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Isolation and partial characterization of feruloylated oligosaccharides from maize bran

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Abstract

Maize bran contains phenolic acids [~ 4% dry matter; mainly ferulic acid (Fe) and also diferulic acid], heteroxylans (~ 50%), and cellulose (~ 20%), but is devoid of lignin. Treatment of maize pericarp with 0.05 M trifluoroacetic acid at 100°C for 2 h released ~ 90% of the heteroxylans and ~ 90% of the ferulic acid and its esters. After fractionation of the products with Amberlite XAD-2 and Sephadex LH-20 three main feruloylated oligosaccharides (F_3–F_7) were isolated. They represented ~ 30% of the ferulic acid, and ~ 2% of the neutral sugars contained in the hydrolysis supernatant. The compositions of F_7, F_6, and F_3 were Fe–Ara (1:1), Fe–Ara–Xyl (1:1:1), and Fe–Ara–Xyl–Gal (1:1:1:1), respectively. The structures of the three oligomers were determined using chemical methods (methylation, acetalation, reduction) and ^{13}C NMR spectroscopy: F_7 was 5-*O*-(*trans*-feruloyl)-L-Ara*f*; F_6 was *O*-β-D-Xyl*p*-(1 → 2)-[5-*O*-(*trans*-feruloyl)-L-Ara*f*]; and F_3 was *O*-L-Gal*p*-(1 → 4)-*O*-D-Xyl*p*-(1 → 2)-[5-*O*-(*trans*-feruloyl)-L-Ara*f*]. F_7 has been previously isolated from other monocots especially from wheat bran and soluble arabinoxylans from wheat flour; this is the first report of feruloylated oligosaccharides F_6 and F_3. Our results suggest that these oligomers are side-chain constituents of heteroxylans in maize bran. Ferulic acid is probably partly responsible for the insolubility of heteroxylans by coupling polysaccharide chains through ferulic acid dimers.

Keywords: Ferulic acid; Feruloylated oligosaccharides; Arabinoxylan; Cell wall; Maize

1. Introduction

The association of ferulic acid and *p*-coumaric acid with hemicellulosic polysaccharides, especially arabinoxylans, has been extensively investigated [1]. Partial enzymic or

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acid hydrolysis of cell walls and subsequent chromatographic fractionation have allowed the isolation from Gramineae tissues of various oligosaccharides ester-linked to hydroxycinnamic acids. The feruloylated trisaccharide derived from arabinoxylans, *O*-[5-*O*-(feruloyl)-α-L-Ara*f*]-(1 → 3)-*O*-β-D-Xyl*p*-(1 → 4)-D-Xyl*p* (FAXX), has been isolated from maize shoots [2], coastal Bermuda grass shoots [3], sugar cane bagasse [4], and barley straw and endosperm [5,6], and the corresponding *p*-coumaroyl compound has been isolated from bamboo shoots [7,8], coastal Bermuda grass shoots [3], and barley straw [5]. The feruloylated disaccharide *O*-[5-*O*-(*trans*-feruloyl)-α-L-Ara*f*]-(1 → 3)-D-Xyl*p* (FAX) [1] has also been isolated from wheat bran [1,9].

Phenolic acids present in the cell wall are thought to play a major part in the linkage of hemicellulosic polysaccharides with other cell-wall components, especially lignin, through ester and ether bonds [10]. In addition dimeric units, such as dehydrodiferulic acid and the truxillic acids, may cross-link polysaccharide chains [10].

Corn bran is made up of several layers of thick-walled cells (aleurone layer, testa, and pericarp) and of residual endosperm tissue. The corn bran is mainly composed of cell wall polysaccharides, e.g., heteroxylans (~ 40%) and cellulose (~ 20%), is almost devoid of lignin, and contains a significant amount of phenolic acids (~ 4%) [11].

In order to study the linkages between phenolics and heteroxylans in maize bran, controlled acid hydrolysis has been carried out. Some feruloylated oligosaccharides have been isolated by chromatography and their structures have been determined using methylation, acetalation, enzymic degradation, and NMR spectroscopy.

2. Results and discussion

Acid hydrolysis of the bran.—The initial starch content of the bran was 12% [11]. It was removed prior to acid hydrolysis by treatment with a heat-stable alpha-amylase; 83% (dry weight basis) of the starting material was recovered. Destarched brans were composed of arabinose (16.8%; dry weight basis), xylose (30.9%), galactose (4.7%), glucose (20.3%), glucuronic acid (5.9%), *p*-coumaric acid (0.4%), ferulic acid (3.1%), and diferulic acid (0.5%). Therefore heteroxylans (the sum of arabinose + xylose + galactose + glucuronic acid) represented ~ 60% of the material.

In order to study the kinetics of release of feruloylated oligosaccharides, the purified bran was hydrolysed at 100°C with 10 mM or 50 mM trifluoroacetic acid or with 32 mM oxalic acid. The amounts of sugars and of free and esterified (linked to sugars) ferulic acid were measured, using orcinol and UV spectroscopy methods, respectively, and are recorded in Figs. 1 and 2. Treatment with 10 mM trifluoroacetic acid released only minor amounts of ferulic acid and sugars. There were only minor differences between 50 mM trifluoroacetic acid and 32 mM oxalic acid treatments. During the first 4 h of hydrolysis, a rapid solubilization of sugars and phenolic acids and their esters was observed. Then a plateau was reached, where ~ 65% of the sugar and ~ 90% of the phenolic acid content of the bran was released. Analysis of the supernatant solution

[1] An incorrect structure was assigned to FAX in ref. [9] (see ref. [5]).

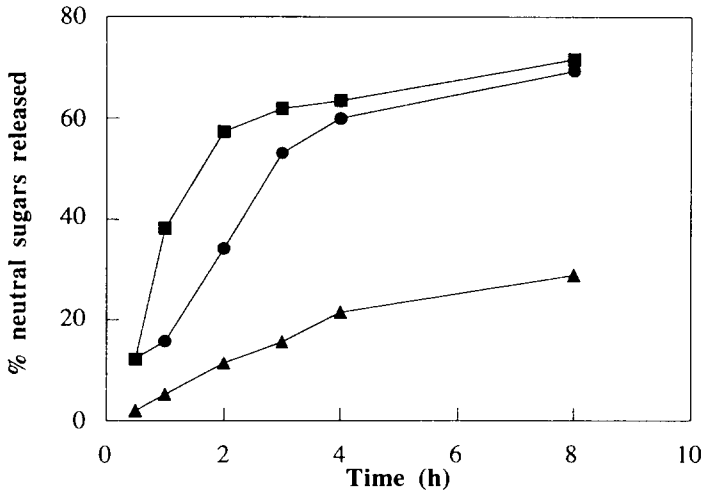


Fig. 1. Kinetics of extraction of neutral sugars with 10 mM CF_3CO_2H (▲), 50 mM CF_3CO_2H, (■), and 32 mM oxalic acid (●) from maize bran at 100°C (neutral sugars determined by the orcinol method).

showed that it was composed mainly of arabinose, xylose, galactose, and glucuronic acid, with very minute amounts of glucose. The arabinose and xylose content in the supernatant solution was also measured by GLC as their alditol acetates without hydrolysis in order to determine the proportion of monomeric sugars. It was observed that even after hydrolysis for only 30 min the proportion of monomeric arabinose was high (70% of released arabinose) and reached 95% within the first three hours of

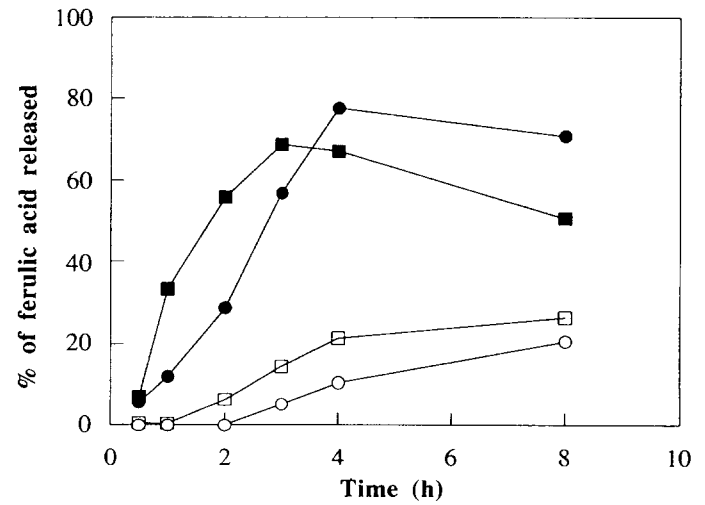


Fig. 2. Kinetics of extraction of phenolic acids with CF_3CO_2H (■ , esterified; □, free) and oxalic acid (●, esterified; ○, free) from maize bran at 100°C (phenolic acids determined by spectrophotometry).

Table 1
Sugar and phenolic acid composition of initial bran and residue [a]

	Bran	Residue
Yield (%) [b]	100.0	30.0
Composition(%) [c]		
Arabinose	16.8	1.1
Xylose	31.0	9.8
Galactose	4.8	1.2
Glucose	20.4	60.7
Glucuronic acid	5.9	1.4
p-Coumaric acid	0.4	0.2
Ferulic acid	3.1	0.4
Diferulic acid	0.5	0.1

[a] Extraction for 50 mM CF_3CO_2H, 100°C, 2 h. [b] Per cent in weight of initial bran. [c] Per cent dry weight.

hydrolysis, whereas the proportion of monomeric xylose (30% of xylose released) was nearly constant during the same period of time and increased for longer hydrolysis times. It was also observed that arabinose was released more rapidly than xylose, galactose, and glucuronic acid which were released at the same rate. Ferulic acid was mainly released in esterified form, but after 4 h significant hydrolysis into the free form occurred (Fig. 2).

Therefore, heteroxylans fragments linked to ferulic acid are released from cell walls by the acidic treatment. A treatment with 50 mM trifluoroacetic acid at 100°C for 2 h was selected since it gave appreciable amounts of feruloylated oligosaccharides with minimal release of free ferulic acid.

Isolation of the feruloylated oligosaccharides.—The yields of neutral sugars and phenolic acids and the composition of the residue are reported in Table 1. Treatment with 50 mM trifluoroacetic acid at 100°C on a preparative scale released ~60% of neutral sugars and ~90% of the phenolic acids and esters of the cell wall. Arabinose (89% of the arabinose from the bran), xylose (80%), galactose (84%), and glucuronic acid (53%) were extensively solubilized in the supernatant solution but glucose was mostly retained in the residue (95%), which demonstrated that heteroxylans were highly

Table 2
Purification on Amberlite XAD-2 of 50 mM CF_3CO_2H hydrolysis (100°C, 2 h) supernatant from maize bran

Elution	AI Water	AII 1:1 MeOH–water	AIII MeOH
Total sugars [a]	42.2	3.4	0.2
Free ferulic acid [a]	0.39	0.14	0.07
Esterified ferulic acid [a]	0.91	1.04	0.06
Arabinose [b]	32	31.4	32.4
Xylose [b]	54.4	59.6	56.7
Galactose [b]	9.4	6.5	6.8
Glucose [b]	4.3	2.5	4.0
Sugar–ferulic acid [c]	40	4	2

[a] g/100 g of bran; determined by spectrophotometry for ferulic acid or the orcinol method for sugars. [b] Mol %. [c] Molar ratio.

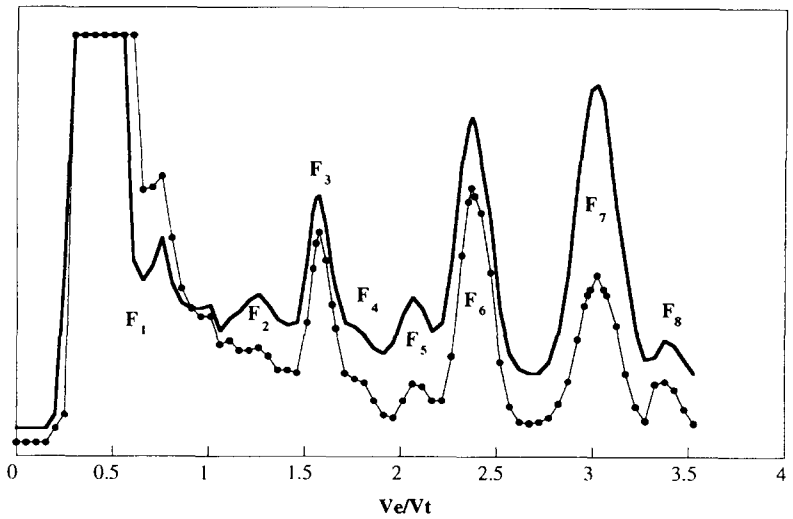


Fig. 3. Elution profile on Sephadex LH-20 eluted with 1:3 methanol–water of fraction II from Amberlite XAD-2 (●, neutral sugar detected with orcinol; ▬, UV at 360 nm).

degraded by the treatment, whereas cellulose was little affected. The proportion of ferulic acid in the esterified form was ~ 80%.

The supernatant solution was loaded onto an Amberlite XAD-2 column which was eluted with water (AI), 1:1 methanol–water (AII), and methanol (AIII). Chromatographic yields were ~ 90%, and the amounts of ferulic acid and neutral sugars in each fraction are reported in Table 2. It was expected that fraction AI would contain mainly sugars, AII feruloylated oligosaccharides, and AIII mainly free ferulic acid, which was partly observed. Indeed, AI contained a high amount of sugar as compared with AII and AIII, but free ferulic acid was also present from UV spectroscopy calculations. However, furfuric products, due to dehydration of sugars in acidic conditions, may have interfered in the UV spectroscopy method. AIII also contained sugars, which possibly may be more tightly bound to the column due to being linked to diferulic acid. However, fraction AII contained the major part of esterified ferulic acid and was therefore used for further purification on Sephadex LH-20. Approximatively 50% of the initial phenolic acids of the bran were found in AII.

Eight fractions (F_1–F_8) were eluted by 1:3 methanol–water from a Sephadex LH-20 column (Fig. 3). The neutral sugar and ferulic acid composition of each fraction are reported in Table 3. The chromatographic yields were 97% and 80% for neutral sugar and phenolic acids, respectively. Fraction F_1, which contained most of the neutral sugars, was eluted in the fractionation range (K_{av} ~ 0.5) of the column which may indicate a high degree of polymerization of the sugars. Other fractions were more strongly absorbed on the column and eluted after the total volume of the column ($K_{av} > 1$). We have isolated three of these fractions which were well separated (F_3, F_6, and F_7). These three fractions represented ~ 50% of the phenolic acids and 13.5% of the neutral sugars of fraction II.

Table 3
Composition of fractions separated on Sephadex LH-20

	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8
Ve/Vt	0.3	1.2	1.6	1.8	2.1	2.4	3	3.4
Total sugars [a]	76.3	5.3	3.2	1.6	1.3	5.9	4.4	2
Ferulic acid [a]	30	8	9	3	4	17	23	6
Composition [b]								
Arabinose	1	1	1	1	1	1	1	1
Xylose	7.6	2	1	1.2	0.7	0.9	0.06	0.7
Galactose	0.4	0.5	0.8	0.3	0.04	0.01	—	0.1
Glucose	0.4	—	—	—	—	—	—	—
Sugar–ferulic acid [c]	18.5	5	3.2	4.5	1.9	2.4	1.2	2.2

[a] Per cent of total amount eluted from LH-20. Determined by spectrophotometry for phenolic acids or the orcinol method for sugars.
[b] Molar ratio relative to arabinose as determined by GLC.
[c] Molar ratio.

Table 4
Glycosyl analysis [a] of acetalated and methylated feruloylated oligomers

Sugar [b]	F_3		F_6		F_7	
	Acetalated	Methylated	Acetalated	Methylated	Acetalated	Methylated
2,3,5-Ara	—	—	—	—	—	1
3,5-Ara	—	1	—	1	—	—
5-Ara	1	—	1	—	1	—
2,3,4-Xyl	—	—	—	1.4	—	—
2,3-Xyl	—	1.2	—	—	—	—
Xyl	1.6	—	1.2	—	—	—
2,3,4,6-Gal	—	1.4	—	—	—	—
Gal	0.8	—	—	—	—	—

[a] Molar ratio relative to arabinose of sugar analysed by GLC as alditol acetates. [b] 2,3,5-Ara: 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol, etc.

Characterization of fractions F_3, F_6, and F_7.—Ferulic acid was the only phenolic acid in the three fractions, as shown by HPLC after deesterification. The feruloylated oligosaccharides were analysed, after deesterification, on a Dionex system. However, when using 2 M sodium hydroxide for 2 h at 35°C, two peaks appeared for each oligosaccharide; this was probably due to rearrangement reactions at the reducing end in the alkaline medium. 0.2 M Sodium hydroxide for 30 min at 25°C was shown to be sufficient to fully deesterify the feruloylated oligosaccharides. Under these conditions, each fraction gave only one peak on the Dionex system, indicating that each fraction was a unique oligosaccharide structure. Fractions F_3, F_6, and F_7 each gave a minor and a major peak (relative proportion 1:13) when chromatographed on Sephadex LH-20 and eluted by water; each peak had a composition and elution time on the Dionex system similar to those of the starting fraction. Similar observations have been previously reported [9], and were attributed to rapid equilibrium reactions of the oligomer. Therefore, the three fractions were pure oligomers, composed of equimolar amounts of ferulic acid and arabinose for F_7; ferulic acid, arabinose, and xylose for F_6; and ferulic acid, arabinose, xylose, and galactose for F_3 (Table 3).

The structures of F_3, F_6, and F_7 were obtained from methylation and acetalation experiments (Table 4). The alkali-labile ferulic ester substituent was assigned to position 5 of arabinose using the acetalation method of De Belder and Norrman [12]. Methylation analysis revealed that the carbohydrate moiety of F_6 was Xyl*p*-(1 → 2)-Ara*f*. Deesterification, reduction, and then hydrolysis and acetylation of F_3 gave only arabinitol pentaacetate, which demonstrated that arabinose was in the reducing position. Therefore the carbohydrate moiety of F_3 was Gal*p*-(1 → 4)-Xyl*p*-(1 → 2)-Ara*f*. α-D-Galactosidase and β-D-galactosidase did not release galactose from deesterified F_3 oligosaccharide. After total acid hydrolysis of F_3, D-galactose was not detected using an enzymatic D-galactose assay kit. We therefore concluded that L-galactose was present in F_3. L-Galactose has previously been reported in polysaccharide material from corn cob [13].

The ^{13}C NMR spectrum of oligomer F_7 was the same as previously reported [14]. Spectra of F_6 and F_3 exhibited common features with F_7 for the ferulic acid and arabinose resonances. Xylose resonances in F_6 were assigned (Table 5) with the help of

Table 5
Chemical shifts (ppm) of the ^{13}C resonances for feruloylated oligosaccharides F_7, F_6, and F_3

Fraction	Unit		C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10
F_7	L-Ara*f*	α	102.1	82.1	76.8	81.5	64.8					
		β	96.1	76.7	75.1	79.4	65.8					
	Ferulic acid		127.3	111.6	148.0	148.4	115.9	123.9	147.0	114.0 [a] 114.1	169.6	56.2
F_6	L-Ara*f*	α	100.9	89.9	75.8	81.2	65.4					
		β	95.7	84.2	73.8	78.9	66.0					
	β-D-Xyl*p*		104.0 [a] 103.1	73.6	76.2	69.8	65.8					
	Ferulic acid		127.1	111.6	148.0	148.4	115.9	123.9	147.0	114.0 [a] 114.1	169.5	56.2
F_3	L-Ara*f*	α	100.3	90.1	nd [b]	nd	63.9					
		β	95.2	84.1	nd	nd	65.2					
	β-D-Xyl*p*		102.9 [a] 102.1	nd	nd	nd	65.1					
	L-Gal*p*		99.1 [a] 98.9	nd	nd	nd	61.6 61.4					
	Ferulic acid		127.2	111.6	148.0	148.4	115.9	123.8	147.0 [a] 146.9	114.1 [a] 114.2	169.4	56.2

[a] Peaks "doubled" by the presence of anomers in the reducing end residue. [b] nd, Not determined.

^{13}C NMR data for oligosaccharides [15,16]. ^{13}C NMR spectroscopy data for F_6 are consistent with the location of ferulic acid on position 5 of L-arabinose, and a β-linked D-xylose residue.

Assignment of the ^1H NMR spectra was not completly achieved. However, the phenolic part of the spectra was similar to those previously published [14,17] for feruloylated oligosaccharides, and H-7 and H-8 of ferulic acid were easily identified (δ: 7.15, 5.96; 7.30, 6.04; 7.50, 6.25; for F_7, F_6, and F_3, respectively). As previously reported [14] the H-7 and the H-8 peaks were "doubled" by the presence of anomers in the reducing end arabinose residue. A coupling constant of $J_{7,8}$ 15.96 Hz indicated that the ferulic acid was present as the *trans* isomer in oligomers F_{7-3}.

Therefore F_7 was 5-*O*-(*trans*-feruloyl)-L-Ara*f*, F_6 was *O*-β-D-Xyl*p*-(1 → 2)-[5-*O*-(*trans*-feruloyl)-L-Ara*f*] and F_3 was *O*-L-Gal*p*-(1 → 4)-*O*-Xyl*p*-(1 → 2)-[5-*O*-(*trans*-feruloyl)-Ara*f*]. While, 5-*O*-feruloyl-L-Ara*f* has been previously isolated from other monocots especially from wheat bran [18], and recently from corn bran [19], the feruloylated oligosaccharides F_6 and F_3 have structures which have not previously been reported.

Feruloylated oligosaccharides containing arabinose and xylose have been isolated from various monocotyledons and especially from wheat bran {FAX: *O*-[5-*O*-(*trans*-feruloyl)-α-L-Ara*f*]-(1 → 3)-D-Xyl*p*} [1,9] and maize coleoptiles {FAXX: *O*-[5-*O*-(feruloyl)-α-L-Ara*f*]-(1 → 3)-*O*-β-D-Xyl*p*-(1 → 4)-D-Xyl*p*)} [2] by enzyme or acid hydrolysis; in these oligomers, the xylose residue is released from the linear 4-linked xylan backbone and is at the reducing end. In contrast, heteroxylans from maize pericarp exhibit a highly branched structure [20], where single arabinose is a major substituent

and Xyl*p*-(1 → 2)-Ara*f* and Gal*p*-(1 → 4)-Xyl*p*-(1 → 2)-Ara*f* have been isolated as side-chains [21]. Therefore, it is likely that acid-labile glycosidic bonds involving arabinose residues are split off during acid treatment leading to the release of side-chain components F_6 and F_3. Ferulic acid is mainly linked to these side-chains, since ca. one-third of the ferulic acid of the cell wall is released in fractions F_3, F_6, and F_7, but other locations cannot be ruled out.

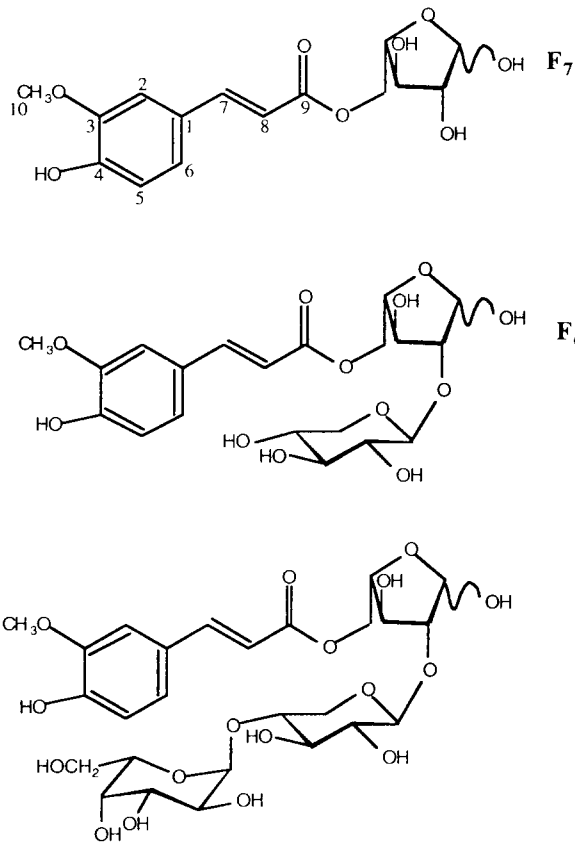
Therefore, ferulic acid is a constituent of native heteroxylans in maize bran and possibly is partly responsible for the insolubility of heteroxylans by coupling polysaccharide chains through ferulic acid dimers. This hypothesis is further confirmed by the identification of diferulic acid in the phenolics extracted from maize pericarp. Indeed, there is 1 mole of ferulic acid per 8 moles of arabinose and 1 mole of diferulic acid for 100 moles of arabinose in corn bran. The weight-average molecular weight of alkali-extracted heteroxylans is ~ 230 000 [11,20] which corresponds to an average dp of ~ 1700 and ~ 500 arabinose residues per heteroxylan molecule. Therefore, assuming that ferulic acid and diferulic acid are exclusively ester-linked to the arabinose residues of heteroxylans, one can calculate that in the cell wall each heteroxylan molecule bears ~ 60 ferulic acid esters and is cross-linked through ~ 5 diferulic bridges.

3. Experimental

Plant material.—Micronized maize brans were provided by ULICE (France). Brans (100 g) were dispersed in distilled water (1 L). Termamyl 120L (20 mL) was added and the mixture was kept in a boiling water bath for 1 h. The residue was recovered by filtration on a sintered glass funnel, washed with water, and dried by washing with EtOH, and then acetone before placing in an oven at 40°C for 24 h.

General.—Neutral sugars were determined by an automated orcinol procedure [22] using D-xylose as standard. Uronic acids were assayed by an automated *m*-phenylphenol method [23], using D-glucuronic acid as standard. Individual neutral sugars were determined after hydrolysis (1 M H_2SO_4, 100°C, 1.5 h) by GLC of their alditol acetate derivatives [24] on a DB-225 (J & W; 30 m × 0.32 mm i.d.) fused-silica capillary column. For insoluble samples (starting bran and residue of extraction) a prehydolysis step with 72% H_2SO_4 was used before neutral sugar analysis by GLC.

Ferulic acid was determined in the supernatant solution by spectrophotometry. Diluted supernatant solution (0.1 mL) was mixed with 0.1 M borate–glycine buffer pH 10 (0.9 mL). The proportion of free and esterified ferulic acid was calculated from the absorptions at 375 and 345 nm, assuming the following molar absorption coefficients (M^{-1} cm^{-1}): ϵ_{345} = 19662, ϵ_{375} = 7630 for free ferulic acid; and ϵ_{345} = 23064, ϵ_{375} = 31430 for esterified ferulic acid. The molar absorption coefficient of bound ferulic acid was obtained from sugar beet pectins of known ferulic acid content [25]. Phenolic acids were also determined after alkaline extraction under Ar with 2 M NaOH at 35°C for 2 h. *p*-Hydroxybenzoic acid was then added as internal standard and the mixture was acidified with HCl to pH 2 and extracted with Et_2O. Samples were evaporated to dryness under a flush of N_2, dissolved in MeOH, and then injected on a CP-Sil-C18 HPLC column eluted at 1 mL/min with MeOH (*A*) and 1% AcOH in water



(*B*) with the following conditions: $t = 0$ min, 20:80 *A*–*B*; linear gradient, $t = 25$ min, 60:40 *A*–*B*; $t = 30$ min, 60:40 *A*–*B*. Detection was made at 280 and 320 nm with response factor (RF) relative to *p*-hydroxybenzoic acid (t_R 10.7 min) at 280 nm: ferulic acid, RF 0.8 (t_R 17.9 min); *p*-coumaric acid, RF 0.45 (t_R 16.9 min.); diferulic acid, RF 0.8 (t_R 24.1 min). The peak at 24.1 min was isolated and directly inserted into a mass spectrometer (Nermag, France); it gave molecular ion at m/z 386, and fragmentation ions at m/z 342, 298, 194, and 150, typical of diferulic acid.

Isolation of feruloylated oligosaccharides.—Destarched brans (100 mg) were treated with CF_3COOH (3 mL, 0.01 M and 0.05 M) for various times (0–8 h) at 100°C.

On a preparative scale, destarched brans (10 g) were mixed in 0.05 M CF_3COOH (500 mL) for 2 h in a boiling water bath. After centrifugation the supernatant solution was filtered (< 10 μm) and evaporated at 40°C under vacuum to dryness, then redissolved in distilled water (200 mL) and evaporated to dryness, and finally dissolved in distilled water (50 mL) and freeze-dried. Amberlite XAD-2, which is a polymeric adsorbent binding aromatic compounds, was used for purification of the supernatant

solution [18]. Aliquots of the solution (2 × 25 mL) were injected on an Amberlite XAD-2 column (50 mL), which was successively eluted by water (150 mL), 1:1 MeOH–water (150 mL), and MeOH (150 mL). The fraction eluted by MeOH–water was evaporated to dryness at 40°C under vacuum, dissolved in 1:3 MeOH–water (6 mL) and fractionated on a Sephadex LH-20 column (71 × 2.6 cm; Vt: 377 mL) eluted at 40 mL/h with 1:3 MeOH–water (Fig. 3). The eluent was analysed at 360 nm and fractions (6 mL) were collected and analysed for their neutral sugar content by the automated orcinol method. The different peaks were concentrated and kept at −20°C or freeze-dried for further analysis. The amounts of oligomers isolated were 27.3 mg for F_3, 50.5 mg for F_6, and 108.9 mg for F_7.

Analysis of oligosaccharides by HPAEC.—Fractions F_3, F_6, and F_7 (0.1 mL; ~ 1 mg/mL) were deesterified with NaOH (0.1 mL, 0.4 M) for 30 min at 25°C prior to analysis by high-performance anion-exchange chromatography with pulse amperometric detection (HPAEC-PAD) on a Dionex BioLC system. Samples (0.1 mL) were injected onto a Carbopac PA1 column (Dionex) eluted at 1 mL/min with solvent *A*: 150 mM NaOH and solvent *B*: 150 mM NaOH containing 600 mM NaOAc, using the following gradient: $t = 0$, A = 90%, B = 10%; linear gradient; $t = 40$ min, A = 35%, B = 65%. The electrode pulse potentials and durations were as follows: $E1 = 0.4$ V, 0.5 s; $E2 = 0.9$ V, 0.8 s and $E3 = -0.3$V, 0.5 s. The signal was integrated during 0.2 s (0.3 to 0.5 s).

Reduction of oligosaccharide F_3.—F_3 (25 μL, 10 mg/mL) was deesterified with a carboxylesterase (25 μL; from porcine liver, Sigma) in phosphate buffer (1 mL; pH 7.7) at 25°C for 16 h. Sodium borohydride (20 mg) was then added, and the mixture was kept for 1 h at 60°C. A drop of AcOH was added to destroy the excess of $NaBH_4$ and the mixture was evaporated to dryness. 9:1 Methanol–AcOH (3 × 0.5 mL) and then MeOH (3 × 0.5 mL) were added and evaporated to dryness after each addition. A solution of inositol (0.25 mg/mL, 0.2 mL) was added to the dried residue and hydrolysis was carried out with CF_3COOH (4 M; 0.2 mL) at 120°C for 1 h. After evaporation to dryness, alditol (arabinitol) was acetylated by a mixture of pyridine (0.25 mL) and Ac_2O (0.25 mL) at 120°C for 20 min, and analysed by GLC.

Enzymic degradation of fraction F_3 by α-D- and β-D-galactosidase.—F_3 (0.2 mL, ~ 15 mg/mL) was first deesterified with NaOH (0.2 mL, 0.4 M) for 30 min at 25°C. The mixture was acidified with HCl (pH < 2), phenolic acids were extracted with Et_2O, and the aqueous phase was then evaporated to dryness at 40°C under reduced pressure. The dried residue was then dissolved in water (1 mL), and aliquots (0.25 mL) were incubated separately with α-D-galactosidase (20 μL; Sigma) and β-D-galactosidase (20 μL; Sigma) with 0.1 M acetate buffer (0.2 mL, pH 4.5) for 24 h at 30°C. The absence of D-galactose in the incubation mixture was shown using a D-galactose enzymatic kit (Boehringer, Mannheim).

Methylation.—The oligosaccharides were methylated according to the method of Hakomori [26]. Dried samples were dissolved in Me_2SO (0.5 mL) and 2 M lithium methylsulfinylmethanide (0.5 mL) was added and kept for 1 h at room temperature under Ar. Methylation was then performed as described [27] using MeI (0.5 mL). Methylated oligosaccharides were hydrolysed with 2 M trifluoroacetic acid (100°C, 2 h) and converted into alditol acetates [24]. The partially methylated alditol acetates were

analysed by GLC on DB-225 and DB-1 fused-silica capillary columns (J & W, USA; 30 m x 0.32 mm i.d.) [28]. Peak areas were corrected by response factors [29]. Identification was based on relative retention times, and confirmed by GLC-MS, by coupling the DB-225 and DB-1 columns to a mass spectrometer (Nermag, France).

Acetalation.—The position of alkali-labile feruloyl groups was determined using an acetalation reaction prior to methylation [30]. Feruloylated oligosaccharides (200 μg) were acetalated with methyl vinyl ether ($\sim$ 0.2 mL) and *p*-toluenesulfonic acid as catalyst in Me_2SO (0.2 mL, 1 mg/mL) using the procedure of De Belder and Norrman [12]. Acetalated samples were then methylated without purification as described above.

^{13}C NMR spectroscopy.—The ^{13}C NMR spectra of F_3, F_6, and F_7 were obtained on a Bruker AM 500 spectrometer operating at 125.76 MHz. Solutions ($\sim$ 20 mg/mL) in D_2O were used. Chemical shifts were expressed in ppm referred to internal Me_2SO (39.6 ppm).

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