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# Step-wise enzymatic preparation and structural characterization of singly and doubly substituted arabinoxylo-oligosaccharides with non-reducing end terminal branches

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ABSTRACT

Shearzyme (GH10 *endo*-1,4-β-D-xylanase) and two different α-L-arabinofuranosidases (AXH-m and AXH-d3) were used stepwise to manufacture arabinoxylo-oligosaccharides (AXOS) with α-L-Ara*f* (1→2)-monosubstituted β-D-Xyl*p* residues or α-L-Ara*f* (1→2)- and (1→3) doubly substituted β-D-Xyl*p* residues from wheat arabinoxylan (AX) in a rather straightforward way. Four major AXOS (d-I, d-II, m-I and m-II) were formed in two separate hydrolyses. The AXOS were purified and the structures were confirmed using TLC, HPAEC-PAD, MALDI-TOF-MS and 1D and 2D NMR spectroscopy. The samples were identified as d-I: α-L-Ara*f*-(1→2)-[α-L-Ara*f*-(1→3)]-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-D-Xyl*p*, d-II: α-L-Ara*f*-(1→2)-[α-L-Ara*f*-(1→3)]-β-D-Xyl*p*-(1→4)-D-Xyl*p*, m-I: α-L-Ara*f*-(1→2)-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-D-Xyl*p* and m-II: α-L-Ara*f*-(1→2)-β-D-Xyl*p*-(1→4)-D-Xyl*p*. To our knowledge, this is the first report on structural $^{1}H$ and $^{13}C$ NMR analysis of xylobiose-derived AXOS d-II and m-II. The latter compound has not been reported previously. The doubly substituted AXOS were produced for the first time in good yields, as d-I and d-II corresponded to 11.8 and 5.6 wt% of AX, respectively. Singly α-L-Ara*f* (1→2)-substituted AXOS could also be prepared in similar yields by treating the doubly substituted AXOS further with AXH-d3.



## 1. Introduction

Plant secondary cell walls consist of three major polymers: cellulose (30–45%), hemicelluloses (25–45%) and lignin (15–30%).[1] Xylans from the hemicellulose group are composed of (1→4)-linked β-D-xylopyranosyl (β-D-Xyl*p*) residues in the linear main chain.[2] Xylans may carry α-L-arabinofuranosyl (α-L-Ara*f*), α-D-glucopyranosyluronic acid or its 4-*O*-methyl ether, and acetyl, feruloyl and *p*-coumaroyl side groups.[3,4] Arabinoxylans (AX), with (1→2)- and/or (1→3)-linked α-L-Ara*f* substituents, are the main cell wall components of cereals. They are often divided into water-extractable and water-unextractable AX fractions.[5] In different cereal species and parts of the plant, AX differ in the manner of substitution of the xylan backbone. α-L-Ara*f* side groups are prevalently (1→3)-linked to β-D-Xyl*p* residues in all cereals.[6–9] AX in wheat flour is relatively highly branched, containing an average of 35–40% of α-L-Ara*f* substituted β-D-Xyl*p* units but an alternation of highly branched and less branched regions was proposed.[10,11] Water-extractable wheat bran AX possesses the highest amounts of doubly (1→2)- and (1→3)-substituted β-D-Xyl*p* units (40%). Monosubstituted β-D-Xyl*p* units with (1→2)-linked α-L-Ara*f* groups are most common in water-unextractable barley AX (10%), but they have been found in small amounts in all cereal AX except those in rye endosperm.[8,12,13]

The AX backbone can be hydrolysed using *endo*-1,4-β-D-xylanases (EC 3.2.1.8) to produce arabinoxylo-oligosaccharides (AXOS). Xylanases from glycoside hydrolase (GH) family 10 are able to produce shorter AXOS than GH11 xylanases.[14,15] In our recent work, GH10 xylanase from *Aspergillus aculeatus* effectively hydrolysed rye AX into short AXOS, the main AXOS being arabinoxylobiose α-L-Ara*f*-(1→3)-β-D-Xyl*p*-(1→4)-D-Xyl*p*.[16] The α-L-arabinofuranosidases (EC 3.2.1.55) are enzymes that cleave terminal α-L-Ara*f* residues from different polysaccharides and oligosaccharides. The α-L-arabinofuranosidases acting on polymeric AX (arabinoxylan arabinofuranohydrolases, AXH) are further divided according to their substrate specificities, since some act on (1→2)- and (1→3)-linked α-L-Ara*f* units on monosubstituted β-D-Xyl*p* residues (AXH-m), whereas others release only (1→3)-linked α-L-Ara*f* units from disubstituted β-D-Xyl*p* residues (AXH-d3).[17] Most α-L-arabinofuranosidases isolated, such as from *Aspergillus awamori*[18], *Pseudomonas fluorescens*[19] and wheat[20], are active only on α-L-Ara*f* residues on singly substituted β-D-Xyl*p* residues (AXH-m). AXH-d3-type α-arabinofuranosidases were isolated from *Bifidobacterium adolescentis*[17,21] and *Humicola insolens*.[22] The first reported α-arabinofuranosidase, able to release α-L-Ara*f* from both singly and doubly substituted β-D-Xyl*p*, was isolated from barley malt.[23] This α-arabinofuranosidase is able to act on linkages at non-reducing terminal

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β-D-Xyl*p* with one or two α-L-Ara*f* substituents, but shows no activity towards doubly substituted internal β-D-Xyl*p*. Highly specific AXH-m and AXH-d3, which act on polymers and oligomers, are excellent tools to use in modifying the branching ratio of AX and AXOS.

AX and AXOS showed promising prebiotic properties in several studies.[24] Some studies investigated the relationship between the structure of AX and the growth of mixed intestinal bacteria cultures.[25–27] Since AXOS differ in degree of polymerization and in degree of substitution, the diversity of these compounds is higher than in other commercial prebiotic carbohydrates. Few attempts have been made to study the prebiotic properties of AXOS.[28–30] In evolving prebiotic compounds, the target is to increase the persistence of prebiotics in the colon, since slow fermentation was related to decreasing the risk of colon cancer.[24] One way to prolong the digestion of prebiotics is to use structurally more complex oligosaccharides, for example, AXOS. Until recently, little information has been available on which AXOS would be the most beneficial. The challenge for these studies is to have enough purified and well-defined AXOS preparations.

We previously prepared arabinoxylobiose with (1→3)-linked α-L-Ara*f*.[16] The aim of the present study was to produce other pure short-chain AXOS with feasible yield in a rather straightforward way. The purpose was to use a combination of different α-L-arabinofuranosidases and *endo*-1,4-β-D-xylanase to manufacture AXOS with α-L-Ara*f* (1→2)-monosubstituted β-D-Xyl*p* residues or α-L-Ara*f* (1→2) and (1→3) doubly substituted β-D-Xyl*p* residues from wheat AX. Another objective was to identify the exact structure of the main AXOS formed.

## 2. Results and discussion

### 2.1. Preparation and isolation of AXOS

The purpose here was to prepare short singly and doubly substituted AXOS using step-wise enzymatic treatments. In the previous study, we prepared α-L-Ara*f*-(1→3)-β-D-Xyl*p*-(1→4)-D-Xyl*p* from rye AX using Shearzyme.[16] In the present study, Shearzyme was used stepwise with two α-L-arabinofuranosidases (AXH-m and AXH-d3) to produce other AXOS from wheat flour AX. Wheat AX was chosen, since it naturally contains a large number of β-D-Xyl*p* units which carry both (1→2)- and (1→3)-linked α-L-Ara*f* residues. Shearzyme contains *A. aculeatus* GH10 *endo*-1,4-β-D-xylanase that possesses high activity towards the xylosidic linkage next to the (1→3)-linked α-Ara*f*-substituted β-D-Xyl*p* unit.[16] To increase the yield and ease the purification of doubly substituted short-chain AXOS, the α-L-Ara*f* groups in the singly substituted β-D-Xyl*p* units were cleaved off, using AXH-m prior to the Shearzyme treatment (Fig. 1). A large number of different AXOS is formed when wheat flour AX is treated as such with *endo*-1,4-β-D-xylanases.[15] The other target was to prepare AXOS with α-L-Ara*f* (1→2)-substituted β-D-Xyl*p* units. This substitution does not exist significantly in commercially available rye and wheat AX. Thus, α-L-Ara*f* (1→2)-substituted AXOS were prepared by further removal of (1→3)-linked α-L-Ara*f*-residue from doubly substituted AXOS by selective AXH-d3 hydrolysis (Fig. 1).

The hydrolysates contained arabinose, xylose, xylobiose, some xylotriose and AXOS (Fig. 2). Two peaks (one with a shoulder) can be seen in the chromatogram, corresponding to doubly substituted AXOS (Fig. 2A) and two for singly substituted AXOS (Fig. 2B). Free arabinose in Figure 2A indicates the activity of AXH-m. The enzyme efficiently hydrolyses polymeric wheat AX, because no peak corresponding to α-L-Ara*f* (1→3)-linked xylobiose, which has retention time of 32–33 min,[16] is detected in the chromatogram. Further treatment of doubly substituted AXOS with AXH-d3 also proceeded well, since increase in free arabinose was detected and peaks corresponding to doubly substituted AXOS disappeared (Fig. 2B).

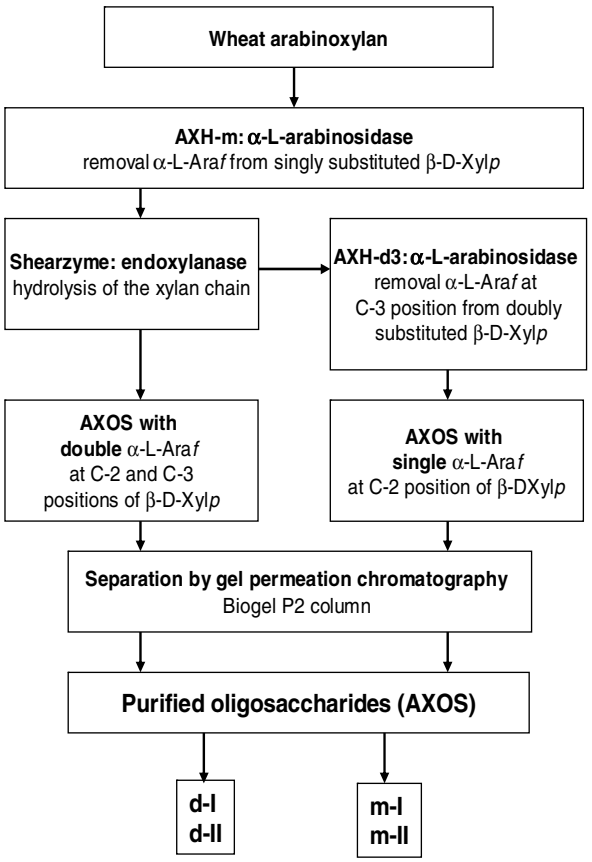


**Figure 1.** Procedure for production of AXOS with doubly (C-2, C-3) and singly (C-2) α-L-arabinofuranosyl (α-L-Ara*f*)-substituted β-D-xylopyranosyl (β-D-Xyl*p*) units from wheat arabinoxylan.

The two hydrolysates were fractionated with gel permeation chromatography (GPC) using a Bio-Gel P2 column. The eluted oligosaccharides were collected in 27-mL fractions, which were screened using thin-layer chromatography (TLC) to determine the fractions containing AXOS. The fractions with AXOS were further analysed with high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Some structurally different AXOS unfortunately have similar retention in TLC and HPAEC. However, since different AXOS co-elute in TLC and HPAEC, by combining information from both TLC and HPAEC-PAD analysis the purest and most concentrated AXOS fractions could be selected for further structural analysis. The isolated AXOS were named according to the expected structures and elution order in GPC: d-I and d-II, first- and second-eluting doubly substituted AXOS; m-I and m-II first- and second-eluting singly substituted AXOS from hydrolysates in Figure 2A and B, respectively.

### 2.2. Analysis of the isolated AXOS by TLC, HPAEC-PAD and MS

Analysis of the selected fractions by TLC and HPAEC-PAD is shown in Figures 3 and 4. In both analyses compounds d-I, d-II and m-I appear quite pure. Compound m-II was not purified completely, and it appears together with compound m-I. The TLC and HPAEC-PAD analysis showed the best fraction from GPC containing m-II. The fraction was further treated with the *Trichoderma reesei* GH5 xylanase, which is able to liberate β-D-Xyl*p* residues from the reducing end of xylo-oligosaccharides,[31] thus hydrolysing m-I

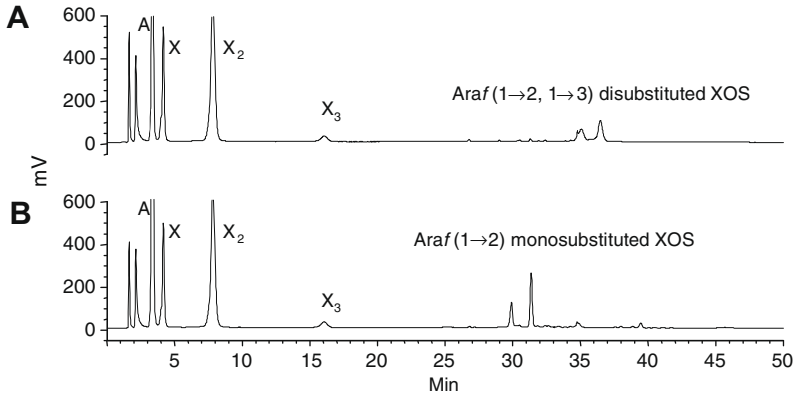


**Figure 2.** HPAEC-PAD chromatograms of wheat AX hydrolysates used as starting materials for AXOS purification. Enzymes AXH-m and Shearzyme (A) and AXH-m, Shearzyme and AXH-d3 (B) were used step-wise in the hydrolysis as presented in Figure 1. A = arabinose, X = xylose, $X_2$ = xylobiose, $X_3$ = xylotriose.

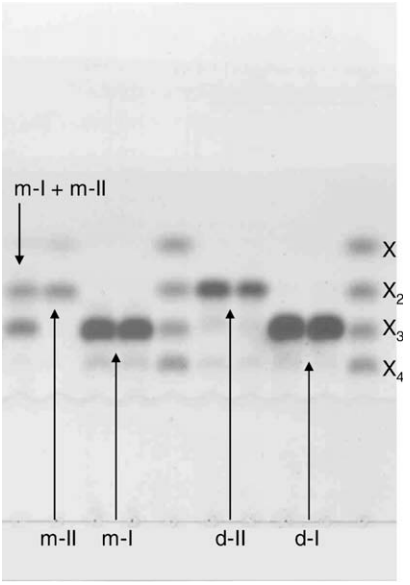


**Figure 3.** TLC analysis of the selected AXOS fractions. Compound m-II after treating the mixture of compounds m-I + m-II with the *Trichoderma reesei* GH5 xylanase. A standard mixture consisted X = xylose, $X_2$ = xylobiose, $X_3$ = xylotriose and $X_4$ = xylotetraose.

to m-II and xylose (Fig. 3). The sample after *T. reesei* GH5 xylanase treatment was used for the nuclear magnetic resonance (NMR) spectroscopic analysis of m-II.

The molecular weights ($M_w$) of the isolated AXOS were analysed with matrix-assisted laser desorption/ionization-time-of-flight-mass-spectrometry with MALDI-TOFMS (Fig. 4). The $M_w$ of compound d-I was 701 (sodium adduct of pentapentaose), that of compounds d-II and m-I was 569 (sodium adduct of pentotetraose) and that of compound m-II was 437 (sodium adduct of pentotriose). The background noise from the matrix is seen at $M_w$ 393. MS confirmed that compound d-I was composed of five pentoses, compounds d-II and m-I of four pentoses and compound m-II of three pentose units.

### 2.3. Monosaccharide composition and quantification of AXOS

Three fractions, each containing one pure AXOS (d-I, d-II and m-I), were hydrolysed completely into monosaccharides using the enzyme mixture designed for wheat AX.[32] The amounts of arabinose and xylose formed were quantified as alditol acetates by gas chromatography (GC). The arabinose to xylose ratios in compounds d-I, d-II and m-I were 0.73, 0.93 and 0.36, respectively. Thus, the pentamer (d-I) and tetramers (d-II, m-I) were composed of three xyloses and two arabinoses (d-I), two xyloses and two arabinoses (d-II) and three xyloses and one arabinose (m-I) unit. Compound m-II was not pure and hence its composition was not measured. Concomitantly, the concentration of AXOS in each sample was also calculated, and the sample was used as a standard in quantification of the content of the corresponding AXOS in the other fractions.

The carbohydrate composition of wheat flour AX (medium viscosity) was previously analysed after degradation with total enzymatic hydrolysis.[32] The carbohydrate yield reported was 84% with an arabinose to xylose ratio of 0.56. Thus, 1.5 g of wheat flour AX used in the hydrolysis contained 1.26 g of AX. The content of each AXOS in the 13–16 best fractions was quantified by HPAEC-PAD. From this, 149 mg (0.22 mmol) of compound d-I and 70 mg (0.13 mmol) of compound d-II, which together constitute 17.4% of AX, were isolated after step-wise hydrolysis by AXH-m and Shearzyme. In the other hydrolysis, which also included the AXH-d3 treatment, 120 mg (0.22 mmol) of compound m-I (11.7% of AX) was obtained. Since m-I was obtained in the same molar amount as d-I, d-I was probably transformed to m-I in good yield by the AXH-d3 treatment. The amount of compound m-II was not quantified due to a lack of pure m-II sample; however, if m-II is similarly transformed from d-II, its amount can be estimated to be 54 mg (0.13 mmol). The contents of arabinose, xylose and xylobiose in the original hydrolysis samples were not quantified. Longer AXOS, which were formed in minor amounts and seen in the TLC analysis of the GPC fractions, were likewise not quantified. The yields of pure AXOS obtained here are higher than previously obtained for arabinoxylobiose from rye AX.[16] The selective removal of part of the α-L-Ara*f*-substituents from AX by the intensive AXH-m treatment created more space for the *endo*-1,4-β-D-xylanase to act, and thus increased the yield of short AXOS. We are not aware of any other studies on isolation of pure AXOS at such a scale and yield. The amounts isolated will enable further studies on prebiotic potential and properties of these AXOS.

### 2.4. NMR analysis of AXOS structures

The 1D- $^1$H spectra and 2D- HSQC experiments were run for all four compounds (d-I, d-II, m-I and m-II). The 1D $^1$H spectra are shown in Figure 5. The spectra show that compounds d-I, d-II and m-I were pure, but for m-II the residual xylose after *T. reesei* GH5 xylanase treatment is also present in the spectrum. The anomeric $^1$H NMR signals of AXOS are found in spectral region of 4.4–5.5 ppm.[6,33] The signals of α-anomeric protons were seen in the spectral region of 5.15–5.30 ppm and β-anomeric protons at

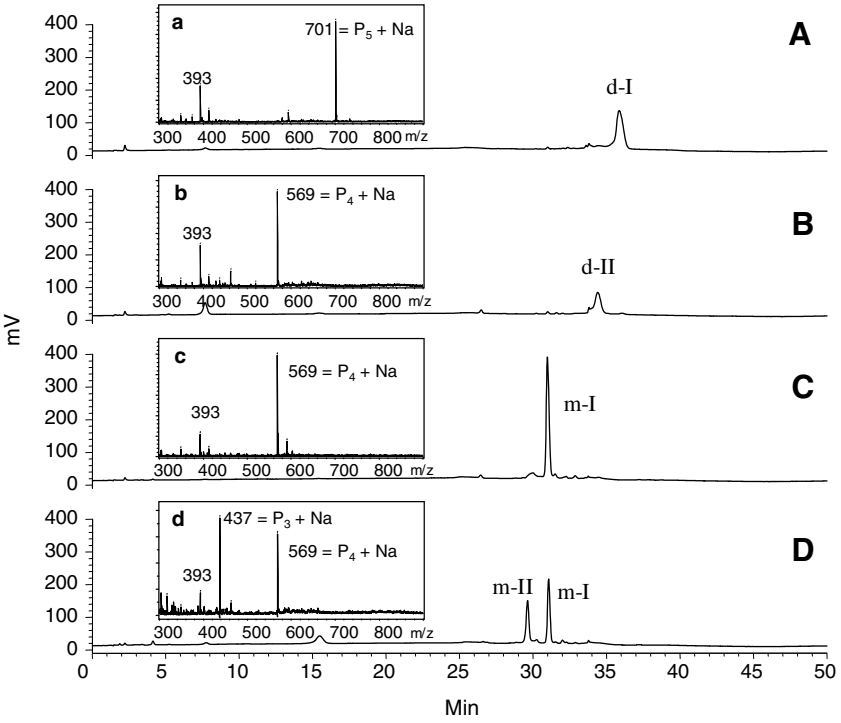


**Figure 4.** HPAEC-PAD chromatograms of isolated AXOS (A–D). The MALDI-TOF-MS analysis of each sample is shown as an insert (a–d). P = pentose unit. Compounds d-I (461 μg/mL) A) and d-II (215 μg/mL) B) are doubly substituted AXOS and compounds m-I (433 μg/mL) C) and m-II are monosubstituted AXOS.

4.40–4.65 ppm. The chemical shifts of the anomeric signals were assigned from the 1 D $^{1}$H spectra and for α-L-Ara*f* substituents also from 2D-DQF-COSY spectra. Resonances for the other ring protons were extracted from the 2D DQF-COSY experiments, beginning with the anomeric protons. In the assignation of some proton chemical shifts, 2D HSQC experiments were also used. The $^{13}$C chemical shifts were acquired using 2D HSQC. The values of the proton chemical shifts are shown in Table 1 and those of $^{13}$C chemical shifts in Table 2.

According to the 1D $^{1}$H spectra of compound d-I, there are three β-D-Xyl*p* signals at 4.4–4.6 ppm while that of compound d-II contains only two β-D-Xyl*p* units. Two partly overlapping signals at 5.23–5.24 ppm in compounds d-I and d-II originate from the (1→3)-linked (A1) and (1→2)-linked (A2) α-L-Ara*f* substituents on a doubly substituted non-reducing terminal β-D-Xyl*p* unit. The signals at 5.28 ppm in compounds m-I and m-II indicate the presence of a single α-L-Ara*f* (A2) residue (1→2)-substituted to β-D-Xyl*p* unit also in the non-reducing terminal position. In compound m-I there are three and in compound m-II only two D-Xyl*p* units. These NMR results obtained are supported by the monosaccharide composition analysis for compounds d-I, d-II and m-I. The proton chemical shifts of compounds d-I and m-I are in good agreement with the data presented earlier for AXOS, in which the α-L-Ara*f* residues are bound to non-reducing terminal β-D-Xyl*p* units and obtained from water-insoluble material of barley grains[34] or for enzymatically derived oligosaccharides from alkali-extractable wheat flour arabinoxylans.[6] Broberg et al.[35] also used $^{1}$H NMR spectroscopy in analysing structures of similar type AX oligomers obtained by enzymatic hydrolysis from high-molecular-weight AX of barley.

The assignments of the $^{13}$C resonances of the samples were based on cross-peaks in the 2D HSQC spectrum (Table 2 and Fig. 6). The $^{13}$C resonances of α-L-Ara*f* (A1 and A2) and terminal reducing D-Xyl*p* (X1) in both the α and β forms were almost similar in all compounds and in good agreement with values published earlier for constituent monosaccharides from water-soluble wheat endosperm AX[5] and also with values of Höije et al.[36] for oligosaccharides obtained from barley husk. The $^{13}$C resonances of internal unsubstituted β-D-Xyl*p* units in compounds d-I and m-I (X2, Table 2) also showed similar values, as published earlier for structures of the same kind.[5,36] In all samples the upfield shifts of C-4 (69.3–70.4 ppm) in β-D-Xyl*p* units, that is, X2 in compounds d-II and m-II or X3 in compounds d-I and m-I (Table 2), were an indication of termination of the β-D-Xyl*p* chain. C-2 and C-3 in the terminal non-reducing β-D-Xyl*p* unit (79.4 and 83.4 ppm, respectively) in compounds d-I and d-II showed a large deshielding effect, indicating the double α-L-Ara*f* substitution. In compounds m-I and m-II, the downfield shift of resonances in C-2 (79.2 and 79.3 ppm, respectively) also confirmed a single substitution of α-L-Ara*f* units in the terminal non-reducing β-D-Xyl*p* unit.

The structures of four different short AXOS (d-I, d-II, m-I and m-II) prepared in this study, using two different α-L-arabinofuranosidases (AXH-m and AXH-d3) and one *endo*-1,4-β-D-xylanase (Shearzyme) are combined in Table 3. All $^{1}$H and $^{13}$C NMR signals for all four AXOS were assigned and used for structural analysis. As far as we are aware, the assignation of the $^{13}$C signals of compounds d-I and m-I and the complete structural NMR analysis of compounds d-II and m-II have not been reported previously. Recently, d-II (α-L-Ara*f*-(1→2)-[α-L-Ara*f*-(1→3)]-β-D-Xyl*p*-(1→4)-D-Xyl*p*) was identified after GH10 *endo*-1,4-β-D-xyanase hydrolysis of wheat AX by the HPLC-MALDI-TOF/TOF-MS/MS analysis.[15] To the best of our knowledge, the compound m-II, with a single (1→2)-linked α-L-Ara*f* residue at the non-reducing β-D-Xyl*p* unit of xylobiose has not been described previously.

All AXOS isolated carried α-L-Ara*f* units on the non-reducing end of the β-D-Xyl*p* residue. Based on the AXOS obtained, the GH10 *endo*-1,4-β-D-xylanase of *A. aculeatus*, present in Shearzyme, is able to hydrolyse efficiently the linkage prior to both singly and doubly α-L-Ara*f*-substituted β-D-Xyl*p* residues. However, the double α-L-Ara*f* substitution partially restricts hydrolysis of the β-(1→4)-xylosidic linkages after the substituted β-D-Xyl*p* residue. The main doubly substituted AXOS (d-I) produced had a backbone

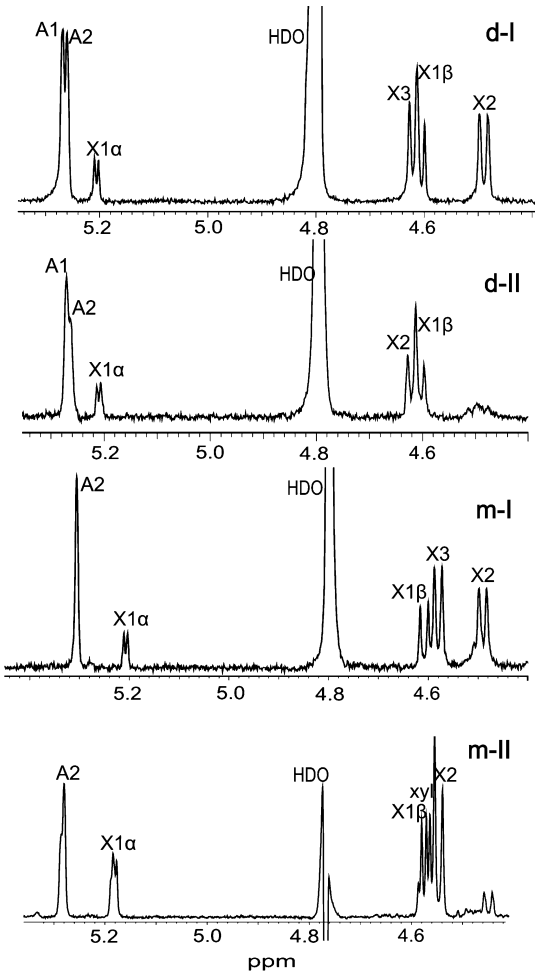


**Figure 5.** The anomeric region of 1D $^1$H NMR spectra of oligomers d-I, d-II, m-I and m-II measured at 500 MHz in $D_2O$. The designations in the spectra are indicated in Tables 1 and 3.

**Table 2**
$^{13}$C NMR chemical shifts in ppm (measured at 500 MHz in $D_2O$; internal acetone, $\delta_{13C}$ 31.55 ppm) of constituent monosaccharide residues of the AXOS d-I, d-II, m-I and m-II derived by enzymatic degradation from wheat AX

| Compound | Residue | Abbr. | C-1 | C-2 | C-3 | C-4 | C-5 |
|---|---|---|---|---|---|---|---|
| d-I | α-L-Ara*f* (1→3) | A1 | 110.0 | 82.4 | 77.8 | 85.3 | 62.4 |
| | α-L-Ara*f* (1→2) | A2 | 110.0 | 82.5 | 77.8 | 85.7 | 62.4 |
| | α-D-Xyl*p* | X1α | 93.3 | 72.8 | 72.2 | n.d. | 60.2 |
| | β-D-Xyl*p* | X1β | 98.0 | 75.0 | 74.8 | 77.7 | 64.3 |
| | β-D-Xyl*p* | X2 | 103.0 | 73.8 | 74.8 | 77.2 | 64.3 |
| | β-D-Xyl*p* | X3 | 101.4 | 79.4 | 83.4 | 69.3 | 66.2 |
| d-II | α-L-Ara*f* (1→3) | A1 | 109.8 | 82.4 | 77.8 | 85.2 | 62.4 |
| | α-L-Ara*f* (1→2) | A2 | 109.8 | 82.4 | 77.9 | 85.6 | 62.4 |
| | α-D-Xyl*p* | X1α | 93.0 | 72.5 | 72.2 | n.d. | 60.2 |
| | β-D-Xyl*p* | X1β | 97.8 | 74.8 | 75.0 | 77.3 | 64.2 |
| | β-D-Xyl*p* | X2 | 101.3 | 79.4 | 83.3 | 69.1 | 66.0 |
| m-I | α-L-Ara*f* (1→2) | A2 | 109.7 | 82.4 | 77.7 | 85.9 | 62.5 |
| | α-D-Xyl*p* | X1α | 93.3 | 72.7 | 72.5 | n.d. | 60.2 |
| | β-D-Xyl*p* | X1β | 97.9 | 75.0 | 75.0 | 77.7 | 64.5 |
| | β-D-Xyl*p* | X2 | 103.2 | 73.8 | 75.0 | 77.7 | 64.5 |
| | β-D-Xyl*p* | X3 | 101.6 | 79.2 | 77.0 | 70.0 | 66.5 |
| m-II | α-L-Ara*f* (1→2) | A2 | 109.8 | 82.4 | 78.0 | 86.0 | 62.5 |
| | α-D-Xyl*p* | X1α | 93.4 | 72.6 | 72.3 | n.d. | 60.0 |
| | β-D-Xyl*p* | X1β | 97.8 | 75.3 | 75.3 | 77.5 | 64.3 |
| | β-D-Xyl*p* | X2 | 101.6 | 79.3 | 76.8 | 70.4 | 66.4 |

n.d. = not detected.

of xylotriose and the amount of xylobiose-derived AXOS (d-II) was about half of this (Fig. 2), whereas the main AXOS formed by Shearzyme with single (1→3)-linked α-L-Ara*f* was arabinoxylobiose.[16]

Short AXOS have been prepared earlier, but in most cases they have not been purified and isolated. Kormelink et al.[37] have hydrolysed AXOS from alkali-extractable wheat flour AX, using β-xylosidase and two different *endo*-1,4-β-D-xylanases purified from *Aspergillus awamori*, and α-L-arabinofuranosidase from *A. niger*. There were several different AXOS with DPs of 3–10 formed and analysed by HPAEC-PAD, including the compound d-I from the present study. The structure of d-I was also prepared and purified from wheat flour AX using *endo*-1,4-β-D-xylanase from *A. tubigensis*.[38] In the study of Swennen et al.[39] *A. aculeatus endo*-1,4-β-D-xylanase was used to produce AXOS with a degree of

**Table 1**
$^1$H NMR chemical shifts in ppm (measured at 500 MHz in $D_2O$; internal acetone, $\delta_H$ 2.225 ppm) of constituent monosaccharide residues of the AXOS d-I, d-II, m-I and m-II derived by enzymatic degradation from wheat AX

| Compound | Residue | Abbr. | H-1 | H-2 | H-3 | H-4 | H-5 eq/H-5 pro*R* | H-5 ax/H-5 pro*S* |
|---|---|---|---|---|---|---|---|---|
| d-I | α-L-Ara*f* (1→3) | A1 | 5.25[a] | 4.19 | 3.98 | 4.20 | 3.82 | 3.71 |
| | α-L-Ara*f* (1→2) | A2 | 5.24[a] | 4.15 | 3.96 | 4.14 | 3.82 | 3.72 |
| | α-D-Xyl*p* | X1α | 5.18 | 3.55 | 3.77 | n.d. | 3.81* | 3.75* |
| | β-D-Xyl*p* | X1β | 4.58 | 3.26 | 3.55 | 3.77* | 4.06 | 3.38 |
| | β-D-Xyl*p* | X2 | 4.47 | 3.30 | 3.57 | 3.80 | 4.15 | 3.42 |
| | β-D-Xyl*p* | X3 | 4.60 | 3.55 | 3.70 | 3.73 | 4.03 | 3.35 |
| d-II | α-L-Ara*f* (1→3) | A1 | 5.25[a] | 4.18 | 3.98 | 4.20 | 3.82 | 3.71 |
| | α-L-Ara*f* (1→2) | A2 | 5.24[a] | 4.15 | 3.96 | 4.13 | 3.82 | 3.71 |
| | α-D-Xyl*p* | X1α | 5.18 | 3.55 | 3.79 | n.d. | 3.85* | 3.77* |
| | β-D-Xyl*p* | X1β | 4.58 | 3.26 | 3.55 | 3.78* | 4.09 | 3.42 |
| | β-D-Xyl*p* | X2 | 4.60 | 3.54 | 3.69 | 3.73 | 4.03 | 3.36 |
| m-I | α-L-Ara*f* (1→2) | A2 | 5.26 | 4.17 | 3.95 | 4.15 | 3.81 | 3.72 |
| | α-D-Xyl*p* | X1α | 5.18 | 3.55 | 3.76 | n.d. | 3.81* | 3.76* |
| | β-D-Xyl*p* | X1β | 4.59 | 3.26 | 3.55 | 3.76* | 4.06 | 3.37 |
| | β-D-Xyl*p* | X2 | 4.47 | 3.30 | 3.57 | 3.79 | 4.14 | 3.42 |
| | β-D-Xyl*p* | X3 | 4.56 | 3.42 | 3.56 | 3.67 | 3.99 | 3.32 |
| m-II | α-L-Ara*f* (1→2) | A2 | 5.28 | 4.17 | 3.97 | 4.14 | 3.82 | 3.72 |
| | α-D-Xyl*p* | X1α | 5.18 | 3.54 | 3.76 | n.d. | 3.84* | 3.77* |
| | β-D-Xyl*p* | X1β | 4.58 | 3.26 | 3.56 | 3.78* | 4.09 | 3.42 |
| | β-D-Xyl*p* | X2 | 4.55 | 3.42 | 3.56 | 3.67 | 4.00 | 3.32 |

* Chemical shifts observed from HSQC spectra. n.d. = not detected.
[a] These assignments might be interchanged.

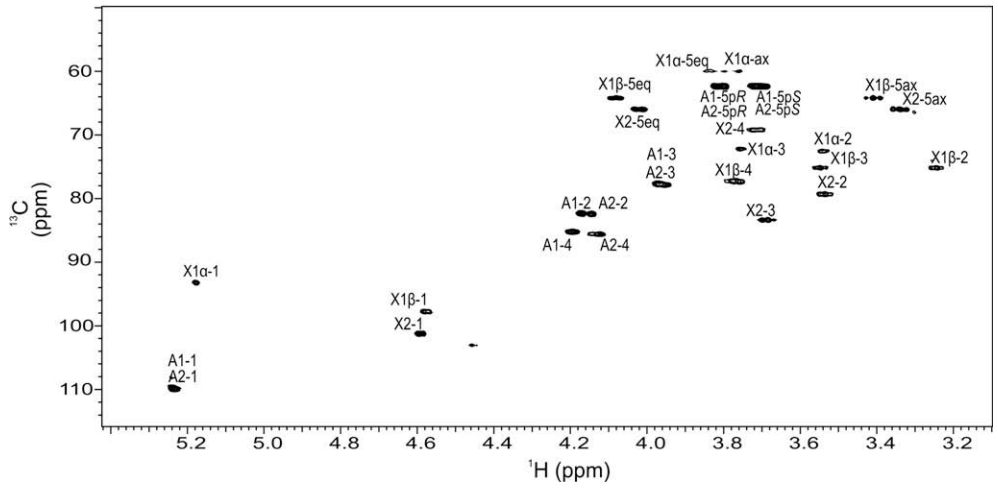


**Figure 6.** HSQC spectrum of compound d-II. The designations of cross-peaks of the monosaccharide residues in the spectrum are indicated in Tables 2 and 3.

**Table 3**
Enzymes used and AXOS formed in the step-wise enzymatic hydrolysis of wheat flour AX

| Enzymes | AXOS Abbr. | Structure | Name |
|---|---|---|---|
| AXH-m, XYN | d-I | AA<br>\ \|<br>X-X-X | α-L-Ara*f*-(1→2)-[α-L-Ara*f*-(1→3)]-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-D-Xyl*p* |
| | d-II | AA<br>\ \|<br>X-X | α-L-Ara*f*-(1→2)-[α-L-Ara*f*-(1→3)]-β-D-Xyl*p*-(1→4)-D-Xyl*p* |
| AXH-m, XYN, AXH-d3 | m-I | A<br>\|<br>X-X-X | α-L-Ara*f*-(1→2)-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-D-Xyl*p* |
| | m-II | A<br>\|<br>X-X | α-L-Ara*f*-(1→2)-β-D-Xyl*p*-(1→4)-D-Xyl*p* |

α-L-Arabinofuranosidase acting on α-L-Ara*f* singly substituted β-D-Xyl*p* residues = AXH-m, α-L-arabinofuranosidase acting on (1→3)-linked α-L-Ara*f* of doubly substituted β-D-Xyl*p* residues = AXH-d3, Shearzyme = *endo*-1,4-β-D-xylanase = XYN. \ = (1 → 3)-linkage, | = (1 → 2)-linkage.

polymerisation (DP) >3 from wheat flour AX, and fractionated by graded ethanol precipitation or ultrafiltration, but isolation of pure AXOS was not obtained. Maslen et al.[15] recently elucidated, using MS, the structures of several AXOS in wheat AX hydrolysates obtained by GH10 and GH11 *endo*-1,4-β-D-xylanases from *Cellvibrio japonicus* and *Neocallimastix patriciarum*, respectively. AXOS with only α-L-Ara*f* (1→2)-substituted β-D-Xyl*p* units are not easy to obtain, because this stucture is not that often present in AX. Only barley AX was reported to have β-D-Xyl*p* units that carry single α-L-Ara*f* substituents at C-2 in relatively high amounts.[40,41] The compounds m-I and d-I were isolated from barley AX after hydrolysis by *A. awamori endo*-1,4-β-D-xylanase, but formation of m-II or d-II was not reported.[34]

## 2.5. AXOS in HPAEC-PAD analysis

In the gradient HPAEC-PAD analysis system, the retention times of linear xylo-oligosaccharides differ according to the homologous oligosaccharide series. The shorter the xylo-oligosaccharides are, the earlier they elute from the column. The same order remains within the groups of singly and doubly α-L-Ara*f*-substituted AXOS, respectively. In the case of monosubstituted AXOS, the linkage position of α-L-Ara*f* on the β-D-Xyl*p* unit also influences the retention order. The retention times are shorter for α-L-Ara*f* (1→2)-linked AXOS (29–30 min for m-II = α-L-Ara*f*-(1→2)-β-D-Xyl*p*-(1→4)-D-Xyl*p*, Fig. 4C) than for corresponding α-L-Ara*f* (1→3)-linked AXOS isomers (32–33 min for α-L-Ara*f*-(1→3)-β-D-Xyl*p*-(1→4)-D-Xyl*p*[16]). The position of the α-L-Ara*f*-substitution in the xylo-oligosaccharide backbone also affects the elution, because arabinoxylotriose with (1→3)-linked α-L-Ara*f* at the internal β-D-Xyl*p* residue elutes before its isomer with non-reducing end terminal (1→3)-linked α-L-Ara*f*. Unfortunately, some AXOS may co-elute in the HPAEC, such as α-L-Ara*f*-(1→3)-β-D-Xyl*p*-(1→4)-D-Xyl*p* and β-D-Xyl*p*-(1→4)-[α-L-Ara*f*-(1→3)]-β-D-Xyl*p*-(1→4)-D-Xyl*p*.[16] The short AXOS with doubly α-L-Ara*f*-substituted β-D-Xyl*p* are retained more in the HPAEC, with retention times above 34 min in the eluent system used (Figs. 2 and 4).

The responses of AXOS on PAD detection are affected by their chemical structure, that is, α-L-Ara*f* substitution. Our studies suggest that the molar responses of α-L-Ara*f* (1→2,1→3)-disubstituted

AXOS are about three times lower than for singly substituted AXOS in the eluent system used. This can be seen by comparing the peak heights or areas in Figure 2A (doubly substituted AXOS) and Figure 2B (singly substituted AXOS), and in Figure 4A and C having 461 μg/mL d-I and 433 μg/mL m-I, respectively. The quite significant differences in the response factors of different AXOS need to be considered in comparing AX hydrolysates, for example, when *endo*-1,4-β-D-xylanase is used for fingerprinting of AX structures,[42] since direct comparison of different peaks in HPAEC-PAD chromatograms will lead to underestimation of α-L-Ara*f*-(1→2,1→3)-disubstituted AXOS levels.

## 3. Conclusions

Using a combination of two different α-L-arabinofuranosidases (AXH-m and AXH-d3) and an efficient glycoside hydrolase family 10 xylanase from *A. aculeatus*, it is possible to prepare singly (1→2)- and doubly (1→2)- and (1→3)-substituted short AXOS with non-reducing end terminal branches in good yields. The AXOS yields were higher than previously reported, due to removal of some of the α-L-Ara*f* units, leaving more space for *endo*-1,4-β-D-xylanase to act. In our previous study the yield of singly (1→3)-substituted arabinoxylobiose was low, since the *endo*-1,4-β-D-xylanase was used to hydrolyse quite densely substituted rye AX without removing any α-L-Ara*f* units.[16] Combining two analytical methods, TLC and HPAEC-PAD, enabled us to obtain a good view of the purity of the samples, since some AXOS cannot be separated by either method. AXOS carrying α-L-Ara*f* (1→2)-linked on the non-reducing terminal β-D-Xyl*p* units elute prior to the (1→3)-linked structural isomers in HPAEC-PAD. The AXOS with doubly α-L-Ara*f*-substituted β-D-Xyl*p* are retained most. In this study, we showed that Shearzyme (*A. aculeatus* GH 10 *endo*-1,4-β-D-xylanase) is also active on the xylosidic linkage next to the double Ara*f* α-(1→2)- and α-(1→3)-substituted β-D-Xyl*p* unit in addition to the xylosidic linkage next to the Ara*f* α-(1→3)-substituted β-D-Xyl*p* unit. To our knowledge this is the first report on structural $^{1}H$ and $^{13}C$ NMR analysis of xylobiose-derived AXOS L-Ara*f*-(1→2)-[α-L-Ara*f*-(1→3)]-β-D-Xyl*p*-(1→4)-D-Xyl*p* and α-L-Ara*f*-(1→2)-β-D-Xyl*p*-(1→4)-D-Xyl*p*. The latter compound has not been reported previously. The prebiotic properties of the AXOS isolated here will undergo further study.

## 4. Experimental

### 4.1. Materials

The medium-viscosity AX from wheat (*Triticum aestivum* L.) flour (WAX) was obtained from Megazyme (Bray, Wicklow, Ireland). The manufacturer states that the wheat AX is 95% pure with a carbohydrate composition of 62% xylose, 38% arabinose and negligible amounts of other sugars, while the remaining 5% contains ash, moisture and proteins.

Water was purified with a Milli-Q-plus-system (Millipore Corporation, Billerica, MA, USA). The NaOH eluent for HPAEC-PAD was prepared from 50% to 52% NaOH (Fluka, Buchs, Switzerland) and the NaOAc eluent from anhydrous NaOAc (Merck, Darmstadt, Germany). The other analytical grade chemicals used were: sodium citrate, $NaBH_4$, $Ac_2O$ and $CH_2Cl_2$ from Merck. The HOAc (99–100%) was purchased from J.T. Baker (Deventer, The Netherlands), 1-methyl imidazole from Fluka and DMSO from Lab-Scan (Dublin, Ireland). Orcinol (3,5-dihydroxy toluene, monohydrate), $H_2SO_4$ (95–97%), 1-butanol (99%) (Merck) and absolute EtOH (Etax A, Primalco, Rajamäki, Finland) were used in the TLC analysis. The monosaccharides D(−)-arabinose, D(+)-glucose and D(+)-xylose from Merck and 1,4-β-D-xylobiose, 1,4-β-D-xylotriose and 1,4-β-D-xylotetraose from Megazyme were used as carbohydrate standards. The *myo*-inositol was purchased from Sigma Chemicals Co. (St. Louis, MO, USA).

Shearzyme 500 L (XYN) (CDN00220), which contains the *Aspergillus aculeatus* GH10 *endo*-1,4-β-D-xylanase as the main activity (49,100 nkat/mL),[16] and α-L-arabinofuranosidase (AXH-m) (14,590 nkat/mL) were obtained from Novozymes A/S (Bagsvaerd, Denmark). The α-L-arabinofuranosidase E-AFAM2 (AXH-d3) from *Bi. adolescentis* (3340 nkat/mL) was purchased from Megazyme. *Trichoderma reesei* GH5 xylanase (9.7 mg protein/mL) was kindly donated by VTT (Technical Research Centre of Finland).

### 4.2. Methods

#### 4.2.1. Production of AXOS

For the production of short wheat AXOS, three different commercial enzymes were used step-wise. The procedure for enzymatic hydrolysis of AX is described in Figure 1.

**4.2.1.1. AXOS with doubly (1→2)- and (1→3)-linked α-L-Araf substituents in Xylp units.** The WAX (5 g/L) in 20 mM NaOAc buffer, pH 5, was first incubated with AXH-m (5000 nkat/g AX) at 40 °C for 24 h to remove all the α-L-Ara*f* units from the singly substituted β-D-Xyl*p* units. Afterwards, the pre-treated AX was further hydrolysed with Shearzyme (10,000 nkat xylanase/g AX) under the same incubation conditions for 48 h.

**4.2.1.2. AXOS with singly (1→2)-linked α-L-Araf substituents in Xylp units.** The WAX was hydrolysed with AXH-m and Shearzyme in the same way as described above and then continued with another α-L-arabinofuranosidase (AXH-d3) treatment. The pH was adjusted to 6.5 by drying the sample and dissolving it into 50 mM Na-phosphate buffer. The sample was then incubated with AXH-d3 (1000 nkat/g AX) at 40 °C for 24 h to remove the α-L-Ara*f* (1→3)-linked substituents from the β-D-Xyl*p* units substituted with two arabinose units. All the hydrolyses were terminated by keeping the samples in a boiling water bath for 10 min.

**4.2.1.3. Purification of AXOS.** For the preparative isolations of AXOS, 1.5 g of WAX was treated enzymatically to prepare a mixture of AXOS. Before the purification, the hydrolysed samples (300 mL) were concentrated to a volume of 40 mL with a rotavapour. The AXOS were separated by gel permeation chromatography (GPC) with Biogel P2 column (8.9 × 135 cm; Bio-Rad, Hercules, CA, USA), using water (10 mL/min) as eluent. Mono- and oligosaccharides were detected from the fractions (27 mL) with thin-layer chromatography (TLC)[16] and high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

Four major AXOS from two separate step-wise AX hydrolyses were obtained after the GPC separations. Three of these were pure (compounds d-I, d-II and m-I,) but compound m-II eluted with the m-I. For the detailed structural NMR analysis of m-II, the mixture of m-II and m-I was further treated with *T. reesei* GH5 xylanase (10 μg protein/mL) at 40 °C in 40 mM NaOAc buffer, pH 5 for 48 h to hydrolyse m-I to m-II.

#### 4.2.2. HPAEC-PAD

The AX hydrolysates and GPC fractions containing oligosaccharides were further analysed with HPAEC-PAD. The HPAEC-PAD system was equipped with an SSI pulse equalizer (Scientific Systems, Inc., model LP 21; State College, PA, USA), two Waters 515 HPLC pumps, a PC Waters pump control module and a cooling Waters 717 autosampler using Millenium[32] software (Waters Corporation, Milford, MA, USA) for instrument control and data handling. The analytical CarboPac PA-100 columns (250 × 4 mm, i.d.) and the

guard column PA-100 (25 × 3 mm, i.d.) (Dionex, Sunnyvale, CA, USA) were maintained at 30 °C. The analyses were carried out using the oligosaccharide method with 100 mM NaOH–1 M NaOAc gradient at a flow rate of 1 mL/min as described in Rantanen et al.[16] A Decade detector with a gold electrode (Antec Leyden, Zoeterwoude, The Netherlands) was used in pulse mode at 30 °C. The pulse potentials and durations were: $E_1$ = 0.15 V, $t_1$ = 400 ms, $E_2$ = 0.75 V, $t_2$ = 120 ms, $E_3$ = −0.8 V, $t_3$ = 130 ms, $t_s$ = 20 ms. A mixture of xylose, xylobiose, xylotriose and xylotetraose was used as an external standard to monitor the stability of the separation. All the compounds in the standard sample had a concentration of 25 μg/mL. The samples were filtered using a 0.45 μm Acrodisc® syringe filter with nylon membrane (Pall Corporation, Ann Arbor, MI, USA), and the injection volume was 10 μL in all the measurements.

#### 4.2.3. Monosaccharide content of isolated AXOS

The arabinose to xylose ratio in the AXOS was determined by hydrolysis and quantifying the amount of arabinose and xylose formed in selected fractions. The concentration of the AXOS was also calculated for each compound. The quantified compounds were used as standards in the analysis of the content of the corresponding AXOS in the other fractions. The top fraction of each purified AXOS (d-I, d-II, m-II) was hydrolysed enzymatically to monosaccharides, using the enzyme mixture prepared from four commercial enzyme preparations.[32] The enzyme mixture used was dosed according to the α-L-arabinofuranosidase activity (1000 nkat/g AX), which was regarded as the limiting enzyme activity in the mixture.[32] The hydrolysis was carried out at 40 °C for 48 h in 20 mM NaOAc buffer, pH 5. The reaction was stopped by maintaining the samples in the boiling water bath for 10 min. After the hydrolysis, the samples were analysed first with the HPAEC-PAD method for oligosaccharides to check the completeness of the hydrolysis. The hydrolysed samples were reduced with $NaBH_4$ thereafter acetylated with acetic $Ac_2O$ according to Blakeney and Johansson,[43,44] and the monosaccharides released were analysed as their alditol acetates by gas chromatography (GC). The monosaccharide standards used were D-xylose, D-arabinose and D-glucose. Quantitation was performed using five concentration levels of each sugar and *myo*-inositol as an internal standard. The amounts of monosaccharides were calculated as anhydro sugars. The GC instrument used was a Hewlett Packard HP5890 series II GC system with a flame ionization detector (FID). The system was equipped with an HP 7673 series injector and autosampler (Hewlett Packard, Palo Alto, CA, USA). The column used was an HP-5 (30 m, 0.32 mm i.d., 0.25 μm film thickness; Agilent Technologies, Foster City, CA, USA). The analysis of acetylated alditols was performed isothermally at 200 °C for 20 min, using the following GC conditions: injector temperature 225 °C, detector temperature 280 °C, injection volume 1 μL with split ratio 1:30, flow rate 1 mL/min and carrier gas helium.

#### 4.2.4. MALDI-TOFMS

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) analysis of selected isolated AXOS fractions was performed using an Ultraflex Instruments (Bruker Daltonics, Wormer, The Netherlands) equipped with a nitrogen laser at 337 nm. The mass spectrometer was selected for positive ions, which were accelerated to a kinetic energy of 12,000 V after a delayed extraction time of 200 ns. Hereafter, the ions were detected using the reflector mode. The lowest laser power required to obtain good spectra was used, and at least 100 spectra were collected. The mass spectrometer was calibrated with a mixture of maltodextrin standards (mass range 365–2309).

The matrix solution was prepared by dissolving 9 mg 2,5-dihydroxybenzoic acid in 1 mL 50% (v/v) acetonitrile-water solution. The samples (1 μL) and the matrix (1 μL) were pipetted onto a MALDI-TOF-plate (Bruker Daltonics), and the mixtures were allowed to dry under a constant stream of warm air.

#### 4.2.5. NMR spectroscopy

The NMR spectra were acquired on a Varian Unity 500 spectrometer (Varian NMR Systems, Palo Alto, CA, USA) operating at 500 MHz for $^1H$. Prior to the NMR experiments the samples were exchanged four times with $D_2O$ (99.8% D; Merck) and finally dissolved in 0.5 mL of $D_2O$. The measurements were performed at 23 °C. The chemical shifts were reported relative to an internal acetone standard at 2.225 ppm for $^1H$ and 31.55 ppm for $^{13}C$. In recording the 1 D $^1H$ proton spectrum, a modification of the water-eliminated Fourier transform (WEFT) sequence was used. The 2D NMR experiments $^1H$–$^1H$ COSY and $^1H$–$^{13}C$ HSQC were run, using standard pulse sequences.

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