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Glucuronoarabinoxylans from maize kernel cell walls are more complex than those from sorghum kernel cell walls

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Abstract

Water-unextractable solids (WUS) were isolated from maize kernels. They contained 7% of protein, 8% of starch and 57% of non-starch polysaccharides (NSP). These NSP were composed mainly of glucose, xylose, arabinose, and glucuronic acid. Sequential extractions with a saturated $Ba(OH)_2$-solution (BE1 extract), and distilled water (BE2 extract) were used to solubilise glucuronoarabinoxylans from maize WUS. Cellulose remained in the insoluble residue. The glycosidic linkage composition of the extracts and their resistance to endo-xylanase treatment indicated that the extracted glucuronoarabinoxylans were highly substituted. In the maize BE1 extract 25% of the xylose was unsubstituted, 38% was monosubstituted and 15% was disubstituted. A new measure for the degree of substitution is defined. The resulting degree of substitution for maize BE1 arabinoxylan (87%) is higher than for sorghum BE1 arabinoxylan (70%). The glucuronoarabinoxylans in maize BE1 can be degraded by a sub-fraction of Ultraflo, a commercial enzyme preparation from *Humicola insolens*. The digest contains a number of series of oligomers: pentose$_n$, pentose$_n$GlcA, pentose$_n$hexose, and pentose$_n$GlcA$_2$.

Keywords: Glucuronoarabinoxylans; Endo-xylanase treatment; Water-unextractable solids

1. Introduction

Cell walls of Gramineae consist predominantly of (glucurono)arabinoxylans and (1,3),(1,4)-β-D-glucans, with smaller amounts of cellulose, heteromannans, protein, and esterified phenolic acids (Evers, Blakeney & O'Brien, 1999). The glucuronoarabinoxylans consist of a β-D-(1,4)-linked xylopyranoside backbone and can be substituted with α-L-arabinofuranose on C2 and/or C3, α-D-glucopyranosyl uronic acid, or its 4-*O*-methyl derivative on C2, acetyl on C2 or C3 of some xylose residues (Brett & Waldron, 1990; Carpita & Gibeaut, 1993; Wilkie, 1979). Ferulic acid and *p*-coumaric acid can occur esterified to the C5 of arabinosyl units of (glucurono)arabinoxylans (Kato & Nevins, 1985; Mueller-Harvey, Hartley, Harris & Curzon, 1986). The degree and pattern of substitution of the (glucurono)-arabinoxylans appears to vary with the source from which they are extracted. These differences are reflected in the ratio of arabinose to xylose, in the relative amounts of the various linkage types of arabinose and xylose, in the presence of other substituents such as (4-*O*-methyl)-glucuronic acid, and the presence of small side chains such as xylopyranosyl-arabinose (Aspinall & Ferrier, 1957) and dimeric sidechains of arabinose (Verbruggen et al., 1998b).

The arabinoxylans from maize kernels have a highly branched structure, as was shown by linkage analysis data (Chanliaud, Saulnier & Thibault, 1995; Saulnier, Mestres, Doublier, Roger & Thibault, 1993). They were shown to meet a lot of the structural characteristics which were described above, like substitution by single units of arabinose or glucuronic acid. In addition, they comprise side-chains containing arabinose, xylose and galactose residues (Saulnier, Vigouroux & Thibault, 1995a; Srivastava & Smith, 1957; Whistler & Corbett, 1955). Some feruloylated oligosaccharides, obtained after acid hydrolysis were identified (Saulnier et al., 1995a) and are suggested to be present as side-chain constituents of the heteroxylans in maize bran.

Most of the information about the structure of maize kernel glucuronoarabinoxylan described so far is obtained by identification of oligosaccharides formed after acid hydrolysis of the heteroxylans. A schematic structure, which takes into account the monosaccharide composition, the linkage profile and the structures of some oligosaccharides obtained by acid hydrolysis, is presented by Saulnier, Marot, Chanliaud and Thibault (1995b). However, due to

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the low specificity of acid hydrolysis, much of the structural information of the polymer is of limited value. The use of specific enzymes can overcome this problem and provide more information about the structure of the polymer, in particular about the distribution of the substituents over the main chain. In this study, we describe the isolation of water-unextractable solids from maize kernels. The WUS is characterised and extracted with bariumhydroxide to obtain rather pure glucuronoarabinoxylans (Gruppen, Hamer & Voragen, 1991). The extracts and their digests obtained after enzymatic degradation are chemically characterised to obtain information about the structure of the maize glucuronoarabinoxylans.

2. Materials and methods

2.1. Materials

In this research whole maize kernels (*Zea mays* L.) harvested in the Alsace region (France) were used for the isolation of maize WUS. Sorghum WUS was isolated by Verbruggen, Beldman, Voragen and Hollemans (1993).

2.2. Isolation of the water-unextractable solids

The isolation of water-unextractable cell wall material from maize kernels was based on the procedure described by Verbruggen et al. (1993). Maize kernels were ground to pass a 0.5-mm sieve. This meal (500 g) was defatted by Soxhlet extraction with 3 l of petroleum ether, refluxing for 6 h. The air-dried residue was extracted with 2 l distilled water containing 0.05% NaN_3 during 2 h at room temperature. The suspension was centrifuged (11 000 g; 30 min). The pellet was resuspended and this procedure was repeated three times.

Subsequently, the protein was extracted from the residue with 1.5 l of 1.5% (w/v) sodium dodecylsulphate solution containing 10 mM 1,4-dithiothreitol, during 3 h at room temperature. After centrifugation (11 000 g; 30 min) this extraction was repeated twice. The final pellet was washed twice with distilled water.

The residue was filtered over a 45 μm sieve by washing with distilled water (6.5 l). The residue, which retained on the sieve, was suspended in 1 l distilled water (pH 5.0) at 85°C and starch gelatinisation was allowed to proceed for 1 h. The residue obtained after centrifugation (11 000 g; 30 min) was suspended in 1 l buffer solution (pH 6.5) containing 10 mM maleic acid, 10 mM NaCl, 1 mM $CaCl_2$ and 0.05% NaN_3. Porcine pancreatic α-amylase (2 mg; Merck art 16312) was added and the mixture incubated at 30°C for 18 h. After centrifugation (11 000 g; 30 min) the residue was washed with 1 l hot distilled water (65°C) and centrifuged again. The α-amylase digestion and hot water washing were repeated once. The remaining unextractable residue was resuspended in distilled water and freeze-dried (WUS).

2.3. Extraction of WUS

Glucuronoarabinoxylans were obtained by the procedure described by Gruppen et al. (1991). Maize WUS (3 g) was extracted with saturated $Ba(OH)_2$ solution containing 260 mM $NaBH_4$ (500 ml for 16 h; 250 ml for 1 h) at room temperature with continuous stirring (BE1). The residue was acidified to pH 5 (acetic acid) and subsequently extracted with distilled water (4 × 300 ml; 1 h) (BE2). After each extraction, solubilised polymers were separated from the insoluble residue by centrifugation (18 900 g; 45 min), and the pH of the extracts was adjusted to 5 with glacial acetic acid before dialysis.

The final residue from the water extraction step was freeze-dried (RES). All extracts were kept at −18°C and aliquots were thawed or freeze dried as needed.

2.4. Enzymatic degradation

Solutions (1.6 mg/ml) of maize BE1 were incubated with a number of purified enzymes. The purified enzymes used were endo-(1,4)-β-xylanase (EC 3.2.1.8, endo-xylanase I; 0.1 μg protein/ml substrate solution), AXH-m (0.05 μg protein/ml substrate solution), and AXH-d3 (an excess amount of enzyme). Endo-xylanase I (Kormelink, Searle-van Leeuwen, Wood, & Voragen, 1993) and AXH-m (Kormelink, Searle-van Leeuwen, Wood, & Voragen, 1991) were purified from *Aspergillus awamori*. AXH-d3 was purified from *Bifidobacterium adolescentis* (van Laere, Beldman & Voragen, 1997).

A solution (1.5 mg/ml) of maize BE1 was also incubated with Ultraflo, a commercial enzyme preparation derived from *Humicola insolens* (Novo Nordisk, Bagsvaerd, Denmark). Before adding to the substrate solution, the enzyme preparation was dialysed against a 50 mM NaAc buffer (pH 5.0) containing 0.01% NaN_3 at 4°C during 2 h. Solutions (1.4 mg/ml) of maize BE1 were incubated with 'Ultraflo-arabinoxylan degrader', a sub-fraction of Ultraflo obtained during the fractionation of the preparation (Düsterhöft, Linssen, Voragen & Beldman, 1997).

The incubations were performed in 50 mM NaAc buffer (pH 5.0) containing 0.01% NaN_3 at 30°C rotating 'head over tail'. Incubations with AXH-d3 were performed in 25 mM $NaPO_4$ buffer (pH 6.5) containing 0.01% NaN_3. All enzymes were inactivated by heating at 100°C for 10 min. Polysaccharide-degrading activities were determined by HPSEC and HPAEC analyses of the digests.

2.5. Ion-exchange chromatography

An amount of the extracts which contains 20 mg of polysaccharides was applied on a column (400 mm × 16 mm) of DEAE Sepharose Fast Flow, which was initially equilibrated in 0.005 M NaAc buffer pH 5.0, using a Hiload System (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Elution was carried out sequentially with 80 ml of 0.005 M NaAc-buffer pH 5.0, a linear gradient from 0.005

Table 1
Composition of whole maize meal and maize WUS (percentage dry weight)

	Whole defatted maize meal	Maize WUS
Yield	100	8.7
Protein content	10.8	7.0
Starch content	62.3	8.1
NSP content	5.5	56.5

to 1 M NaAc-buffer pH 5.0 (160 ml), a linear gradient from 1 to 2 M NaAc-buffer pH 5.0 (80 ml) and 40 ml 2 M NaAc-buffer pH 5.0. Residual bound polysaccharides were washed from the column with 80 ml of 0.5 M NaOH. The elution rate was 5 ml/min except for the first step, in which the sample was applied onto the column using an elution rate of 1 ml/min. Fractions (5 ml) were collected and assayed by automated methods for uronic acid (Thibault, 1979) and neutral sugar content (Tollier & Robin, 1979).

2.6. Analytical methods

Neutral sugar composition was determined by gas chromatography according to Englyst and Cummings (1984), using inositol as an internal standard. The samples were pre-treated with 72% (w/w) H_2SO_4 (1 h, 30°C) followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100°C and the constituent sugars were analysed as their alditol acetates.

Uronic acid content was determined by the automated colorimetric *m*-hydroxydiphenyl assay (Blumenkrantz & Asboe-Hansen, 1973; Thibault, 1979) using an auto-analyser (Skalar Analytical BV, Breda, The Netherlands). Corrections were made for interference by neutral sugars present in the sample as measured by the orcinol–sulfuric acid method (Tollier & Robin, 1979).

Starch was determined enzymatically using a test kit (Boehringer).

Glycosidic linkage analysis. Carboxyl groups in all fractions were reduced according to Taylor and Conrad (1972) using $NaBH_4$, and the reduced fractions were subsequently methylated using a modification of the Hakomori method (Sandford & Conrad, 1966), dialysed and dried in a stream of air. The reduction and methylation steps were repeated in order to improve the extent of both reactions. Partially methylated alditol acetates were prepared and analysed as described by Verbruggen, Beldman and Voragen (1995). The derivatives were quantified according to their effective carbon response (ECR) factors (Sweet, Shapiro & Albersheim, 1975). The identity of the compounds was confirmed by gas chromatography-mass spectrometry (GC-MS, Hewlett Packard, USA) using a mass selective detector (MSD) 5973 coupled to a HP 6890 gas chromatograph equipped with a fused silica column (CPSIL 19CB, 25 m × 0.25 mm; 0.2 μm; Chrompack). The temperature program was 160 → 185°C at 0.5°C/min, 185 → 230°C at 10°C/min and 230°C isothermal for 5.5 min.

High-performance size-exclusion chromatography (HPSEC) was performed on a SP8800 HPLC (Spectra Physics) equipped with three columns (each 300 × 7.5 mm^2) of Bio-Gel TSK in series (60XL, 40XL and 30XL; Bio-Rad Labs., Richmond, CA, USA) in combination with a TSK guard column (40 × 6 mm^2) and elution at 30°C with 0.4 M sodium acetate buffer pH 3.0 at 0.8 ml/min. The eluate was monitored using a combined RI detector and viscometer (model 250, Viscotek Corporation, Houston, TX, USA), and a Right Angle Laser Light-Scattering detector (RALLS, Viscotek, LD 600). Molecular masses were calculated using the light scattering and universal calibration modules of the Trisec software (Viscotek). Calibration was performed using pullulans, ranging from 6 to 788 kDa (Polymer Laboratories, Amherst, MA, USA).

High-performance anion-exchange chromatography (HPAEC) was performed on a Dionex Bio-LC system as described by Schols, Voragen and Colquhoun (1994). The gradient was obtained by mixing solutions of 0.1 M NaOH and 1 M sodium acetate in 0.1 M NaOH.

For the determination of arabinoxylan oligomers, the (4 × 250 mm^2) CarboPac PA-1 column was equilibrated with 0.1 M NaOH. Twenty microlitres of the sample was injected and a linear gradient to 0.2 M sodium acetate in 0.1 M NaOH in 30 min, and a linear gradient from 0.2 to 0.6 M sodium acetate in 0.1 M NaOH in the next 10 min was applied. The column was washed for 5 min with 1 M sodium acetate in 0.1 M NaOH and equilibrated again for 15 min with 0.1 M NaOH. Calibration was performed with standard solutions of arabinose, xylose, glucuronic acid, and a series of xylan oligomers.

Matrix-assisted laser-desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS). The matrix solution was prepared by dissolving 9 mg 2,5-dihydroxybenzoic acid and 3 mg 1-hydroxy isoquinoline in 700 μl distilled water and 300 μl acetonitril. A 1 μl volume of this solution was placed on the sample plate together with 1 μl of the sample solution and allowed to dry at room temperature. The sample plate was then placed in the instrument.

MALDI-TOF mass spectra were recorded on a Voyager-DE RP Biospectrometry Workstation (PerSeptive Biosystems, Inc., Framingham, MA, USA) equipped with a nitrogen laser operating at 337 nm (3-ns pulse duration), a single stage reflector, and delayed extraction. The accelerating voltage used was 12 kV and the delay time setting was 200 ns. Each spectrum was produced by accumulating data from 100–256 laser shots. Mass spectra were calibrated with an external standard containing galacturonic acid oligomers (degree of polymerisation 2–9).

3. Results and discussion

3.1. Yield and composition of the WUS

The first step in the isolation of maize WUS from whole maize meal was the removal of lipids. Extraction with

Table 2
Molar sugar compositions of fractions from maize WUS and sorghum WUS

Sample	Yield (% WUS)	Sugar composition						Carbohydrate content (% (w/w))
		ara	xyl	man	gal	glc	glcA	
Maize WUS	100	23.0	30.3	0.9	4.5	32.9	8.5	82.6
Maize BE1	21.1	38.4	48.3	0.1	4.3	0.7	8.3	84.3
Maize BE2	18.1	34.3	43.5	0.3	7.6	6.2	8.3	86.2
Maize RES	46.3	16.4	22.9	1.6	3.7	47.6	7.8	92.9
Recovery	85.5							
Sorghum WUS	100	25.2	23.9	1.6	2.3	38.2	9.0	72.2
Sorghum BE1	26.0	45.8	40.9	0.2	1.8	1.7	9.8	75.2
Sorghum BE2	14.5	29.3	23.1	1.0	2.9	31.1	12.0	80.1
Sorghum RES	48.7	9.6	28.2	1.4	1.2	54.1	5.7	74.7
Recovery	89.2							

petroleum ether removed 8.1% of the original material. Table 1 presents the composition of defatted whole maize meal and isolated maize WUS.

The protein content of the defatted meal (10.8%) agrees well with results published by Watson (1987), who found the protein content to vary from 8 to 12%. The starch content of the meal (62.3%) is lower than the average value found by Watson (1987) (71.7%). The defatted whole maize meal contained 5.5% non-starch polysaccharides. The analyses performed here account for only 78% of the maize meal. Another component, which is very likely to be present, is lignin. The lignin content is not determined, but lignin is a common component of secondary thickening in the pericarp of all cereal grains (Evers et al., 1999). In addition, maize kernels also contain phytate, tannins, mineral elements, vitamins, and other chemical compounds in low concentrations (Evers et al., 1999; Watson, 1987).

The WUS fraction still contained 7% of protein, this might be present as structural proteins, glycoproteins or intracellular proteins (Fry, 1988). Only 5.6% of the protein originally present in the meal was found in the WUS fraction. Starch could not be removed completely. The WUS fraction contained 8% of starch, representing approximately 1% of the starch in the meal. This might be due to certain physical changes in the starch component introduced by the isolation procedure which may cause resistance to degradation by α-amylases (Englyst & Kingman, 1990) or due to the presence of resistant starch which can not be degraded by α-amylase (Englyst, Wiggins & Cummings, 1982). The WUS contains 57% of NSP, representing 89% of the NSP originally present in the WUS fraction. A small part of the residual 28% consists of acetyl, ferulic acid, and coumaric acid groups, the remainder can not be explained.

A substantial proportion of the polysaccharides in the cell walls of maize kernels consists of arabinoxylans and cellulose, as can be concluded from the high contents of arabinose, xylose and glucose in maize WUS (Table 2). In addition, phenolic acids were determined in the maize WUS; coumaric acid (0.1% (w/w)) and ferulic acid (1.6% (w/w)) may be involved in oxidative cross-linking of polysaccharides and other cell wall components. Acetyl groups have also been determined in maize WUS (4.9% (w/w)), indicating the presence of 36 acetyl groups per 100 xylose residues.

3.2. Extraction of glucuronoarabinoxylans from maize WUS

Glucuronoarabinoxylans can be selectively extracted from cell wall material with saturated barium hydroxide solutions (Bergmans, Beldman, Gruppen & Voragen, 1996; Gruppen et al., 1991). Extraction of maize and sorghum WUS with a $Ba(OH)_2$ solution resulted in two fractions for each starting-material, BE1 and BE2, both containing hemicellulosic material. Verbruggen et al. (1995) also extracted glucuronoarabinoxylan from sorghum WUS, but they used a more extensive extraction procedure. Gruppen et al. (1991) followed the same extraction procedure for wheat flour WUS. The BE1 fraction was released from the WUS directly, being extractable in $Ba(OH)_2$. The BE2 fraction was only released from the WUS-residue after lowering the pH and washing with distilled water. A cellulose-enriched residue (RES) remains. The sugar composition of the fractions is shown in Table 2. Saturated $Ba(OH)_2$ extracted 37% of the arabinose and xylose from maize WUS. In the event of wheat flour WUS, saturated $Ba(OH)_2$ extracted the bulk (80%) of the arabinose and xylose (Gruppen et al., 1991), while only 50% of the arabinose and xylose could be extracted from sorghum WUS (this research). BE1 from maize, sorghum, and wheat flour contained a rather pure (glucurono)arabinoxylan population, only minor amounts of other neutral sugars were present in these extracts.

The structures of the arabinoxylans in both maize and sorghum are much more complex than those found in other cereals. The degree of substitution in maize and sorghum (molar arabinose: xylose ratio is 0.80 and 1.12, respectively) is higher than in wheat flour (0.54) (Gruppen, Hamer & Voragen, 1992), barley (0.72) (Viëtor, Angelino & Voragen, 1992), and wheat bran (0.71) (Bergmans et al., 1996) arabinoxylans. Moreover the arabinoxylans in maize

Table 3
Linkage composition (mol%) of $NaBH_4$ reduced barium hydroxide extracts of maize and sorghum WUS

Component	Linkage type	Maize BE1	Maize BE2	Sorghum BE1	Sorghum BE2
2,3,5-Me_3-Ara	(Araf)1 →	24.7	24.3	32.9	24.2
3,5-Me_2-Ara	→ 2(Araf)1 →	3.9	4.8	2.7	1.9
2,3-Me_2-Ara	→ 5(Araf)1 →	3.9	4.3	5.2	5.6
2,3,4-Me_3-Xyl	(Xylp) →	9.6	11.2	1.6	1.2
2,3-Me_2-Xyl	→ 4(Xylp)1 →	15.4	7.6	21.1	12.0
2,4-Me_2-Xyl	→ 3(Xylp)1 →	3.6	4.1		3.0
2-Me-Xyl	→ 4(Xylp)1 → 3↑	18.6	17.0	19.2	14.0
3-Me-Xyl	→ 4(Xylp)1 → ↑2	5.2	4.0	6.4	4.7
Xyl	→ 4(Xylp)1 → 3↑ ↑2	9.5	9.9	6.0	4.6
2,3,4,6-Me_4-Glc	(Glcp)1 →	2.0[a]	2.9[a]	2.0[a]	3.6[a]
2,3,6-Me_3-Glc	→ 4(Glcp)1 →			0.6	20.6
2,4,6-Me_3-Glc	→ 3(Glcp)1 →				3.4
2,3,4,6-Me_4-Gal	(Galp)1 →	3.1	5.6	1.1	1.4
2,3,6-Me_3-Gal	→ 4(Galp)1 →	0.5	4.2	1.0	
Ratio terminal/branching		0.92	1.08	1.00	1.09

[a] (Partially) originating from terminally-linked glucuronic acid.

and sorghum are additionally substituted with uronic acid (8.3 and 9.8 mol%, respectively). HPAEC of H_2SO_4 hydrolysed samples (Verbruggen et al., 1995) of both maize and sorghum showed that the only uronic acid present in the samples was glucuronic acid. Uronic acids are almost absent in wheat flour (Gruppen et al., 1992) and barley (Viëtor et al., 1992) arabinoxylans, wheat bran arabinoxylan contains 2.6 mol% uronic acid (Bergmans et al., 1996).

The BE2 extracts of maize and sorghum also contained predominantly arabinoxylan, but glucose-containing polysaccharides were co-extracted, especially in sorghum BE2. This co-extracted glucose in sorghum and wheat flour BE2 was present mainly in the form of (1,3),(1,4)-β-D-glucans (Gruppen et al., 1992; Verbruggen et al., 1995). The yields of these fractions for maize, sorghum and wheat flour, (18.1, 14.5, and 10.7%, respectively) were lower than the yield of the BE1 extracts. Glucose was by far the most important sugar in the residues, presumably present as cellulose and (1,3),(1,4)-β-D-glucans.

3.3. Glycosidic linkage composition

The results of linkage analysis of the extracted polysaccharides from maize and sorghum are presented in Table 3. The barium hydroxide extracts of maize appeared to comprise highly substituted arabinoxylans. Assuming that the backbone consists of only (1,4)-, (1,2,4)-, (1,3,4)-, and (1,2,3,4)-linked xylopyranosyl residues, 32% of the xylopyranosyl residues in the (1,4)-xylan backbone in maize BE1 was unsubstituted, and 49% was monosubstituted, with branch points mainly at the C3 position. In addition, the xylan backbone contained a large amount (19%) of C2,C3-disubstituted xylopyranosyl residues. The amount of unsubstituted xylose in maize BE1 (32%) is lower than in sorghum BE1 (40%) and wheat flour BE1 (63%) (Gruppen et al., 1992).

The degree of substitution of the xylan backbone can be expressed as the number of sugar substituents per 100 xylose residues in the backbone, by calculating the ratio of the number of branches attached to xylose residues to the total number of xylose residues in the backbone. It turns out that the degree of substitution in maize BE1 is much higher (87%) than in sorghum (70%) and wheat flour BE1 (56%). This is a better measure for the degree of substitution than the arabinose:xylose ratio, which is highest for sorghum BE1. This can be explained by terminally linked xylose residues being present in side chains (Kusakabe, Ohgushi, Yasui & Kobayashi, 1983; Saulnier et al., 1995a; Whistler & Corbett, 1955), and the existence of oligomeric side chains of arabinose (Nishitani & Nevins, 1989; Verbruggen et al., 1998b). However, this measure does not give any information about the distribution of the substituents over the main chain.

In addition to terminally linked arabinofuranosyl residues, the arabinoxylans in maize BE1and BE2 also contain (1,2)- and (1,5)-linked arabinofuranosyl residues, indicating complicated structural features of its side chains. Short side chains containing these sugar linkages have been isolated from enzymatic and acid hydrolysates of some arabinoxylans (Kusakabe et al., 1983; Nishitani & Nevins, 1989; Saulnier et al., 1995a; Verbruggen et al., 1998b; Whistler & Corbett, 1955; Wilkie, 1979). The above results indicate that similar structural units are also present in maize BE1.

In these studies, glucuronic acid was reduced with $NaBH_4$ prior to methylation, hydrolysis and derivatisation, and therefore, was determined as glucose. The originally extracted polysaccharides (not reduced with $NaBH_4$) from maize did not contain any terminal glucose residues, the

Table 4
Average molecular weight, polydispersity and intrinsic viscosity of the barium hydroxide extracts of maize and sorghum WUS

Extract	M_w (kDa)	Polydispersity (M_w/M_n)	$[\eta]$ (dl/g)
maize BE1	171	1.41	2.33
Maize BE2	400	4.23	2.61
Maize BE1	132	2.13	2.36
Sorghum BE2	326	3.89	2.34

content of terminal glucose residues in the originally extracted sorghum polysaccharides was very low. This indicates the presence of terminal glucuronic acid residues in the original polysaccharide. Previously isolated and identified glucuronic acid-containing oligomers from glucuronoarabinoxylan hydrolysates showed that glucuronic acid is linked to the (1,4)-xylan backbone at the C2 position of xylose (Shibuya, Misaki & Iwasaki, 1983; Verbruggen et al., 1998b). The linkage composition of the glucose in the sorghum BE2 extract confirms the presence of a (1,3),(1,4)-β-D-glucan, as shown before (Verbruggen et al., 1995). Comparison of the linkage analysis data with the sugar composition shows that the amounts of terminal glucose (after reduction) were lower than the amounts of uronic acids determined directly in the extracts. The discrepancy between sugar and linkage composition suggests an incomplete reduction of the uronic acids resulting in a substantial underestimation of the glucose originating from glucuronic acid content in the linkage composition.

Comparison of the linkage compositions of the BE2 to those of the BE1 extracts shows that the glucuronoarabinoxylans in BE2 have a higher degree of substitution than those in BE1 for both maize and sorghum. The portion of unsubstituted xylose in the backbone of the BE2 extracts of maize and sorghum (20 and 34%, respectively) is lower than that in the backbone of the BE 1 extracts (32 and 40%, respectively). The degree of substitution (calculated as described above) is 107% for maize BE2 and 79% for sorghum BE2.

3.4. Homogeneity of the barium hydroxide extracts

The homogeneity of the extracts was studied by high-performance size-exclusion chromatography (HPSEC). As can be seen from the elution patterns in Fig. 1, all extracts contained polysaccharides varying widely in hydrodynamic volume. The weight-averaged molecular masses of the extracts of maize are slightly higher than those of the extracts of sorghum, as is shown in Table 4. The RI-elution patterns of all extracts showed no distinct peaks, although the extracted polymers were clearly polydispersed. This was confirmed by their M_w to M_n ratio (Table 4), which is a measure for the heterodispersity. The BE1 fraction of both sorghum and maize is more homogeneous than the BE2 fraction, which was already found by Verbruggen et al. (1995) for the sorghum extracts. The maize BE1 fraction is the most homogeneous extract. The intrinsic viscosity of all extracts, from both maize and sorghum, are in the same order of magnitude, varying between 2.33 and 2,61 dl/g.

The extracts from both maize WUS and sorghum WUS were further studied for homogeneity based on their charge density. The results of anion-exchange chromatography (not shown) suggest that all extracts are very homogeneous. Almost all material was bound to the column, due to the presence of glucuronic acids in all the arabinoxylan molecules. The glucuronic acids are probably evenly distributed over the arabinoxylan molecules, since the material eluted in one peak with similar uronic acid:neutral sugar ratios throughout the whole peak. Only a small proportion (2% for sorghum BE1, 7% for sorghum BE2, <1% for maize BE1 and 2% for maize BE2) of the recovered material was not bound. Another small proportion (3% for sorghum BE1, 10% for sorghum BE2, 1% for maize BE1 and 5% for maize BE2) was eluted with 0.5 M NaOH. The high homogeneity of the sorghum BE1 fraction was in good agreement with the results found by Verbruggen et al. (1995). Chanliaud et al. (1995) also found that a major fraction of alkali extracted heteroxylans from maize bran, containing all the uronic acid, was bound to the column and was eluted in a gradient with sodium acetate buffer.

3.5. Enzymatic degradation of arabinoxylans from maize

The BE1 fraction of maize was digested with pure and well-defined enzymes, and compared with the degradation of sorghum glucuronoarabinoxylan (Verbruggen et al. 1998b). Degradation of maize BE1 by endo-xylanase I only, resulted in a small shift in molecular weight distribution on HPSEC (results not shown). Supplementation of the reaction mixture with AXH-m did not change the molecular weight distribution of the maize arabinoxylans in fraction BE1. However, degradation of sorghum arabinoxylan with endo-xylanase I and AXH-m showed a reasonable shift in the molecular weight distribution of the sorghum arabinoxylans, as was shown by Verbruggen et al. (1998b). The arabinoxylans in the maize BE1 fraction appear to be more resistant to enzymatic degradation than those in the sorghum BE1 extract, probably due to the relatively low amount of unsubstituted xylose residues and the relatively high amount of disubstituted xylose residues, which are expressed in their higher degree of substitution (87% for maize BE1 and 70% for sorghum BE1). In addition, the *distribution* of the substituents can also play an important role in the resistance to enzymatic degradation.

Since the combined action of endo-xylanase I and AXH-m was not able to degrade maize BE1 to oligomeric degradation products, and the relatively high amount of disubstituted xylose is mentioned as a possible cause, incubation of maize BE1 with an arabinofuranohydrolase (AXH-d3 (van Laere et al., 1997; van Laere, Voragen, Kroef, van den Broek, Beldman & Voragen, 1999)) was performed. It was expected that the removal of some arabinofuranosyl groups could increase the susceptibility of the substrate

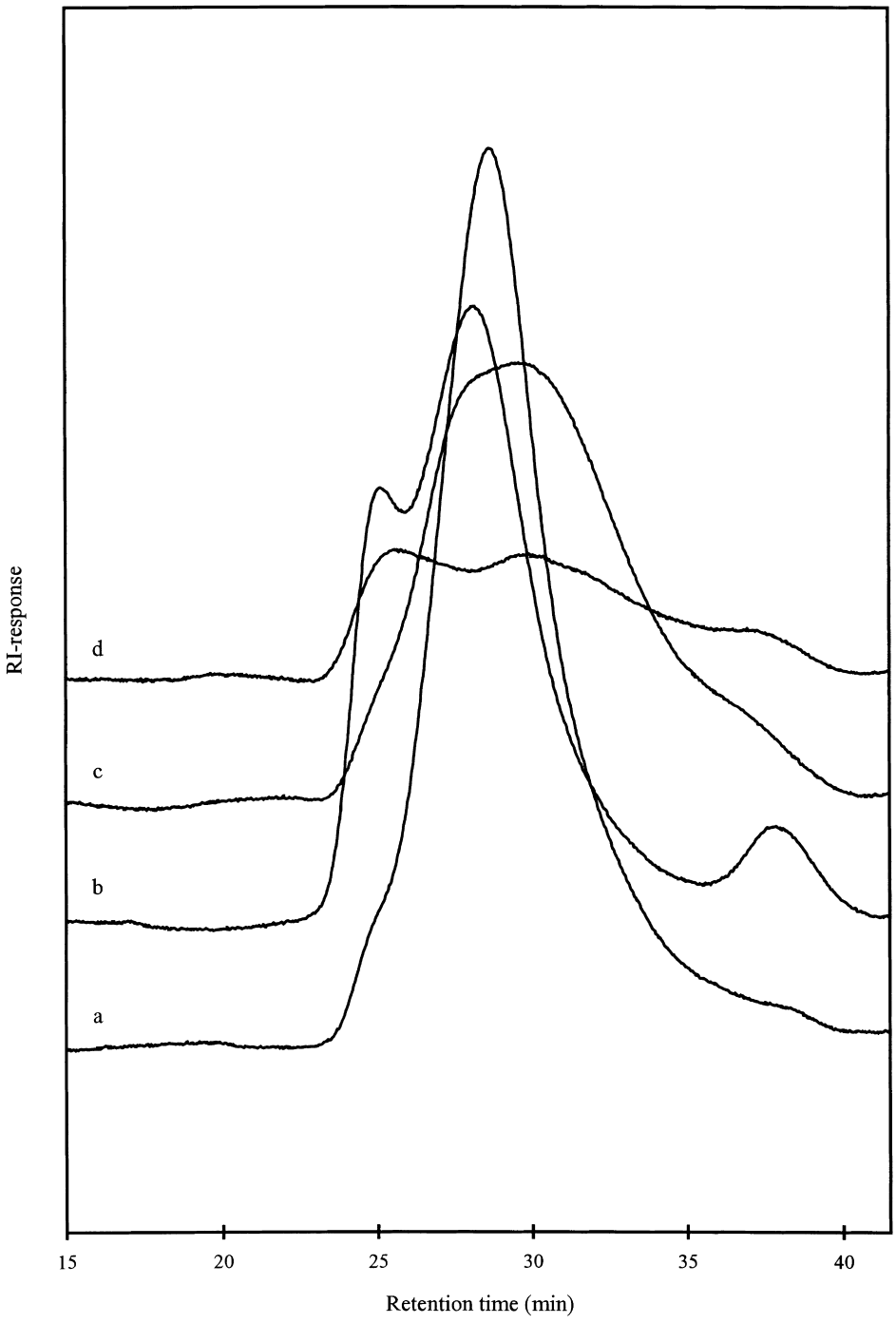

Fig. 1. HPSEC elution profiles of: (a) maize BE1; (b) maize BE2; (c) sorghum BE1; and (d) sorghum BE2.

for AXH-m and endo-xylanase I. However, this enzyme showed no activity towards the maize BE1 extract.

The search for more powerful arabinoxylan degrading enzymes leads to a commercial enzyme preparation derived from the thermophilic fungus *H. insolens*. The degradation of the maize BE1 extract by Ultraflo goes fast and is almost complete. The main degradation products are arabinose, xylose and glucuronic acid. The amounts of arabinose and xylose *monomers* released after 20 h of incubation are about 85 and 100% of the arabinose and xylose present in the substrate. As is shown in Fig. 2, the larger part of the arabinose is released within the first hour of the incubation. The release of xylose is more moderate; indicating that arabinose has to be removed first before xylose can be released.

Probably because of the large variety of enzymes present in Ultraflo, the maize BE1 extract is almost completely degraded to monomers, by which all the information about the structure of the substrate is lost. Therefore,

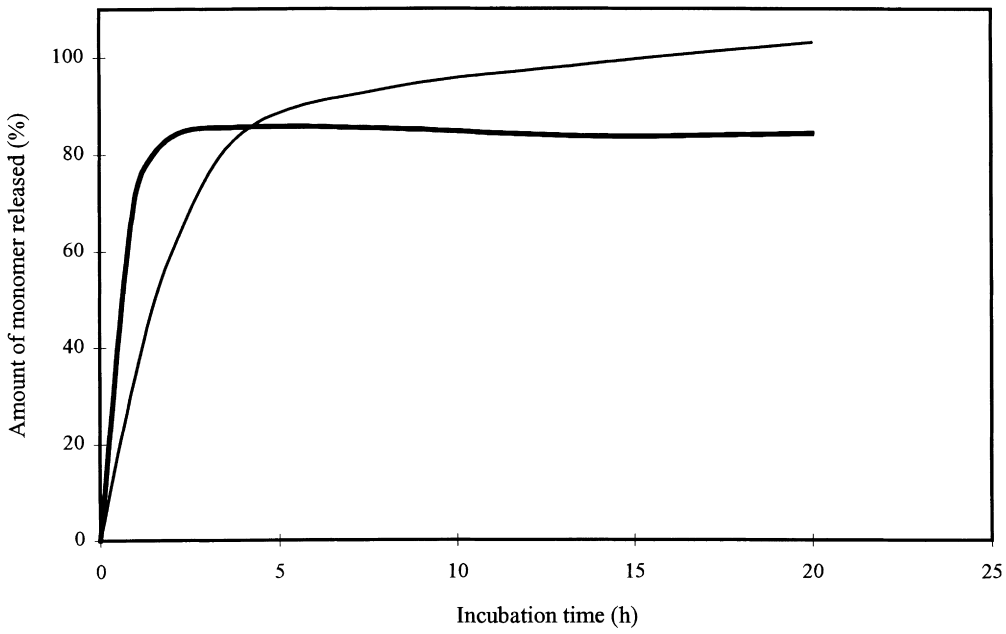

Fig. 2. Release of arabinose (thick line) and xylose (thin line) monomers during incubation of maize BE1 with Ultraflo.

maize BE1 was incubated with a number of sub-fractions of Ultraflo, obtained during the isolation of two xylanases by Düsterhöft et al. (1997). One of these sub-fractions, designated 'Ultraflo-arabinoxylan degrader' here, is able to degrade maize BE1 and form a large variety of oligomeric degradation products. Although the 'Ultraflo-arabinoxylan degrader' is still a mixture of enzymes, it can be used as a tool for the production of oligosaccharides.

Incubation of maize BE1 with 'Ultraflo-arabinoxylan degrader' degrades all polymeric material in the extract (Fig. 3). Incubation of this digest with endo-xylanase I did not result in further degradation of the digest. After incubation, arabinose is released as the main degradation product (Fig. 4). After 20 h of incubation, about 40% of the arabinose, about 3% of the xylose, and only about 7% of the glucuronic acid originally present in the substrate is released as monomeric degradation products. Furthermore, a peak of xylobiose, xylotriose, and a lot of peaks representing unknown compound can also be seen in the elution profile of the digest.

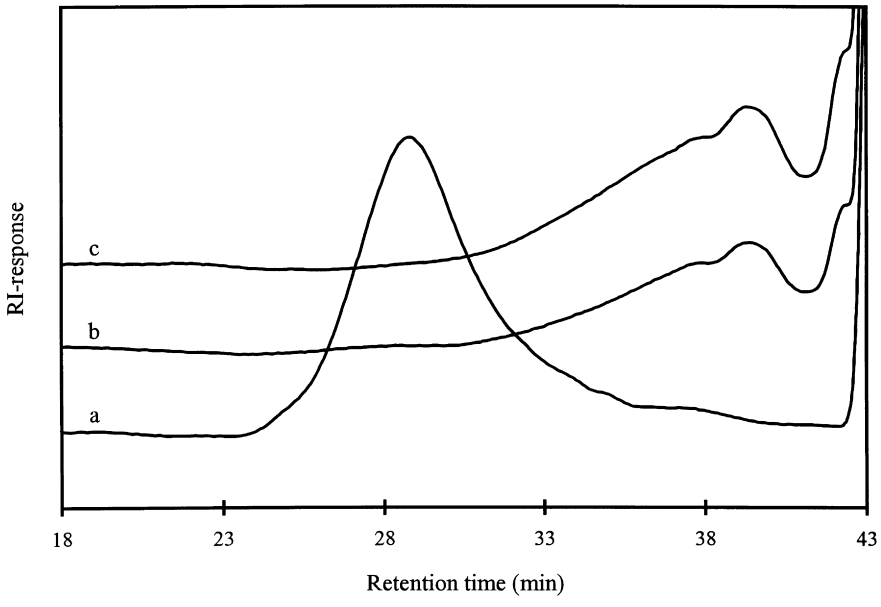

Fig. 3. HPSEC elution profiles of: (a) maize BE1 blank; (b) digest of maize BE1 after incubation with 'Ultraflo-arabinoxylan degrader' for 24 h; and (c) digest of maize BE1 after sequential degradation with 'Ultraflo-arabinoxylan degrader' for 24 h and endo-xylanase I for 24 h.

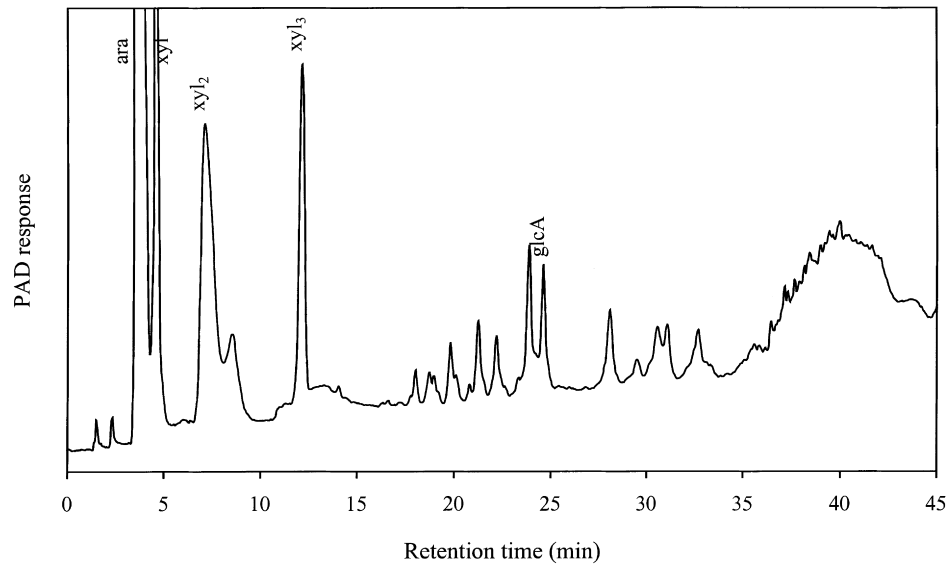

Fig. 4. HPAEC elution profile of the digest of maize BE1 after incubation with 'Ultraflo-arabinoxylan degrader' for 20 h.

In an earlier study, it was determined that this subfraction was able to degrade both wheat arabinoxylan and the disubstituted oligomer β-Xyl*p*-(1 → 4)[α-Ara*f*-(1 → 2)][α-Ara*f*-(1 → 3)] β-Xyl*p*-(1 → 4) β-Xyl*p*-(1 → 4) Xyl*p*. So it contains an arabinofuranohydrolase which can release arabinose from disubstituted xylose residues (Düsterhöft; unpublished results). It is not sure whether this enzyme resembles the AXH-d3 (van Laere et al., 1997, 1999). AXH-d3 was unable to release arabinose from maize BE1, probably because it is hindered by the (4-*O*-methyl)glucuronic acid substituents or the high degree of branching (van Laere et al., 1999). The presence of α-glucuronidase (glcAase) (Verbruggen, Beldman & Voragen, 1998a), exo-glucuronase or other accessory enzymes in the 'arabinoxylan degrader' could possibly enable AXH-d3 to work. About 7% of the glucuronic acid was released as a monomeric degradation product, thus this possibility can not be ruled out. Another possibility is the existence of for example an AXH-d2, which would enable other enzymes to work. In addition to this, the presence of β-xylosidases, endo-xylanases and α-L-arabinofuranosidases able to remove arabinose substituents at C2 and/or C3 in the

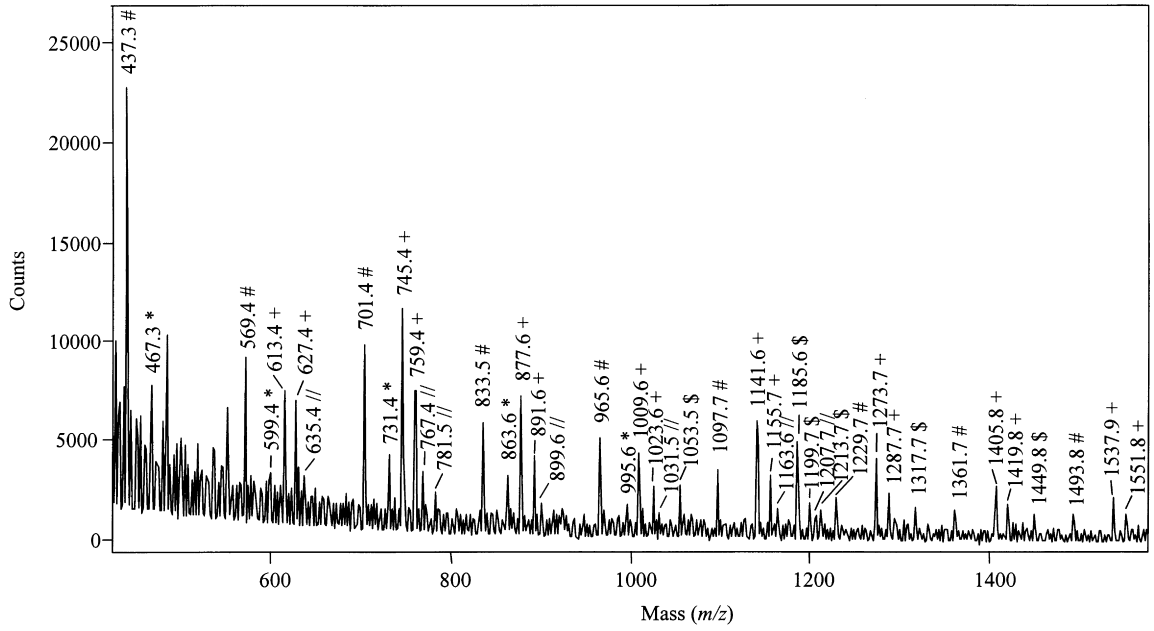

Fig. 5. MALDI-TOF mass spectrum of the digest of maize BE1 after incubation with 'Ultraflo-arabinoxylan degrader' for 20 h. Signals belonging to the same homologues series of sodium-cationised oligosaccharides; # = $pent_n$, * = $pent_n$hex, + = $pent_n Ome_m hexA$($m = 0$ or 1), $ = $pent_n Ome_m hexA_2$($m = 0,1$, or 2), and // indicates doubly sodiated oligomers.

arabinoxylan degrader is demonstrated (Düsterhöft; unpublished results).

To obtain more information about the composition of the oligomers formed after incubation with 'Ultraflo-arabinoxylan degrader', the digest was analysed by MALDI-TOF MS. Although the spectrum, shown in Fig. 5, appears to be rather complex, a few series of analogous oligomers can be distinguished. Firstly, oligomers containing only pentoses (xylose or arabinose; indicated with #) are present with a degree of polymerisation up to 11. Secondly, oligomers containing one hexuronic acid (glucuronic acid) in addition to pentoses (signals marked with +). The number of pentoses in these oligomers ranges from 3 to 10. In the MALDI-TOF mass spectrum a signal of equivalent oligomers containing an additional methoxyl group was also shown (also marked with +). This methoxyl group is linked to C4 of the glucuronic acid residue. Thirdly, a series of oligomers containing pentoses and *two* glucuronic acids was present (signals marked with $). The number of pentose residues in these oligomers ranges from 5 to 8. A number of these oligomers are also present in which one or both glucuronic acid residues contain an additional methoxyl group. Finally, a series of oligomers can be detected, which consist of one hexose in addition to the pentoses (signals marked with *). The number of pentoses in these oligomers ranges from 2 to 6.

The presence of oligosaccharides containing pentoses of which the non-reducing terminal xylose unit is substituted with α-D-glucuronopyranosyl at C2 was previously shown in a sorghum GAX digest (Verbruggen et al., 1998b), and a hexose-containing pentosan oligomer (Gal*p*-(1 → 4) Xyl*p*-(1 → 2) [5-*O*-(*trans*-feruloyl)] ara*f*) was already found in an acid hydrolysate of maize bran (Saulnier et al., 1995a). Further analysis of the larger oligomers in this series could show whether this oligomer is present as a side-chain directly attached to the xylan backbone as suggested by Saulnier et al. (1995a) or not. However, we will only be able to detect the oligomer without the ferulic acid substituent, because the ester linkage was saponified during the extraction of the arabinoxylans.

This is the first time that the presence of arabinoxylan oligomers containing two glucuronic acids is indicated. The presence of these oligomers, but also of oligomers containing one glucuronic acid shows that the glucuronic acid substituents were evenly distributed over the xylan backbone. This is in agreement with the fact that fractionation based on differences in charge density was not possible. Blockwise distribution of the glucuronic acid substituents over the xylan backbone and the fact that only 7% of the glucuronic acid was removed during the enzymatic degradation, would give a large amount of oligomers containing pentose only and probably some remaining glucuronic acid-rich polymeric material. In addition, it is shown that MALDI-TOF MS is an adequate tool to show the presence of methoxyl ethers of glucuronic acid residues. HPAEC analysis of a digest cannot directly show the presence of these methoxyl groups, since it is not known how the retention time of a component is affected by the presence of an additional methoxyl group, and the elution pattern of an enzymatically obtained digest is very complex.

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