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Isolation and structural determination of two 5,5′-diferuloyl oligosaccharides indicate that maize heteroxylans are covalently cross-linked by oxidatively coupled ferulates

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Abstract

Large amounts of ferulic acid (~ 2.6% w/w) and dehydrodiferulic acids (~ 1.3% total, w/w) were detected in saponified extracts of maize bran. After treatment of maize bran by mild acid hydrolysis and fractionation of the solubilised products by liquid chromatography, two dehydrodiferuloylated oligosaccharides (F8 and F9) were isolated. The compositions of F8 and F9 were shown to be 1:2:1 5,5′-diferulic acid (5,5′-diFA)–arabinose–xylose and 1:2 5,5′-diFA–arabinose, respectively. Their structure was determined using chromatography and LCMS and confirmed by ^{1}H and ^{13}C NMR spectroscopy. Fraction F9 was composed of 5,5′-diFA esterified at both carboxylic groups by a 5-*O*-L-arabinofuranoside moiety, while F8 was similar but with one of the arabinofuranosyl residues further substituted with a (1→2)-linked xylopyranosyl residue. These results provide direct evidence that the heteroxylans in maize cell walls are covalently cross-linked through dehydrodiferulates.

Keywords: Ferulic acid; Dehydrodiferulic acid; Arabinoxylan; Cell wall

1. Introduction

The cell walls of some plants, namely graminaceous monocots and caryophylaceous dicots, contain significant quantities of esterified phenolic compounds (e.g., ferulates and *p*-coumarates) [1]. It has been shown that ferulates can form dimers through oxidative cross-linking [2], and this may serve to cross-link cell-wall polymers and contribute to the mesh-like network of the cell wall [3], potentially affecting the digestibility [4,5] and mechanical properties [6]. For example, there is some evidence that increased levels of dehydrodimers are associated with decreases in the rate and extent of cell-wall degradation by ruminant microbes [5–7] and fungal enzymes [8]. Hence, dehydrodiferulates are unique bifunctional elements which can cross-link cell wall polymers. Dehydrodiferulates have been identified and quantified in various plant tissues [1–9]. However, although dehydrodiferulic acids have been isolated and identified

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Table 1
Content in ferulic and dehydrodiferulic acids of maize bran

Hydroxycinnamic acid [a]	g/100 g Bran
p-Coumaric acid	0.38
Ferulic acid	2.61
5,5′	0.29
8-*O*-4′	0.57
8,5′NC	0.16
8,5′C	0.31
Σ Dimers	1.33

[a] NC, non cyclic; C, cyclic.

following saponification of plant cell-wall materials [10,11], the isolation of dehydrodiferulates linked to neutral sugars — hence providing direct evidence for these cross-links in the cell wall — has only been reported for bamboo-shoot cell walls following enzymatic digestion of cell walls [12].

Maize bran, a by-product of the maize industry, is a rich source of ferulates and diferulates [9–13]. Maize bran is composed mainly of secondary cell walls of pericarp tissues; it contains some cellulose (~ 22% of the bran), but the major polysaccharide (~ 50% of the bran) is a heteroxylan composed mainly of arabinose and xylose with small amounts of galactose, glucuronic acid and phenolics. Heteroxylans consist of a β-(1→4)-linked xylan backbone within which the xylose residues are highly substituted, with (1→2)- and/or (1→3)-linked monomeric side-chains of arabinofuranose or glucuronic acid, or by oligomeric side-chains containing arabinose, xylose and sometimes galactose [14,15]. It has been estimated that ferulates comprise some 3% (w/w) of maize bran [9–13]. Following mild acid hydrolysis and identification of feruloylated oligosaccharides isolated from maize bran, it was apparent that ferulic acid is esterified to O-5 of arabinofuranosyl residues which are substituents of the xylan backbone [13], and that mild acid hydrolysis split the relatively weak glycosidic linkages involving arabinose residues, while leaving the ester linkages involving hydroxycinnamic acids intact. It has been estimated that, on average, maize bran heteroxylans have a DP of about 2000 [16]. After assuming that all the ferulic acid esterified arabinose residues in the bran, it was calculated that each heteroxylan molecule bore 60–75 ferulic acid molecules [13].

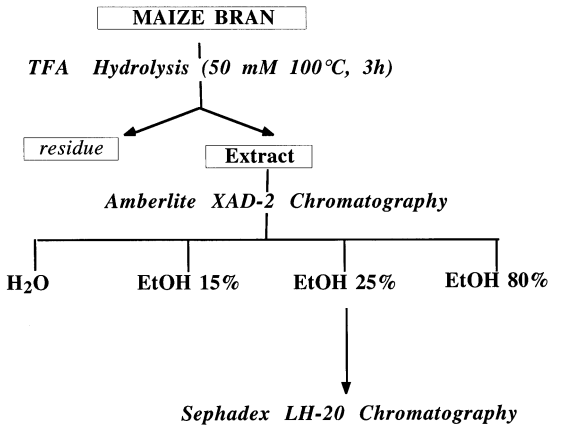


Fig. 1. Isolation scheme of dehydrodiferulic oligosaccharides.

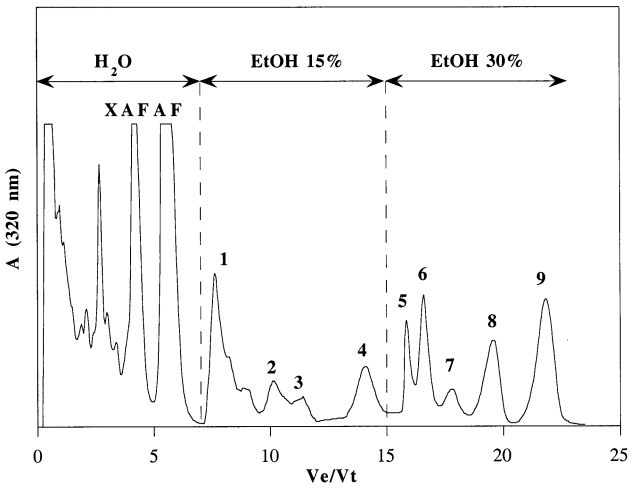


Fig. 2. Elution profile on Sephadex LH-20 of EtOH 25% fraction from Amberlite XAD-2.

Table 2
Neutral sugar and ferulic acid content of fractions separated on Amberlite XAD-2

XAD2 fractions	Water	15% EtOH	25% EtOH	80% EtOH
Neutral sugars [a]	41.6	5.0	3.2	1.2
Esterified ferulic acid [a]	0.9	0.8	1.8	0.3
Neutral sugars/ferulic acid [b]	58.0	8.0	2.3	5.5

[a] g/100 g bran. Determined by spectrophotometry for ferulic acid or orcinol method for sugars.
[b] Molar ratio.

Table 3
Composition of fractions isolated on Sephadex LH-20 (molar ratio) [a]

LH-20 fractions	Ara	Xyl	Gal	Ferulic acid	Dimers	
					5, 5′	8-*O*-4′
Water						
XAF	1.0	1.2	0.0	1.0	0.0	0.0
AF	0.9	0.0	0.0	1.0	0.0	0.0
EtOH 15%						
1	7.8	5.5	1.4	0.0	0.5	1.0
2	5.8	4.0	0.9	0.0	0.9	1.0
3	4.2	3.4	0.6	0.0	0.7	1.0
4	1.9	0.6	0.2	0.0	0.3	1.0
EtOH 30%						
5	1.9	2.2	0.6	0.0	1.0	0.0
6	2.0	1.3	0.7	0.0	1.0	0.0
7	1.9	1.2	0.7	0.0	1.0	0.0
8	1.7	1.0	0.0	0.0	1.0	0.0
9	1.7	0.1	0.0	0.0	1.0	0.0

[a] Neutral sugars and hydroxycinnamic acids were determined by GLC and HPLC methods, respectively.

In this paper, we describe the quantification of alkali-extractable dehydrodiferulates in maize bran, and the isolation and structure determination of two dehydrodiferulate oligosaccharides derived from the bran following mild acid hydrolysis, which provides direct evidence for covalent cross-linking by dehydrodiferulates in maize cell walls.

2. Experimental

Materials.—Maize bran was provided by ULICE (France). The destarching and deproteinising of the maize bran, and the preparation of 5-*O*-(*trans*-feruloyl)-L-Ara*f* (AF) and *O*-β-D-Xyl*p*-(1→2)-[5-*O*-(*trans*-feruloyl)-L-Ara*f*] (XAF) have been described previously [13].

Isolation of feruloylated and diferuloylated oligosaccharides.—Controlled acid hydrolysis of maize bran was carried out according to a published procedure [13] but with minor modifications. Destarched bran (80 g) was mixed with 0.05 M trifluoroacetic acid (1.2 L) for 3 h in a boiling water bath. After centrifugation, the supernatant was filtered (<10 μm) and concentrated to 300 mL at 40 °C in vacuo. The pH was adjusted to 4.0 and the solution was centrifuged (10,000*g*, 30 min). Samples

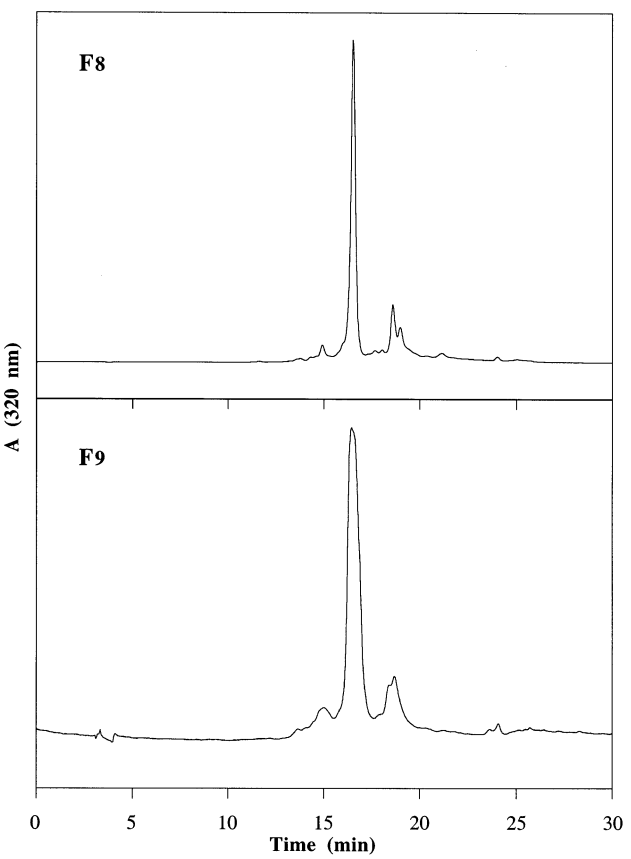


Fig. 3. HPLC chromatogram on C18 reverse phase chromatography of Fractions F8 and F9.

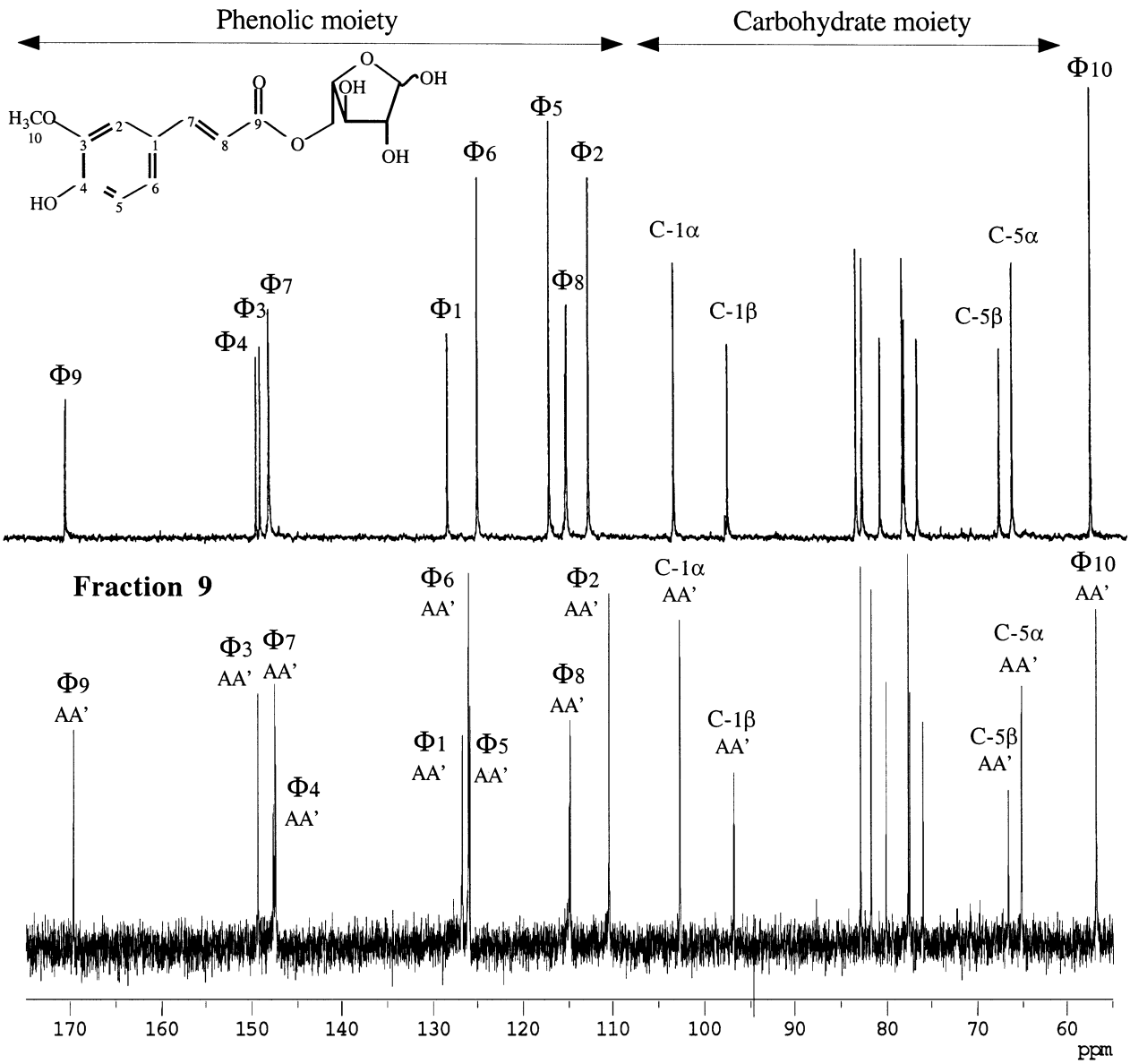


Fig. 4. ^{13}C NMR spectra at 100.62 MHz of 5-*O*-(*trans*-feruloyl)-L-Ara*f* and Fraction F9 from Sephadex LH-20 (see Fig. 5 for attribution of letters A and A′).

(150 mL) of the clarified supernatant were injected onto a column (58 × 2.6 cm) of Amberlite XAD-2 and eluted at 150 mL/h by, successively, water (9 column vol), 15% EtOH (3 column vol), 25% EtOH (3 column vol) and 80% EtOH (3 column vol). The fraction eluted by 25% EtOH was evaporated to dryness at 40 °C in vacuo, redissolved in water, and fractionated on a column of Sephadex LH-20 (85 × 2.6 cm; $V_t = 456$ mL) eluted at 60 mL/h, successively, by water (7 column vol), 15% EtOH (7 column vol) and 30% EtOH (7 column vol). The absorbance of the eluent was monitored continuously at 320 nm and fractions (15 mL) were collected and analysed for total neutral sugars. Fractions corresponding to separate peaks of absorbance at 320 nm were pooled, concentrated (40 °C in vacuo), and freeze-dried for further analysis.

Carbohydrate analysis.—Total neutral sugars were determined by an automated orcinol procedure [17], using xylose as standard. Individual neutral sugars were quantified after hydrolysis (1 M H_2SO_4, 100 °C, 1.5 h) and derivatisation to their corresponding alditol acetates [18] by GLC on a BP-225 (SGE; 30 m × 0.32 mm i.d.) fused-silica capillary column.

Identification and quantification of ferulic and dehydrodiferulic acids.—Ferulic and dehydrodiferulic acids were determined after alkaline treatment with NaOH (2 M) at 35 °C for 30 min. *o*-Coumaric acid was added as an internal standard, the mixture was acidified to pH 2.0 with HCl, and then extracted with Et_2O. Ether extracts were evaporated to dryness at 40 °C and analysed for ferulic and dehydrodiferulic acids using HPLC. Samples were dissolved in 1:1 MeOH–water, injected onto a C18 column (Purospher, E. Merck Germany), and eluted at 0.7 mL/min with an increasing gradient of MeOH in acidified water as follows: $t = 0$ min, 20% solvent A (1% AcOH in MeOH)/80% solvent B (1% AcOH in water); $t = 20$ min, 60% A/40% B; $t = 21$ min, 80% A/20% B; $t = 30$ min, 80% A/20% B. The absorbance of the eluate was monitored continuously at 320 nm. Dehydrodiferulate diethyl diesters were synthesised according to the method of Ralph et al. [2]. The corresponding dehydrodiferulic acids were obtained following saponification of the diethyl diesters and purification by preparative HPLC [10], and were used to calculate response factors (R_f) for HPLC. Response factors (R_f) relative to *o*-coumaric acid (R_t 19.7 min) at 320 nm were: ferulic acid, 0.54 (16.1 min); *p*-coumaric acid, 0.40 (15.4 min); 5,5′-diFA, 0.57 (21.0 min); 8,5′-diFA (non-cyclic), 1.02 (16.5); 8,5′-BendiFA (cyclic), 1.22 (22.5 min); 8-*O*-4′-diFA, 1.17 (21.9 min); 8-8′-diFA(aryltetralin), 1.31 (17.8 min) [2].

Analysis of oligosaccharides by high-performance anion-exchange chromatography.—Fraction or XAF (0.1 mL; ~1 mg/mL) were saponified with NaOH (0.1 mL, 0.4 M) for 30 min at 35 °C, neutralised with HCl (0.1 mL, 0.4 M), and analysed by HPAEC with pulsed amperometric detection using a Dionex BioLC system. Samples (0.1 mL) were injected onto a Carbopac PA1 column (Dionex) with the temperature maintained at 25 °C. The column was eluted isocratically at 1 mL/min using water/aq NaOH (10 mM) in aq NaOAc (40 mM)/aq NaOH (100 mM) (3:1:1) as eluent and with the following electrode pulse potentials and durations: 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 over 0.2 s (0.3 to 0.5 s). Under these conditions, the elution times for *O*-β-D-Xyl*p*-(1→2)-L-Ara*f*, Ara*f*, Xyl*p*, xylobiose and xylotriose were 16.6, 4.5, 6.2, 11.4 and 18.1 min, respectively.

NMR spectroscopy.—^1H and ^{13}C NMR spectra of isolated fractions were analysed using a Bruker AM 400 spectrometer at 303 K. Samples (~10 mg/mL) were dissolved in a 1:1 mixture of CD_3OD–D_2O. Chemical shifts were expressed in ppm with reference to CD_3OD multiplets at 49.05 ppm for ^{13}C (7) and CD_2HOD multiplets at 3.30 ppm for ^1H (5).

Table 4
^{13}C NMR chemical shift of fractions isolated on Sephadex LH-20

Fraction	Residue	Unit [a]	C-1	C-2	C-3	C-4	C-S	C-6	C-7	C-8	C-9	C-10
8	L-Ara*f*	α A	102.83	83.02	77.56	81.78	65.18					
		β A	96.89	77.78	76.08	80.19	66.72					
	L-Ara*f*	α B	101.76	90.88	76.82	81.17	64.85					
		β B	96.50	84.69	74.62	79.76	66.63					
	β-D-Xyl*p*	B	104.18	74.38	77.17	70.58	66.49					
	5,5′	A&B	126.61	110.41	149.91	147.71 [b]	126.27	126.49	147.60 [b]	114.68 114.82	169.74 169.84	56.90
9	L-Ara*f*	α A&A′	102.80	82.93	77.45	81.77	65.22					
		β A&A′	96.85	77.72	76.09	80.13	66.69					
	5,5′	A&A′	126.82	110.53	149.41	147.73	125.96	126.16	147.47 147.53	114.90 115.00	169.71 169.78	56.93

[a] See Fig. 5 for letter meaning.
[b] Overlapping peaks.

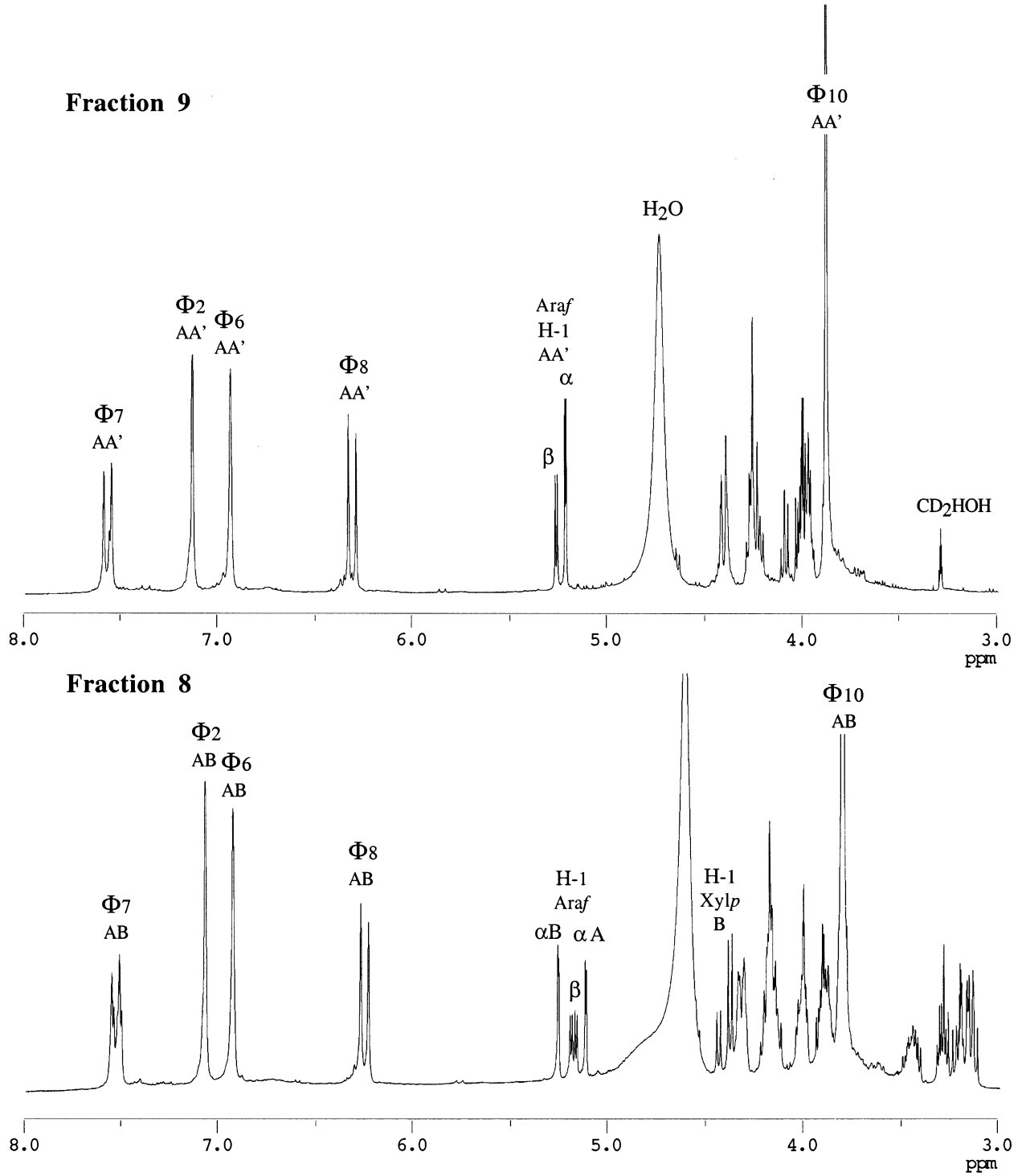


Fig. 5. ^{1}H NMR spectra at 400.13 MHz of Fractions F8 and F9 from Sephadex LH-20.

Liquid chromatography mass spectrometry.—Samples were separated [19] using a Philips PU 4100 HPLC (Cambridge, UK). Spectra were acquired using a Micromass Quattro II mass spectrometer (Manchester, UK) equipped with a Z-spray™ Electrospray (ES)/Atmospheric Pressure Chemical Ionisation (APCI) ion source working in negative-ion mode. ES mass spectra were acquired continuously every 3 s with an interscan delay of 0.1 s under the following conditions: probe temperature, 400 °C; source block tempera-

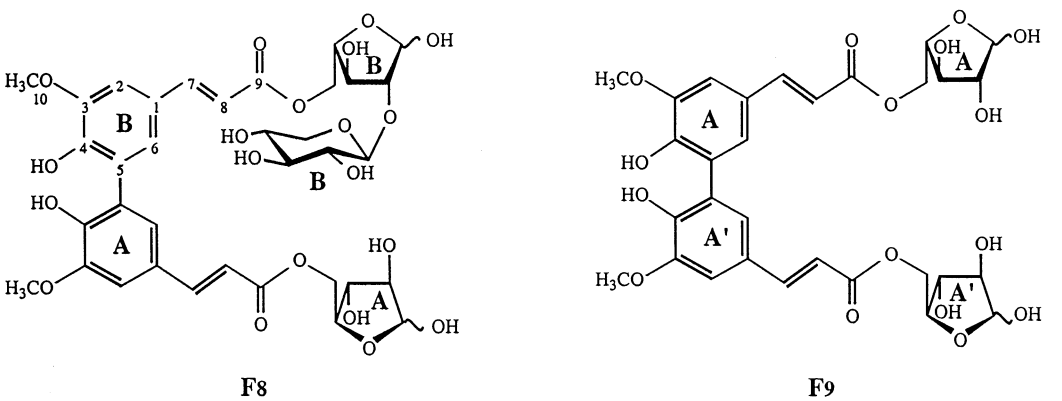
ture, 150 °C; capillary voltage ~2.8 kV; cone voltage ~30 V.

3. Results

Hydroxycinnamic acid composition of maize bran.—Ferulic acid was the most abundant hydroxycinnamic acid detected in the bran, but *p*-coumaric acid was also present in significant quantities; together these monomeric phenolics accounted for ~3% (w/w) of the bran (Table 1). Four different dehydrodimers of ferulic acid were detected in saponified extracts of maize bran, these being products of 5,5′-, 8,5′- and 8-*O*-4′-couplings. The most abundant dimer was 8-*O*-4′-diFA (43% of total dehydrodimers detected), with 8,5′-diFA (35%) and 5,5′-diFA (22%) present in lower amounts. We were not able to detect products of 8,8′-coupling, but this may be due to the relatively large peak of ferulic acid eluting with an R_t value very similar to that found for synthetic standards of 8,8′-diFA (cyclic and non-cyclic). In total, the dehydrodiferulic acids accounted for ~1.3% (w/w) of maize bran.

Isolation of dehydrodiferulate oligosaccharides.—The conditions for acid hydrolysis, optimised for release of ferulic acid from esterified sugar residues, were described in a previous paper [13]. The proportions of ferulic and dehydrodiferulic acids in saponified extracts of the soluble fraction following mild acid hydrolysis of the bran, were similar to those obtained for the whole bran (data not presented). The overall fractionation procedure is presented in Fig. 1. Four fractions were obtained after chromatography on Amberlite XAD2 (Table 2), and all four fractions contained ferulic and dehydrodiferulic acids esterified to sugars. The fraction obtained by eluting with 25% ethanol, which contained the highest amount of esters of ferulic acid, was selected to isolate dehydrodiferulate oligosaccharides using Sephadex LH-20 chromatography.

Sephadex LH-20 chromatography separates molecules based on both their molecular weight and interaction with phenolics. After testing a range of elution conditions, it was found that a stepwise gradient using first water, then 15% ethanol, and finally 30% ethanol gave a good separation of ferulate oligosaccharides from dehydrodiferulate oligosaccharides, with feruloylated oligosaccharides eluted with water, while dehydrodiferulate oligosaccharides were eluted with 15 or 30% ethanol (Fig. 2). Fractions corresponding to peaks of absorbance at 320 nm were pooled and analysed for their sugar and hydroxycinnamic acid contents (Table 3). The peaks eluted with water contained ferulic acid as the only phenolic constituent, with the two major peaks corresponding to feruloylated oligosaccharides, AF and XAF, whose isolation and structural determination has been described elsewhere [13]. Four distinct peaks were obtained by elution of the column with 15% ethanol. Each of these four peaks contained a mixture of 8-*O*-4′-diFA and 5,5′-diFA dimers, and arabinose, xylose and galactose residues. 8,5′-Dehydrodiferulates were not detected in this fraction. Subsequent elution of the



Scheme 1. Structure of Fractions F8 and F9 isolated from Sephadex LH-20.

column with 30% ethanol yielded five further peaks of absorbance at 320 nm. Only 5,5′-diFA was detected in these peaks. Peaks 5, 6 and 7 also contained arabinose, xylose and galactose, peak 8 arabinose and xylose, and peak 9 arabinose only. No further dehydrodiferulates were eluted with 80% ethanol. Peaks eluted with 15% ethanol appeared to comprise of oligomers linked to different dehydrodiferulic acids, and were not studied further. Peaks 8 (F8) and 9 (F9), eluted by 30% ethanol, gave good absorbances at 320 nm and were well separated. Fractions corresponding to F8 and F9 were pooled and assessed for purity.

Purity of Fractions F8 and F9.—Fractions F8 and F9 were injected onto a C18 HPLC column and eluted using the conditions described for the separation of hydroxycinnamic acids. For both fractions, a major peak accounting for $\sim 80\%$ of the absorbance at 320 nm was observed, with minor peaks accounting for the remaining 20% (Fig. 3).

Determination of the structure of Fractions F8 and F9.—Samples of F8 and F9 were incubated in the presence of an excess of a feruloyl esterase from *Aspergillus niger* [21,22], which is able to release 5,5′-diFA from solubilised wheat and barley cell walls [22,23]. Following an extended period of incubation, products were analysed by HPLC with diode array detection [19] and compared with samples of unhydrolysed material. The results indicated that feruloyl esterase hydrolysed all the material present in F8 and F9, and that the only phenolic product was 5,5′-diFA.

Analysis of F8 and F9 by LCMS gave ion masses $[M-H]$ for the major peaks at m/z 781 and 649, respectively, with ion masses for the major contaminant ($R_t = 18$ min) of m/z 649 and 517, respectively. 5,5′-DiFA has a molecular mass of 386, so the ion mass for F8 would appear to comprise of one unit of 5,5′-diFA and three sugar residues (xylose and/or arabinose), while the ion mass for F9 appears to comprise of one unit of 5,5′-diFA and two sugar residues. The masses for the major impurities in F8 and F9 could be accounted for by the loss of one neutral sugar residue (xylose or arabinose) from the ion masses obtained for the respective major peaks. The contaminating compounds were not detected by NMR, probably due to their low concentration in the samples. In addition, the LCMS data suggested that the contaminants were mono-esterified derivatives of F8 and F9, and we would predict that their NMR spectra would be similar to those for the fully esterified dimers.

Fractions F8 and F9 were dissolved in a mixture of 1:1 methanol-d_4–deuterium oxide for ^{1}H and ^{13}C NMR spectroscopy. If samples were dissolved in pure deuterium oxide, spectra of poor quality were obtained, possibly due to poor solubility of the compounds. The ^{13}C NMR spectrum of F9 is shown in Fig. 4. Peaks arising between 170 and 110 ppm were attributed to carbons of the phenolic moiety of the oligomers, and corresponded (with small differences in chemical shifts) to data published previously for 5,5′-diFA [2]. Peaks arising between 105 and 65 ppm corresponded to carbons of the carbohydrate moiety. This part of the spectrum was similar to that corresponding to the carbohydrate component of the spectrum of 5-*O*-(*trans*-feruloyl)-L-Ara*f*, as shown in Fig. 3; some slight differences may be ascribed to solvent effects, since AF spectra were obtained in pure deuterium oxide. Hence, all resonances have been assigned (Table 4) with the help of previously published data [2–13]. The molecule was symmetrical, and the ratio of integrated peak areas between the phenolic and carbohydrate moieties were similar in AF and F9, which suggests this oligomer contains 1 mol of 5,5′-diFA acid and 2 mol of arabinofuranose.

The ^{1}H NMR spectra of F9 is shown in Fig. 5. The assignment of peaks was achieved only for protons of the phenolic moiety and for anomeric protons of the carbohydrate moiety (Table 5). The coupling constant ($J_{7,8}$) of 15.8 Hz indicated that ferulic acid was present as the trans isomer. Chemical shifts and $J_{1,2}$ coupling constants for anomeric protons from arabinose were in good agreement with those reported previously for AF [13]. An H-5 (α,β) proton was observed at 4.45 ppm, considerably downfield from the value of 3.8 ppm expected for a free hydroxymethyl group in the Ara*f* ring, which indicates that Ara*f* units were ester linked at O-5 to 5,5′-diFA. Integrals for the anomeric peaks of arabinose and for

Table 5
^{1}H NMR chemical shift of fractions isolated on Sephadex LH-20

Fraction	Residue	Unit [a]	H-1		F-2		F-6		F-7		F-8		F-10	
			δ	I [c]	δ	I	δ	I	δ	I	δ	I	δ	I
8	L-Ara*f*	α B	5.12(2.5) [b]	0.6										
		β B	5.18(4.5)	0.4										
	L-Ara*f*	α A	5.26(1.8)	0.6										
		β A	5.20(4.4)	0.4										
	β-D-Xyl*p*	A	4.38(7.7)	0.8										
			4.44(7.6)	0.4										
	5,5′	A&B			7.08	2.0	6.94	2.1	7.54(15.8)	2.0	6.26(15.8)	1.7	3.89	6.4
9	L-Ara*f*	α A&A′	5.22(2.5s) [b]	1.0										
		β A&At	5.27(4.5s)	0.7										
	5,5′	A&A′			7.14	2.0	6.94	1.9	7.58(15.8)	1.9	6.32(15.8)	1.7	3.89	6.3

[a] See Fig. 5 for meaning of letters.
[b] Values in brackets correspond to coupling constant J_{12} or J_{78}.
[c] *I*, peak integral; a value of 1 corresponds to the signal of 1 proton.

the proton of the phenolic ring are reported in Table 5; a value of about 2 protons (1.7–2.0) was calculated for each peak of the phenolic ring (6 for the OCH_3) as well as for the sum of the anomeric peaks of arabinose (1.7), which confirmed that F9 was comprised of 1 mol of 5,5′-diFA (2 mol of ferulic acid) and 2 mol of arabinose. This result was in good agreement with the chemical composition (Table 3). The structure of F9 is shown in Scheme 1.

The spectra of F8 were more complex due to the presence of a xylose residue. The ^{13}C NMR spectrum of F8 gave the peaks observed in F9, and in addition, peaks which corresponded to the carbohydrate moiety of *O*-β-D-Xyl*p*-(1→2)-[5-*O*-(*trans*-feruloyl)-L-Ara*f*]. The presence of *O*-β-D-Xyl*p*-(1→2)-L-Ara*f* in F8 was further confirmed by saponification of F8 and chromatography of the products using HPAEC, where only arabinose and an oligomer with an R_t similar to that for *O*-β-D-Xyl*p*-(1→2)-L-Ara*f* were detected. The carbons of each phenolic ring had similar chemical shifts and were not affected by the presence of different substituents on the ring, namely Ara*f* on ring A and *O*-β-D-Xyl*p*-(1→2)-L-Ara*f* on ring B. The ^{1}H NMR spectrum gave the same chemical shifts for the protons of each phenolic ring, which confirmed that chemical shifts for the phenolic rings were not affected by differences in their esterification pattern. Anomeric peaks of Ara*f* and β-D-Xyl*p* were fully attributed (Table 5). The integrals of the peaks corresponded to a ratio of 2 mol of arabinose for 1 mol of xylose and 1 mol of 5,5′-diFA (2 mol of ferulic acid), which was in good agreement with the chemical composition data (Table 3). The structure of F8 is depicted in Scheme 1.

Minor contaminating monoesters were not observed during NMR experiments, probably due to similar chemical shifts as compared with the fully esterified dimers. However, their presence explain perfectly the slight discrepancies observed between chemical composition or integration of NMR signals and expected structure, e.g., Ara:DiF calculated molar ratio was 1.7:1 instead of 2:1 for F9 and Ara:Xyl:DiF molar ratio was 1.7:1:1 instead of 2:1:1 for F8.

4. Discussion

We have shown that maize bran is a rich source of dehydrodiferulates. Indeed, our results show that maize bran contains some 10-fold more dehydrodiferulates (1.3% w/w) than have been detected in wheat bran, which is reported to contain 0.09–0.18% (w/w) total dehydrodiferulates [10–22].

By controlled acid hydrolysis, it was possible to solubilise dehydrodiferulic acids still esterified to sugars. We were able to isolate and determine the structure of two dehydrodiferulate oligomers (Scheme 1), and it was shown that the dehydrodiferulic acid is esterified through both carboxylic acid groups to the side-chains of heteroxylans. Hence, maize heteroxylans are cross-linked through 5,5′-diFA in the cell wall. We have described the isolation of dehydrodiferulate oligosaccharide diesters containing only the 5,5′-linked ferulate dimer. However, even though the saponifiable dehydrodiferulates present in maize bran were also present in saponified extracts of the supernatant obtained by mild acid hydrolysis of the bran, and the 8-*O*-4′- and 8,5′-linked dehydrodimers are more abundant in maize than 5,5′-diFA, it appeared more difficult to attempt isolation of these esterified compounds. In the fraction eluted by 25% ethanol from Amberlite XAD-2, oligosaccharides containing 8-*O*-4′-diFA were detected, but eluted on Sephadex LH-20 as a complex mixture with 5,5′-diFA-containing oligomers, and this appeared to be a difficult sample for attempting isolation of significant amounts of pure dehydrodiferulate-containing oligomers. 8,5′-DiFA was not detected in any of these fractions. In the fraction eluted by water from Amberlite XAD-2, large oligosaccharide fragments containing 8,5′-, 8-0-4′- and 5,5′-coupled dimers were detected, but subsequent acid hydrolysis of these large oligosaccharide fragments gave mixture of 8,5′, 8-*O*-4′ and 5,5′ esterified dimers we were not able to separate using LH-20 chromatography.

Static and dynamic light-scattering studies [16] indicate that maize heteroxylans have an average molecular weight of $\sim 2.8 \times 10^5$ g/mol, which corresponds to a DP of ~ 2000 residues/molecule. Based on our data for the dehydrodiferulate composition of maize bran, we have calculated that for each heteroxylan molecule there are ~ 15 dehydrodiferulate molecules. Assuming that all dehydrodiferulates in maize are esterified to heteroxylans, we estimate that each heteroxylan molecule in the bran is cross-linked on average through ~ 15 diferulic bridges. In previous papers, we have estimated a lower [13] or higher [9] number of bridges per heteroxylan molecule, but these values were obtained using data generated with response factors that have since been shown to be inaccurate.

The maize bran cell walls are very resistant to enzymatic degradation [25]. The high level of cross-linkings through diferulic bridges as well as the high level of ferulic acid esterified to heteroxylan are probably the main factors that restrict enzyme accessibility and the highly branched character of the heteroxylan might be less decisive in the resistance to enzyme degradation. As a matter of fact, wheat bran, which is also constituted of a highly branched heteroxylan [26], but exhibits lower level of ferulic and diferulic acids as compared with maize bran, is readily degraded by a mixture of ferulate esterases and xylanases [21–27].

In conclusion, we have isolated and determined the structure of two oligosaccharide diesters from maize bran cell walls, both of which contain 5,5′-diFA. This has provided direct evidence that the heteroxylans of maize are cross-linked through diphenolic bridges. We have calculated that, on average, each heteroxylan molecule is cross-linked through about 15 dehydrodiferulate bridges, and speculate that this will have profound effects on the physical and chemical properties of maize cell walls.

References

[1] T. Ishii, *Plant Sci.*, 127 (1997) 111–127.
[2] J. Ralph, S. Quideau, J.H. Grabber, R.D. Hatfield, *J. Chem. Soc., Perkin Trans. 1*, (1994) 3485–3498.
[3] K. Iiyama, T.B.T. Lam, B.A. Stone, *Plant Physiol.*, 104 (1994) 315–320.
[4] W.S. Borneman, R.D. Hartley, D.S. Himmelsbach, L.G. Ljungdahl, *Anal. Biochem.*, 190 (1990) 129–133.
[5] F. Eraso, R.D. Hartley, *J. Sci. Food Agric.*, 51 (1990) 163–170.
[6] S. Kamisaka, S. Takeda, K. Takahashi, K. Shibata, *Physiol. Plant*, 78 (1990) 1–7.

[7] K. Iiyama, T.B.T. Lam, P.J. Meikle, K. Ng, D. Rhodes, B.A. Stone, in H. Jung, D. Buxton, R. Hatfield, J. Ralph (Eds.), *Forage Cell Wall Structure and Digestibility*, American Society of Agronomy, Madison, WI, 1993, pp. 621–683.
[8] J.H. Grabber, R.D. Hatfield, J. Ralph, *J. Sci. Food Agric.*, 77 (1998) 193–200.
[9] L. Saulnier, J.-F. Thibault, *J. Sci. Food Agric.*, 79 (1999) 396–402.
[10] M.T. Garcia-Conesa, G.W. Plumb, K.W. Waldron, J. Ralph, G. Williamson, *Redox Report*, 3 (5/6) (1997) 319–323.
[11] V. Micard, J.H. Grabber, J. Ralph, C.M.G.C Renard, J.-F. Thibault, *Phytochemistry*, 44 (1997) 1365–1368.
[12] T. Ishii, *Carbohydr. Res.*, 219 (1991) 15–22.
[13] L. Saulnier, J. Vigouroux, J.-F. Thibault, *Carbohydr. Res.*, 272 (1995) 241–253.
[14] E. Chanliaud, L. Saulnier, J.-F. Thibault, *J. Cereal Sci.*, 21 (1995) 195–203.
[15] L. Saulnier, C. Marot, E. Chanliaud, J.-F. Thibault, *Carbohydr. Polym.*, 26 (1995) 279–287.
[16] E. Chanliaud, P. Roger, L. Saulnier, J.-F. Thibault, *Carbohydr. Polym.*, 31 (1996) 41–46.
[17] M.-T. Tollier, J.-P. Robin, *Ann. Technol. Agric.*, 28 (1979) 1–15.
[18] H.N. Englyst, J.H. Cummings, *J. Assoc. Off. Anal. Chem.*, 71 (1988) 808–814.
[19] K.W. Waldron, A.J. Parr, A. Ng, J. Ralph, *Phytochem. Anal.*, 7 (1996) 305–312.
[20] C.B. Faulds, G. Williamson, *Microbiology*, 140 (1994) 779–787.
[21] R.P De Vries, B. Michelsen, C.H. Poulsen, P.A. Kroon, R.H.H. Van den Heuvel, C.B. Faulds, G. Williamson, J.P.T.W. Van den Hombergh, J. Visser, *Appl. Environ. Microbiol.*, 63 (1997) 4638–4644.
[22] B. Bartolome, C.B. Faulds, M. Tuohy, G.P. Hazlewood, H.J. Gilbert, G. Williamson, *Appl. Biochem.*, 22 (1995) 65–73.
[23] P.A. Kroon, M.T. Garcia-Conesa, I.J. Fillingham, G.P. Hazlewood, G. Williamson, *J. Sci. Food Agric.*, 79 (1999) 428–434.
[24] I.J. Colquhoun, M.-C. Ralet, J.-F. Thibault, C.B. Faulds, G. Williamson, *Carbohydr. Res.*, 263 (1994) 243–256.
[25] C.B. Faulds, P.A. Kroon, L. Saulnier, J.-F. Thibault, G. Williamson, *Carbohydr. Polym.*, 27 (1995) 187–190.
[26] J.-M. Brillouet, J.-P. Joseleau, J.-P. Utille, D. Lelievre, *J. Agric. Food Chem.*, 30 (1982) 488–495.
[27] C.B. Faulds, B. Bartolome, G. Williamson, *Ind. Crop. Prod.*, 6 (1997) 367–374.