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Preparation of arabinoxylobiose from rye xylan using family 10 *Aspergillus aculeatus endo*-1,4-β-D-xylanase

Helena Rantanen [a,*], Liisa Virkki [a], Päivi Tuomainen [a], Mirjam Kabel [b], Henk Schols [b], Maija Tenkanen [a]

[a] *Department of Applied Chemistry and Microbiology, Faculty of Agriculture and Forestry, P.O. Box 27, FIN-00014 University of Helsinki, Finland*
[b] *Department of Agrotechnology and Food Sciences, Wageningen Agricultural University, Bomenweg 2, NL-6703 HD Wageningen, The Netherlands*

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Abstract

Commercial xylanase preparation Shearzyme®, which contains the glycoside hydrolase family 10 *endo*-1,4-β-D-xylanase from *Aspergillus aculeatus*, was used to prepare short-chain arabinoxylo-oligosaccharides (AXOS) from rye arabinoxylan (AX). A major AXOS was formed as a hydrolysis product. Longer AXOS were also produced as minor products. The pure GH10 xylanase from *A. aculeatus* was used as a comparison to ensure that the formed AXOS were consequence of the endoxylanase's function instead of some side enzymes present in Shearzyme. The major AXOS was purified and the structure confirmed with various analysis methods (TLC, HPAEC-PAD, MALDI-TOF-MS, and one- and two-dimensional NMR spectroscopy with nano-probe) as α-L-Ara*f*-(1 → 3)-β-D-Xyl*p*-(1 → 4)-D-Xyl*p* (arabinoxylobiose). This is the first report on ^{13}C NMR data of pure arabinoxylobiose. The yield of arabinoxylobiose was 12% from the quantified hydrolysis products. In conclusion, GH10 endoxylanase from *A. aculeatus* is thus able to cut efficiently the xylosidic linkage next to the arabinofuranosyl-substituted xylose unit which is not typical for all the GH10 endoxylanases. Interestingly, pure *A. aculeatus* xylanase showed notably activity towards *p*-nitrophenyl-β-D-xylopyranose. In previously studies longer AXOS have been produced with Shearzyme but the formation of short-chain AXOS by *A. aculeatus* GH10 xylanase has not been studied before.

Keywords: Arabinoxylan; Arabinoxylobiose; *Aspergillus aculeatus*; Glycoside hydrolase family 10; Shearzyme; Xylanase; Xylo-oligosaccharides

1. Introduction

Functional foods and new food ingredients are currently under intensive study. Various oligosaccharides are included among these new food ingredients. Oligosaccharides have many beneficial physiological properties, including protection against colon cancer by forming short-chain fatty acids (SCFA) in the large intestine during fermentation (Edwards, 1995; Voragen, 1998) and a prebiotic effect promoting the growth of beneficial intestinal bacteria (*Bifidobacterium* and *Lactobacillus*) (Crittenden & Playne, 1996; Gibson & Roberfroid, 1995; Voragen, 1998). Arabinoxylo-oligosaccharides (AXOS) are non-digestible oligosaccharides but are fermented in the large intestine by the intestinal flora (Gibson & Roberfroid, 1995; Van Laere, Hartemink, Bosveld, Schols, & Voragen, 2000). A study by Van Laere et al. (2000) revealed that branched AXOS are fermented by some health-promoting bifidobacteria and by the predominant intestinal bacteria, *Bacteroides* spp., but harmful *Clostridium* spp. showed low utilization of branched AXOS. (Kabel, Kortenoeven, Schols, & Voragen, 2002b) reported that AXOS are specific substrates for lactobacilli and bifidobacteria.

AXOS can be produced with enzymes from cereal arabinoxylans (AX) (Tenkanen, 2003; Vázquez, Alonso, Domínguez, & Parajó, 2000). Purified enzymes allow the hydrolysis end-products to be successfully regulated, while using commercial enzymes the products usually contain a combination

* Corresponding author. Tel.: +358 9 191 58241; fax: +358 9 191 58475.
E-mail address: helena.pastell@helsinki.fi (H. Rantanen).

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of different enzymes that may affect the hydrolysis pattern. The backbone of AX is constructed from 1,4-linked β-D-xylopyranosyl (Xyl*p*) residues, which are substituted at positions C3 and/or C2 with α-L-arabinofuranosyl Ara*f* units, thus resulting in single- and double-substituted Xyl*p* residues. The xylan backbone can also carry 4-*O*-methylglucopyranosyl uronic acid and acetyl substituents (Aspinall, 1959; Tharanathan, 2002). Furthermore, phenolic acids (mainly ferulic acid) may be esterified to some of the Ara*f* units (Vázquez et al., 2000). The structure of AX varies between plant species and even between different parts of the same plant. The AX backbone can be randomly hydrolysed using *endo*-1,4-β-D-xylanases (EC 3.2.1.8), producing a mixture of various oligosaccharides. Arabinose side groups are liberated by α-L-arabinofuranosidases (EC 3.2.1.55) and *exo*-1,4-β-D-xylosidases (EC 3.2.1.37) release xylose units from the non-reducing end of xylo-oligosaccharides (Biely, 1985).

Most xylanases are classified based on the structural and sequence classification in glycoside hydrolase (GH) families GH10 and GH11 (afmb.cnrs-mrs.fr/gazy/GAZY/index/html). GH10 and GH11 xylanases also possess distinctive catalytic properties. The xylo-oligosaccharides resulting from the activity of GH10 xylanases are shorter than those produced by GH11 xylanases. GH11 xylanases are hindered by the presence of side groups, whereas GH10 xylanases are able to act near the substitution, forming oligosaccharides that carry subsituents at the non-reducing terminal xylopyranose residue (Beaugrand et al., 2004; Biely, Vršanská, Tenkanen, & Kluepfel, 1997). Pell et al. (2004) and Fujimoto et al. (2004) observed that the family GH10 contains enzymes with slightly diverse catalytic sites, resulting in small differences in their hydrolytic end-products. The shortest AXOS produced were 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*. The feruloyl substituents do not impede the action of xylanases as feruloyl arabinoxylobiose (FAXX) is the shortest feruloylated oligosaccharide liberated by GH10 xylanase (Vardakou et al., 2003). The most comprehensive studies on isolation and structural characterization of AXOS were carried out after hydrolysis of wheat and barley endosperm AX with *Aspergillus awamori* xylanases I and III, which unfortunately have not been assigned GH families but most probably are members of GH10 and GH11, respectively (Gruppen et al., 1992; Kormelink et al., 1993; Viëtor et al., 1994).

The aim of this study was to prepare, for further fermentation studies, the most abundant short AXOS from cereal AX, using the commercial enzyme Shearzyme®, which contains the *Aspergillus aculeatus* GH10 xylanase. The objective was also to define the exact structure of the main AXOS formed.

2. Materials and methods

2.1. Chemicals and enzymes

Sodium hydroxide (NaOH) and sodium acetate (NaAc) solutions were prepared from 50–52% NaOH (Fluka, Buchs, Switzerland) and anhydrous NaAc (Merck, Darmstadt, Germany), respectively. Milli-Q-water (Milli-Q-plus, Millipore, Billerica, MA, USA) was used to prepare all the solutions. The other chemicals used were: hydrochloric acid (HCl) and pyridine (Sigma–Aldrich Riedel-de-Haën, Seelze, Germany), anhydrous methanol and chlorotrimethylsilane (Fluka), sulphuric acid (H_2SO_4 95–97%) and bis-trimethyltrifluoroacetamide (Merck, Darmstadt, Germany), orcinol (3,5-dihydroxy toluene, monohydrate) and 1-butanol (99%) (Merck, Schuchardt, Germany), and absolute ethanol (Etax A, Primalco, Rajamäki, Finland). D(+)-xylose, D(−)-arabinose, D(+)-glucose, D(−)-fructose, D(+)-galactose, D(+)-mannose (Merck, Darmstadt, Germany), 1,4-β-D-xylobiose, 1,4-β-D-xylotriose, 1,4-β-D-xylotetraose (Megazyme, Bray, Ireland) and D(+)-raffinose pentahydrat (Fluka) were used as carbohydrate standards.

Oat (*Avena sativa*) spelt AX was from Sigma–Aldrich Chemie (Steinheim, Germany). Low- and high-viscosity AX from wheat (*Triticum aestivum*) flour (P-WAXYL, P-WAXYH) and high-viscosity AX from rye (*Secale cereale*) flour (P-RAXY) were from Megazyme. The manufacturer states that the low- and high-viscosity wheat flour AXs were both 94% pure with a sugar composition of 61% xylose, 37% arabinose and 3% other sugars, while the remaining 6% is ash, moisture and proteins. Rye flour AX is 95% pure with a sugar composition of 48% xylose, 49% arabinose and 3% other sugars. The manufacturer reports that the oat spelt AX contains 10% arabinose and 15% glucose, while the remaining 75% is presumed to be xylose.

Shearzyme 500 L was obtained from Novozymes A/S (Bagsvaerd, Denmark). Shearzyme is produced by *Aspergillus oryzae* which carries the gene encoding GH10 xylanase from *A. aculeatus*. α-Arabinofuranosidase from *Aspergillus niger* (E-AFASE, 6970 nkat/ml) was purchased from Megazyme. β-Xylosidase from *Trichoderma reesei* (2340 nkat/ml) and xylanase p*I* 6.9 from *A. oryzae* (1850 nkat/ml) were kindly donated by VTT (Technical Research Centre of Finland). Pure xylanase from *A. aculeatus* (121,530 nkat/ml) was a kind gift from Novozymes A/S of Denmark.

2.2. Carbohydrate composition analysis of rye flour arabinoxylan

2.2.1. HCl and H_2SO_4 acid hydrolyses

Rye flour AX (25 mg) was hydrolysed using 2 M HCl (4 ml) for 4 h at 100 °C or 1 M H_2SO_4 (5 ml) for 30 min at 120 °C. The HCl method was modified from that used by Houben, de Ruijter, & Brunt (1996) and the H_2SO_4 method from Lebet, Arrigoni, & Amadò (1997). The hydrolyses were carried out with four samples in parallel. After the hydrolysis the samples were first neutralized with 4 M NaOH (2 ml) or 10 M NaOH (1 ml), respectively, after which the pH was adjusted to 7–9. The final volumes of the samples were adjusted to 20 ml. The standards (25 mg/ml) D-Xylose, D-arabinose and D-glucose, were treated the same way as the AX samples and the dilutions for the standard curves were made from these monosaccharide solutions.

High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) method for monosaccharides was used to analyse the samples.

2.2.2. Methanolysis

Methanolysis was performed according to the method of Sundberg, Sundberg, Lillandt, & Holmbom (1996). The gas chromatogram (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 × 0.25 μm; Agilent Technologies, Foster City, CA, USA). The samples were analysed using the following GC conditions: oven temperature 150 °C (5 min), 2 °C/min to 260 °C, injector temperature 225 °C, FID temperature 280 °C, injection volume 1 μl, split ratio 1:30, flow rate 1 ml/min and carrier gas helium. The samples and standards were analysed in triplicate. The same monosaccharide standards were used as in the acid hydrolyses described earlier.

2.3. Enzyme activity assays

endo-1,4-β-D-Xylanase activity was determined using the method of Bailey, Biely, & Poutanen (1992) with 1% birchwood (*Betula alba*) xylan (Roth 7500; Carl Roth RG, Karlsruhe, Germany) as a substrate. The β-glucanase activity was measured with 1% barley (*Hordeum vulgare*) β-glucan (medium viscosity, P-BGBM; Megazyme) as a substrate as described by Zurbriggen, Bailey, Penttilä, Poutanen, & Linko (1990). The α-arabinofuranosidase, β-xylosidase and β-glucanase activities were measured using 2 mM *p*-nitrophenyl-α-L-arabinofuranoside (N3641; Sigma), 5 mM *p*-nitrophenyl-β-D-xylopyranoside (N2132; Sigma) and 1 mM *p*-nitrophenyl-β-D-glucopyranoside (N7006; Sigma) as substrates according to Poutanen, Rättö, Puls, & Viikari (1987), Poutanen & Puls (1988) and Bailey & Nevalainen (1981), respectively.

2.4. Production of AXOS

The arabinoxylans (5 g/l) in 20 mM NaAc buffer, pH 5, were incubated with Sheazyme or purified *A. aculeatus* xylanase (10,000 nkat xylanase/g AX) at 40 °C for 48 h. The hydrolyses were terminated by keeping the sample in the boiling water bath for 10 min, after which the samples were analysed with HPAEC-PAD. For preparative isolation of AXOS 1.5 g of rye flour AX was treated by Shearzyme in the same way. Before the purification, the hydrolysed sample (300 ml) was concentrated to a volume of 50 ml with rotavapor. The AXOS were separated by gel permation chromatography (GPC) with a Biogel P2 column (8.9 × 135 cm, Bio-Rad, Hercules, CA, USA) eluted by water at 10 ml/min and 27-ml fractions collected. The elution of mono- and oligosaccharides was detected with thin-layer chromatography (TLC). The fractions containing oligosaccharides were further analysed by HPAEC-PAD. The structure of the main AXOS formed was elucidated from the most concentrated fraction using MS and NMR analysis.

2.5. Thin-layer chromatography

The TLC was carried out using silica gel 60 plates (Merck, Darmstadt, Germany). A mixture of 1-butanol/ethanol/water (3:2:2 v/v) was used as an eluent. The carbohydrates were detected by spraying with 2% (w/v) orsinol dissolved in a solution of ethanol/H_2SO_4/H_2O (80:10:10%), after which the plates were heated at 105 °C for 10 min.

2.6. 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-1 and CarboPac PA-100 columns (250 × 4 mm, i.d.), and the guard column PA-1 and PA-100, respectively, (25 × 3 mm, i.d.) (Dionex, Sunnyvale, CA, USA) were maintained at 30 °C. All 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.

The mobile phases were filtered with 0.45 μm GH Polypro membrane filters (Pall Corporation) and degassed using helium. The eluents for gradient analysis of the oligosaccharides were A: 1 M NaAc in 100 mM NaOH and B: 100 mM NaOH. The first isocratic phase was 15 min in 100% B, followed by a linear gradient from 100% B to 88% B and 12% A in 20 min. The second isocratic phase was 5 min in 12% A and 88% B. The second linear gradient was back to 100% B in 5 min. The last isocratic phase was 5 min in 100% B, in which the column was stabilized before the next injection. The total analysis time was 50 min and the flow rate was 1 ml/min. The eluents for gradient analysis of the monosaccharides were A: H_2O and B: 50 mM NaOH. The gradient and the flow rates are described in Table 1. The total analysis time was 45 min. The method was

Table 1
The gradient used in the HPAEC-PAD method for monosaccharides

Time (min)	Flow rate (ml/min)	% A	% B
	0.7	97	3
21	0.7	97	3
22	1.1	97	3
24	1.1	0	100
30	1.1	0	100
32	1.1	97	3
33	0.7	97	3
45	0.7	97	3

A, water; B, 50 mM NaOH.

modified from that of Johansson et al. (2006). In both oligo- and monosaccharide methods, 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, $E3 = -0.8$ V, $t_3 = 130$ ms, $t_s = 20$ ms.

Both internal and external standard methods were used in oligosaccharide analysis. Raffinose (50 μg/ml) used as an internal standard was added to the samples before the HPAEC-PAD analyses to allow accurate quantification. A mixture of xylose, xylobiose, raffinose, xylotriose and xylotetraose was used as an external standard. All the compounds in the standard sample had a concentration of 25 μg/ml, except for raffinose which was 50 μg/ml. The concentration of raffinose was higher due to the detector's lower response for raffinose than for the other standards. For the monosaccharides an external standard mixture containing arabinose, galactose, glucose, xylose (50 μg/ml) and fructose and mannose (100 μg/ml) was used; an internal standard was not used in monosaccharide analysis. The external standard was injected after every three samples in both methods to monitor the retention times.

2.7. Mass spectrometry

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) analysis was performed using an Ultraflex instrument (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 10 mg 2,5-dihydroxybenzoic acid in a 1 ml of 30% (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.

2.8. Enzymatic hydrolysis of AXOS

The isolated AXOS were hydrolysed with α-arabinofuranosidase (5000 nkat/g of substrate) and β-xylosidase (1000 nkat/g of substrate) for 24 h at 40 °C in 20 mM NaAc buffer, pH 5. After treatment, the sample was analysed first with the HPAEC-PAD method for oligosaccharides to check the completeness of the hydrolysis. After that the arabinose to xylose ratio in the AXOS was determined by quantifying the amount of arabinose and xylose formed using the HPAEC-PAD method for monosaccharides. Concomitantly, information was obtained on the concentration of AXOS in the sample, which then was used as a standard in the quantification of its content in other samples.

2.9. NMR-spectroscopy

Prior to the NMR experiments the sample was exchanged four times with D_2O (99.8% D, Merck, Darmstadt, Germany) and finally dissolved in 40 μl of D_2O. The NMR experiments were carried out on a Varian Unity 500 spectrometer (Varian NMR Systems, Palo Alto, CA, USA) operating at 500 MHz for ^{1}H. The measurements were performed at 23 °C using a gHX nano-NMR probe. In recording the ^{1}H proton spectrum, a modification of the water-eliminated Fourier transform (WEFT) sequence was used. The spectrum was accumulated with 64 transients, a spectral width of 6000 Hz and a repetition time of 4 s. The ^{1}H chemical shifts were referenced to an HDO signal at 4.78 ppm. For the double-quantum filtered correlated spectroscopy (DQF-COSY) experiment, eight transients per t_1 value were collected, yielding a data matrix consisting of 256×1024 complex points. A spectral width of 3400 Hz was employed in both dimensions, and the relaxation delay was 1.0 s. The data were zero-filled to 512×2048 matrix prior to Fourier transform, and a shifted sine-bell window function was applied in both dimensions. For the heteronuclear multiple-quantum correlation (HMQC) spectrum 32 transients per t_1 value were collected. The data matrix consisted of 256×1024 complex points (zero-filled to 512×2048). A spectral width of 15,000 Hz was employed in t_1 and 3400 Hz in t_2. The delay between transients was 1.0 s and the average ^{1}H–^{13}C coupling constant was estimated to be 140 Hz. A shifted sine-bell window function was applied in both dimensions.

3. Results and discussion

3.1. Hydrolysis of cereal AX with Shearzyme

The enzyme activity assay revealed that in addition to β-xylanase activity (49 100 nkat/ml), Shearzyme contains some β-xylosidase (860 nkat/ml), α-arabinofuranosidase (40 nkat/ml), β-glucanase (660 nkat/ml) and β-glucosidase (55 nkat/ml) activities. The main side activity detected was β-xylosidase, which could further act on the xylo-oligosaccharides formed by the β-xylanase. The α-arabinosidase activity was very low. Thus, Shearzyme was a suitable commercial β-xylanase preparation for the production of AXOS.

Formation of AXOS from four commercial cereal AXs (oat spelt, wheat low- and high-viscosity and rye high viscosity) by an extensive Shearzyme hydrolysis was first compared to determine the most suitable raw material for the production of short-chain AXOS. HPAEC-PAD analysis showed that a major AXOS was produced from all AXs by Shearzyme (Fig. 1). Its amount was the highest from rye flour AX and the lowest from oat spelt AX. Furthermore, less xylobiose and other short-chain AXOS were formed from rye flour AX than from either of wheat flour AXs.

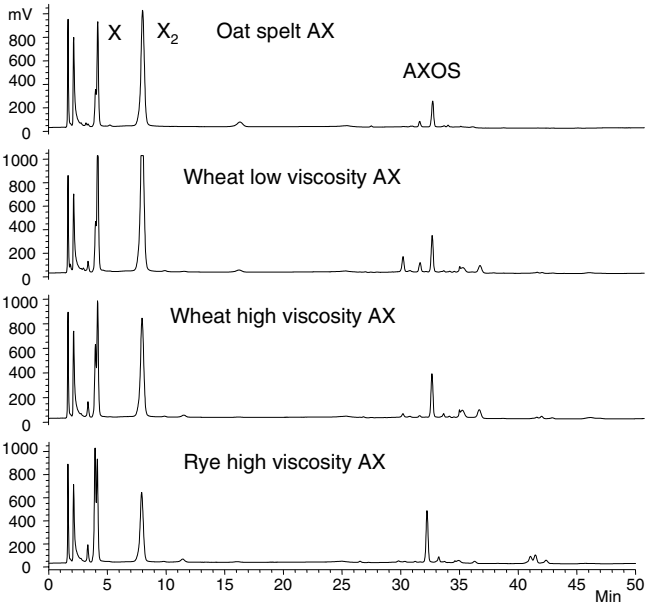

Fig. 1. HPAEC-PAD chromatograms (PA-100 column) of four cereal arabinoxylans (AXs) hydrolysed by Shearzyme. X, xylose; X_2, xylobiose and AXOS, arabinoxylo-oligosaccharides.

Thus, rye flour AX was chosen as a raw material for isolation of the main AXOS.

3.2. Compositional analysis of rye flour AX

The carbohydrate composition of rye flour AX was analysed in detail after degradation with three different methods. HPAEC-PAD was used for analysis after acid hydrolyses with 2 M HCl (100 °C, 4 h) and with 1 M H_2SO_4 (120 °C, 30 min). The hydrolysis conditions were selected after preliminary tests. After methanolysis the monosaccharides were analysed with GC. The three methods tested gave variable results. The highest carbohydrate yield was obtained after H_2SO_4 hydrolysis (92%). The amount of xylose was the lowest after HCl hydrolysis and the highest after H_2SO_4 hydrolysis, while the concentration of arabinose remained almost constant (Table 2). Thus the ratio of arabinose to xylose was the highest after HCl hydrolysis (0.68). The information provided by the manufacturer indicated an even higher arabinose content, with arabinose to xylose ratio of 1.0. The highest content of glucose impurity (11%) was detected after methanolysis.

Surprisingly, none of the three methods tested resulted in the carbohydrate composition provided by the manufacturer. The results given by the H_2SO_4 hydrolysis, which gave the highest carbohydrate yield, were used in further calculations. According to that, the total monosaccharide content of the sample was 92%, with a monosaccharide composition of 30% arabinose, 65% xylose and 5% glucose.

Table 2
Comparison of three methods for monosaccharide analysis of rye flour arabinoxylan

	Monosaccharide composition			Yield (%)	Ara/Xyl
	Arabinose (%)	Xylose (%)	Glucose (%)		
Methanolysis 2 M HCl in methanol 100 °C, 3 h	27	63	10	80	0.42
1 M H_2SO_4 120 °C, 30 min	30	65	5	92	0.46
2 M HCl 100 °C, 4 h	39	55	6	77	0.68

The yield is calculated from xylan dry weight.

3.3. Isolation of the main AXOS

Shearzyme hydrolysis of 1.5 g of commercial rye flour AX, which according to our analysis contained 1.3 g of AX, forming 13 mg arabinose, 140 mg xylose, 152 mg xylobiose, 4 mg xylotriose, 8 mg xylotetraose and 44 mg of the main, later isolated AXOS. These quantified products accounted for 28% of the AX. Although there were some unidentified longer AXOS (Fig. 2a; 35–50 min), we concluded that large part of the sample remained non-hydrolysed, although high enzyme dosage and long hydrolysis time were used. The yield of the main AXOS from AX was 3% and from quantified products 12%. Previously Yoshida, Kuno, Saito, Aoyama, & Kusakabe (1998) isolated four AXOS from an enzymatic hydrolysate of bamboo grass AX with a 5% yield from the starting material. Viëtor et al. (1994) reported a 24% yield for all quantified mono- and oligosaccharides obtained from barley AX after a xylanase treatment. The β-xylosidase detected in Shearzyme did not act efficiently, since the xylobiose had not been degraded to xylose.

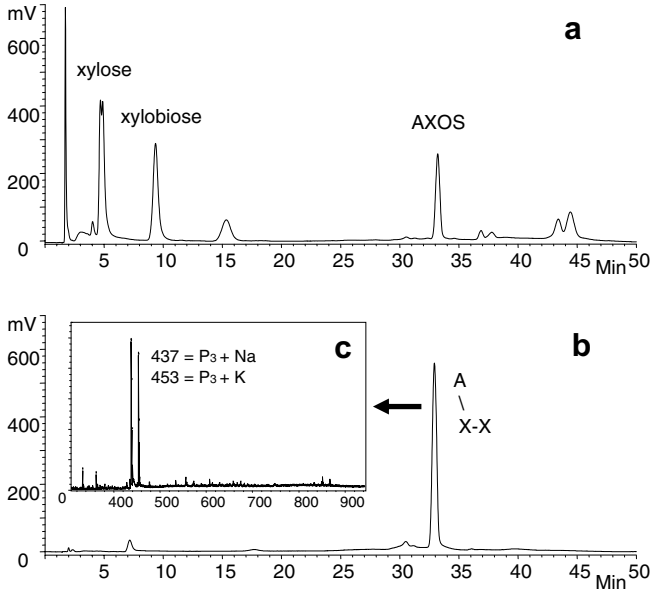

Fig. 2. HPAEC-PAD chromatogram (PA-100 column) of enzymatically hydrolysed rye flour AX (a), and of the purified oligosaccharide (b). MALDI-TOF-MS analysis of the purified oligosaccharide (c). P, pentose unit; X, xylose and A, arabinose.

β-Xylosidase activity of the purified *A. aculeatus* xylanase was also measured and the specific activity using *p*-nitrophenyl-β-D-xylopyranoside as a substrate was surprisingly high, 50 nkat/mg of protein. In comparison, the specific activity of the purified *T. reesei* β-xylosidase is 470 nkat/mg of protein (Poutanen & Puls, 1988). However, β-xylosidase activity of *A. aculeatus* endoxylanase is artificial since this endoxylanase is not evidently able to hydrolyse xylobiose. α-Arabinofuranosidase in Shearzyme liberated low amounts of arabinose. Some glucose (167 mg) was also formed from rye flour AX during the Shearzyme treatment. This result is in agreement with our carbohydrate analysis, which also revealed a clear glucose impurity in rye flour AX (Table 2).

The main AXOS formed from rye flour AX was isolated with GPC, using a Bio-Gel P2 column. The AXOS was collected in ten 27-ml fractions. The purest and the most concentrated fraction (Fig. 2b), which was used for the structural analysis, contained 132 μg/ml of AXOS. The 10 fractions together contained 25 mg of AXOS.

3.4. Structural analysis of the purified AXOS

The purified oligosaccharide was first analysed with MS. The molecular weight (M_w) of AXOS was 437 (sodium adduct of pentotriose) and 453 (potassium adduct of pentotriose) (Fig. 2c). MS confirmed that the isolated oligosaccharide was composed of three pentoses. To analyse its carbohydrate composition in more detail, the AXOS was hydrolysed completely with a mixture of α-arabinofuranosidase and β-xylosidase, after which the ratio of arabinose to xylose was analysed by HPAEC-PAD. The results illustrated that the trimer was composed of one arabinose and two xylose units. Thus the purified AXOS was confirmed as arabinoxylobiose.

The structure of the AXOS was further elucidated by NMR spectroscopy. After purification the fractions were not pooled and the amount of oligosaccharide in the most concentrated fraction was at nanomole level (12 nmol). The nano-probe technique used in the NMR measurements enabled structural elucidation by different 1D and 2D NMR techniques.

The anomeric signals of AXOS in the ^{1}H NMR spectra are found in spectral region 4.4–5.5 ppm (Gruppen et al., 1992; Hoffmann, Leeflang, de Bares, Kamerling, & Vliegenthart, 1991). In the 1D ^{1}H NMR spectrum the signals of the anomeric protons confirmed the presence of arabinoxylobiose (Fig. 3). The signal at 5.33 ppm indicates that the α-L-arabinofuranosyl group is (1 → 3)-linked to a β-D-xylopyranosyl group. The chemical shifts of the anomeric signals were determined directly from the ^{1}H spectra and those for the other ring protons were extracted from 2D ^{1}H–^{1}H DQF-COSY experiments (Fig. 4). The values of the chemical shifts are shown in Table 3. The proton chemical shifts were in good agreement with the data presented earlier for (1 → 3)-linked arabinoxylobiose obtained from wheat flour AX (Hoffmann et al., 1991) and also from barley AX (Viëtor et al., 1994).

Assignments of ^{13}C resonances of cross-peaks in the HMQC spectrum were based on the proton signals assigned (Fig. 5). The ^{13}C chemical shifts thus obtained are not very accurate (Table 2). The resonances of Ara*f* and unbranched Xyl*p* were in good agreement with values published earlier for constituent monosaccharides of (1 → 3)-linked arabinoxylan units isolated from water-soluble AX from wheat endosperm (Hoffmann, Kamerling, & Vliegenthart, 1992). In terminal non-reducing Xyl*p* unit the resonance of C3 (81.9 ppm) showed a large deshielding indicating the α-L-arabinofuranosyl substitution. This resonance value was higher than the values determined for Xyl*p* units in the internal substituted arabinoxylans (78.4 ppm) (Hoffmann et al., 1992). The chemical shift of C4 (68.1 ppm) was an indication of termination of the xylopyranosyl chain. To the best of our knowledge the ^{13}C NMR data of arabinoxylobiose have not been published earlier.

From these NMR data we concluded that NMR spectroscopy confirmed all the results from the previous analysis. In addition to the information that the oligosaccharide was arabinoxylobiose, the NMR analysis demonstrated that the arabinose subunit was bound to position C3 of the β-D-xylopyranosyl unit at the non-reducing end of the xylobiose. The final structure of the isolated oligosaccharide is α-L-Ara*f*-(1 → 3)-β-D-Xyl*p*-(1 → 4)-D-Xyl*p* (Fig. 6).

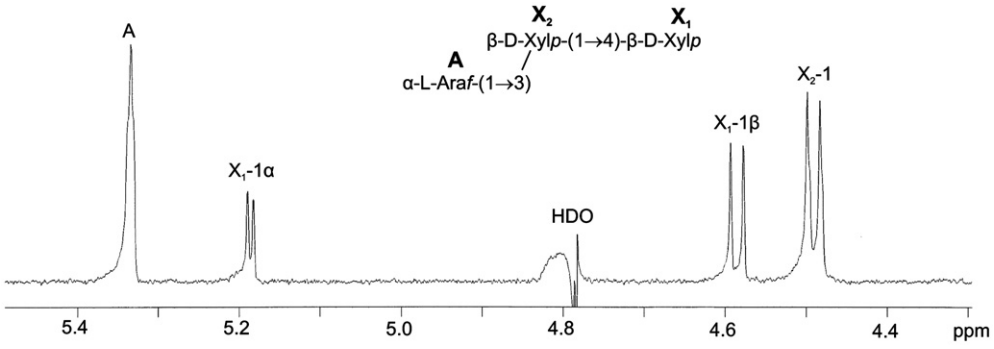

Fig. 3. Anomeric region of the 1D ^{1}H NMR spectrum of arabinoxylobiose. The measurements were performed at 500 MHz using the gHX nano-NMR probe. The designations in the spectrum are indicated in the structure.

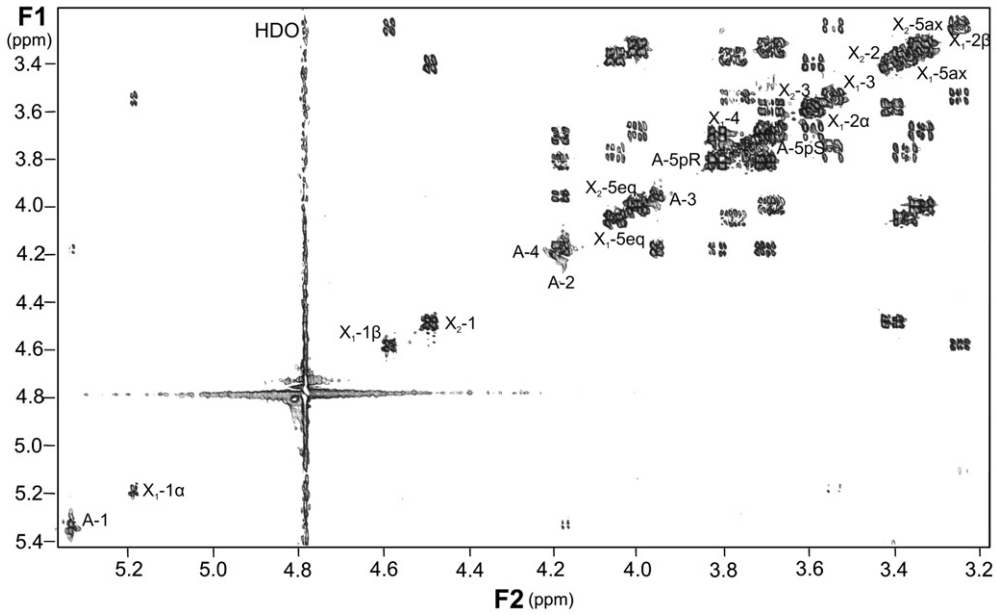

Fig. 4. DQF-COSY spectrum of arabinoxylobiose. The designation of diagonal peaks of the monosaccharide residues in the spectrum refers to the structure in Fig. 3 and Table 3.

Table 3
NMR data on the constituent monosaccharide residues of the arabinoxylobiose derived by enzymatic degradation of rye xylan

Residue	Assigned C,H position	^{1}H chemical shifts (ppm)	^{13}C chemical shifts (ppm)
Ara*f* (A)	1	5.33	108.5
	2	4.19	82.0
	3	3.96	77.1
	4	4.18	85.1
	5pro*R*	3.82	61.9
	5pro*S*	3.71	61.9
Xyl*p* 1 (X_1)	1α	5.18	92.7
	1β	4.58	97.2
	2α	3.55	74.9
	2β	3.25	74.9
	3	3.55	75.0
	4	3.75	77.0
	5*eq*	4.05	63.7
	5*ax*	3.38	n.d.
Xyl*p* 2 (X_2)	1	4.49	102.6
	2	3.41	73.7
	3	3.59	81.9
	4	3.69	68.1
	5*eq*	4.00	65.6
	5*ax*	3.34	65.6

Arabinoxylobiose was also produced in earlier studies using enzymatic hydrolysis. Dekker & Richards (1975) already reported in 1975 that hemicelluloses from various sources were hydrolysed to arabinoxylobiose by *Ceratocystis paradoxa* xylanase. Anand & Vithayathil (1996) were further able to hydrolyse short arabinoxylo-oligomers made from sugarcane bagasse xylan and presumably form some arabinoxylobiose using xylanase from *Humicola lanuginosa*. Kabel et al. (2002a, 2002c) reported arabinoxylobiose as a minor hydrolysis product of brewery's spent grain, wheat bran and corn cobs by xylanase I from *A. awamori*. In these reports, arabinoxylobiose was detected in HPAEC-PAD and MALDI-TOF-MS. It was not the main hydrolysis product and was not purified. Arabinoxylobiose with an α-1,3 bond between the Ara*f* and Xyl*p* units was also isolated from enzymatic hydrolysates for further studies using GPC (Bio-Gel P2) alone (Pell et al., 2004) or in combination with HPAEC (Gruppen et al., 1992; Viëtor et al., 1994) or with a charcoal column (Yoshida et al., 1998).

3.5. Activity of *A. aculeatus* GH10 xylanase

GH10 xylanases have previously been reported to produce α-L-Ara*f*-(1 → 3)-β-D-Xyl*p*-(1 → 4)-D-Xyl*p* (arabinoxylobiose) and β-D-Xyl*p*-(1 → 4)[α-L-Ara*f*-(1 → 3)]-β-D-Xyl*p*-(1 → 4)-D-Xyl*p* (arabinoxylotriose) as the shortest AXOS, with the ratio depending on the xylanase. Arabinoxylobiose was reported as the main arabinofuranoside-substituted product formed by *Cellvibrio mixtus* Xyn10B (Pell et al., 2004) and *Streptomyces olivaceoviridis* Xyn10A (Fujimoto et al., 2004; Yoshida et al., 1998). Arabinoxylotriose was the predominant AXOS produced by *A. awamori* xylanase I, which presumably belongs to glycoside hydrolase family 10 (Kabel et al., 2002c), and *A. oryzae* xylanase p*I* 6.9. (Tenkanen, 1999).

The main AXOS produced by Shearzyme was arabinoxylobiose. However, formation of arabinoxylotriose is difficult to analyse by HPAEC-PAD, due to its almost simultaneous elution with arabinoxylobiose (Kabel et al., 2002a). TLC analysis also revealed small amounts of arabinoxylotriose in Shearzyme hydrolysate of rye flour AX (Fig. 7). The hydrolysate formed by *A. oryzae* xylanase pI 6.9 was used as a reference sample for arabinoxylotriose.

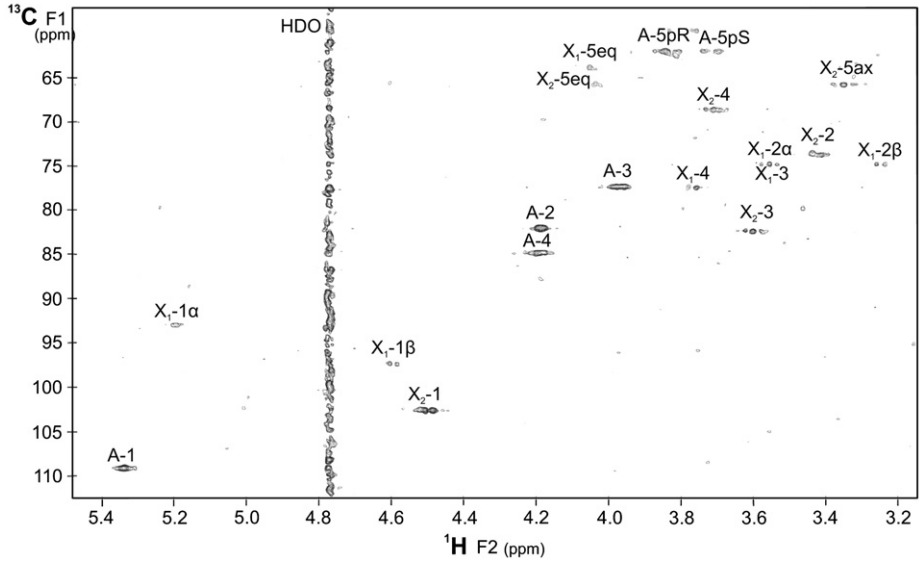

Fig. 5. HMQC spectrum of arabinoxylobiose. The designation of cross-peaks of the monosaccharide residues in the spectrum refers to the structure in Fig. 3 and Table 3.

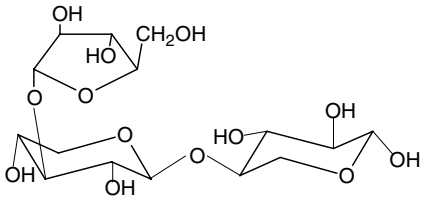

Fig. 6. Arabinoxylobiose (α-L-Ara*f*-(1 → 3)-β-D-Xyl*p*-(1 → 4)-D-Xyl*p*).

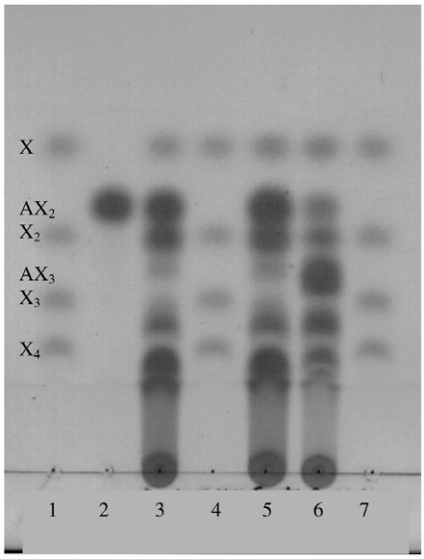

Fig. 7. TLC analysis of hydrolysed samples. 1, 4 and 7, standards X–X_4; 2, purified arabinoxylobiose (AX_2), 3, *A. aculeatus* hydrolysate of rye flour AX, 5, Shearzyme hydrolysate of rye flour AX and 6, *A. oryzae* hydrolysate of rye flour AX.

Since Shearzyme also contains other enzymes, the activity patterns and products formed by the pure *A. aculeatus* GH10 xylanase was determined. TLC analysis revealed that Shearzyme and pure xylanase resulted in the same product pattern when hydrolysing rye flour AX (Fig. 7). We concluded that the ability of Shearzyme to produce α-L-Ara*f*-(1 → 3)-β-D-Xyl*p*-(1 → 4)-D-Xyl*p* as the major AXOS occurs solely from the function of *A. aculeatus* GH10 xylanase, which clearly possesses high activity towards xylosidic linkage after an Ara*f*-substituted xylose residue and is thus able to accommodate α-1,3-linked Ara*f*-substituted xylose unit in its +1 subsite. The ability of *A. aculeatus* GH10 xylanase to produce very short-chain AXOS may originate from catalytic site properties similar to those descibed for *C. mixtus* Xyn10B (Pell et al., 2004). Pure *A. aculeatus* GH10 xylanase was previously used to produce a mixture of longer AXOS from wheat flour AX by using more limited hydrolysis than carried out in the present work (Swennen, Courtin, Van der Bruggen, Vandecasteele, & Delcour, 2005).

4. Conclusions

Rye arabinoxylan was intensively hydrolysed with Shearzyme and the main short AXOS formed was confirmed to be arabinoxylobiose (α-L-Ara*f*-(1 → 3)-β-D-Xyl*p*-(1 → 4)-D-Xyl*p*). Arabinoxylobiose was also preparatively isolated. Previously, this oligosaccharide was isolated from the enzymatic hydrolysates of other cereal (wheat and barley) arabinoxylans. The yield of arabinoxylobiose was, however, rather low, presumably due to the limited hydrolysis of densely substituted arabinoxylan. Hydrolysis could be theoretically intensified by removing some of the arabinofuranosyl units from the double-substituted xylopyranosyl units. The ability of *A. aculeatus* GH10 xylanase, which is heterologously produced in Shearzyme, to form arabinoxylobiose as the main product may originate from the catalytical site properties similar to those described for *C. mixtus* xylanase

(*Cm*Xyn10B) (Pell et al., 2004). The prebiotic properties of arabinoxylobiose need to be further studied.

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