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Cell wall polysaccharide interactions in maize bran

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Sequential extractions with alkali have been carried out in order to study the nature of linkages which hold heteroxylans in maize bran cell walls. Treatment with 0·5 M sodium hydroxide at 30°C for 2 h released all the phenolic acids (*p*-coumaric, ferulic, and diferulic) but extracted only ~30% of heteroxylans (S_1); further treatment with 1·5 M potassium hydroxide at 100°C for 2 h released the remaining heteroxylans (S_2). The heteroxylans from S_1 and S_2 had a similar neutral sugar composition and structure, but their weight average molecular weights were 270 kDa ($M_w/M_n=2$) and 370 kDa ($M_w/M_n=2{\cdot}8$), respectively. Proteins (5%) are found with polysaccharides from S_1 and S_2, with different amino acid composition. The results suggest that covalent linkages through phenolic acids are only partly responsible for the associations of maize bran heteroxylans in the cell wall and that linkages to structural cell wall proteins were probably the main cause of heteroxylan insolubility.

INTRODUCTION

Maize bran is a composite plant material consisting of thick walled cells originating from the aleurone layer, testa, pericarp and residual endosperm tissue. The walls of the various component cell types are composed of polysaccharides, e.g. heteroxylans and cellulose, but also contain significant amounts of phenolic acids, some proteins and are almost devoid of lignin (Chanliaud *et al.*, 1994).

Covalent bridges, ionic and hydrogen bonding between components of the cell wall have been postulated (Carpita & Gibeaut, 1993). Phenolic acids present in the cell wall are thought to play an important part in the connection of polysaccharides with other cell wall components, especially lignin and proteins, through ester and ether bonds, but also in the cross-linking of polysaccharide chains (Jung & Deetz, 1993). Feruloylated oligosaccharides from the side chains of heteroxylans have been previously isolated from maize bran by acid hydrolysis (Saulnier *et al.*, 1994), and the cross-linking of heteroxylan chains through ferulic acid is strongly supported by the presence of diferulic acid in bran (Saulnier *et al.*, 1994). It has been calculated that in the cell wall, each heteroxylan macromolecule carried ~60 ferulic acid esters and can be cross-linked through approximately five diferulic bridges (Saulnier *et al.*, 1994).

Treatment of maize bran with alkali (2 M, 2 h, 30°C) cleaves feruloyl ester linkages and more severe alkaline conditions (1·5 M, 2 h, 100°C) may release up to 90% of the heteroxylans of the wall (Chanliaud *et al.*, 1994). However, the mild conditions (2 M, 2 h, 30°C) generally used for release of phenolic acids (including diferulic acid) allow the extraction of only ~35% of heteroxylans (Chanliaud *et al.*, 1994). Therefore, the insolubility of the heteroxylan cannot be only ascribed to diferulic acid bridges. To obtain a better insight into the structure of the cell wall and the linkages between polysaccharides and other cell wall polymers we now report the use of various sequential extraction procedures, and the characterization of the extracted materials.

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EXPERIMENTAL

Plant material

Commercial micronized (particle size < 80 μm) corn bran were provided by ULICE (Riom, France).

Enzymes

The alkaline protease from *Bacillus licheniformis* was from Sigma (p6670), the heat-stable amylase Termamyl 120L from Novo Nordisk (Denmark), and the α-L-arabinofuranosidase from *Aspergillus niger* from Megazyme (Australia).

Purification of bran cell walls

Starch and proteins from contaminating endosperm tissue were removed using a procedure adapted from Brillouet *et al.* (1988). Bran (50 g) was suspended in 0·1 M phosphate buffer (pH 7·5, 1 l) containing sodium lauryl sulfate (1%), 2-mercaptoethanol (1%) and sodium azide (0·02%). A solution (25 ml) of alkaline protease (5 mg/ml) was added and the mixture stirred for 2 h at 40°C. After centrifugation, the residue was rinsed with distilled water and suspended again in 1 l of distilled water. The mixture was boiled for 15 min, then 10 ml of Termamyl 120 L was added and the mixture was stirred for 1 h at 95°C. The residue was collected on a sintered glass filter, rinsed with hot water (500 ml) and then dried by solvent exchange (ethanol, acetone) and kept overnight in an oven at 40°C. The purified bran cell walls (37 g) represented a yield of 74%.

Chemical extraction

Sequential extraction (Fig. 1)

Purified brans (25 g) were suspended in 500 ml of 0·5 M sodium hydroxide and stirred for 2 h at 30°C. Solids were separated from solution by centrifugation (5000 **g**, 15 min). The residue was rinsed with 500 ml of distilled water, centrifuged and rinsed again with 2 × 250 ml of distilled water. The extract and washings were pooled, acidified to pH 3–4 with 12 M hydrochloric acid and then concentrated in a rotary evaporator at 40°C to ~200 ml, dialysed against distilled water, and concentrated again up to ~200 ml. The pH was adjusted to 3·5 with concentrated acetic acid and polysaccharides were isolated by ethanol precipitation (4 vol, overnight at 4°C) to give S_1 (4·8 g). The residue was dried by solvent exchange (ethanol, acetone) and kept overnight in an oven at 40°C to give R_1 (15 g).

R_1 (10 g) was suspended in 1·5 M potassium hydroxide (200 ml) and stirred for 2 h at 1000°C. The residue was rinsed with distilled water (200 ml) collected by centrifugation and rinsed with 2 × 100 ml of distilled water. The final residue was dried as described above to give R_2 (3·5 g). The supernatant and washings were treated as described above to give S_2 (4·7 g).

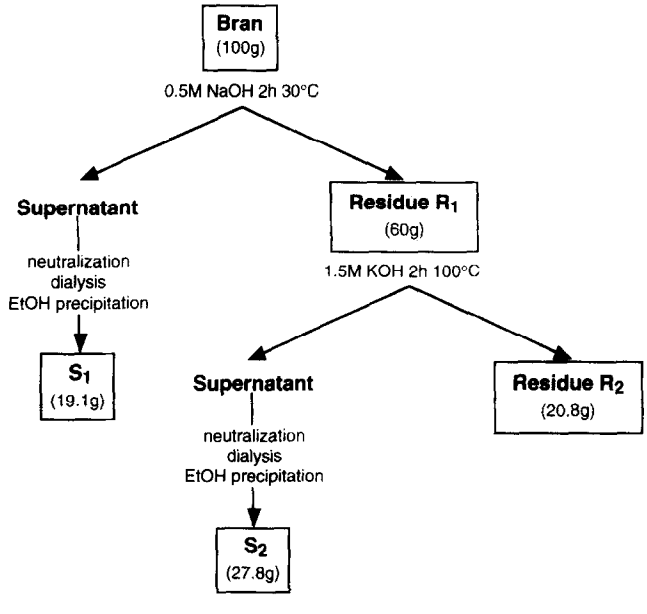

Fig. 1. Alkaline sequential extraction of purified maize bran (weights were recalculated starting from 100 g bran).

Other extractions

Purified bran was treated with 0·25 M methanolic sodium methoxide at 25°C and then extracted with water at 30°C as described by Morrison (1977). Other extractants applied to residue R_1 to solubilize the heteroxylans, were water at 100°C for 2 h, 8 M urea at 25°C for 2 h, 1·5 M potassium hydroxide containing 1% sodium borohydride at 100°C for 2 h, and acidified (pH 3·4) 0·3% sodium chlorite at 70°C for 1 h. In all extractions, liquid to solid ratios were similar to the ratio used in the sequential extraction procedure; residues and supernatants were isolated as described above.

Analytical methods

Individual neutral sugars in the extracted polysaccharides were determined after hydrolysis (1 M sulfuric acid, 100°C, 1·5 h) by gas liquid chromatography (glc) of their alditol acetate derivatives (Englyst & Cummings, 1988) on a DB-225 (J&W; 30 m × 0·32 mm i.d.) fused-silica capillary column. For the starting bran and the extraction residues, a prehydrolysis step with 72% sulfuric acid (25°C, 2 h) was used before neutral sugar analysis as described above.

Uronic acids were assayed by an automated *m*-phenylphenol method (Thibault, 1979), using glucuronic acid as standard on water-soluble samples. Insoluble samples (starting bran and residue of extraction) were first prehydrolysed with 95% sulfuric acid as described by Ahmed and Labavitch (1977).

Proteins were determined by semi-automated micro-Kjeldhal method (N × 5·7) or by the method of Lowry *et al.* (1951). Starch was determined by the enzymatic method of Karkalas (1985). Phenolic acids were determined by HPLC after alkaline extraction under argon with 2 M sodium hydroxide at 30°C for 2 h as previously described (Saulnier *et al.*, 1994). Acetic acid was determined after saponification by HPLC (Voragen *et al.*, 1986). Amino acid composition was determined by the HPLC Pico-Tag method (Waters Associates) after acid hydrolysis of samples (2 mg) by 6 M hydrochloric acid (1 ml) at 110°C for 20 h under argon atmosphere.

Methylation

Polysaccharides were methylated according to the method of Hakomori (1964) as previously described (Saulnier *et al.*, 1993). Methylated polysaccharides were hydrolysed with 2 M trifluoroacetic acid (100°C, 2 h) and converted into alditol acetates (Englyst & Cummings, 1988). The partially methylated alditol acetates were analysed by GLC on DB-225 and DB-1 fused-silica capillary columns (J&W, USA; 30 m × 0·32 mm i.d.) as previously described (Saulnier *et al.*, 1993).

Degradation with α-L-arabinofuranosidase

A solution of polysaccharide (10 mg) in 0·1 M acetate buffer (2 ml; pH 4·0) was incubated at 40°C for 48 h with successive addition of α-L-arabinofuranosidase (50 μl; 108·8 U/ml; 1 Unit hydrolyses 1 μmol of *p*-nitrophenyl-α-L-arabinoside per minute at pH 4·0 and 40°C) at 0 h and 24 h. The arabinose liberated was determined by the NAD^+-galactose dehydrogenase system (Melrose & Sturgeon, 1983).

Chromatography

Ion-exchange chromatography was performed on a DEAE-Sepharose CL-6B column (gel volume 20 ml) eluted at 50 ml/h by acetate buffer (pH 5). 2·5 ml solutions of S_1 and S_2 (2 mg/ml) were loaded onto the column which was washed with 50 ml of 0·05 M acetate buffer. The column was then eluted with a 50 ml linear gradient of acetate buffer pH 5 (0·05 M → 1 M). Fractions (3·2 ml) were collected and analysed by automated orcinol (Tollier & Robin, 1979) and *m*-phenylphenol (Thibault, 1979) methods.

Size-exclusion chromatography was performed with solutions of polysaccharides in water (2 mg/ml). Filtered solutions (0·45 μm) were injected on a Shodex OH-Pack 805 B column, eluted at 0·8 ml/min with 50 mM sodium nitrate. Double detection was performed with a variable wavelength UV detector (LDC, Spectromonitor 3000) and a differential refractometer (Erma ERC 7510, Japan).

Molecular weight determinations were performed by HPSEC-MALLS. The high-pressure size-exclusion chromatography (HPSEC) system comprised three Shodex OH-Pack columns (KB-806-805-804) eluted with 50 mM sodium nitrate at 1 ml/min and on-line molecular weight determination was performed using a multi-angle laser-light scattering (MALLS) detector (DawnF, Wyatt Technology, USA) and a differential refractometer (Erma ERC 7510, Japan).

Viscosity

Viscosity measurements were carried out on polysaccharide solutions in 0·1 M sodium chloride with an Ubbelohde capillary viscometer (capillary diameter: 0·46 mm). Solutions were filtered (0·45 μm) and intrinsic viscosities ($[\eta]$) were obtained in 0·1 M sodium chloride at 25°C by extrapolation to zero concentration of the reduced and inherent viscosities obtained for concentrations ranging from 5 to 0·7 mg/ml, using the Huggins and Kraemer equations.

RESULTS AND DISCUSSION

Purification of maize bran cell walls

The composition of crude and purified maize brans are shown in Table 1. The native bran contained some starch and proteins arising from endosperm tissue, but the purification treatment eliminated all the starch and ~65% of the protein. The purified bran was composed mainly of heteroxylans (67·5%; Ara + Xyl + Gal + GlcA) and cellulose (22·5%). More than 90% of arabinose, xylose, galactose and glucuronic acid of the initial bran was recovered in the purified cell walls indicating that most of the heteroxylans were insoluble in the aqueous extractant. Phenolic acids and acetic acid were significant components of the cell wall. Ferulic acid was the main phenolic acid of maize bran cell-wall and was esterified to arabinosyl in heteroxylan side chains (Saulnier *et al.*, 1994). *p*-Coumaric acid which was found in lower amounts is also known to be esterified to arabinoxylans in graminaceous cell walls (Hartley *et al.*, 1990). The occurrence of diferulic acid in walls of grasses is well known (Iiyama *et al.*, 1994), and Ishii (1991) has reported its role as a cross-linking molecule between arabinoxylans in bamboo shoots' cell wall. It is likely that diferulic acid also bridge

Table 1. Composition of crude and purified maize brans (% dry weight)

	Crude	Purified
Arabinose	17·8	22·8
Xylose	24·3	34·3
Galactose	5·0	5·6
Glucuronic acid	4·2	4·8
Glucose[a]	24·8	22·5
Starch	4·0	0·0
Protein (N ×5·7)	5·0	2·4
p-Coumaric acid	0·4	0·4
Ferulic acid	3·1	3·2
Diferulic acid	0·5	0·7
Acetic acid	3·6	4·2

[a]Total glucose measured by glc. method minus glucose from starch.
Data are mean of duplicate analyses. Coefficient of variation is 3% for neutral sugars and 5% for phenolic acids.

Table 2. Influence of extraction conditions on phenolic acids content of maize brans (% dry weight)

			p-Coumaric acid	Ferulic acid	Diferulic acid	Heteroxylan solubilization[a]
30°C	2 h	0·5 M	0·4	3·3	0·7	20·4
		1 M	0·4	3·1	0·7	38·3
		2 M	0·4	3·2	0·7	34·3
30°C	24 h	0·5 M	0·4	3·1	0·6	20·4
		1 M	0·4	3·4	0·7	38·3
		2 M	0·4	3·3	0·7	34·3
100°C	2 h	2 M	0·4	3·6	0·7	83·3

Data are means of duplicate analysis. CV 5%.
[a]% of heteroxylan content of bran.
Calculated from Chanliaud *et al.* (1994).

heteroxylans chains in maize bran. Acetic acid is known to be esterified to xylans (Aspinall, 1980).

Sequential extraction

Prior to developing the sequential extraction procedure for maize bran heteroxylans, we determined the conditions in which ester-linked phenolic acids are quantitatively released. Hartley and Morrison (1991) have showed that most of saponifiable feruloyl groups were released from grass cell walls by treatment at 25°C with 0·1 M sodium hydroxide for 1 h and then for 24 h under the same conditions. Our results (Table 2) showed no significant differences in the solubilization of phenolic acids (including diferulic acid) between treatment with 0·5 M sodium hydroxide for 2 h at 30°C and 2 M sodium hydroxide for 24 h at 30°C or 2 h at 100°C. However, these treatments released very different amounts of heteroxylans from the bran (Table 2; Chanliaud *et al.*, 1994). Therefore, we initially extracted the bran with 0·5 M sodium hydroxide at 30°C for 2 h to release any heteroxylans cross-linked by diferulic ester bridges. The residue was further extracted with 1·5 M potassium hydroxide at 100°C for 2 h to release the remaining bound heteroxylans probably held in the walls through other non-ester linkages. The use of potassium hydroxide at 100°C for 2 h instead of sodium hydroxide was previously shown to provide optimum extraction of heteroxylans from maize bran (Chanliaud *et al.*, 1994).

The composition of the extracts and the residues are reported in Table 3. Ninety-one per cent of heteroxylans were extracted by the sequential procedure and the final residue R_2 was highly enriched in glucose polymers, presumably cellulose. S_1 and S_2 contain mainly polysaccharides (90–95%). The neutral sugar content of S_1 was slightly lower than for S_2, however, they both had very similar molar compositions (Xyl: Gal: GlcA: Ara, 1·62: 0·21: 0·15: 1·00, and 1·61: 0·22: 0·16: 1·00, for S_1 and S_2, respectively). In S_1, 27·6% of the heteroxylan of the initial bran was recovered and 36·4% was recovered in S_2. The recovery of heteroxylans from S_1 was slightly higher than predicted by the experimental design (20·8%, Chanliaud *et al.*, 1994), which might be due to the purification treatment. The total recovery of heteroxylans ($S_1 + S_2 + R_2$) from initial purified bran was 73%, as there was some loss of material (~20% for S_1 and S_2) during ethanol precipitation. Proteins in the purified bran were co-extracted with polysaccharides. S_1 and S_2 contained ~4–6% of proteins, whereas the final residue of extraction R_2 was depleted in proteins (Table 3).

Table 3. Composition of supernatant and residue of sequential alkaline extraction (% dry weight)

	S_1	S_2	R_1	R_2
Yield[a]	19·1	27·8	60·0	20·8
Arabinose	31·6	27·8	19·0	8·1
Xylose	51·4	45·9	30·7	13·6
Galactose	8·4	7·8	4·8	3·6
Glucuronic acid	6·2	6·0	4·4	3·1
Glucose	tr.	1·6	34·0	78·9
Protein[b]	5·5	3·8	1·8	0·4
M_w	270 kDa	370 kDa	–	–
$[\eta]$ ml/g	159	181	–	–

Neutral sugar (CV 3%) and protein (CV 4%) content are means of duplicate.
[a]% weight of purified bran.
[b]Determined by Lowry methods for S_1 and S_2 and micro-Kjelhdal for residue R_1 and R_2.

Characterization of S_1 and S_2

Physico-chemical characterization

Ion-exchange chromatogoraphy of S_1 and S_2 gave similar elution patterns (Fig. 2; S_2 not shown). A minor proportion of the polysaccharides was not bound to the column (5%) and a major fraction, containing all the uronic acid, was bound and eluted with ~0·2 M acetate buffer, as previously reported (Chanliaud *et al.*, 1994). It has been previously observed that proteins were coeluted with the bound polysaccharide fraction (Chanliaud, 1995).

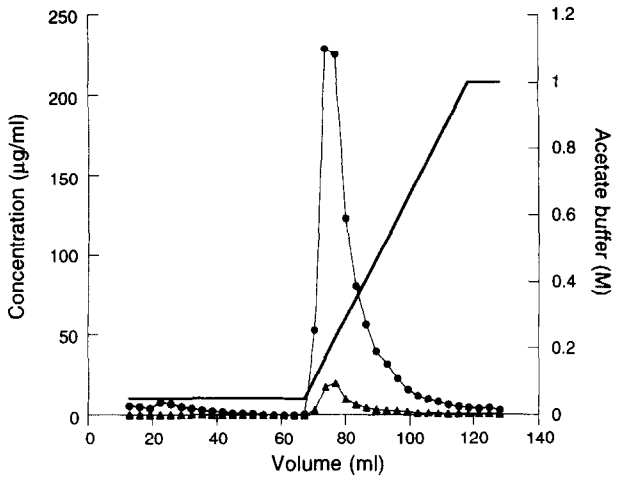

Fig. 2. Ion-exchange chromatography on DEAE-Sepharose CL-6B of fraction S_1. (—●—, neutral sugars; —▲—, uronic acid.)

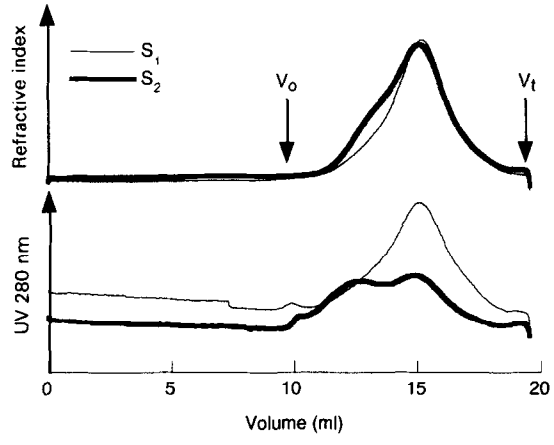

Fig. 3. Size-exclusion chromatography on Shodex OH-pack 805B column of fraction S_1 and S_2.

The size-exclusion chromatography behaviour of S_1 and S_2 was different (Fig. 3). Refractive index detection showed that S_1 polysaccharides were eluted as a single homogeneous peak, whereas S_2 polysaccharides showed a shoulder on the leading edge indicating the presence of higher molecular weight components. UV detection showed that absorbing material co-eluted with the polysaccharide component; this absorption was ascribed to proteins.

The weight-average molecular weight of S_1 and S_2 was measured using HPSEC-MALLS (Table 3). S_2 had a higher weight-average molecular weight ($M_w = 366{,}000$) and a higher polydispersity index ($I = 2{\cdot}8$) than S_1 ($M_w = 267{,}000$; $I = 2$). Intrinsic viscosities [η] were 159 and 181 ml/g for S_1 and S_2, respectively, which confirmed the higher weight-average molecular weight of S_2 components. The Huggins coefficients were 0·446 and 0·48 for S_1 and S_2, respectively, which indicated that 0·1 M sodium chloride was a good solvent for the heteroxylans.

Table 4. Methylation analysis of supernatant S_1 and S_2 (mol %)

Methyl ether	Linkage	S_1	S_2
2,3,5-Ara	Terminal	21·7	20·6
2,5-Ara	(1→3)	4·7	5·2
3,5-Ara	(1→2)	4·6	4·8
2,3-Ara	(1→5)	2·3	2·4
2,3,4-Xyl	Terminal	19·0	18·4
2,3-Xyl	(1→4)	9·7	9·8
2(3)-Xyl	(1→3(2).4)	22·2	22·8
Xyl	(1→2.3.4)	10·6	10·6
2,3,4,6-Gal	Terminal	4·3	4·3

Structural characterization

The structure of the polysaccharides in S_1 and S_2 were studied by methylation. Both samples showed identical linkage distribution (Table 4), similar to maize bran heteroxylans extracted in various conditions (Saulnier *et al.*, 1993; Chanliaud *et al.*, 1994). This structure was typical of highly branched heteroxylans. Indeed, assuming that all 4-linked xylose residues (2,3-Xyl; 2(3)-Xyl and Xyl, Table 4) belonged to the xylan backbone, only 23% of the xylose in the backbone (23-Xyl) did not bear side-chains.

Despite the high proportion of terminal arabinose, the α-L-arabinofuranosidase from *Aspergillus niger* released only ~20% of total arabinose (30% of terminal residue) in both samples. The kinetics of the hydrolysis were similar for the two samples. Although, monomeric non-reducing arabinose units were the main side chains of the xylan backbone, various oligomeric side chains, containing xylose, arabinose and galactose have been reported in maize bran heteroxylans (Whistler & Corbett, 1955; Srivastava & Smith, 1957; Saulnier *et al.*, 1994). Therefore, the low degradation may have been due to steric hindrance of arabinosidase attack. To test this hypothesis, other arabinofuranosidases should be used as very high specificity have been reported for arabinosidase degrading arabinoxylans (Andrewartha *et al.*, 1979; Kormelink *et al.*, 1991). The amino acid composition of the brans, the extracts and the residue R_1 are reported in Table 5. The amino acid compositions of proteins in S_1 and S_2 are characterized by an abundance in hydroxyproline, threonine, alanine and proline, which is characteristic of 'extensin' from maize pericarp (Hood *et al.*, 1988). Although, the amino acid composition of S_1 was similar to the purified cell walls and residue R_1, the proteins from S_2 were significantly different.

Other extractions

Treatment of purified brans with 0·5 M sodium hydroxide at 30°C was realized to release selectively ester-linked heteroxylans. Morrison (1977) reported the extraction of a galactoarabinoxylan from ryegrass by

Table 5. Amino acid composition of proteins from maize bran fractions (mol %)

Amino acid	Crude bran	Purified bran	S_1	S_2	R_1
Asx	6·3	4·5	6·2	6·3	4·2
Glx	10·2	6·1	7·2	5·8	7·2
Hyp	6·9	13·9	11·3	5·7	13·8
Ser	5·8	5·5	5·4	1·6	5·6
Gly	9·6	10·8	7·8	4·7	9·5
His	2·3	2·3	2·4	1	2·6
Arg	3·3	2	2·3	0	1·9
Thr	7·1	10·6	8·8	8·7	9·3
Ala	11·3	7·9	9·4	15·9	9·5
Pro	10·7	12·8	12·3	17·5	12·5
Tyr	2·3	2·8	3·2	5·3	2·2
Val	5·5	5	6·3	9·9	5·5
Met	0·4	1·7	2·2	6·8	0·8
Cys	0·2	0·2	0·3	2·3	0
Ile	3	2	2·3	1·6	2·4
Leu	7·4	4	4·8	3·4	5·2
Phe	2·8	2·2	2·8	1·9	2·7
Lys	4·7	5·7	5	1·8	5·2

Data are means of duplicate. Coefficient of variation, 5%.

treatment with 0·25 M methanolic sodium methoxide and subsequent extraction with water; he explained this result by hydrolysis of the ester bonds involving phenolic acids. When this treatment was applied to the maize bran only a very low amount of material (1%) was released. Although methanolic methoxide did not remove all the phenolic acids (~60% of ferulic acid and ~50% of diferulic and *p*-coumaric acids), this result suggested that the sodium hydroxide treatment might have cleaved other linkages.

R_1 was extracted further by other agents. Water at 100°C or 8 M urea released only minute amounts of material (7·3% and 3·4% from R_1, respectively), as compared with potassium hydroxide at 100°C (46·4%), which suggested that increasing the temperature by itself has a very limited effect on the extraction and that hydrogen bonds (e.g. to cellulose) were not involved in the insolubility of heteroxylans. R_1 was also extracted at 100°C with 1·5 M potassium hydroxide containing sodium borohydride, to avoid possible degradation of polysaccharide during alkaline treatment by oxidative peeling from the reducing end (Fincher & Stone, 1986). Similar yields were obtained with or without sodium borohydride (Table 6). Treatment with acidic sodium chlorite, which is known to cleave isodityrosine cross-links of extensin (Biggs & Fry, 1990) released substantial amounts of heteroxylans (Table 6).

The materials extracted with acidic chlorite and alkaline borohydride were further characterized. The neutral sugar compositions were similar to those obtained for polysaccharides in the sequential extraction (Table 6). Size-exclusion chromatography of the two extracts (Fig. 4) showed that both samples were of lower molecular weight than S_2, which was confirmed by the

Table 6. Composition of supernatant and residue isolated after treatment of R_1 by sodium chlorite or potassium hydroxide containing sodium borohydride

Extraction	Sodium chlorite		Sodium borohydride	
	Supernatant	Residue	Supernatant	Residue
Yield[a]	10·6	40·7	28·2	17·6
Arabinose	31·0	15·5	31·4	1·6
Xylose	49·2	24·3	50·9	4·4
Galactose	7·6	5·4	6·6	0·8
Glucose	trace	42·7	3·9	86·9

Data are means of duplicate. Coefficient of variation, 3%.
[a] % weight purified bran.

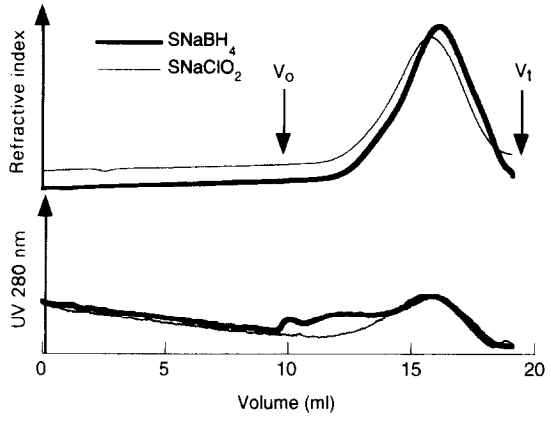

Fig. 4. Size-exclusion chromatography on Shodex OH-pack 805B column of fraction $SNaClO_2$ and $SNaBH_4$.

intrinsic viscosities values of 114 and 98 ml/g, for acidic chlorite and alkaline borohydride extracts, respectively. Refractive index showed that the alkaline borohydride extract was less polydisperse than S_2, and UV detection showed that proteins were present.

CONCLUSIONS

Cell walls of the tissues of maize bran are mainly composed of heteroxylans and cellulose. The results of treatment with different extractants showed that the monosaccharide composition, and linkage profile of the extracted heteroxylans are similar whatever the extractant used, indicating a highly homogeneous nature of the native polysaccharide. A schematic structure, which takes into account previous studies on maize heteroxylans (Whistler & Corbett, 1955; Montgomery *et al.*, 1956, 1957; Srivastava & Smith, 1957; Saulnier *et al.*, 1994) is proposed for the polysaccharide moiety of the heteroxylan (Fig. 5).

Proteins were co-extracted with polysaccharides by the different treatments, and these protein components were coeluted with polysaccharide components in both

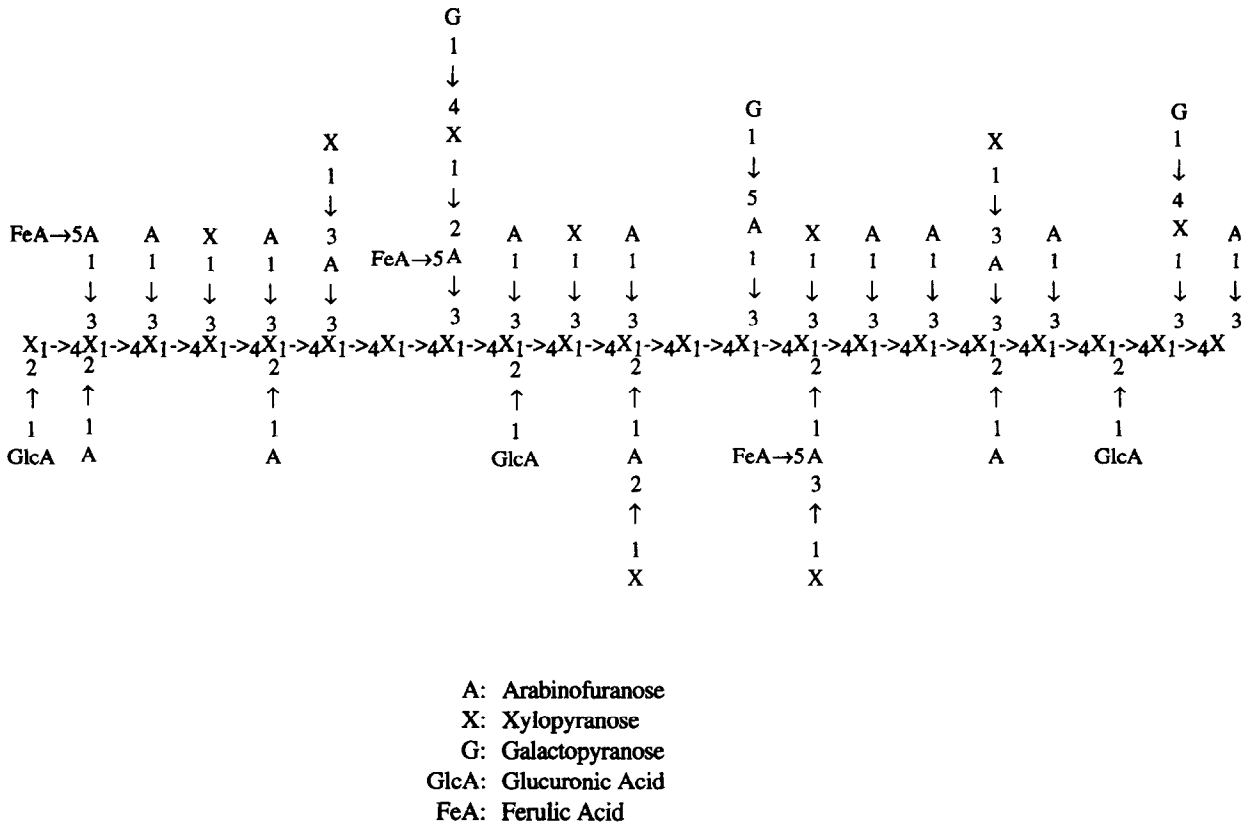

Fig. 5. Schematic structure of the sugar moiety of heteroxylans from maize bran.

gel permeation and ion-exchange chromatographies. The high proportion of hydroxyproline and proline suggest that these proteins are possibly related to maize hydroxyproline-rich glycoprotein (HRGP).

The sequential extraction used was supposed to extract first heteroxylans only linked through ester linkages (S_1) to the cell wall and then heteroxylans (S_2) linked by other linkages (H-bonding). However, S_1 and S_2 components only differed by their molecular weight and their amino acid composition; 0·5 M sodium hydroxide at 30°C probably has another role other than removing ester linkages, as suggested by the inefficiency of sodium methoxide treatment. Urea failed to extract heteroxylans indicating that hydrogen bonding was not involved in heteroxylan insolubility. Indeed the highly branched structure of maize bran heteroxylans excludes the possibility of hydrogen bonding with cellulose, in opposition to the general assumption concerning heteroxylans (xyloglucans) association in the cell wall. Heteroxylans in walls of other grasses, e.g. wheat endosperm cell walls, have a much simpler structure (less substituted) which may allow non-covalent associations between heteroxylan chains and on the surface of cellulose microfibrils.

On the other hand, treatment with sodium chlorite has released heteroxylans from the cell wall, suggesting that proteins linked to heteroxylans might be responsible for the insolubility of heteroxylans. Previous studies (Hood *et al.*, 1988, 1991) have shown that HRGPs were present in maize pericarp and that the amount of HRGPs was positively correlated with pericarp thickness suggesting that HRGPs contributed to the structural integrity of pericarp tissue. The decrease in weight-average molecular weight of alkaline borohydride extract as compared to S_2 might be explained by the reduction of disulphide bonds of associated proteins of S_2 (cysteine is detected in amino acids from S_2) by sodium borohydride and further sustained the importance of protein–polysaccharide interactions in maize bran. The highly branched structure of heteroxylans prevented degradation by oxidative peeling, and, therefore, the presence of sodium borohydride was not necessary for this purpose.

In conclusion, our results show that the insolubility of heteroxylans in the cell wall cannot be explained only by diferulic bridges. It is suggested that protein–polysaccharide linkages might be the main cause of insolubility of maize bran heteroxylans. Diferulic acid bridges between heteroxylan chains probably reinforced the basic protein–polysaccharide network, rather than insolubilized a specific population of heteroxylans in the cell wall. Alkali acts in our sequential extraction as a deesterifying agent but may also cleave isodityrosine bridges of cell wall protein, thus allowing the release of heteroxylans. The severe conditions of the second step (1·5 M potassium hydroxide, 100°C) of the sequential

extraction would break down more highly cross-linked proteins than the first step (0·5 M) sodium hydroxide, 30°C). The actual nature of protein–polysaccharide linkages is not known. Glycosidic bonds through hydroxyproline are known to be alkali-resistant but further studies are needed to determine the nature of the linkage between protein and heteroxylan.

Interconnection of heteroxylans with a protein network is probably a good interim way of describing the situation until more evidence for or against covalent linkages is adduced. In this context, the highly branched nature of maize bran heteroxylan, together with the interconnection with a cell wall protein network further reinforced by diferulic bridges between polysaccharide chains, probably explains the extreme resistance of bran tissue to enzyme attack by pure xylanase and ferulic esterase or mixtures of cell wall degrading enzymes (Faulds *et al.*, 1994).

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