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End-products of enzymic saccharification of beet pulp, with a special attention to feruloylated oligosaccharides

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Sugar-beet pulp was hydrolysed by a 'pectinase' preparation ('SP 584' from Novo Nordisk A/S). High degradation of pectins was observed with release of ~75–85% of the pectic sugars and 70% of the ferulic acid. Of the sugars and ferulic acid initially present in the pulp, ~60–70 and ~40% were released as monomers, respectively. The solubilised material was composed of these monomers and also of some resistant materials, rhamnogalacturonans and feruloylated oligosaccharides. Rhamnogalacturonans, representing only ~5% of the galacturonic and neutral sugars from the pulp, were separated on a Bio-Gel P-4 column eluted by sodium acetate buffer. Two-thirds of these rhamnogalacturonans still carried residual side-chains, the remaining was only constituted of rhamnose and galacturonic acid in a ratio of 1:2. Feruloylated compounds were purified on Sephadex LH-20 and Bio-Gel P-2 columns eluted by water. The main compound was a feruloylated galactobiose, which represented 14% of the initial ferulic acid amount in the pulp. Feruloylated arabinobiose and galactose were also identified, representing only ~3% each of the ferulic acid initially present in pulp. © 1997 Elsevier Science Ltd

INTRODUCTION

Sugar-beet pulp is the main by-product of the sugar refining industry. Galacturonic acid (21%), arabinose (~21%), glucose (~21%), galactose (~5%) and rhamnose (~2·5%) are its main components (Micard *et al.*, 1994). They are the constitutive monomers of cellulose and pectins. Pectins are characterized by alternating 'smooth' (homogalacturonans) and 'hairy' regions, rich in rhamnose and side-chains. These side-chains are mainly constituted of long and ramified arabinans and rather short linear galactans (Guillon *et al.*, 1989). Some of the neutral sugars of the side-chains were shown to carry ferulic acid groups (Rombouts and Thibault, 1986). Only spinach (Fry, 1979) and glasswort (Renard *et al.*, 1993) pectins also have this characteristic feature.

The pulp is an abundant (14×10^6 tonnes/year in the European Community) and very cheap by-product, mainly used in cattle feeding (Rombouts and Thibault, 1986). Other potential uses are currently studied, e.g. production of polymers (pectins, arabinans...) or monomers. Monomers of sugars and phenolic components of the pectins could be of great interest. Galacturonic acid can be transformed in surface active agents by esterification with various fatty acids (Petit *et al.*, 1993). L-Arabinose can be used, for example, for diagnostic purposes in bacteriology and has anti-Parkinson properties (Vogel, 1991). Rhamnose and ferulic acid are potential aroma precursors. Rhamnose can be used to produce 'furaneol' (applications in caramel, roasted, and fruit flavours) by chemical transformation (Wong *et al.*, 1983). Ferulic acid can be converted by lignolytic microorganisms into vanillin (Falconnier *et al.*, 1994). If these compounds are released from sugar-beet pulp by enzymic treatment, the use of chemicals is avoided and the energy impact may be low, leading to environmental advantages. Furthermore, the production of all the pectic monomers in one unique set of experimental conditions may be possible by enzymic saccharification and not by chemical treatment. Another advantage is that the monomers produced and biotransformed can be considered as 'natural' (EC directive 88/388; Lesage-Meessen *et al.*, 1995).

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With this objective in mind, we have previously screened a number of enzymic complexes to saccharify pectins from sugar-beet pulp. To simplify the separation of the monomers, we have selected a preparation which did not degrade cellulose (Micard *et al.*, 1994, 1996). This preparation ('SP 584' from Novo Nordisk A/S) released ~75–85% of the pectic sugars and 70% of the ferulic acid. Of the sugars initially contained in the pulp, ~60–70% were released as monomers, showing that a part of the pectin structure was resistant to the action of pectinolytic activities. Ferulic acid was also not totally released as free acid by the enzyme(s), the so-called ferulate-esterase(s), from 'SP 584'.

We report now on the purification and identification of the end-products of the action of 'SP 584' on sugar-beet pulp. Special attention will be paid to the feruloylated compounds which were not degraded, in order to elucidate the resistant structures and to identify the enzymes needed for a complete saccharification.

EXPERIMENTAL

Materials

Sugar-beet pulp (dry matter = 89.9%) was from Sofalia (Chappes-France). Alcohol-insoluble residue was prepared as described by Micard *et al.* (1996).

Enzymic preparation 'SP 584' was from Novo Nordisk A/S (Bagsvaerd, Denmark).

Standards

Ferulic acid and vanillin were obtained from Fluka (Switzerland). 5,5′-Dehydrodiferulic acid from maize bran and feruloylated oligomers from sugar-beet pulp (feruloylated arabinose, mono-, di- and tri-saccharides and feruloylated galactobiose) were from the laboratory collection (Saulnier *et al.*, 1995; Ralet *et al.*, 1994a, b). Arabinan and galactan oligomers were obtained as described by Micard *et al.* (1996).

Methods

Hydrolysis from sugar-beet pulp by 'SP 584' was carried out according to Micard *et al.* (1996). The supernatant was concentrated approximately 20 times on a rotary evaporator. Hydrolysis of 100 g of dried pulp gave 142 g of concentrated supernatant.

Determination of ferulic acid

Spectrophotometry. Free and esterified ferulic acids were quantified from the absorbance at 350 and 375 nm, pH 10, according to Micard *et al.* (1994).

HPLC analysis of ferulic acid. Phenolic and diphenolic acids were extracted and analysed as described by Micard *et al.* (1996). Vanillin (amount two times higher than the estimated amount of ferulic acid, in 0.2 M NaOH) was used as an internal standard and added to the sample after the deesterification step. The RF of dehydrodiferulic acid relative to vanillin was considered to be identical to that of ferulic acid (Saulnier *et al.*, 1995). Elution was carried out by a gradient of 2% acetic acid in water (A)→2% of acetic acid in methanol (B). For the fractions A–G from Sephadex LH-20, the following gradient was applied: 0 min, B = 25%; 10 min, B = 50%; 14 min, B = 60%; 15–20 min, B = 25%. Dehydrodiferulic acids eluted between 15.9 and 17.9 min probably depending on their fine structure; total diferulic acid was calculated from the total area of the peaks in this region which had a ratio of absorbances at 280 and 320 nm of approximately 0.5, as shown for ferulic acid and dehydrodiferulic acid standards. Other fractions from Bio-Gel P-2 and Sephadex LH-20 were analysed with the following gradient: 0 min, B = 20%; 20 min, B = 60%; 23 min, B = 80%; 24 to 30 min, B = 20%. Feruloylated oligomers were injected without prior deesterification with the following gradient: 0 min, B = 20%; 25 min, B = 60%; 30 min, B = 60%; 31 to 35 min, B = 20%).

Sugars analysis

Uronic acids were determined by an automated meta-hydroxybiphenyl method (Thibault, 1979), using galacturonic acid as a standard. Monomeric galacturonic acid was determined by HPLC on an Aminex HPX 87H column (30×7.8 mm), with a precolumn (Bio-Rad), eluted by 0.0005 M H_2SO_4, at a flow rate of 0.6 ml/min, at room temperature, with a refractometric detection.

Total neutral sugars were determined by an automated orcinol procedure (Tollier and Robin, 1979), using arabinose as a standard, after correction for the galacturonic acid interference.

Individual neutral sugars were analysed by GLC (Englyst *et al.*, 1982) after 2 M TFA hydrolysis at 121°C during 1 h. Neutral sugars present as monomers were assayed by GLC without acid hydrolysis. Oligomeric neutral sugars were analysed on a Carbopack PA 1 column (Dionex, 4×250 mm) with PAD detection, after deesterification (0·2 M NaOH, 30 min, 25°C, under argon and protected from light). Total neutral sugars concentration of sample must not exceed 100 μg/ml. The gradient was A: 150 mM→B: 150 mM NaOH + 600 mM NaOAc (0 min, A = 90%; 10 min, A = 40%: 11 to 20 min, A = 90%).

Chromatography on Sephadex LH-20

The sample (3×25 g of concentrated supernatant from the hydrolysis of the pulp) was loaded onto the column (840×25 mm) and the gel was washed ascendingly by water at a flow rate of 40 ml/h, during 15 column volumes. The bound material was eluted with

25% methanol, 50% methanol and pure methanol (2 column volumes each) at a flow rate of 30, 30 and 20 ml/h, respectively. Fractions (18 min/tube) were collected.

Another column (410×16 mm), was used for preparation of the fractions C_3 and C_4 and was eluted ascendingly by deionised water at a flow rate of 20 ml/h, during 2.5 column volumes. Fractions (4 ml) were collected.

Elutions were continuously monitored by the absorbance at 320 or 360 nm.

Chromatography on Bio-Gel P-2

The Bio-Gel P-2 column (785×25 mm) was eluted ascendingly by water at 40°C and at a flow rate of 20 ml/h. Fractions (~5 ml) were collected.

Chromatography on Bio-Gel P-4

The Bio-Gel P-4 column (870×25 mm) was eluted ascendingly by sodium acetate buffer (0.1 M, pH 4.5) at 30°C and at a flow rate of 8.6 ml/h. Fractions (~2 ml) were collected.

Nuclear magnetic resonance

Purified feruloylated fractions, containing approximately 100 μg–3 mg of ferulic acid were exchanged twice with 99.9 deuterium oxide before solubilisation by 0.5 ml of 100% deuterium oxide (feruloylated sugars) or 100% deuterated methanol (free ferulic acid). Spectra were taken with a Jeol GX-400 spectrometer, at 27°C (^{1}H: 400 MHz, ^{13}C: 100 MHz). Chemical shifts of protons and carbons were referenced against acetone at 1.217 (^{1}H) and 31.07 (^{13}C) ppm.

RESULTS

Degradation of the pulp

Sugar-beet pulp (100 g/3 l of water) was treated by 1 g of protein from 'SP 584' (Novo Nordisk A/S) as described by Micard *et al.* (1996). The soluble material was isolated by filtration and concentrated. Hydrolysis of 100 g of dried pulp gave 142 g of concentrated supernatant. Its composition is shown in Table 1. 'SP 584' degraded efficiently the pectins from sugar-beet pulp, and the supernatant was especially rich in galacturonic acid and arabinose residues and to a lesser extent in galactose and rhamnose residues (76, 86, 80 and 84% of their respective initial content in the pulp). Little glucose was released (15% of the initial content in the pulp), in agreement with the low cellulase activity in 'SP 584' (Micard *et al.*, 1996). Analysis on HPAEC and GLC without hydrolysis showed that all the sugars were mainly released as monomers. For galacturonic acid, arabinose, rhamnose and galactose, 71, 67, 61 and 57% of their initial content in the pulp were recovered as monomers, respectively, confirming previous data (Micard *et al.*, 1996).

Table 1. Composition of the pulp (g/100 g of dry matter) and soluble material (g/100 g of dried pulp) obtained by enzymic degradation of sugar-beet pulp

	Pulp[a]	Soluble material
Ferulic acid	0.8	0.56
Diferulic acid	0.04	0.03
Galacturonic acid	21.1	16.0
Rhamnose	2.4	2.0
Arabinose	20.9	18.0
Galactose	5.1	4.1
Glucose	21.1	3.2

[a]Data from Micard *et al.* (1996).

'SP 584' also released 70% of the ferulic acid initially present in the pulp (Table 1), and analysis by spectrophotometry showed that 43% was in the free form and 57% still esterified (analysed by spectrophotometry). After deesterification, the supernatant was analysed by HPLC and a peak, eluted at a retention time close to that of a 5,5′-dehydrodiferulic acid standard, was observed. As sugar-beet pulp was shown by the same method to contain 0.04% diferulic acids, it can be calculated that 66% of the diferulic acids of pulp were solubilised (Micard *et al.*, 1996).

Fractionation of the solubilised products

The purification steps are summarised in Fig. 1. The solubilised products were injected after concentration (75 g) on a Sephadex LH-20 column eluted first with water (during 15 column volumes) and then with methanol 25, 50 and 100% (v/v) (during 2 column volumes each), in order to recover the phenolic materials retained onto the column. All the galacturonic acid residues and almost all the neutral sugars were eluted by water, together with 90% of the ferulic acid. Only diferulic acids and traces of ferulic acid were eluted by methanol. Seven fractions were obtained: A, B, C and D, eluted by water (Fig. 2a), E and F eluted by 50% methanol, and G by 100% methanol (Fig. 2b).

Fraction A, eluted after 0.4 column volumes, contained the bulk of the neutral sugars and galacturonic acid (Table 2). In this fraction, 90, 93, 89 and 54% of galacturonic acid, arabinose, galactose and rhamnose, respectively, were in the monomeric form.

Fractions B and C were eluted with 1.7 and 3.3 column volumes of water. These two fractions contained ferulic acid and neutral sugars (Table 2).

Fraction D was eluted with 11.3 column volumes of water and contained ~50% of the ferulic acid eluted and no neutral sugars (Table 2). When analysed without deesterification by HPLC on a C-18 column, this fraction was shown to be composed only of ferulic acid mainly in its *trans* form. The presence of *trans*-ferulic

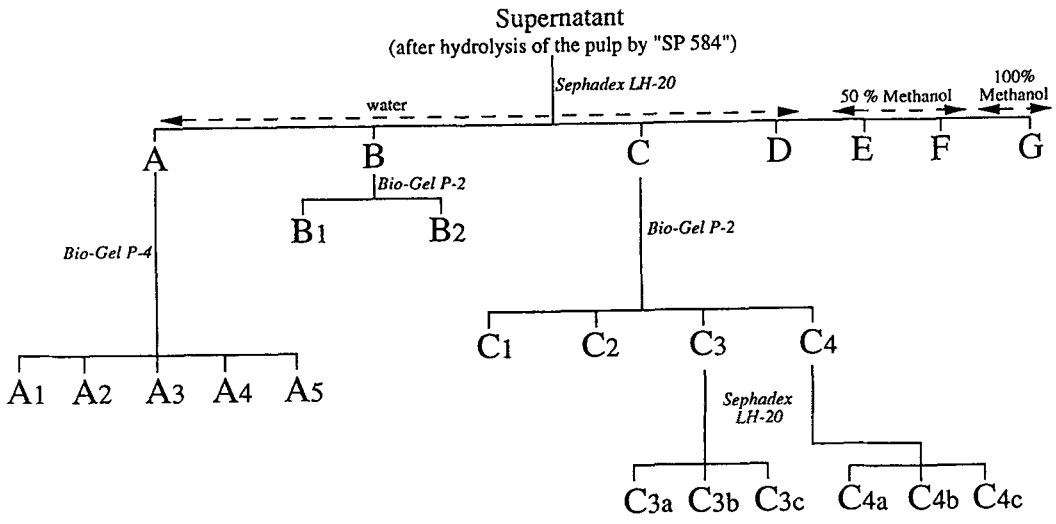


Fig. 1. Steps of the purification of the supernatant from pulp hydrolysis by 'SP 584'.

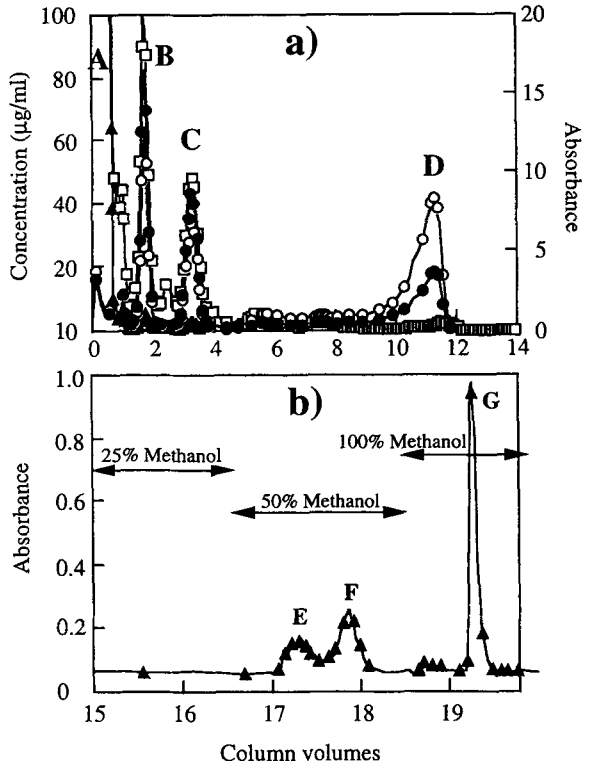


Fig. 2. Fractionation of the soluble material of beet pulp after enzymic hydrolysis on Sephadex LH-20. (a) eluted by water: ▲, galacturonic acid; □, total neutral sugars; ○, absorbance at 350 nm; ●, absorbance at 375 nm. (b) Eluted by methanol: ▲, absorbance at 360 nm.

acid was confirmed by the value (15.9 Hz) of the proton coupling constant between H-7 and H-8 of the ferulic acid group ($J_{7,8}$) (Kato and Nevins, 1985; Azuma *et al.*, 1990; Mueller-Harvey *et al.*, 1986).

Fractions E and F were eluted with 50% methanol (v/v) at 17.3 and 17.8 column volumes, respectively. The HPLC analysis of the deesterified fractions E and F showed they contained 49 and 45% of the diferulic acids from the sugar-beet pulp, respectively (Table 2). Fraction E contained traces of ferulic acid probably resulting from contamination by fraction D.

Fraction G was eluted with pure methanol at 19.2 column volumes. The HPLC analysis of this deesterified fraction showed it contained 6% of the diferulic acids from the pulp (Table 2).

Fraction A, containing the bulk of pectic sugars, and fractions B and C containing the feruloyl esters released by enzymic treatment of the pulp, were further studied.

Study of fraction A

Fraction A contained all the galacturonic acid and 99% of the neutral pectic sugars. This fraction was injected on Bio-Gel P-4 eluted with sodium acetate buffer (0·1 M, pH 3.6) in order to identify the fractions resistant to saccharification.

The elution profile is presented in Fig. 3. Recovery in neutral sugars and galacturonic acid were 95 and 92%, respectively. Five fractions (A_1, A_2, A_3, A_4 and A_5) were identified. Fractions A_1 and A_5, eluted at the void volume and near the total volume, respectively, were the two major fractions. The fraction A_5 represented 94 and 96% of recovered galacturonic and neutral sugars (mainly arabinose), respectively, which were therefore in their monomeric form. A_1 contained 2.9 and 1.7% of the recovered galacturonic acid and neutral sugars, respectively, with arabinose, galactose and rhamnose in the molar ratio 2:1:1 (Table 3). The fraction A_2, eluted at K_{av} 0.25, represented 1% of recovered acidic and neutral sugars, and had a composition close to that of A_1. The two fractions A_3 ($K_{av}=0.44$) and A_4 ($K_{av}=0.61$) represented, each, 1% of the galacturonic and neutral sugars. Rhamnose was the main neutral sugar, with a GalA/Rha molar ratio of 2:1 (Table 3).

Table 2. Composition of the fractions obtained by fractionation of the soluble products on LH-20 (% of each compound eluted, w/w)

Fractions	Eluted by	Column volumes	GalA[a]	Total neutral sugars	Ferulic acid	Diferulic acid
A	water	0.4	100	99.0	0	
B	water	1.7		0.6	25	
C	water	3.3		0.4	13	
D	water	11.3		0.0	52	
E	50% methanol	17.3		0.0	traces	49
F	50% methanol	17.8		0.0	0.0	45
G	100% methanol	19.2		0.0	0.0	6

[a]Galacturonic acid.

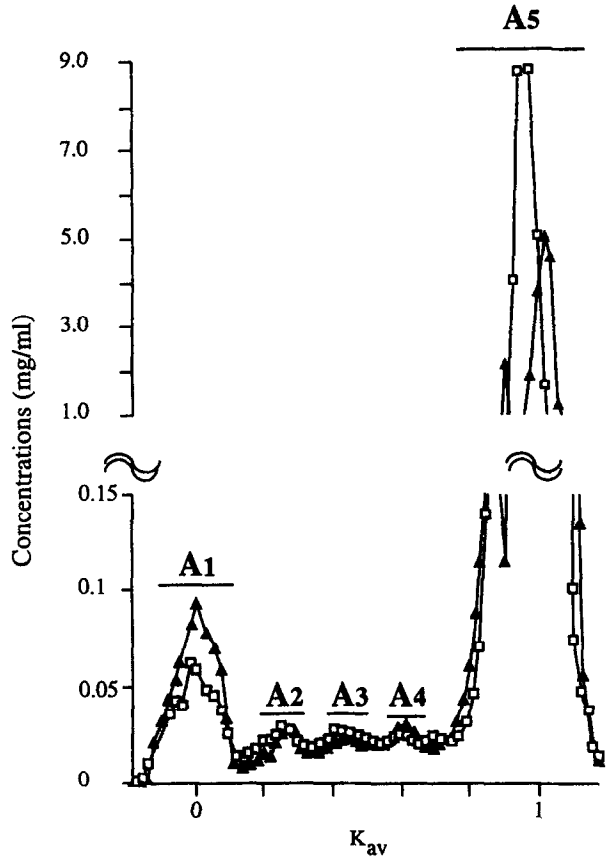


Fig. 3. Chromatography on Bio-Gel P-4 of fraction A, eluted by 0.1 M sodium acetate buffer (pH 3.6): □, total neutral sugars; ▲, galacturonic acid.

Table 3. Sugar composition (molar ratios) of the fractions eluted from Bio-Gel P-4 column

	Fractions					
Sugars	A	A_1	A_2	A_3	A_4	A_5
Rha	5	12	13	20	25	5
Fuc	0	0	2	2	0	0
Ara	48	24	23	10	6	49
Xyl	1	3	2	3	2	0
Man	1	3	7	14	4	0
Gal	9	13	11	9	7	8
Glc	6	4	2	3	6	6
GalA	30	41	40	39	50	32

Study of fraction B

Fraction B mainly consisted of ferulic acid and galactose with a small amount of arabinose (Table 4). At pH 10, this fraction presented an absorbance maximum around 375 nm, showing that feruloyl esters were present. After alkaline deesterification, only ferulic acid was observed (90.5% under the *trans* configuration) by HPLC analysis on a C-18 column. The sugars released by the alkaline deesterification were identified by HPAEC mainly as monomers and dimers of galactose, and to a lesser extent, dimers of arabinose. It was concluded that fraction B consisted of feruloylated galactose, feruloylated galactobiose and lower amounts of feruloylated arabinobiose.

Fractionation of fraction B

Fraction B was further separated on Bio-Gel P-2 eluted in water (Fig. 4). The recoveries in neutral sugars and ferulic acid were 87.4 and 88.0%, respectively. One major feruloylated population (B_2) (representing 72% of B) was observed, with a second incompletely resolved fraction (B_1) (representing 13% of B). B_1, eluted at K_{av} 0·95, contained 16 and 14% of recovered neutral sugars and ferulic acid, respectively. B_2 contained the main part of the neutral sugars and ferulic acid (57 and 82%

Table 4. Molar ratios to FA of Ara and Gal in the fractions

	Molar ratio to ferulic acid	
Fractions	Ara	Gal
B	0.3	1.5
B_1	0.5	1.8
B_1'	0	1.8
B_2	0	2.4
C	0.8	0.9
C1	2.6	0.3
C2	1.5	0.2
C3	0.1	1.1
C4	0	1.1
C3a	0	1.8
C4a	0	1.6
C3b	0	1.9
C4b	0	1.5
C3c	0	1.5
C4c	0	1.0

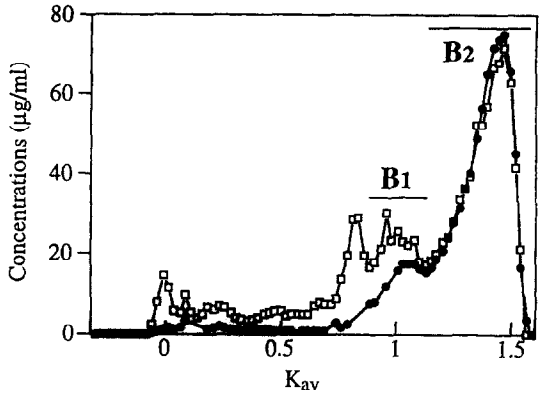


Fig. 4. Chromatography on Bio-Gel P-2 of fraction B, eluted by water: □, total neutral sugars; ●, ferulic acid (calculated from the absorbance at 375 nm).

of recovered amounts, respectively) and was eluted at $K_{av} = 1.45$ as an asymetric peak, confirming affinity between phenolic compounds and the Bio-Gel matrix (Guillon and Thibault, 1989).

B_1 (Table 4) was constituted of ferulic acid, galactose and arabinose in the molar ratio 1:1.8:0.5. It was purified by further chromatography on Bio-Gel P-2 (not shown), giving fraction B_1' with a yield of 60%. The purity of this peak was confirmed by HPLC on a C-18 column. It had a molar ratio ferulic acid:Gal of 1:1.8 suggesting the presence of a feruloylated galactobiose (Gal_2F).

The major fraction B_2 was composed of ferulic acid and galactose in the molar ratio 1:2.4. When injected on HPLC on a C-18 column, B_2 gave one single peak with a retention time (13.9 min) identical to that of the Gal_2F standard.

Fraction B was therefore mainly constituted of Gal_2F. This compound constituted all of the sub-fraction B_2 and 60% of the sub-fraction B_1, where it was mixed with low amounts of feruloylated oligomers of arabinose. Indeed, fraction B_1 formed a shoulder on peak B_2 (cf. Fig. 4) and was contaminated by Gal_2F.

NMR studies

Fractions B_1 and B_2 were analysed by ^1H NMR before and after purification. Fraction B_2 was also analysed by ^{13}C NMR.

The ^1H spectra showed the presence of *trans*-ferulic acid ($J_{7,8}$ was 15.9 Hz) in both fractions. The composition of the fractions was determined by comparison of the spectra with those of the pure components Ara_1F, Ara_2F, Ara_3F, and Gal_2F (Colquhoun *et al.*, 1994). The main fraction B_2 gave ^1H (Table 5) and ^{13}C (Table 6) chemical shifts very close to those of the Gal_2F stan-

Table 5. Chemical shifts (ppm) of the ^1H resonances of purified fractions

Compounds	Unit		H-1	H-2	H-3	H-4	H-5	H-6	H-7	H-8	H-10
Standards											
Ferulic acid	FA			7.18			6.85	7.04	7.39	6.42	
Methyl ferulate	FA			7.26			6.93	7.16	7.64	6.41	
Ara_2F[a]	FA			7.17			6.90	7.10	7.60	6.33	
Gal_2F[a]	FA			7.18			6.81	7.11	7.62	6.30	
Samples											
B_1	FA			7.26			6.85	7.17	7.70	6.40	
B_1'	α-Gal*p*	A	5.27	3.88	3.96	4.23	4.13	3.75/3.83			
	β-Gal*p*	A	4.61	3.57	3.75	4.17	n.d.	n.d.			
	Gal	B	4.59	3.59	3.66	3.91	n.d.	n.d.			
	FA			7.28			6.94	7.15	7.34	6.40	
B_2 (mixture)											
1 (major)	FA			7.20			6.91	7.12	7.60	6.35	
	α-Gal*p*	A	5.25	n.d.	n.d.	n.d.	n.d.	n.d.			
	β-Gal*p*	A	4.56	3.53	3.69	4.06	n.d.	n.d.			
	Gal*p*	B	4.54	3.58	n.d.	n.d.	3.87	4.42/4.30			
2 (minor)	FA			7.22			6.90	7.10	7.31	6.36	
C_1	FA			7.28			6.94	7.18	7.70	6.43	
C_2	FA			7.23			6.91	7.16	7.66	6.39	3.86
	α-Ara*f*	A		4.04	4.05	4.25	3.79/3.89				
	β-Ara*f*	A		4.10	4.10	3.96	3.79/3.87				
	α-Ara*f*	B		5.08	4.20	4.19	3.77/3.88				
$C_{(3+4)c}$											
1 (major)	FA			7.25			6.92	7.13	7.42	6.37	
2 (middle)	FA								7.66	6.41	
	β-Gal*p*		4.59	3.50	3.65	3.98	3.96	4.31/4.37?			
	α-Gal*p*		5.27	3.81	3.87	4.05	?	?			
3 (minor)	FA								7.73	6.47	

FA: ferulic acid; A: reducing sugar; B: non-reducing sugar; n.d.: not determined.
[a]Data from Colquhoun *et al.* (1994).

Table 6. Chemical shifts (ppm) of the ^{13}C resonances of purified fractions

Fractions	Unit		C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10
B_2	FA		127.61	111.90	148.44	148.68	116.38	124.24	147.24	112.10	169.79	56.69
	Gal*p*	α-A	93.11	69.69	70.43	79.89	70.65	62.01				
		β-A	97.21	73.10	74.00	78.84	75.17	61.84				
	β-Gal*p*	B	105.34[a]	72.14[a]	73.47	69.41	73.52	64.45				
C_1 & C_2	FA		127.61	112.36	148.58	148.97	116.56	124.35	147.75	114.72	169.13	56.81
	Ara*f*	α-A	101.99	82.19	76.69	82.19	67.48					
		β-A	96.16	76.88	75.27	80.22	68.84					
	α-Ara*f*	B	106.35[a]	84.34[a]	75.93[a]	85.27[a]	61.74[a]					
$C_{(3+4)c}$	Gal*p*	α	93.23									
	Gal*p*	β	97.19									

[a]Peaks 'doubled' by anomeric effect (free reducing-end) of neighbouring residue.

dard. After further attempted purification, however, two distinct sets of signals (relative intensities 60 and 40%) were evident in the ferulic acid region of the ^1H spectrum. The largest chemical shift difference found was between the H-7 resonances of the major (7.60 ppm) and minor (7.31 ppm) components. The former signal was at the same position as in Gal_2F, whilst the latter could be assigned to free ferulic acid by comparison with a standard (Table 5: the H-7 shift is probably somewhat pH dependent in the free acid). It was concluded that the fraction originally contained only Gal_2F, but that this had been partially deesterified by subsequent treatment to give 60% Gal_2F together with 40% β-Gal-(1→4)-Gal and free ferulic acid.

In agreement with the chemical analysis, NMR showed that the original B_1 fraction contained Gal_2F (major component) and an arabinose oligosaccharide, almost certainly Ara_2F (there was too little material for a full characterization). Subsequent purification removed the arabinose compound but also completely deesterified the Gal_2F to give β-Gal-(1→4)-Gal (parameters reported under 'B_1'' in Table 5) and free ferulic acid. The only ferulic acid H-7 signal was at 7.34 ppm, and the loss of ferulic acid from the β-Gal O-6 position was also evident from the disappearance of the very prominent H-6,6′ resonances which are at 4.3–4.4 ppm in Gal_2F.

Study of fraction C

Fraction C was composed of arabinose, galactose and ferulic acid in very similar proportions (Table 4). At pH 10, its maximal absorption was at 375 nm showing it was constituted of feruloylated esters. Analysis of this fraction by HPLC on a C-18 column showed the presence of *trans*-ferulic acid. The sugars released by the alkaline deesterification were identified by HPAEC mainly as galactose and dimers of arabinose: fraction C would therefore be constituted of feruloylated arabinobiose and feruloylated galactose.

Fractionation of fraction C

Fraction C was further separated on Bio-Gel P-2 eluted by water (recovery: neutral sugars 100%, ferulic acid

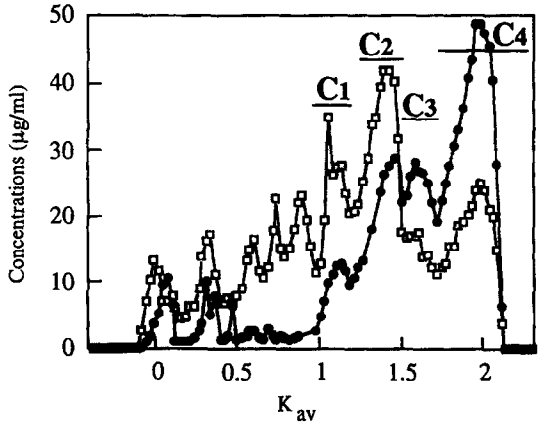


Fig. 5. Chromatography on Bio-Gel P-2 of fraction C, eluted by water: □, total neutral sugars; ●, ferulic acid (calculated from the absorbance at 375 nm).

93%) (Fig. 5). Four main distinct and asymetric peaks (C_1, C_2, C_3 and C_4) containing feruloyl groups were eluted at $K_{av} \geq 1$ due to affinity with the Bio-Gel matrix. Fractions C_1– C_4 represented 7, 21, 15 and 44% of the initial fraction C, respectively.

C_1, eluted at K_{av} 1.1, contained 13.4 and 7.2% of total recovered neutral sugars and ferulic acid, respectively. It was mainly constituted of arabinose and ferulic acid, with small amount of galactose (Table 4).

C_2, eluted at K_{av} 1.4, contained 27.3 and 22.7% of total recovered neutral sugars and ferulic acid, respectively. Injection on C-18 column without deesterification showed a main peak, eluted at the same retention time as the Ara_2F standard.

C_3 and C_4, eluted at K_{av} 1.6 and 2, respectively, represented 7.1 and 18.6% of total neutral sugars, and 15.7 and 47.2% of ferulic acid recovered, respectively. In C_3 and C_4, a molar ratio of ~1 between ferulic acid and galactose was consistent with the presence of Gal_1F (Table 4). However, C_3 and C_4 without deesterification gave several peaks when analysed by HPLC on a C-18 column, showing that both fractions were mixtures of feruloylated esters.

The fractions C_3 and C_4 from Bio-Gel P-2 were

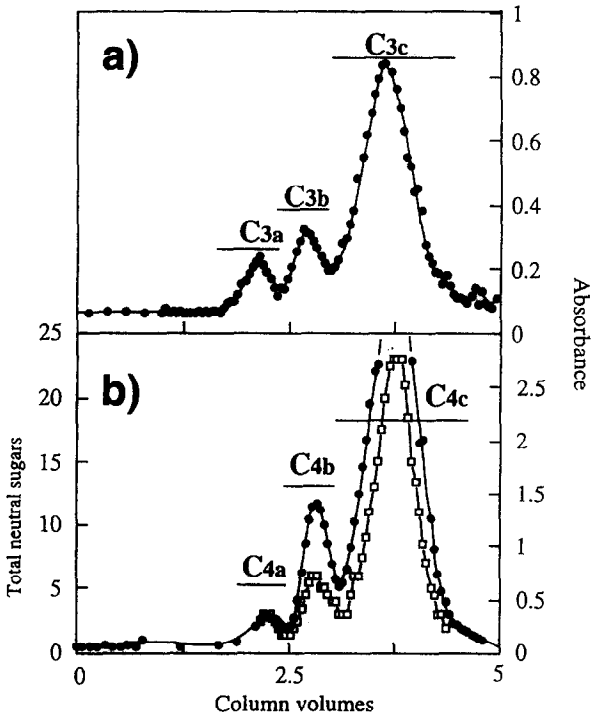


Fig. 6. Chromatography of fractions C_3 and C_4 on Sephadex LH-20, eluted by water. (a) Fraction C_3: ●, absorbance at 320 nm. (b) Fraction C_4: □, peaks high (mm) (measured during the colorimetric assay of total neutral sugars); ●, absorbance at 320 nm.

therefore further purified on Sephadex LH-20 eluted by water (Fig. 6a and b). Each gave three peaks C_{3a}, C_{3b}, C_{3c} and C_{4a}, C_{4b}, C_{4c}. The two main fractions were C_{3c} and C_{4c}, representing 48 and 65%, respectively of the initial fractions C_3 and C_4.

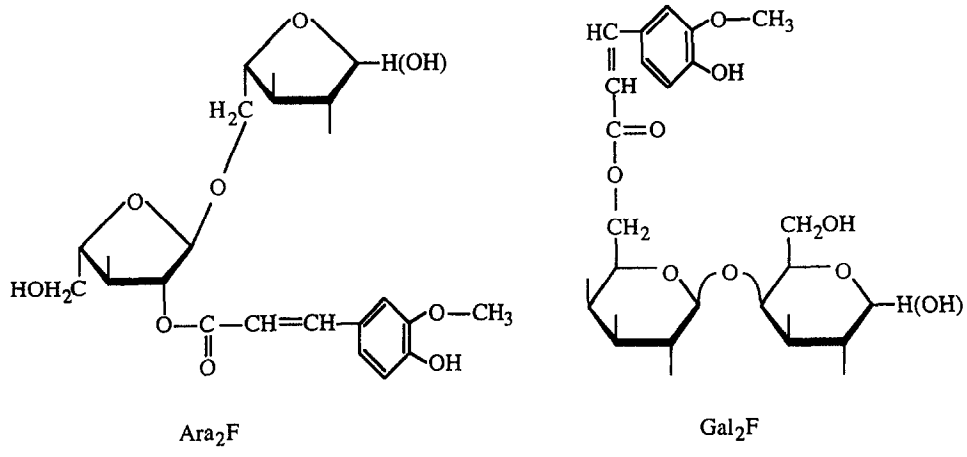
The fractions C_{3a} and C_{4a} were eluted at the same column volumes (2.25) and each represented 6% of total neutral sugars, and 15 and 4% of ferulic acid eluted, respectively. Fractions C_{3b} and C_{4b} were eluted at 2.9 column volumes and represented 5 and 15.5% of neutral sugars, and 13 and 11% of ferulic acid eluted, respectively. The molar ratio (Table 4) of ~1·7 between galactose and ferulic acid of the two fractions C_{3b} and C_{4b} was consistent with the presence of Gal_2F. Fractions C_{3c} and C_{4c}, eluted with 3·75 column volumes, represented 89 and 78% of neutral sugars, and 72 and 84% of ferulic acid eluted, respectively. Their molar ratio (Table 4) of 1.5 and 1, respectively, between galactose and ferulic acid, indicated the presence of Gal_1F. Apparently, the same compounds were present in different proportions in the fractions C_3 and C_4. In fact, these two fractions showed major overlap on Bio-Gel P-2 (Fig. 5).

Therefore, the Sephadex LH-20 fractions from C_3 and C_4 were recombined for further analysis and the fraction C gave five fractions: C_1, C_2, $C_{(3+4)a}$, $C_{(3+4)b}$ and $C_{(3+4)c}$. The fractions C_2 and $C_{(3+4)c}$ were the major ones.

NMR identification

Fractions C_1, C_2 and $C_{(3+4)c}$ were analysed by ^1H and ^{13}C NMR (Tables 5 and 6). Only a partial analysis was possible for the last fraction which contained a mixture of components. C_1 and C_2, however, each contained only one component. The ^1H spectra of these two fractions showed that each molecule contained a single *trans*-ferulic acid group. The spectra of both fractions showed great similarity to the spectrum of Ara_2F obtained previously (Colquhoun *et al.*, 1994). Whereas C_2 was almost identical to Ara_2F, there were slight differences of ^1H chemical shift between C_1 and C_2 (or Ara_2F) in the ferulic acid and anomeric regions. Because of this, ^{13}C spectra of C_1 and C_2 were obtained and compared with the earlier spectrum of Ara_2F. No differences could be seen between the spectra of the three samples and it was concluded that both fractions contained Ara_2F (see Fig. 7 for structure). The reasons for the slight differences in the ^1H spectra or, indeed, for the appearance of C_1 and C_2 as separate fractions are not yet understood.

The fraction $C_{(3+4)c}$ was a mixture as shown by the



Ara_2F Gal_2F

Fig. 7. Structures of the feruloylated arabinobiose (Ara_2F) and galactobiose (Gal_2F).

observation of three sets of signals for ferulic acid in the ^1H spectrum. The strongest component had the H-7 chemical shift at 7.42 ppm and therefore corresponds to free ferulic acid (see above). Two other components had H-7 shifts of 7.66 and 7.73 ppm and must correspond to sugar-linked ferulic acid groups. In the anomeric region of the ^{13}C spectrum, only peaks at 93.23 and 97.19 ppm could be assigned to reducing end α- and β-Gal, respectively. No signal was detected in the region of 105.3 ppm where the C-1 resonance of the non-reducing Gal appears in Gal_2F. The ^1H signal could be partly assigned (Table 5) with the aid of COSY experiment to the monosaccharides α- and β-Gal feruloylated at O-6. This is indicated by the downfield shift (4.3–4.4 ppm) of the H-6,6′ signals. More than half of the cross-peaks in the COSY spectrum were still unassigned and must arise from, as yet, unidentified components.

We can conclude that the fraction C eluted from Sephadex LH-20 contained Ara_2F and probably Gal_1F.

DISCUSSION

'SP 584', a commercial pectinolytic preparation, was selected to saccharify the pectins of sugar-beet pulp (Micard *et al.*, 1996). High degradation was observed (~75–85%) and the majority of the sugars were released as monomers (~60–70% of their initial pulp content). Ferulic acid and diferulic acids were also released (~70% each of their initial content). A part of ferulic acid (43%) was released in the free form showing that 'SP 584' contained one or several ferulate esterases (Micard *et al.*, 1996). The fractions which were not released as monomers were separated by different types of chromatographies.

Oligomers and polymers from the 'hairy' regions of the pectins were identified. These fractions represented only 6 and 5% of the galacturonic acid and total neutral sugars of the pulp, respectively. The major part (two-thirds) was constituted by rhamnogalacturonans still carrying portions of side-chains. The other part was oligomers of low degree of polymerisation containing only rhamnose and galacturonic acid in a molar ratio of 1:2. Part of the backbone of 'hairy' regions of pectins therefore resisted the action of 'SP 584'. It is known that rhamnogalacturonans are highly resistant to the enzymic degradation. Schols *et al.* (1990b) showed that only one enzymic complex, among numerous pure enzymes and commercial preparations tested, degraded a similar area in apple pectins to oligomers. The enzyme was identified and called 'rhamnogalacturonase' (Schols *et al.*, 1990a). Its optimal pH and temperature were 3–4 and 40–50°C, respectively. 'SP 584' released approximately 60% of the rhamnose from the pulp as monomers, and one-third of the soluble resistant fragments of rhamnogalacturonans were oligomers. It is likely that 'SP 584' contained a 'rhamnogalacturonase'. This enzyme can act in our experimental conditions because hydrolysis by 'SP 584' was carried out at 40°C in pure water, allowing the pH to decrease rapidly to 3.5–4 (Micard *et al.*, 1996) and then allowing this enzyme to act.

'SP 584' also released 70% of the ferulic initially present in the pulp; 43% was free ferulic acid, showing that some ferulate esterase(s) are present in the preparation and that they are not able to release all ferulic acid. Indeed, ferulic acid was released as various feruloylated oligosaccharides. These compounds were identified by NMR after several purification steps, and were mostly feruloylated arabinobiose and feruloylated galactobiose (Fig. 7):

$-O-[2-O-(trans-\text{feruloyl})-\alpha\text{-L-Ara}f]$
$-(1\rightarrow 5)\text{-L-Ara}f(Ara_2F)$;
$-O-[6-O-(trans-\text{feruloyl})-\beta-\text{D}$
$-\text{Gal}p]-(1\rightarrow 4)-\text{D-Gal}p(Gal_2F)$

A feruloylated galactose (Gal_1F) also seemed to be released with the linkage on the O-6 position of the galactose.

These two feruloylated dimers were also the end-products of the hydrolysis of sugar-beet pulp (Ralet *et al.*, 1994a; Colquhoun *et al.*, 1994) or of the spinach leaf cell-walls (Ishii and Tobita, 1993) by another commercial preparation ('Driselase').

Gal_2F was the main product (14% of the initial ferulic acid content of the pulp) resisting the action of 'SP 584'. Ara_2F and Gal_1F represented only 3.5 and 3.2% (of the initial ferulic acid content of the pulp), respectively. Recent studies (Micard *et al.*, 1994; Ralet *et al.*, 1994a) have shown that ferulic acid from beet is preferentially linked (~50–60%) to the arabinose residues rather than to the galactose residues. Therefore, the higher proportion of feruloylated galactose oligomers as end-products indicated that the ferulate esterase(s) from 'SP 584' were mainly active on ferulic acid linked to the O-2 of arabinofuranose residues.

Gal_1F seemed to be released at a much lower content than Gal_2F. It has been previously reported that only chemical (acid) treatment of the pulp was able to release feruloylated monomers (Ralet *et al.*, 1994a).

Two ferulate esterases (FAE I and CinnAE) active on oligomeric substrates from sugar-beet pulp have been purified from *Aspergillus niger* culture (Ralet *et al.*, 1994b; Kroon *et al.*, 1996). In these substrates, ferulic acid is linked to the O-2 of arabinofuranose and O-6 of galactopyranose residues. FAE I degraded feruloylated monomers of arabinose as well as of galactose, but the hydrolysis of the feruloylated galactobiose was much less than that of the feruloylated arabinobiose (Ralet *et al.*, 1994b). Therefore, the action of the ferulate esterase(s) from 'SP 584' could be closely related to the action of the FAE I.

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