C	5.127	4.3	4.1	4.32	3.85	3.96	110.27 ^c	81.97 ^d	85.17	84.51	69.38 ^g
T ₃	5.164	4.15	3.96	4.05	3.73	3.84	109.96	84.08	79.40	86.77 ^f	63.96 ^h
T ₅	5.097	4.14	3.96	4.12	3.73	3.84	110.2	83.79 ^e	79.44	86.83	64.00 ^h
Pool <i>VIII</i> ₁₀₀	H-1	H-2	H-3	H-4	H-5R	H-5S	C-1	C-2	C-3	C-4	C-5
R α	5.26	4.04	4.05	4.24	3.77	3.86	104	84.2	78.60	84.2	69.8
R β	5.31	4.09	4.1	3.96	3.77	3.86	98.2	78.9	77.20	82.1	71.2
A	5.10 [†]	4.14	4.04	4.22	3.9	3.807	110.34	84.08 ⁱ	79.40	85.0 [†]	69.21
B	5.13	4.29	4.12	4.32	3.86	3.96	110.24	82.05	85.12	84.44	68.86
T ₃	5.162	4.15	3.96	4.05	3.73	3.84	110.02	84.08 ⁱ	79.40	86.75	63.95
C	5.273	4.33	4.261	4.344	3.86	3.96	109.11	87.63	83.07	84.19	68.86
T ₂	5.191	4.14	3.97	4.08	3.73	3.85	109.68	84.21	79.40	86.92	63.95
T ₃	5.162	4.15	3.96	4.05	3.73	3.84	109.78	84.11 ⁱ	79.40	86.87	63.95
T ₅	5.092	4.14	3.96	4.12	3.73	3.84	110.26	83.96 ⁱ	79.47	86.68	63.95

[†] Signal broadening or splitting due to anomerization effect; superscript values may have to be interchanged.

Apparently, both pools contain the same AOS (denoted 3.1) with a purity of >90% determined by HPAEC. NMR analysis was carried out with pool *III*₃₀. The major component (3.1) could be assigned as a dimeric α-(1,5)-linked arabinan backbone with an α-(1,3)-linked arabinose residue at the non-reducing end (Table 3; structure 3.1) due to the following NMR characteristics: compared to the data for α-(1,5)-arabinobiose, the α-(1,3)-linkage

Table 3 (continued)

Series 1		Series 2	
Structures of identified arabino-oligosaccharides from sugar beet arabinan			
DP6		VI ₃₀	6.2
DP7		VII ₃₀	7.1
DP8		VIII ₁₀₀	8.1

of a third arabinose residue (Table 3, T-residue) is indicated by a cross-peak in the HMBC between H-1 of this T-residue and the C-3 of the A-residue (Fig. 4, T1/A3). The downfield shift of 5.6 ppm for C-3 in the arabinose A-residue relative to the C-3 of the T-residue of an α -(1,5)-arabinobiose and the smaller upfield shifts for C-2 and C-4 of 1.4 ppm and 0.8 ppm, respectively, confirm the α -(1,3)-linkage of the arabinose T-residue (Table 2; ^{12–14}). To enable the distinction between the linear α -(1,5)-linked arabinotriose (3.0) and the novel branched arabinotriose, the peak at 19.1 min was denoted 3.1.

2.2.3. Tetramers

Pools *IV*₃₀ and *IV*₁₀₀ contain oligomers of DP4 as analyzed with MALDI-TOF MS (Figs. 2B and 3B, inset tables). HPAEC analysis of *IV*₃₀ showed one major peak, which elutes at the retention time of the linear α -(1,5)-linked arabinotetraose (Fig. 2B; 22.3 min). To investigate if a co-eluting branched AOS is present, pool *IV*₃₀ has been analyzed by NMR. In the ¹³C-spectra of pool *IV*₃₀ the downfield shift of 5.4 ppm for the C-5 of the A-residue (Tables 2 and 3; structure 4.2) compared to the A-residue of the component 3.1 in pool *III*₃₀ indicates the presence of an additional α -(1,5)-linked residue. An upfield shift of 1.5 ppm for the C-4 of the A-residue (Table 2) and a HMBC cross-peak between H-1 of the T5-residue and C-5 of the A-residue confirms the presence of an α -(1,5)-linked T5-residue (Table 2). Following these NMR data, the component present in pool *IV*₃₀ (denoted 4.2) could be assigned as a trimeric α -(1,5)-linked arabinan backbone with an α -(1,3)-linked arabinose residue attached at the middle arabinose unit (Table 3; structure 4.2). Thus, NMR data reveal that the main tetrameric component in the D-30 digest is a branched AOS (4.2) instead of the linear α -(1,5)-linked AOS (4.0). These two structures are apparently co-eluting in HPAEC with the separation conditions used.

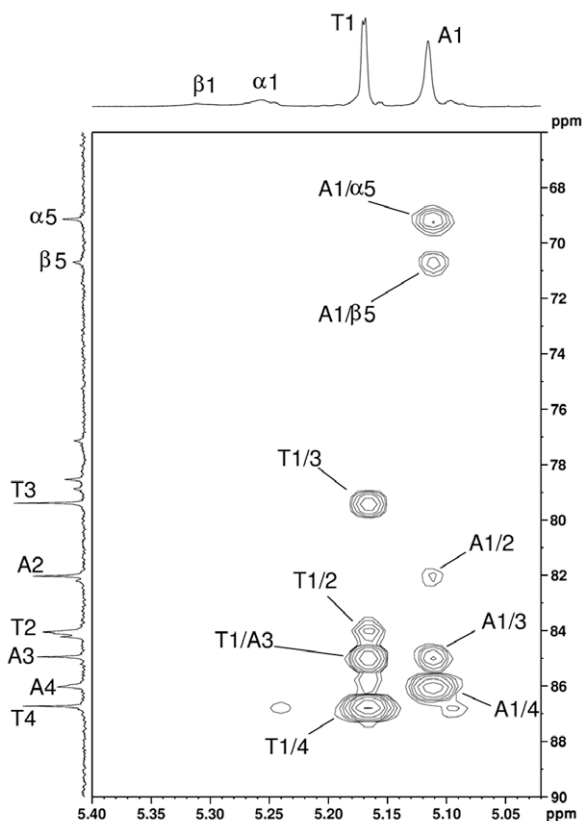


Figure 4. [¹H,¹³C]-HMBC spectrum of pool *III*₃₀ (zoom at 5.40–5.00 ppm (¹H) and 66–90 ppm (¹³C), respectively); T and A as indicated in Tables 2 and 3.

According to HPAEC analysis, the pool *IV*₁₀₀ contains two major peaks (Fig. 3B; 18.2 min (denoted 4.1) and 22.3 min (4.0 or 4.2)) next to a number of minor peaks. NMR analysis confirms the presence of two major components. The first component is identical to the one assigned in pool *IV*₃₀ (4.2). The second component could be identified having an H-1 signal shifted downfield to 5.253 ppm of the A-residue (Tables 2 and 3; structure 4.1). The HMBC shows a cross-peak with the C-2 of this residue (arabinose-A), and from this C-2 a cross-peak with another H-1 can be found in the HMBC, indicating an α -(1,2)-linkage. Signals for an α -(1,3)-linked T3-residue were also found. Compared to pool *III*₃₀ (3.1), the C-2 of the A-residue is shifted downfield with 6.0 ppm and the C-3 and C-1 are shifted upfield with 2.0 ppm and 1.1 ppm, respectively (Table 2), confirming the α -(1,2)-linkage of the T2-residue in pool *IV*₁₀₀ (4.1). Conclusively, the NMR data reveal the second component (denoted 4.1) as a dimeric α -(1,5)-linked arabinan backbone with an α -(1,2)-linked and an α -(1,3)-linked arabinose residue at the non-reducing end (Table 2; structure 4.1).

2.2.4. Pentamers

MALDI-TOF MS reveals that only oligomer(s) with DP5 are present in both pools (*V*₃₀ and *V*₁₀₀, inset table in Figs. 2B and 3B). According to HPAEC, pool *V*₃₀ consists of two major oligosaccharides present in about equal amounts (Fig. 2B; denoted 5.1 and 5.2; 22.6 min and 26.5 min, respectively), whereas pool *V*₁₀₀ showed the presence of only one major peak at 22.6 min (denoted 5.1; Fig. 3B).

The first component (5.1; 22.6 min) elutes close to the retention time of the linear α -(1,5)-linked arabinotetraose (Fig. 3B; 4.0; 22.3 min). The second component, present in *V*₃₀, represents another DP5-AOS (denoted 5.2; 26.5 min; Fig. 3B) with substantially different retention behavior compared to AOS 5.1, but with a similar retention behavior compared to the linear α -(1,5)-linked arabinopentaose (5.0; 25.6 min). For further characterization of the AOS 5.1, the pool *V*₁₀₀ was analyzed by NMR as this pool contains the AOS in high purity. In pool *V*₁₀₀ all signals for the A-residue typical for (1,2), (1,3), and (1,5)-linkages as identified in *IV*₃₀ and *IV*₁₀₀ are present. Firstly, the H-1 at 5.26 ppm and the C-2 at 87.8 ppm indicate a (1,2)-linkage. Secondly, the chemical shift of C-3 at 83.12 ppm, which results from the combination of a downfield shift due to a (1,3)-linkage and a small upfield shift due to a (1,2)-linkage, indicates a (1,3)-linkage in combination with a (1,2)-linkage. Thirdly, the C-5 at 68.88 ppm indicates a (1,5)-linkage (Table 2). These data are in good agreement with Capek et al.¹¹ In the HMBC cross-peaks between all three terminal residues (T2, T3, and T5) and the A-residue could be assigned (Fig. 5), resulting in a structure as shown in Table 3 (structure 5.1). The cross-peaks denoted as X (most likely C-4) and Y are not belonging to the main component as is clear from the ¹³C-spectrum, in which the signals belonging to this cross-peak are too low. The signals are visible in the HMBC due to the higher sensitivity of this proton detected 2D experiment and due to the high intensity of cross-peaks between H-1 and C-4 in arabinoses.

Although a mixture of two major compounds was present in pool *V*₃₀, with component 5.1 (pool *V*₁₀₀) as one of them, it was possible to determine the structure of the second compound, because of the presence of three characteristic signals: A signal at 86.08 ppm, assigned as C-4 of the B-residue with only an α -(1,3)-linked arabinose attached to it (compare with pool *III*₃₀ (3.1)), and two signals at 85.18 ppm and 84.95 ppm for the A-residue and B-residue, respectively (Tables 2 and 3). This is typical for C-3 signals in arabinoses with only α -(1,3)-linked arabinose attached to it. Due to proximity of these two C-3 signals, the almost identical assignments for the two T3-residues and the lower resolution of the 2D HMBC experiment, only one combined cross-peak in the HMBC confirms the two α -(1,3)-linkages. A cross-peak between

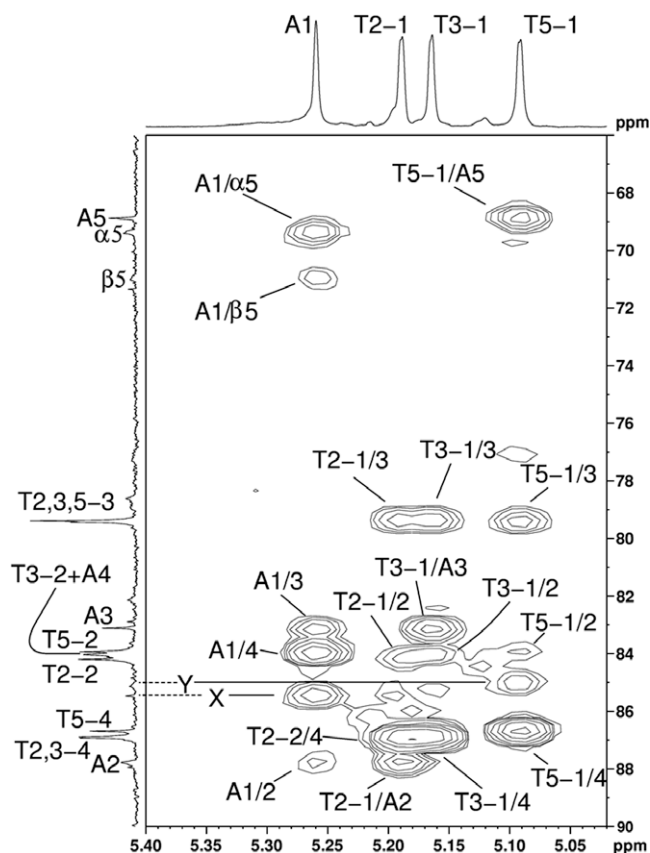


Figure 5. ^1H , ^{13}C -HMBC spectrum of pool V_{100} (zoom at 5.40–5.00 (^1H) and 66–90 (^{13}C), respectively); T_n , A, B and C as indicated in Tables 2 and 3.

H-1 of the B-residue and C-5 of the A-residue connects the two α -(1,3)-substituted arabinoses (data not shown). Cross-peaks between the A-residue and the reducing end arabinose complete the assignment of this structure, resulting in the structure as shown in Table 3 for component 5.2.

2.2.5. Hexamers

HPAEC analysis of the pools V_{30} and V_{100} reveals the presence of each one major peak at 28.4 min and 25.5 min, respectively (Figs. 2B and 3B). As MALDI-TOF MS shows the presence of only oligomers of DP6, these AOS are assigned as 6.2 and 6.1, respectively. NMR analysis shows that the pool V_{30} is similar to pool V_{30} with respect to the presence of two (1,3)-linked residues as indicated by two signals for C-3 at 85.13 and 85.21 ppm (Table 2) together with a combined cross-peak with H-1 of the T3-residues, and a cross-peak between H-1 of the B-residue and C-5 of the A-residue (data not shown). In pool V_{30} the C-4 signal of the B-residue indicates the presence of an additional (1,5)-linked T5-residue, confirmed by a cross-peak between H-1 of the T5-residue and C-5 of the B-residue in the HMBC (data not shown). Following the NMR data, compound 6.2 could be assigned as tetrameric α -(1,5)-linked arabinan backbone with α -(1,3)-substitution of single arabinose residues at the two middle arabinose units as depicted in Table 3 (structure 6.2).

In pool V_{100} signals similar to those in pool V_{30} could be assigned (Table 2), indicating that the same structural element, an arabinose with (1,2)- and (1,3)-linked arabinose residues attached to it, must be present. The two residues between this element and the reducing end, needed to complete the structure could not be assigned due to a large heterogeneity in the spectra. Thus, even though HPAEC showed only one major peak at 25.5 min, NMR analysis revealed that more than one component must be present, indicating insufficient separation of HPAEC for these compounds.

2.2.6. Heptamers

The pool V_{30} shows one major peak at 31.2 min during HPAEC analysis (Fig. 2B; denoted 7.1), co-eluting with the linear α -(1,5)-linked AOS (7.0). MALDI-TOF MS analysis shows the presence of an oligomer of DP7. NMR analysis shows that the pool V_{30} has all the features of pool V_{30} : two (1,3)-linked arabinose residues (T3-residues) and one (1,5)-linked T5-residue (Table 2). An additional signal at 85.08 ppm, assigned as C-4, indicates the presence of an additional (1,5)-linked arabinose in the backbone. Two positions for this additional residue are possible: Between the two (1,3)-substituted arabinoses (T3) or between the first (1,3)-substituted arabinose (T3) and the reducing end (Table 3; structure 6.2). The first possibility with (1,3)-linked residues on the A-residue and the C-residue, respectively, represents the main component in this pool (7.1). HMBC cross-peaks between the H-1 of the C-residue and the C-5 of the B-residue confirm this assignment (data not shown), resulting in the structure as depicted in Table 3 (structure 7.1). The latter possibility would result in slightly different signals for the C-5 of the reducing end and is found for the component in V_{100} (see discussion there).

According to HPAEC and MALDI-TOF MS analysis the pool V_{100} contains a mixture of components with DP6 and DP7, thus, no further NMR analysis was done for pool V_{100} .

2.2.7. Octamers

HPAEC analysis of pool V_{100} reveals the presence of one major peak at 31.2 min, co-eluting with the linear α -(1,5)-linked arabinooctaose (7.0) and component 7.1. MALDI-TOF MS results show the presence of mainly DP8, thus, the unknown component represents an arabinooctaose (denoted 8.1) with a substantially different retention behavior compared to linear α -(1,5)-linked arabinooctaose (8.0; 33.4 min; Fig. 3B). NMR analysis shows that in pool V_{100} all signals of a triple substituted arabinose are present as were assigned for V_{100} (compare pool V_{100} with V_{100} in Table 2). In the HMBC at the position of H-1 of the T3-residues two cross-peaks are found with two different C-3 signals: at 83.07 ppm (C-residue, Tables 2 and 3), characteristic for a (1,3)-linkage in combination with a (1,2)-linkage as mentioned in pool V_{100} , and at 85.12 ppm (B-residue, Tables 2 and 3), indicating a (1,3)-linkage without (1,2)-substitution at the same residue (similar to V_{30} (4.2), V_{30} (5.2), V_{30} (6.2), and V_{30} (7.1)). As in V_{30} a C-4 signal at 85.0 ppm indicates the presence of an additional (1,5)-linked arabinose residue, which is located next to the reducing end due to a clear anomerization effect of this signal (A-residue, Tables 2 and 3). This is, furthermore, substantiated by cross-peaks in the HMBC between the H-1 of this A-residue and the C-5 signals of the reducing end (R α/β). The chemical shifts of these C-5 carbons are slightly different for those of all structures with a (1,3)-substituted A-residue, but resembles the chemical shifts found for α -(1,5)-arabinobiose, confirming the presence of an arabinose residue attached to the reducing end with no (1,3)-substitution (Table 2). Following all the NMR data, component 8.1, which is present in V_{100} , has a structure as depicted in Table 3.

2.3. Overview of AOS identified from sugar beet arabinan

In Table 3 an overview of the structures of all identified branched AOS derived from sugar beet arabinan is given as based on extensive NMR analysis. All of them consist of an α -(1,5)-linked backbone of L-arabinosyl residues. Two main structural features could be identified among all identified AOS, varying in their type of linkages and the degree of substitution. AOS of the first series contain a structure with double substituted α -(1,2)- and α -(1,3)-linked L-arabinosyl residues (4.1, 5.1, 8.1; Table 3, series 1). An additional single substituted α -(1,3)-linked L-arabinosyl residue

may be present within the same molecule as identified in component 8.1 (Table 3). AOS of the second series carry single substituted α -(1,3)-linked arabinose(s) (Table 3; series 2). Components with either one or two α -(1,3,5)-linkages were identified (3.1, 4.2 and 5.2, 6.2, 7.1, respectively).

None of the identified structures was substituted at the arabinose at the reducing end, which is in contrast with the synthesized methyl 2-O, methyl 3-O- and methyl 5-O- α -L-arabinofuranosyl- α -L-arabinofuranosides as described by Kaneko et al.¹⁵ The isolated component 3.1 is similar to an earlier described feruloylated arabinose-oligosaccharide with an α -L-arabinosyl residue linked at O-3 and a ferulic acid attached at O-2 of the non-reducing end of an α -(1,5)-linked dimeric backbone of L-arabinosyl residues, which has been isolated from spinach leaves¹⁶ and sugar beet pulp.¹⁷

Almost all AOS of the second series (Table 3; 3.1, 4.2, 5.2, 6.2, and 7.1) were exclusively present in the D-30 digest, while the three isolated AOS belonging to the first series were mainly present in the D-100 digest. This indicates a different degradability of the structures by the arabinofuranosidase Abn4. Abn4 seems to be able to remove α -(1,3,5)-linked L-arabinosyl residues (present in the AOS of the second series; Table 3), whereas a α -(1,2,3,5)-linked double substituted structure as present in the AOS of the first series (Table 3) is not degradable by the arabinofuranosidase Abn4. Further investigation of the precise mode of action and specificity of the arabinohydrolases is currently ongoing.

3. Conclusions

Eight novel neutral branched AOS have been isolated from sugar beet arabinan after enzyme digestion with two different mixtures of the *C. lucknowense* arabinohydrolases Abn1, Abn2, and Abn4. NMR analysis revealed two series of branched AOS varying in the type of linkage. To the best of our knowledge, this is the first characterization of such branched AOS. These AOS may now be used for (further) characterization of arabinan-specific enzymes as well as for possible exploration of their prebiotic potential.

4. Experimental

4.1. Materials

Branched sugar beet arabinan was obtained from British Sugar (McCleary¹⁸). The arabinose content is 66% (w/w%), the remaining part consists of hairy regions (rhamnose, galacturonic acid, and galactose) and glucans (glucose).⁹

Linear AOS (DP2–8) have been purchased from Megazyme International Ltd (Bray, Ireland), and were denoted 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0, respectively.

Branched AOS were denoted according to elution order during HPAEC analysis.

4.2. Enzymatic degradation of sugar beet arabinan

For fractionation and isolation of branched AOS two times 1 g of branched sugar beet arabinan was digested in 200 mL water set at pH 5 (30 °C) with the arabinohydrolases Abn1, Abn2, and Abn4 (*C. lucknowense* strain C1⁹). One arabinan batch (D-100) was incubated for 15 h with an overdose of Abn4 (1.1 U), whereas another batch (D-30) was incubated for 15 h with 0.22 U Abn4 ($t = 15$ h) resulting in about 30% of maximal degradation by Abn4. This enzyme dosage was calculated based on the fact that about 18% of arabinose present can be degraded by Abn4.⁹ The degradation was monitored by arabinose monomer release by HPAEC. After inactivation of the enzyme by boiling for 10 min, both incubations were followed by an end-point degradation of Abn1 and Abn2

($t = 24$ h, 4.8 U and 4.4 U, respectively). After enzyme incubation, the samples were freeze-dried.

4.3. High performance anion exchange chromatography (HPAEC, pH 12)

Arabinose and AOS were determined by HPAEC with pulsed amperometric detection (PAD). A HPAEC system (ICS-3000, Dionex Corporation, Sunnyvale, CA, USA) was equipped with a CarboPac PA-1 separation column (2 mm ID \times 250 mm; Dionex Corporation) and a CarboPac PA-1 guard column (2 mm ID \times 25 mm; Dionex Corporation). A flow of 0.3 mL/min was used and the temperature was kept at 20 °C. AOS (injection volume 10 μ L; 10–100 μ g/mL) were separated using a gradient with 0.1 M NaOH (solution A) and 1 M NaOAc in 0.1 M NaOH (solution B): 0–36 min from 0% B to 42% B, 36–42 min at 100% B, and 42–57 min at 0% B.

4.4. Fractionation based on size: Biogel P2

Freeze-dried material of both digests was each dissolved in 22.5 mL water and subsequently centrifuged. Fractionation of the supernatants was performed on an Äkta Explorer system (Amersham Biosciences, Uppsala, Sweden) equipped with a Bio-Gel P2 column (porous polyacrylamide, 1000 \times 26 mm, 200–400 mesh, Bio-Rad Laboratories, Hercules, CA, USA) thermostated at 60 °C and eluted with Millipore water at 1.0 mL/min. Twenty milliliters of each sample (50 mg/mL) were injected. The column efflux was first led through a refractive index detector (Shodex RI72, Showa Denko K.K., Tokyo, Japan) and secondly collected in fractions of 3.5 mL by a fraction collector (Superfrac, GE Amersham, Uppsala, Sweden). Appropriate fractions were pooled and freeze-dried for further analysis.

4.5. Determination of neutral sugar and uronic acid content of the Biogel P2 fractions

The total neutral sugar and uronic acid content was determined with automated colorimetric assay analyzer. The total neutral sugar content was determined by using the orcinol–sulfuric acid color assay with arabinose (25–200 μ g/mL) as standard curve.¹⁹ The uronic acid content was determined with the metahydroxy-biphenyl assay based on a standard curve of galacturonic acid (12.5–100.0 μ g/mL).²⁰

4.6. MALDI-TOF MS

Each sample was desalted with AG 50W-X8 Resin (Bio-Rad Laboratories, Hercules, CA, USA) and 1 μ L of the desalted sample solution was mixed on a MALDI-plate (Bruker Daltonics, Bremen, Germany) with 1 μ L matrix solution of 12 mg/mL 2,5-dihydroxy benzoic acid (Bruker Daltonics) in 30% acetonitrile and dried under a stream of air.²¹ MALDI-TOF MS analysis was performed using an Ultraflex workstation (Bruker Daltonics) equipped with a nitrogen laser of 337 nm and operated in positive mode. After a delayed extraction time of 350 ns, the ions were accelerated to a kinetic energy of 22,000 V and detected using reflector mode. The lowest laser power required to obtain good spectra was used and 200 spectra were collected for each measurement. The mass spectrometer was calibrated with a mixture of maltodextrins (Avebe, Foxhol, The Netherlands; MD20; mass range m/z 500–2000). The data were processed using Bruker Daltonics flexAnalysis version 2.2.

4.7. NMR analysis

Samples (1–6 mg) have been exchanged with D₂O (99.9 atom % D, Sigma–Aldrich, St. Louis, MO, USA) and subsequently dissolved

in 0.5 mL D₂O (99.9 atom % D, Sigma–Aldrich) containing 0.75% 3-(trimethylsilyl)-propionic-2,2,3,3-*d*₄ acid, sodium salt (TMSP, Sigma–Aldrich). NMR spectra were recorded at a probe temperature of 300 K on a Bruker Avance-III-600 spectrometer, equipped with a cryo-probe located at Bionalys (Wageningen, The Netherlands). Chemical shifts are expressed in parts per million (ppm) relative to internal TMSP at 0.00 ppm. 1D and 2D COSY, TOCSY, HMBC, and HMQC spectra were acquired using standard pulse sequences delivered by Bruker. For the ¹H-COSY and -TOCSY spectra, 400 experiments of two scans were recorded, resulting in measuring times of 0.5 h. The mixing time for the TOCSY spectra was 100 ms. For the [¹H, ¹³C]-HMBC and -HMQC spectra 800 experiments of 32 scans and 512 experiments of 8 scans, respectively, were recorded, resulting in measuring times of 8.7 h and 2.5 h, respectively.

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References

- Albersheim, P.; Darvill, A. G.; O'Neill, M. A.; Schols, H. A.; Voragen, A. G. J. In *Progress in Biotechnology 14: Pectins and Pectinases*, Proceedings of an International Symposium, Wageningen (NL), 1995; Visser, J., Voragen, A. G. J., Eds.; Elsevier: Amsterdam (NL), 1996; pp 47–55.
- Ralet, M. C.; Thibault, J. F. *Biomacromolecules* **2002**, *3*, 917–925.
- Ridley, B. L.; O'Neill, M. A.; Mohnen, D. A. *Phytochemistry* **2001**, *57*, 929–967.
- Voragen, A. G. J.; Pilnik, W.; Thibault, J. F.; Axelos, M. A. V.; Renard, C. M. G. C. In *Food Polysaccharides and their Applications*; Stephen, A. M., Ed.; Marcel Dekker: New York, Basel, Hong Kong, 1995; pp 287–339.
- Beldman, G.; Schols, H. A.; Pitson, S. M.; Searle-van Leeuwen, M. F.; Voragen, A. G. J. In *Advances in Macromolecular Carbohydrate Research*; Sturgeon, R. G., Ed.; JAI Press, 1997; Vol. 1, pp 1–64.
- Weinstein, L.; Albersheim, P. *Plant Physiol.* **1979**, *63*, 425–432.
- Ishii, T. *Plant Cell Physiol.* **1994**, *35*, 701–704.
- Levigne, S. V.; Ralet, M. C. J.; Quemener, B. C.; Pollet, B. N. L.; Lapiere, C.; Thibault, J. F. *Plant Physiol.* **2004**, *134*, 1173–1180.
- Kühnel, S.; Hinz, S. W. A.; Pouvreau, L.; Visser, J.; Schols, H. A.; Gruppen, H. *Bioresour. Technol.*, accepted for publication.
- Suzuki, Y.; Tanaka, K.; Nano, T.; Asakura, T.; Muramatsu, N. *Jpn. Soc. Hortic. Sci.* **2004**, *73*, 574–579.
- Cros, S.; Imbert, A.; Bouchemal, N.; Dupenhoat, C. H.; Perez, S. *Biopolymers* **1994**, *34*, 1433–1447.
- Capek, P.; Toman, R.; Kardosova, A.; Rosik, J. *Carbohydr. Res.* **1983**, *117*, 133–140.
- Dourado, F.; Cardoso, S. M.; Silva, A. M. S.; Gama, F. M.; Coimbra, M. A. *Carbohydr. Polym.* **2006**, *66*, 27–33.
- Cardoso, S. M.; Silva, A. M. S.; Coimbra, M. A. *Carbohydr. Res.* **2002**, *337*, 917–924.
- Kaneko, S.; Kawabata, Y.; Ishii, T.; Gama, Y.; Kusakabe, I. *Carbohydr. Res.* **1995**, *268*, 307–311.
- Ishii, T.; Tobita, T. *Carbohydr. Res.* **1993**, *248*, 179–190.
- Colquhoun, I. J.; Ralet, M. C.; Thibault, J. F.; Faulds, C. B.; Williamson, G. *Carbohydr. Res.* **1994**, *263*, 243–256.
- McCleary, B.V.; Cooper, J.M.; Williams, E.L. Pat. Application, GB 88283809, 1989.
- Tollier, M. T.; Robin, J. P. *Ann. Technol. Agric.* **1979**, *28*, 1–15.
- Ahmed, E. R. A.; Labavitch, J. M. *J. Food Biochem.* **1978**, *1*, 361–365.
- Verhoef, R.; Beldman, G.; Schols, H. A.; Siika-Aho, M.; Ratto, M.; Buchert, J.; Voragen, A. G. J. *Carbohydr. Res.* **2005**, *340*, 1780–1788.