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Ultrafiltration and ethanol precipitation for isolation of arabinoxylooligosaccharides with different structures

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Abstract

Ultrafiltration was investigated as possible alternative to ethanol precipitation for isolation of arabinoxylooligosaccharides (AXOS) with different structures from enzymically produced arabinoxylan (AX) hydrolysates. Incubation of wheat flour AX with an *Aspergillus aculeatus* endoxylanase yielded mixtures of AX poly- and oligosaccharides. Their fractionation by graded ethanol precipitation or ultrafiltration membranes with different molecular mass cut-off yielded fractions, which differed both in degree of polymerization (DP) and degree of substitution (DS). Although the ultrafiltration fractions were more heterogeneous than the ones obtained by ethanol precipitation, AXOS fractions with similar DP and DS could be obtained with both methods.

Keywords: Arabinoxylooligosaccharides; Arabinoxylans; Ethanol precipitation; Ultrafiltation

1. Introduction

There is an increasing interest in plant cell wall polysaccharides as sources of novel prebiotic oligosaccharides (OS) (Rastall and Maitin, 2002). In this context, e.g. xylooligosaccharides (XOS), linear OS with low molecular mass (MM) [degree of polymerization (DP) < 4], have received attention (Rastall and Hotchkiss, 2003). XOS are typically produced by enzymic and/or chemical degradation of lignocellulosic materials, such as hard woods, brewery spent grain, corn cobs and wheat bran, having xylan as major hemicellulose component (Parajó Garrote, Cruz, & Domínguez, 2004). Although, the prebiotic effectiveness of these low MM XOS has intensively been investigated in both in vitro (Rycroft, Jones, Gibson, & Rastall, 2001; Kabel, Kortenoeven, Schols, & Voragen, 2002) and in vivo experiments (Okazaki, Fujikawa, & Matsumoto, 1990; Suwa, et al., 1999; Vázquez, Alonso, Domínguez, & Parajó, 2000), little is known about the effects of substituted XOS in general or arabinoxylooligosaccharides (AXOS) in particular. The latter can be formed from wheat flour arabinoxylans (AX). Their AX are built of a backbone of (1→4)-β-linked D-xylopyranosyl units with substitutions of α-L-arabinofuranosyl units at position C-(O)-2 and/or C-(O)-3 (Gruppen, Hamer, & Voragen, 1992) and are divided into water-extractable AX (WE-AX) and water-unextractable AX (WU-AX) (Courtin and Delcour, 2001). Acid treatment or endo-β-(1,4)-xylanases (EC 3.2.1.8), further referred to as endoxylanases, can hydrolyse AX. Mild acid treatment of AX mostly yields very small OS and monosaccharides, in particular α-L-arabinofuranose (Zhang, Zhang, & Whistler, 2003). Endoxylanases attack the AX backbone internally,

Abbreviations used A, arabinose; AX, arabinoxylans; (A)(X)OS, (arabino)(xylo)oligosaccharides; AZCL-AX, azurine cross-linked AX; DP, degree of polymerisation; DS, degree of substitution; GC, gas–liquid chromatography; HPAEC-PAD, high-performance anion exchange chromatography with pulsed amperometric detection; HPSEC, high-performance size-exclusion chromatography; MM, molecular mass; MMCO, molecular mass cut-off; S-AX, solubilised AX; SQF, squeegee fraction; U, unit; WE-AX, water-extractable AX; WU-AX, water-unextractable AX; XAA, *Aspergillus aculeatus* endoxylanase; X_1, xylose; X_2, xylobiose; X_3–X_6, XOS with DP of 3–6.

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dependent on the arabinose substitution pattern. They solubilise WU-AX and degrade WE-AX and solubilised AX (S-AX) yielding series of oligomeric and polymeric AX with different DP (Courtin and Delcour, 2001; Maes, Vangeneugden, & Delcour, 2004). As polymeric AX, as a result of their characteristic property to induce high viscosities in solution, and arabinose and xylose monomers have anti-nutritive effects on monogastric animals (Annison, 1993; Austin, Wiseman, & Chesson, 1999), it is useful to separate AXOS from these components. Fractionation of low and high MM components in general (Defloor, Vandenreyken, Grobet, & Delcour, 1998; Ku et al., 2003; Moerman, van Leeuwen, & Delcour, 2004), and of AX in particular (Courtin and Delcour, 1998) is mostly carried out by precipitation with alcoholic solutions. However, recently, ultrafiltration, a well-established separation process in the biotechnology and fermentation industries (Grandison, Goulas, & Rastall, 2002), was used to separate OS and polysaccharides (Freixo and Norberta de Pinho, 2002; Kim, Kim, & Hwang, 2003; Czermak, et al., 2004; Moerman, van Leeuwen, & Delcour, 2004).

In this study, AXOS were separated and fractionated from their monomeric building blocks, arabinose and xylose, and longer chain analogues, AX, by ethanol precipitation and ultrafiltration. The fractions obtained during both fractionations were compared with respect to AX yield, average DP and degree of substitution (DS). In view of nutritional functionality, AXOS are here defined to encompass molecules with a DP up to 60 or a MM up to approximately 8000 Da, thus deviating from the chemical definition of OS maintaining that they have a DP between 3 and 10 (Voragen, 1998).

2. Materials and methods

2.1. Materials

Wheat (cultivar Legat, harvest 2002) was obtained from AVEVE (Landen, Belgium) and milled at a moisture content of 14.5% on a Bühler MLU-202 laboratory mill (Uzwil, Switzerland) according to AACC method 26-31 (Anonymous, 2000). Enzymes used were heat-stable α-amylase (Termamyl 120 L, Novozymes, Bagsvaerd, Denmark), amyloglucosidase (Megazyme, Bray, Ireland), bacterial protease (Neutrase 0.8 L, Novozymes), and pure *Aspergillus aculeatus* endoxylanase (XAA) (Puratos N.V., Groot-Bijgaarden, Belgium; glycosyl hydrolase family 10, optimum temperature 70 °C, optimum pH 4.0, MM 46.58 kDa, pI 5.45). All chemicals and reagents were of at least analytical grade and supplied by Sigma-Aldrich (Bornem, Belgium) unless specified otherwise. Shodex standard P-82 pullulans were purchased from Showa Denko K.K. (Tokyo, Japan) and XOS standards from Megazyme. Azurine cross-linked AX tablets (AZCL-AX) were obtained from Megazyme.

2.2. Standard analyses

Moisture and ash contents were analysed according to the AACC methods 44-19 and 08-01, respectively (Anonymous, 2000). Protein contents were determined using the Dumas combustion method, an adaptation of the AOAC Official Method (1995) to an automated Dumas protein analysis system (EAS varioMax N/CN, Elt, Gouda, The Netherlands), using 5.7 as a factor for conversion from nitrogen to protein content.

2.3. Analysis of total sugar and reducing end sugar content

Total sugar content was determined by gas-liquid chromatography (GC) of alditol acetates as described by Courtin, Van den Broeck, & Delcour (2000). Flour and squeegee starch samples (approximately 150 mg) on the one hand, and AX by-streams (approximately 15 mg) on the other were hydrolysed with 2.0 N trifluoroacetic acid (TFA, 5.0 ml) at 110 °C for 2 and 1 h, respectively. Hydrolysis of AX containing supernatants (2.5 ml) was with 4.0 N TFA (2.5 ml) at 110 °C for 1 h. The resulting monosaccharides were reduced and converted to alditol acetates which were separated on a Supelco SP-2380 column (30 m × 0.32 mm ID, 0.2 μm film thickness) (Supelco, Bellefonte, PA, USA) with helium as carrier gas in a Agilent 6890 series chromatograph (Agilent, Wilmington, DE, USA) equipped with autosampler, splitter injection port (split ratio 1:20), and flame-ionisation detector. Separation was at 225 °C with injection and detection temperatures at 270 °C. β-D-Allose was used as internal standard and calibration samples, containing the expected monosaccharides, were included with each set of samples. The AX content was calculated as 0.88 times the sum of the monosaccharides xylose and arabinose while the DS was calculated as the arabinose-to-xylose ratio.

Analysis of reducing end sugar contents was very similar to those of total sugar. However, samples (2.5 ml) were reduced prior to hydrolysis and acetylation to alditol acetates (Courtin, Van den Broeck, & Delcour, 2000). Combined with total sugar analysis, these results allowed to calculate the average DP of AX rich materials as the sum of the total xylose and arabinose divided by the amount of reducing end xylose.

2.4. High-performance size-exclusion chromatography

Apparent MM distributions were determined using High-Performance Size-Exclusion Chromatography (HPSEC) on a High Performance Liquid Chromatography system (Kontron Instruments, 325 pump systems, Kontron, Milan, Italy) equipped with autoinjection. Aliquots of supernatants were filtered (0.45 μm membrane filter) and injected (20 μl) on a Shodex SB 800P guard column (50 mm × 6 mm) (Showa Denko K.K.) attached to a Shodex SB 802.5 or SB 803 HQ HPSEC column (300 mm × 8 mm, separation

ranges 100–10,000 and 100–100,000 Da, respectively). Elution was with sodium acetate (25 mM, pH 4.7) (0.5 ml/min, 30 °C) and monitored with a refractive index detector (VSD Optilab, Berlin, Germany). Molecular mass markers were Shodex standard P-82 pullulans (2.0 mg/ml) with a MM of 11.2×10^4, 4.73×10^4, 2.28×10^4, 1.18×10^4 and 0.59×10^4 Da, XOS standards with MM of 810, 678, 546, 414, and 282 Da, and glucose.

2.5. High-performance anion-exchange chromatography with pulsed amperometric detection

High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) was performed with a Dionex DX-500 chromatography system (Sunnyvale, CA, USA) equipped with an ED-40 electrochemical detector, a GP-50 gradient pump and an AS-3500 autosampler. Aliquots of supernatants were filtered (0.20 μm membrane filter) and injected (25 μl) on a CarboPac PA-100 guard column (25×4 mm) attached to a CarboPac PA-100 anion-exchange column (250×4 mm). Elution (1.0 ml/min) was with a linear gradient of 0–250 mM sodium acetate in 100 mM sodium hydroxide for 30 min, followed by a linear gradient of 250–400 mM sodium acetate in 100 mM sodium hydroxide for 15 min. The gradients ended by 5 min washing with 100 mM sodium hydroxide. The elution was monitored using the ED-40 detector in the pulsed amperometric detection mode with following potentials and time periods: E_1, +0.05 V ($t_1=400$ ms); E_2, +0.75 V ($t_2=200$ ms); E_3, −0.15 V ($t_3=400$ ms). Arabinose (A), xylose (X_1), xylobiose (X_2) and XOS with DP 3–6 (X_3–X_6) were used as references.

To obtain information on the distribution of xylan backbone chain lengths in the supernatants, mild acid hydrolysis was performed, resulting in mostly selective removal of arabinose substituents. Freshly prepared hydrochloric acid (0.1 M) was used to adjust the pH to 2.8. The acid solutions were further heated in a sealed tube for 24 h at 90 °C. After cooling, the reaction mixtures were neutralized with sodium hydroxide (0.1 M) and centrifuged (9000 g, 15 min, 18 °C). The obtained supernatants were analysed by GC and HPAEC-PAD as described above.

2.6. Determination of endoxylanase activity

Endoxylanase activity was determined using insoluble AZCL-AX as described in Megazyme Data Sheet 9/95. XAA was diluted in sodium acetate buffer (25 mM, pH 4.7) containing bovine serum albumin. Appropriate enzyme dilutions were incubated with an AZCL-AX tablet. The absorbance at 590 nm was measured with an Ultraspec III UV/Visible spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). One Unit (U) of enzyme activity was the amount of enzyme required to yield an absorbance of 1.0 at 590 nm under the assay conditions (40 °C, pH 4.7).

2.7. Preparation of WU-AX

Wheat flour (1600 g) was fractionated into gluten, prime starch, a water soluble and a squeegee fraction (SQF, containing most of the flour WU-AX) using the procedure of McRitchie (1985). The SQF (400 g) was further suspended in deionised water (1:6 w/v), and starch was hydrolysed with Termamyl 120L (30 μl/g SQF, 60 min, 90 °C) followed by amyloglucosidase treatment (20 μl/g SQF, 16 h, 60 °C, pH 4.5). Following centrifugation (10,000 g, 30 min, 18 °C), proteins were hydrolysed with Neutrase 0.8L (20 μl/g SQF, 20 h, 50 °C, pH 5.0). The residue was recovered and washed several times with deionised water and ethanol [95% (v/v)]. After air-drying, the obtained material, further referred to as WU-AX, was ground and sieved to pass a 250 μm sieve.

2.8. Enzymic hydrolysis of WU-AX

Preliminary experiments were carried out to assess the enzyme dosage needed for solubilising a maximal amount of WU-AX with sufficient breakdown of the polymeric structure of S-AX. To this end, three enzyme dosages (0.8, 1.6 and 5 U, each 8 ml) were added to WU-AX (approximately 720 mg) suspended in 232 ml sodium acetate buffer (25 mM, pH 4.7). The mixtures were incubated under continuous stirring during 105, 225, 465 and 1425 min at 30 °C, and centrifuged (9000 g, 15 min, 18 °C). The supernatants were then placed in boiling water for 30 min. The residue was washed with 60 ml of the same sodium acetate buffer, suspended in deionised water, lyophilised and analysed by GC. The wash waters were inactivated (30 min, 100 °C) and combined with the supernatants. After filtration, the obtained solutions were analysed by GC, HPSEC and HPAEC-PAD.

2.9. Fractionation by ethanol precipitation

The enzymically prepared AXOS (see Section 2.8), were separated into three fractions by graded ethanol precipitation. Aliquots of ethanol were added under continuous stirring to a final concentration of 60% (v/v). The mixtures were then stirred for an additional 30 min and kept overnight at 4 °C. Precipitated materials were recovered by centrifugation (10,000 g, 20 min, 4 °C), dissolved in deionised water and lyophilised to obtain fraction $F_{0–60\%}$. The ethanol concentration of the supernatant was further increased to 90% (v/v) and the precipitated fraction, referred to as fraction $F_{60–90\%}$, was recovered as described above. Ethanol was removed from the remaining supernatant by rotary evaporation. The remaining fraction was lyophilised and recovered as fraction $F_{>90\%}$. Fractions $F_{0–60\%}$, $F_{60–90\%}$ and $F_{>90\%}$ were analysed by GC, HPSEC and HPAEC-PAD.

2.10. Fractionation by ultrafiltration

Dead-end ultrafiltration was carried out using a HP 4750 Stirred Cell ultrafiltration device (Sterlitech Corporation, Kent, OH, USA), a unique designed 316L stainless steel construction operational at pressures up to 69 bar and hold-up volumes as low as 1.0 ml. Concentration polarization at the membrane surface was minimized by a Teflon-coated magnetic stir bar mechanism which was centrally positioned in the cell and had a stirring rate of 700 rpm. The pressure source was a compressed nitrogen gas cylinder. The pressure was controlled by a pressure regulator and a gauge attached to the pressure inlet allowed readings of the system pressure. A decompression valve allowed filtration to be stopped.

Commercial membranes with a molecular mass cut-off (MMCO), also known as molecular weight cut-off, of 5 kDa (P005F) (Celgard, Wiesbaden, Germany), 10 kDa (PES-10) (Synder Filtration, Vacaville, CA, USA) and 30 kDa (PES-030H) (Celgard) were used. All three membranes were cut to size and soaked overnight in deionised water prior to use.

Filtration experiments were carried out at a constant pressure of 4 bar. The dead-end ultrafiltration cell was filled with 300 ml of enzymically hydrolysed WU-AX (see Section 2.8) and ultrafiltration was allowed to proceed for 5–8 h, depending on the used membrane, at room temperature. In general, during the filtration process, fractions of average MMs less than the MMCO of the used membrane passed through the membrane (=permeate) while those having larger MMs were collected as retentate. When approximately 200 ml of permeate was collected, filtration was stopped. Both permeate and retentate solutions were analysed by GC, HPSEC and HPAEC-PAD.

3. Results and discussion

3.1. Chemical composition of wheat flour, SQF and WU-AX

Table 1 presents fractionation yields and analytical data of Legat flour, SQF and WU-AX. The isolated WU-AX are rich in AX [approximately 70% of dry matter (dm) and DS 0.54]. The remaining part of the sample consists mainly of proteins (3% dm), mannose (3% dm), polymeric glucose (4% dm) and some galactose (1% dm). As the isolated WU-AX are cell wall components, it can be expected that the sample also contains ash, cellulose, lignin and/or ferulic acid.

3.2. Enzymic hydrolysis of WU-AX

Table 2 represents the relative amount of S-AX (%), average DS and DP of components formed after incubation of WU-AX with 0.8, 1.6 and 5 U XAA during 2, 4, 8 and 24 h. Irrespective of the 'enzyme dosage—time' combination, maximally 89% of WU-AX were solubilised. The higher the enzyme dosage, the sooner this maximum was reached. Under all incubation conditions, HPSEC profiles (Fig. 1) showed AX fragments with apparent peak MMs of 1100, 500 and 300 Da. Higher enzyme dosage and/or longer incubation times yielded higher levels of these fragments. The same features were observed in HPAEC profiles (not shown).

Based on the above, it was decided to work with the AXOS enriched solution obtained after 4 h of incubation of WU-AX with 1.6 U XAA, further referred to as AXOS containing stock solution. This solution contained a considerable level of S-AX with an average DP of 8. Approximately 30% of the S-AX had MMs exceeding 8000 Da [Fig. 1(b)], which classified them outside the range of AXOS.

3.3. Fractionation by ethanol precipitation

The AXOS containing stock solution was fractionated with ethanol into three fractions, precipitating at ethanol concentrations of 60% ($F_{0–60\%}$), between 60 and 90% ($F_{60–90\%}$), and above 90% ($F_{>90\%}$). Fig. 2 shows the HPSEC profiles of the fractions obtained. With increasing levels of ethanol, the MM systematically decreased. The $F_{0–60\%}$ fraction contained AX components with an average DP of 53 (Table 3) and represented only 15%

Table 1
Yields (expressed as % starting material, dry matter (dm)), protein contents (% dm), ash contents (% dm), monosaccharide compositions (% dm), arabinoxylan (AX) content (% dm), degree of substitution (DS) and starch content (% dm) of Legat flour, squeegee fraction (SQF) and water-unextractable arabinoxylans (WU-AX)

Fraction	Yield	Protein	Ash	Ara[a]	Xyl[a]	Man[a]	Gal[a]	Glc[a]	AX[b]	DS[c]	Starch[d]
Legat flour	100.0	11.7	0.5	0.9	1.5	0.2	0.3	82.9	2.1	0.62	76.7
SQF	28.0	1.3	1.3	1.9	3.2	0.3	0.1	86.9	4.5	0.58	78.2
WU-AX	4.8	3.4	n.d.[e]	27.8	51.9	3.4	0.5	4.3	70.1	0.54	3.8

[a] Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose.
[b] AX = 0.88 × (Ara + Xyl).
[c] DS = Ara/Xyl.
[d] Starch = (0.9 × Glc).
[e] n.d., not determined.

Table 2
Solubilised arabinoxylan (S-AX) material (expressed as % of starting material), average degree of substitution (DS) and degree of polymerization (DP) of arabinoxylan fractions obtained after incubation of water-unextractable arabinoxylans during 2, 4, 8 and 24 h with 0.8, 1.6 and 5 Units (U) Aspergillus aculeatus endoxylanase

	2 h			4 h			8 h			24 h		
	0.8 U	1.6 U	5 U	0.8 U	1.6 U	5 U	0.8 U	1.6 U	5 U	0.8 U	1.6 U	5 U
S-AX	63.9	78.0	88.9	76.6	84.4	88.2	84.5	86.0	88.0	88.9	88.6	88.7
DS	0.50	0.49	0.50	0.49	0.50	0.50	0.49	0.50	0.49	0.50	0.49	0.49
DP	14	10	7	12	8	6	7	6	6	6	6	5

of the S-AX in the AXOS containing stock solution. Fig. 2 showed that a considerable part of these molecules appeared in the void volume of the Shodex SB-802.5 HQ column, and thus represented the previously mentioned 30% S-AX in the AXOS containing stock solution [Figs. 1(b) and 2]. Detection of these components with HPAEC-PAD was impossible. Fraction $F_{60–90\%}$ contained medium MM AXOS with an average DP of 23 (Table 3) and an average peak MM of approximately 5000 Da (Fig. 2). They represented approximately 29% of the S-AX present in the AXOS containing stock solution and appeared in the HPAEC profile as a 'bump' [Fig. 3(a)]. Fraction $F_{>90\%}$ contained the largest part of S-AX population in the AXOS containing stock solution (approximately 47%). These low MM AXOS components had an average DP of 5 (Table 3) and peak MMs varying between 300 and 2000 Da (Fig. 2). The HPAEC profile [Fig. 3(b)], showed that this low MM AXOS fraction contained small levels of A, X_1, X_2 and XOS with DP from 3 to 6 (X_3–X_6) and larger levels of unidentified AXOS. Since AXOS standards are not available, identification of these unidentified AXOS is difficult. Mild acid hydrolysis of this fraction resulted in the release of approximately 90% of the arabinofyranosyl units and 8% of the xylopyranosyl units, the latter entailing a small underestimation of XOS DP in the HPAEC profiles. HPAEC [Fig. 3(d)] showed that this fraction contained A, X_1 and X_2, as well as a well separated range of peaks for XOS. As the profile resembled profiles obtained for linear dextrins (White, Hudson, & Adamson, 2003), we reasonably assume that a consistent range of DPs was obtained, and consequently carried out peak assignments

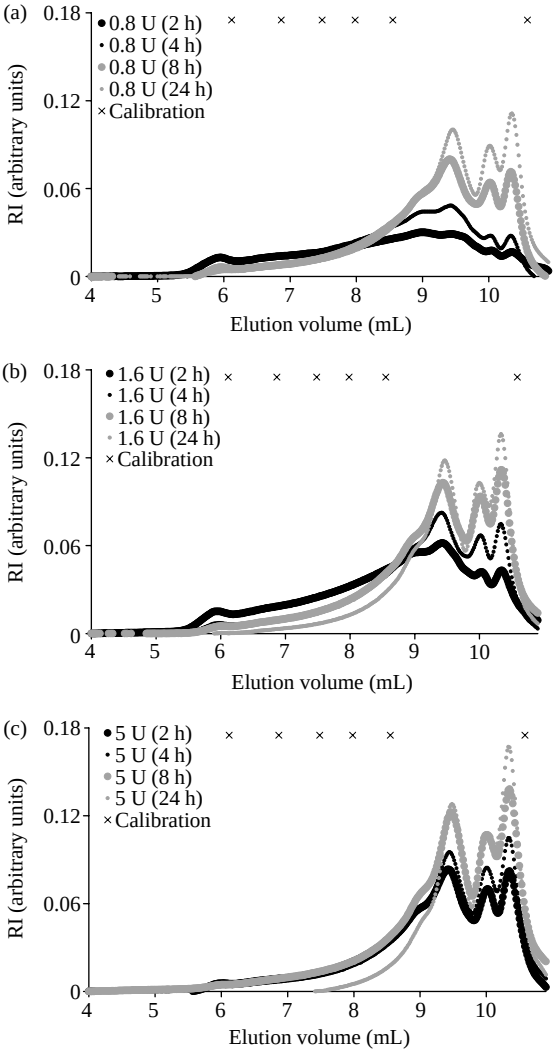

Fig. 1. HPSEC molecular mass profiles obtained after 2, 4, 8 and 24 h incubation of WU-AX with 0.8 (a), 1.6 (b) and 5 U (c) XAA. Elution volumes of pullulan standards (×) of molecular mass of 11.2×10^4, 4.73×10^4, 2.28×10^4, 1.18×10^4, 0.59×10^4 Da and glucose are indicated from left to right. Column is a Shodex SB-803 HQ.

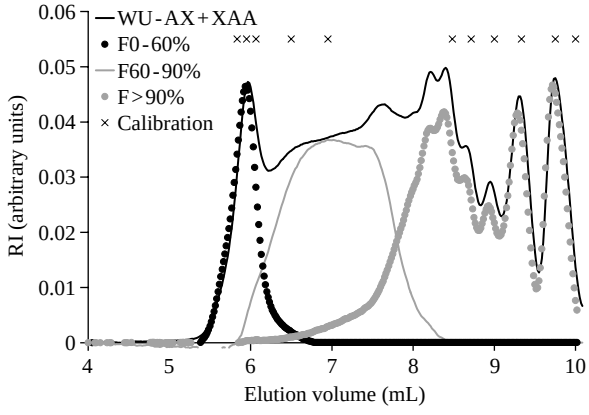

Fig. 2. HPSEC molecular mass profiles of fractions $F_{0–60\%}$, $F_{60–90\%}$ and $F_{>90\%}$ obtained after fractionation of the AXOS containing stock solution by ethanol precipitation. Elution volumes of standards (×) of molecular mass of 11.2×10^4, 4.73×10^4, 2.28×10^4, 1.18×10^4, 0.59×10^4, 0.081×10^4, 0.0678×10^4, 0.0546×10^4, 0.0414×10^4, 0.0282×10^4 Da and glucose are indicated from left to right. Column is a Shodex SB-802.5 HQ.

Table 3
Arabinoxylan (AX) yield, average degree of substitution (DS) and degree of polymerization (DP) of AX in fractions $F_{0-60\%}$, $F_{60-90\%}$ and $F_{>90\%}$, obtained after fractionation of the AXOS containing stock solution by ethanol precipitation

	$F_{0-60\%}$	$F_{60-90\%}$	$F_{>90\%}$
AX yield	15.1	29.2	47.2
DS	0.78	0.64	0.37
DP	53	23	5

[Fig. 3(d)]. HPAEC analysis of acid treated fraction $F_{60-90\%}$ [Fig. 3(c)] showed that the AXOS present in this fraction were of higher MM than the ones present in fraction $F_{>90\%}$, as larger levels of XOS and AXOS with high DP were detected.

The DS of the fractions obtained after ethanol precipitation decreased with increasing ethanol concentration. These results are in contrast to those published by Cleemput and co-workers (1995) for unmodified wheat WE-AX. They found DS values increasing from 0.36 to 0.82 in a range going from 15 to 65% ethanol. Similar observations were made by Gruppen and co-workers (1992) and Izydorczyck and Biliaderis (1993), who used ethanol and ammonium sulphate, respectively, for fractionation of wheat AX. However, the results are in line with the observation by Courtin and Delcour (1998) that enzymically degraded AX and native AX have different precipitation behaviour. The researchers observed a drop in DS from 0.70 to 0.49 for ethanol concentrations rising from 60 to 80%. This observation led them to suppose that more substituted AX, which are less susceptible to enzyme degradation, have higher MMs and for that reason precipitate first.

3.4. Fractionation by ultrafiltration

3.4.1. One step ultrafiltration

To judge the usefulness of ultrafiltration for fractionation of enzymically produced AXOS, ultrafiltration experiments with membranes of different MMCOs were first carried out.

With the membrane of lowest MMCO, i.e. 5 kDa, the HPSEC profile of the permeate fraction ($PER_{5\ kDa}$) [Fig. 4(a)] mainly showed OS with peak MMs varying between 180 and 700 Da. In contrast, AXOS in the retentate fraction ($RET_{5\ kDa}$), which contained 86% of the S-AX in the AXOS containing stock solution (Table 4), were very polydisperse. HPAEC profiles (not shown) clearly showed a $PER_{5\ kDa}$ fraction containing OS of low DP, and a $RET_{5\ kDa}$ fraction containing OS of low and higher DPs. The presence of components with low DP in the $RET_{5\ kDa}$ fraction may result from their retention as a result of reduced permeate fluxes that themselves are the result of the presence of high MM compounds (Jorda, Mare chal, Rigal, & Pontalier, 2002). A broad distribution of pore sizes in the membrane, as is often the case for ultrafiltration, also results in a non-perfect separation. If the permeate volume is increased, it can be expected that less components with low DP are present in the retentate, but the permeate would be more polydisperse.

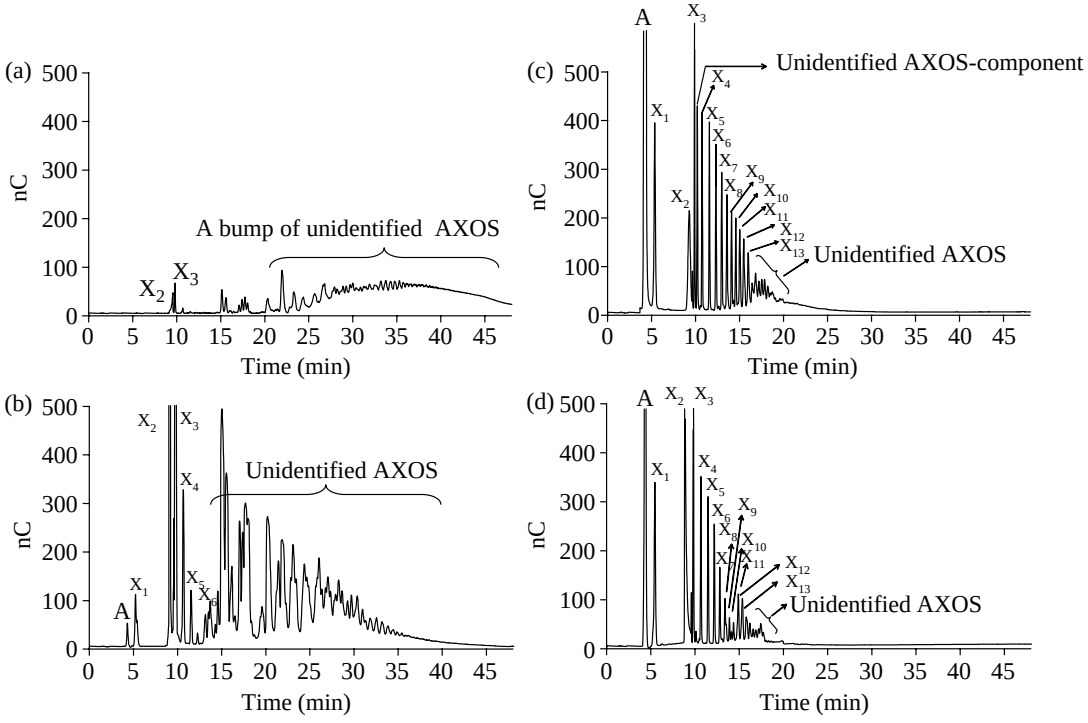

Fig. 3. HPAEC profiles of fractions $F_{60-90\%}$ (a) and $F_{>90\%}$ (b) obtained after fractionation of the AXOS containing stock solution by ethanol precipitation and HPAEC profiles of the above described fractions followed by acid hydrolysis (c) and (d), respectively.

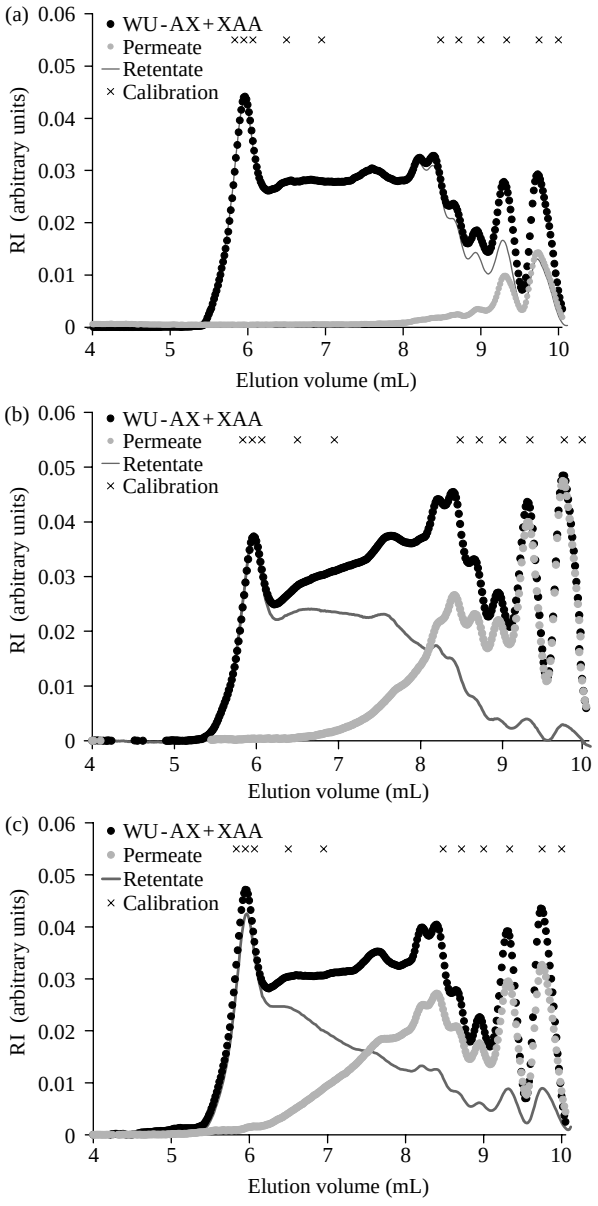

Fig. 4. HPSEC molecular mass profiles of fractions obtained after one step ultrafiltration of the AXOS containing stock solution with a membrane with MMCO of 5 (a), 10 (b) and 30 kDa (c). Elution volumes of standards (×) of molecular mass of 11.2×10^4, 4.73×10^4, 2.28×10^4, 1.18×10^4, 0.59×10^4, 0.081×10^4, 0.0678×10^4, 0.0546×10^4, 0.0414×10^4, 0.0282×10^4 Da and glucose are indicated from left to right. Column is a Shodex SB-802.5 HQ.

With membranes of increased MMCO [10 and 30 kDa; Figs. 4(b) and (c)], the levels of low and medium MM compounds in permeate fractions ($PER_{10\ kDa}$ and PER_{30kDa}) increased with AX yields respectively, while those in retentate fractions ($RET_{10\ kDa}$ and $RET_{30\ kDa}$) decreased with AX yields respectively. HPAEC results (not shown) were in line with the above.

Table 4 showed that ultrafiltration, like ethanol precipitation, separated fractions of different DS. In the permeate fractions ($PER_{5\ kDa}$, $PER_{10\ kDa}$ and $PER_{30\ kDa}$), OS enriched

Table 4
Arabinoxylan (AX) yield, average degree of substitution (DS) and degree of polymerization (DP) of AX in permeate (PER) and retentate (RET) fraction, obtained after one step ultrafiltration of the arabinoxylooligosaccharides containing stock solution with a membrane with molecular mass cut-off of 5, 10 and 30 kDa

	$PER_{5\ kDa}$	$RET_{5\ kDa}$
AX yield	11.2	86.1
DS	0.24	0.55
DP	3	11
	$PER_{10\ kDa}$	$RET_{10\ kDa}$
AX yield	34.2	64.8
DS	0.33	0.58
DP	5	12
	$PER_{30\ kDa}$	$RET_{30\ kDa}$
AX yield	52.4	45.7
DS	0.40	0.61
DP	6	15

components with relative low DS, varying from 0.24 to 0.40 depending on the used membrane, were found while the retentate fractions ($RET_{5\ kDa}$, $RET_{10\ kDa}$ and $RET_{30\ kDa}$) contained more substituted components.

Each of the permeate fractions ($PER_{5\ kDa}$, $PER_{10\ kDa}$ and $PER_{30\ kDa}$) was to a large degree comparable to fraction $F_{>90\%}$ as they contained low DS material of low DP. The AXOS in the retentate fractions resembled the material precipitating below 90% ethanol, i.e. fractions $F_{0–60\%}$ and $F_{60–90\%}$, as, in these fractions, more highly substituted fractions with high and medium MMs respectively, were found.

3.4.2. Two step ultrafiltration

The $RET_{10\ kDa}$ fraction was subjected to ultrafiltration with a MMCO 30 kDa membrane. Fig. 5 shows the HPSEC profile of the starting material and its permeate

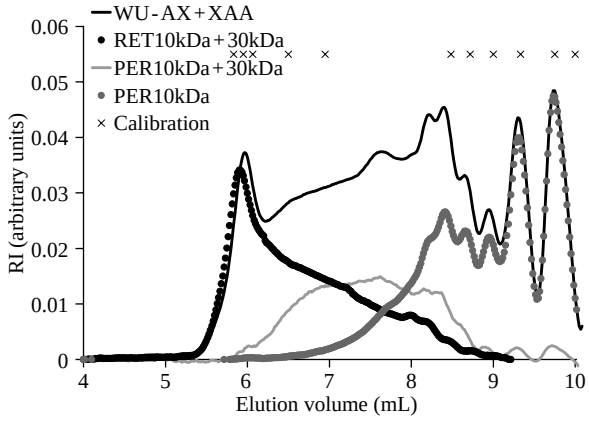

Fig. 5. HPSEC molecular mass profiles of fractions $PER_{10\ kDa}$, $PER_{10+30\ kDa}$ and $RET_{10+30\ kDa}$, obtained after double step ultrafiltration of the AXOS containing stock solution with membranes with MMCO of 10 and 30 kDa. Elution volumes of standards (×) of molecular mass of 11.2×10^4, 4.73×10^4, 2.28×10^4, 1.18×10^4, 0.59×10^4, 0.081×10^4, 0.0678×10^4, 0.0546×10^4, 0.0414×10^4, 0.0282×10^4 Da and glucose are indicated from left to right. Column is a Shodex SB-802.5 HQ.

Table 5
Arabinoxylan (AX) yield, average degree of substitution (DS) and degree of polymerization (DP) of AX in permeate ($PER_{10\ kDa}$ and $PER_{10+30\ kDa}$) and retentate ($RET_{10+30\ kDa}$) fractions after double step ultrafiltration of the arabinoxylooligosaccharides containing stock solution with membranes molecular mass cut-off of 10 and 30 kDa

	$PER_{10\ kDa}$	$PER_{10+30\ kDa}$	$RET_{10+30\ kDa}$
AX yield	34.2	25.2	36.6
DS	0.33	0.50	0.65
DP	5	7	18

($PER_{10+30\ kDa}$) and retentate ($RET_{10+30\ kDa}$) fractions. The $RET_{10+30\ kDa}$ fraction contained relative high MM components which eluted in the void volume of the Shodex SB-802.5 HQ column. In contrast, the $PER_{10+30\ kDa}$ and $PER_{10\ kDa}$ fractions, which represented approximately 25 and 34% of the S-AX in the AXOS containing stock solution (Table 5), respectively, had much lower MMs (Fig. 5), as also clear from the HPAEC profiles [Figs. 6(a–c)] which showed an increase in low MM material when comparing the $RET_{10+30\ kDa}$, $PER_{10+30\ kDa}$ and $PER_{10\ kDa}$ fractions. Figs. 6(d–f) show

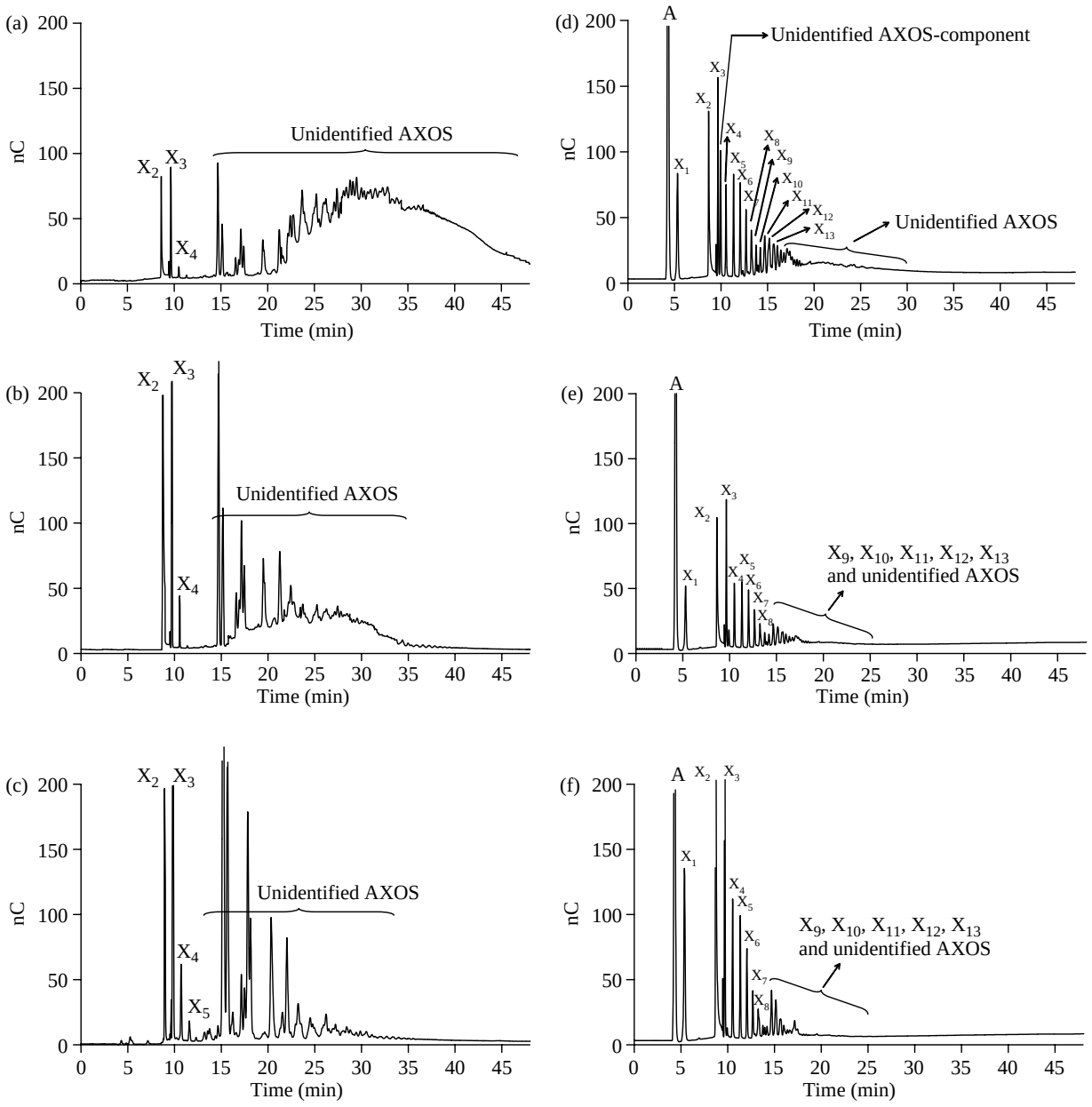

Fig. 6. HPAEC profiles of fractions $RET_{10+30\ kDa}$ (a), $PER_{10+30\ kDa}$ (b) and $PER_{10\ kDa}$ (c) obtained after double step ultrafiltration of the AXOS containing stock solution with membranes with MMCO of 10 and 30 kDa. The HPAEC profiles of the earlier described fractions followed by acid hydrolysis are shown in (d)–(f), respectively.

the HPAEC profiles of the corresponding acid treated fractions and reveal the presence of A, X_1, X_2 and a well separated range of peaks for XOS.

As mentioned earlier, the DS of the material impacts on both ultrafiltration and ethanol precipitation. More substituted components, which are less susceptible to enzyme degradation and thus have higher MMs, were found in the $RET_{10+30\ kDa}$ fraction while less susbstituted components with lower MMs were found in fractions $PER_{10+30\ kDa}$ and $PER_{10\ kDa}$ (Table 5).

The $RET_{10+30\ kDa}$ fraction was more polydisperse than the fraction $F_{0–60\%}$ (Figs. 2 and 5). The AXOS yield of the former (37%) was higher than that of the latter (15%), while the average DP was lower (18 versus 53) (Table 5). As a consequence, components in fraction $RET_{10+30\ kDa}$ were partly detected with HPAEC [Fig. 6(a)], in contrast to compounds present in fraction $F_{0–60\%}$, which could not be detected at all. Conclusions for fractions $RET_{10+30\ kDa}$ and $F_{0–60\%}$ were also valid for fractions $PER_{10+30\ kDa}$ and $F_{60–90\%}$. Although the latter had comparable AXOS yields, the $PER_{10+30\ kDa}$ fraction was more heterogeneous than the $F_{60–90\%}$ fraction. Besides medium MM AXOS, it also contained low MM AXOS, as reflected in its DP value (Table 5) and, HPSEC (Fig. 5) and HPAEC [Fig. 6(b)] profiles.

In contrast to the earlier mentioned fractions, fractions $PER_{10\ kDa}$ and $F_{>90\%}$ had similar DP values. Although GC, HPSEC (Figs. 2 and 5) and HPAEC [Figs. 3(b) and 6(c)] results showed that fraction $F_{>90\%}$ contained more AXOS components than fraction $PER_{10\ kDa}$, these fractions had comparable OS distributions.

4. Conclusion

In this work, graded ethanol precipitation and ultrafiltration were used to fractionate an AXOS containing stock solution into fractions of different size and structure, with emphasis on AXOS. Gradual ethanol precipitation yielded AX containing fractions with a relatively narrow MM distribution. At low ethanol concentrations, high average DP and high DS AX precipitated, while with increasing solvent concentrations, lower average DP and DS components precipitated. This indicated that the solubility of enzymically degraded AX depends on the DP and, only to a lesser degree, on the DS of these compounds. From the reported experiments, it can be concluded that ethanol precipitation is effective for isolation of AXOS fractions with different structures from AX hydrolysates. It is however, not clear whether application of this technique on industrial scale, e.g. as in the isolation process of pectins, is technically and economically attractive. The process involves large volumes of ethanol and the dissolved OS, which have been separated-off, have to be recovered from dilute solutions.

Depending on the fractions wanted, one or two step ultrafiltration procedures with different types of membranes can be used for fractionation of AXOS. The obtained fractions were, however, more heterogeneous and polydisperse than the ones obtained with ethanol precipitation, resulting in less strict separation and more overlap between the different ultrafiltration fractions. However, as the ultrafiltration separation is already used for several years as method for purification of fructooligosaccharides, pectate oligosaccharides, human milk oligosaccharides and soybean oligosaccharides, it can be expected that by optimising the process and choosing the right membrane, an economically viable separation process can be developed. Preferably, ultrafiltration membranes with a narrow pore size distribution should be used although traditional membranes in special configurations, which result in a more selective separation, could also be used (Feins and Sirkar, 2004, 2005).

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