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ENZYMIC HYDROLYSIS OF THE "HAIRY" FRAGMENTS OF SUGAR-BEET PECTINS*

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ABSTRACT

The "hairy" fragments of acid-soluble (HP) and alkali-soluble (OHP) beet pectins have been treated with an arabinofuranosidase, an endoarabinanase, a β-D-galactosidase, and an endogalactanase from *Aspergillus niger* separately, in sequence, in combination, and prior to mild hydrolysis by 0.05M trifluoroacetic acid. The products were analysed by h.p.l.c. and by gel-permeation chromatography on Bio-Gel P-2 and Sepharose CL-6B. The side-chains occur as branched structures attached to the rhamnogalacturonan backbone. They consist mainly of (1→5)-linked α-arabinans with branches attached to positions 3 randomly distributed along the main core, (1→4)-linked β-galactans of low d.p., and highly branched (1→3,6)-linked galactans. Of the feruloyl groups, 20–30% are carried by the arabinans and are probably responsible for their limited degradation by arabinofuranosidase. The remaining feruloyl groups are attached to the galactose residues and may contribute to the low activity of the galactanases. Some of the feruloyl groups and part of the galactose are not released by a more drastic treatment by acid. A tentative structure for the "hairy" fragments from sugar-beet pectins is presented.

INTRODUCTION

The extraction of pectic substances from sugar-beet pulp, the isolation of the "hairy" fragments, and their chemical composition have been described[1–3]. The structure of the neutral side-chains has been investigated by methylation analysis

*Structural Investigation of the Neutral Sugar Side-Chains of Sugar-Beet Pectins, Part II. For Part I, see preceding paper.

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and characterisation of the products obtained after mild acid hydrolysis[4]. Special attention was paid to the location of the feruloyl groups. Sugar-beet pectins consist[2,3] of (*a*) homogalacturonan ("smooth") regions and (*b*) rhamnogalacturonan backbone carrying neutral sugar side-chains and the feruloyl groups ("hairy" fragments). Highly branched (1→5)-linked arabinans were the preponderant neutral sugar side-chains followed by fairly linear (1→4)-linked galactans. The feruloyl groups were assumed to be ester-linked at the non-reducing arabinose or galactose termini, and ~30% were released together with ~70–80% of the arabinose and ~30–40% of the galactose on hydrolysis by 0.05M trifluoroacetic acid. A further 30% was removed together with ~80% of the arabinose and 60–80% of the galactose by hydrolysis with 0.1M trifluoroacetic acid[4].

We now report on the use of purified glycanases in order to obtain information on the structure of neutral sugar side-chains and the location of the feruloyl groups.

EXPERIMENTAL

Pectins. — Pectic substances were extracted sequentially from an alcohol-insoluble residue by hot dilute acid (→HP) and cold dilute alkali (→OHP) as described[3]. "Hairy" fragments of the purified pectins were isolated by gel-filtration chromatography on Sephacryl S-200 from digests obtained by degradation of the de-esterified and deacetylated pectins with an endopolygalacturonase.

Enzymes. — α-L-Arabinofuranosidase B (Araf-ase, EC 3.2.1.55), endo-(1→5)-α-L-arabinanase (endo-Ara-ase, EC 3.2.1.99), β-D-galactosidase (Gal-ase, EC 3.2.1.23), and endo-(1→4)-β-D-galactanase (endo-Gal-ase, EC 3.2.1.89) were isolated from a pectinase preparation from *Aspergillus niger*[5,6]. Endopolygalacturonase (endo-PG-ase, EC 3.2.1.15) was purified from a preparation of *Aspergillus niger*[7].

Analytical methods. — All the values were calculated on a moisture-free basis. Uronic acids (as galacturonic acids) were determined by the automated 3-hydroxybiphenyl method[8]. Neutral sugars were determined by the automated orcinol method, using arabinose as the standard[9]. Corrections were made for interference from galacturonic acid. Neutral sugars were analysed by g.l.c. of their alditol acetates after hydrolysis with sulphuric acid[10]. Feruloyl groups were determined spectrophotometrically at 375 nm on freshly prepared solutions of pectins in 0.05M glycine–NaOH (pH 10) buffer, using a molar extinction coefficient[11] of 31,600. Feruloyl groups in column eluates were monitored by spectrophotometry at 375 nm, the pH of the fraction being adjusted to 10 with M NaOH. When the concentration of the fraction was too low, the content of ferulic acid was measured in buffer at pH 4 at 325 nm. Under these conditions, the extinction coefficient was equal[4] to 22,900.

Reducing sugars were determined by the method of Nelson[12], using D-galacturonic acid or L-arabinose or D-galactose as standard.

H.p.l.c. — The products of enzymic degradation were analysed by h.p.l.c., using an Aminex HP X 87P column (Bio-Rad) and elution with water[13].

Gel-filtration chromatography. — Columns of Sepharose CL 6-B (50 × 1.6 cm), Sephacryl S-200 (79 × 2.0 cm), or Bio-Gel P-2 (203 × 1.6 cm) were used. The Sepharose CL 6-B was eluted (ascending) with 0.1M sodium acetate buffer (pH 4) at 20 mL/h. Samples (6 mg) dissolved in the same buffer were applied to the column and fractions of 4 mL were collected. The Sephacryl S-200 was eluted (descending) with 0.05M sodium acetate buffer (pH 4) at 20 mL/h. Samples (10 mg) were loaded onto the column and fractions of 3 mL were collected. The results were expressed as a function of $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_e, V_o, and V_t are the elution volume of the fraction, the void volume, and the total volume of the column, respectively. V_o and V_t are the elution volumes of *O*-(carboxymethyl)cellulose and D-galacturonic acid, respectively. Chromatography on Bio-Gel P-2 was performed as described[4].

Enzymic degradation. — Arabinanases and galactanases were used on the "hairy" fragments separately, in sequence, or in combination. Each reaction medium contained 4 mg/mL (final concentration) of "hairy" fragments, enzyme(s) (arabinanases at 20 μg/mL of protein, and galactanase at 1 μg/mL of protein) in 0.05M sodium acetate buffer (pH 5). Incubation was carried out at 30° and each reaction was monitored by the increase in reducing power. The reaction was stopped by heating at ~100° for 5 min. The digest was dialysed against distilled water and then concentrated under vacuum at 35°. The results are expressed relative to the corresponding initial contents of "hairy" fragments.

For sequential hydrolysis, the "hairy" fragments were subjected successively to the action of Ara*f*-ase for 1.5 and 24 h, endo-Ara-ase for 1.5 h, and endo-Gal-ase for 1.5 h. After each hydrolysis, the digest was dialysed and the retentate was incubated with the following enzyme.

Endo-PG-ase degradation was carried out in 0.05M sodium acetate buffer (pH 4.2) for 24 h at 30° with 2 nkat/mL of the enzyme.

Mild acid treatment. — Hydrolysis with 0.05M trifluoroacetic acid was performed[4] on the "hairy" fragments, previously degraded with Ara*f*-ase, at 100° in a sealed tube for 1 h. The digest was concentrated *in vacuo*.

RESULTS

Enzymic degradations. — The results of the action of Ara*f*-ase on the "hairy" fragments are summarised in Table I. The enzyme was efficient in degrading the arabinose side-chains as shown by the final values of hydrolysis of 55–56%. The release of arabinose from HP and OHP "hairy" fragments was a two-step process (Fig. 1) and followed Michaelis kinetics (Fig. 2) with values of K_m of 1.4 and 1.2 (mg/mL of arabinose residues), and V_{max} of 63.5 and 60.4 (nmol/min/μg of protein), for HP and OHP "hairy" fragments, respectively.

The percentage of arabinose liberated by the endo-Ara-ase after 1.5 h was

TABLE I

AMOUNTS OF ARABINOSE OR GALACTOSE RELEASED FROM "HAIRY" FRAGMENTS BY VARIOUS ENZYMES

Treatment	*Time*	*HP*	*OHP*
Separate digestion			
Araf-ase	1.5	23.0[a]	29.0
	24	36.0	41.0
	48	55.0	55.8
endo-Ara-ase	1.5	2.0	3.0
Gal-ase	1.5	7.5	15.1
endo-Gal-ase	1.5	6.5	15.8
Sequential digestion			
Araf-ase	1.5	23.0	25.6
+endo-Ara-ase	1.5	20.0	34.1
+endo-Gal-ase	1.5	10.5	10.9
Araf-ase	24	35.7	39.7
+endo-Ara-ase	1.5	26.8	47.4
+endo-Gal-ase	1.5	16.3	19.2

[a]Results are expressed in % (wt) of the initial contents in arabinose or galactose of the "hairy" fragments.

only 2–3% and did not increase. The products were arabinobiose and arabinotriose. This reaction followed Michaelis kinetics (Fig. 2) with values of K_m of 0.42 and 0.45, and V_{max} of 6.1 and 4.6, for HP and OHP "hairy" fragments, respectively.

Gal-ase released 7.5 and 15.1% of the galactose from the HP and OHP "hairy" fragments, respectively, after 1.5 h and there was no further increase. The product was mainly galactose. Endo-Gal-ase did not show more activity, as only 6.5 and 15.8% of the initial galactose were removed from HP and OHP "hairy"

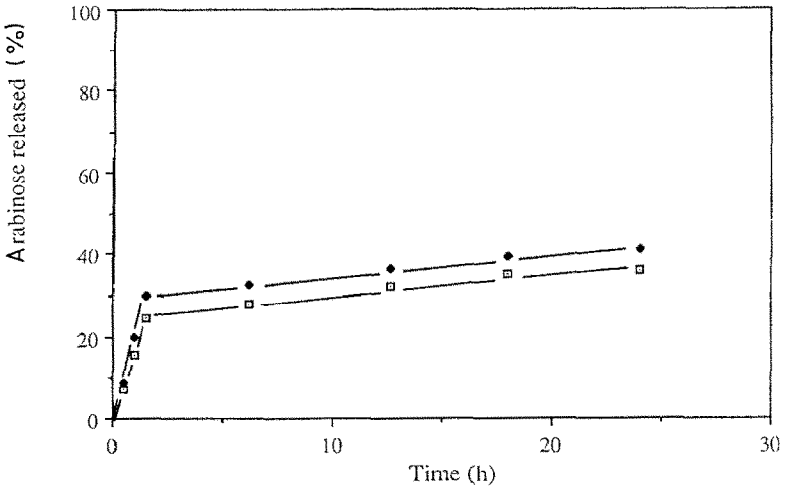

Fig. 1. Hydrolysis of the "hairy" fragments from sugar-beet pectins by Araf-ase. The extent of degradation was measured from the increase in the percentage of arabinose released: —◆—, HP; —□—, OHP.

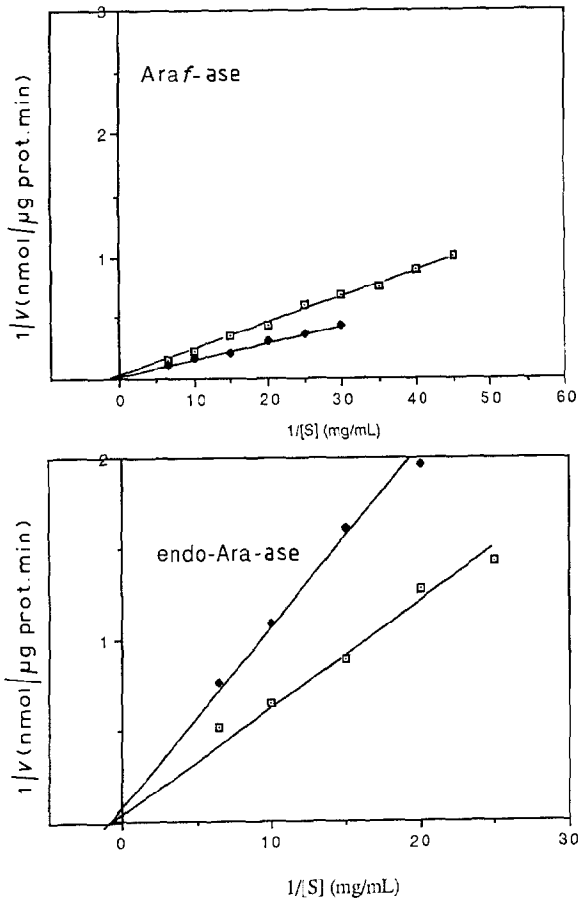

Fig. 2. Lineweaver–Burk plots of Araf-ase and endo-Ara-ase on "hairy" fragments of sugar-beet pectins: —□—, HP; —◆—, OHP.

fragments, respectively, after 1.5 h. Galactobiose and galactotriose were the main products of reaction.

Sequential hydrolysis. — Araf-ase, endo-Ara-ase, and endo-Gal-ase were applied successively to the "hairy" fragments (Table I). On incubation for 90 min with Araf-ase then with endo-Ara-ase, 43 and 59.7% of the arabinose were removed together with 10 and 11% of the feruloyl groups from the HP and OHP "hairy" fragments, respectively. H.p.l.c. showed that Araf-ase released arabinose and that endo-Ara-ase liberated arabinotriose and arabinobiose as the main products. The endo-Gal-ase removed 10.5 and 10.9% of the galactose with 9.2 and 10.2% of the feruloyl groups from the HP and OHP "hairy" fragments, respectively.

When the incubation with Araf-ase was extended to 24 h, the percentages of arabinose released increased to 62.5 and 87.1%, and those of the feruloyl groups

were 26.9 and 21.5%, for HP and OHP "hairy" fragments, respectively. In addition to arabinose, the dialysable products of the action of endo-Ara-ase also contained galactose (2.9 and 4.5% of the neutral sugar released from HP and OHP "hairy" fragments, respectively), which could be part of a dimer with an arabinosyl unit as suggested by h.p.l.c. Endo-Gal-ase liberated 16.3 and 19.2% of the galactose from HP and OHP "hairy" fragments, respectively, whereas the amount of feruloyl groups remained close to that obtained in the preceding experiment. The feruloyl-glycosyl complexes were not characterised because the quantities were too small.

The HP and OHP residues from the first experiment contained 69.5 and 63.3% of the initial neutral sugars, and 79.8 and 76.7% of the initial feruloyl groups, respectively. These percentages decreased to 56.9 and 47.7% for the neutral sugars and to 63.3 and 67.5% for the feruloyl groups in the second experiment. The contents of galacturonic acid, rhamnose, and galactose increased at the expense of that of arabinose. However, Araf-ase and endo-Ara-ase did not release all of the arabinose, particularly from the HP "hairy" fragments. The relative enrichment in feruloyl groups was ascribed to the concomitant decrease in neutral sugars.

The residues showed similar elution patterns on Sepharose CL6-B (Fig. 3). They eluted at K_{av} 0.5–0.6, which is similar to that of the initial "hairy" fragments. The ratios of galacturonic acid/rhamnose, and galacturonic acid/neutral sugar were constant. Feruloyl groups were eluted together with neutral sugars.

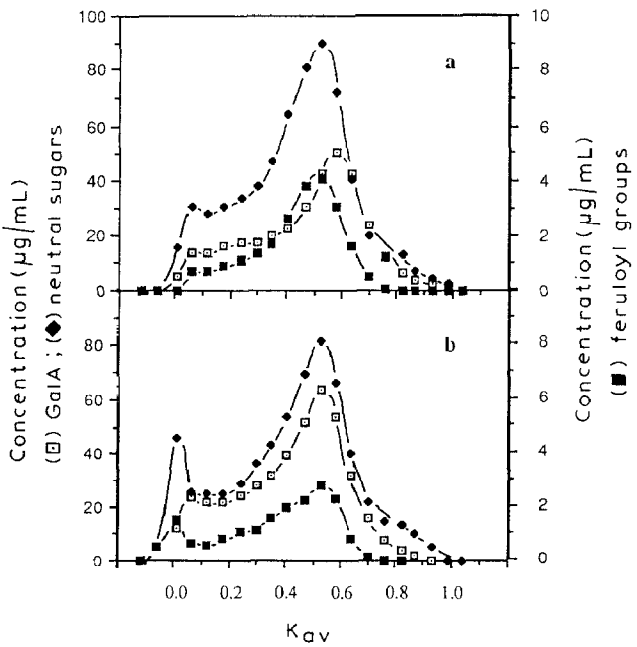

Fig. 3. Chromatography on Sepharose CL-6B of (*a*) retentate from HP "hairy" fragments degraded successively by Araf-ase (1.5 h), endo-Ara-ase, and endo-Gal-ase, (*b*) retentate from OHP "hairy" fragments as in (a).

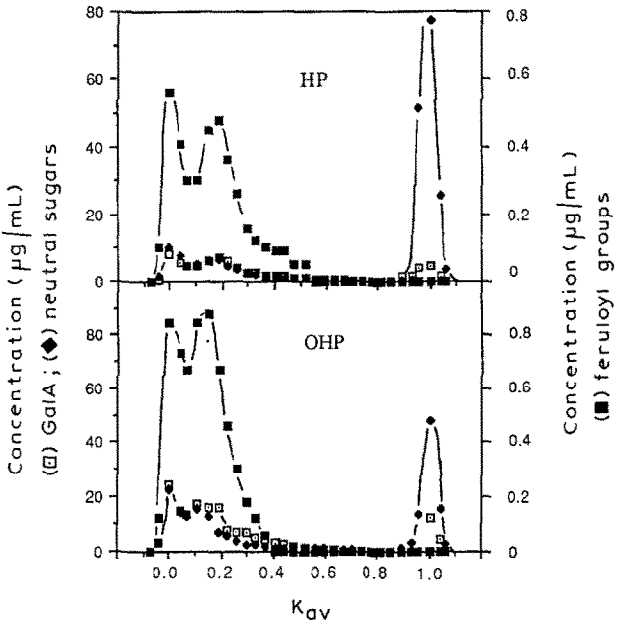

Fig. 4. Chromatography on Sephacryl S-200 of the products released from HP and OHP "hairy" fragments by hydrolysis with Araf-ase, endo-Ara-ase, and endo-Gal-ase in combination.

Hydrolysis of the side-chains by mixtures of the enzymes. — The "hairy" fragments were incubated for 1.5 h with Araf-ase, and endo-Gal-ase in combination. Chromatography of the products on Sephacryl S-200 (Fig. 4) gave a peak near or at the void volume (K_{av} <0.2), which contained almost all of the galacturonic acid together with part of the neutral sugars and of the feruloyl groups, and a peak eluted in the total volume which was rich in neutral sugars and in feruloyl groups and poor in galacturonic acid; 43.4 and 41.1% of the inital neutral sugars and 33.6 and 43.3% of the initial feruloyl groups of HP and OHP "hairy" fragments, respectively, were recovered in the material at the void volume (Table II). Feruloyl groups were removed from "hairy" fragments in higher amount by enzymes in combination than by enzymes in sequence. The proportion of arabinose released by the enzymes in combination (~80% of the initial arabinose) was similar for HP and OHP "hairy" fragments and was higher for HP than that obtained by enzymes in sequence. In contrast, galactose was liberated in similar amounts (10.3–41% of the HP and OHP initial galactose). When the residues were hydrolysed by the endo-PG-ase and the products were chromatographed on Sephacryl S-200, minor amounts of material were recovered at the total volume of the column, which indicates that binding sites for the enzyme were not generated.

Mild acid hydrolysis of Araf-ase-degraded "hairy" fragments. — HP and OHP "hairy" fragments were first hydrolysed by Araf-ase for 24 h. After dialysis, the retentates were hydrolysed with 0.05M trifluoroacetic acid for 1 h at 100°, and the

TABLE II

SUGAR AND FERULIC ACID CONTENTS OF THE "HAIRY" FRAGMENTS AND OF THEIR RESIDUES FROM ACID OR ENZYMIC HYDROLYSIS

	HP[a]					*OHP*[a]				
	1	*2*	*3*	*4*	*5*	*1*	*2*	*3*	*4*	*5*
Yield (%)[b]		66.3	58.8	50.5	39.3		60.9	46.2	50.4	43.7
GalA	19.6	25.8	29.5	30.7	43.0	21.2	36.5	33.9	35.7	45
Rha	8.0	10.8	12.2	14.1	22.4	7.3	12.5	11.9	13.3	16.1
Ara	51.0	39.1	31.4	21.1	17.7	38.7	12.2	11.5	16.8	21.1
Xyl	0.9	1.2	1.4	2.3		0.8	0.1	1.1		
Gal	19.8	23.1	25.5	31.8	16.7	31.5	38.7	41.6	34.2	17.8
Glc	0.7					0.7				
Feruloyl group	1.8	2.2	1.9	1.2	1.5	1.3	1.6	1.5	2.1	1.7

[a]"Hairy" fragments: 1, initial; 2, after treatment in sequence with Ara*f*-ase (1.5 h), endo-Ara-ase, endo-Gal-ase; 3, after treatment in sequence with Ara*f*-ase (24 h), endo-Ara-ase, endo-Gal-ase; 4, after treatment with Ara*f*-ase, endo-Ara-ase, and endo-Gal-ase in combination; 5, after treatment with Ara*f*-ase, then with 0.05M CF_3 COOH (1 h, 100°). [b]Relative to the initial "hairy" fragments.

products were chromatographed on Bio-Gel P-2 (Fig. 5) to give 4 peaks and excluded material. The compositions of the fractions are given in Table III. Peak 1 contained arabinose and galactose together with feruloyl groups (18.2 and 20.7% of the initial feruloyl groups in HP and OHP "hairy" fragments, respectively). Their

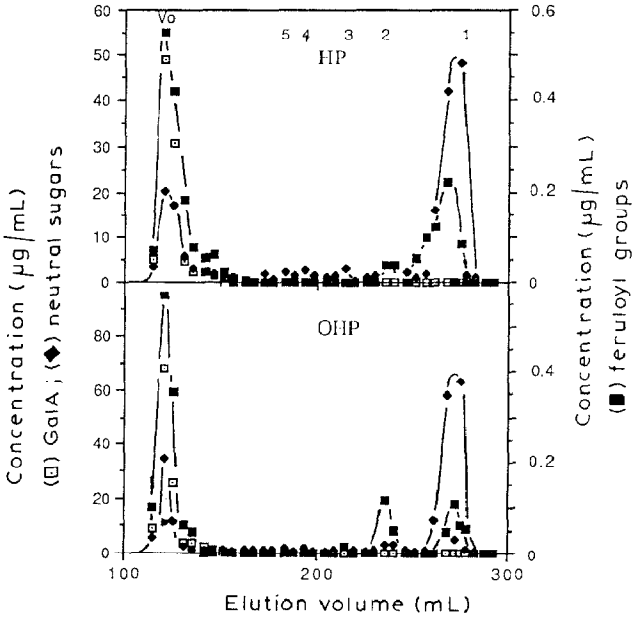

Fig. 5. Chromatography on Bio-Gel P-2 of HP and OHP "hairy" fragments degraded by 0.05M trifluoroacetic acid (100°, 1 h) after hydrolysis by Ara*f*-ase (for 24 h).

TABLE III

SUGAR AND FERULIC ACID CONTENTS OF THE FRACTIONS OBTAINED FROM BIO GEL P-2 OF THE 0.05M CF_3CO_2H HYDROLYSATE FROM Araf-ase-DEGRADED "HAIRY" FRAGMENTS

	HP[a]						*OHP*[a]					
	1	*2*	*3*	*4*	*5*	V_o	*1*	*2*	*3*	*4*	*5*	V_o
Recovery (%)[b]	27.6	3.1	1.8	1.3	1.8	41.5	34.4	1.9	1.5	0.9	0.4	45.5
GalA						43.0						45.0
Rha	2.0[c]			0.9	2.1	22.4	1.5					16.1
Ara	62.4	21.0		5.8	8.5	17.7	61.9	19.0	6.1	1.6	6.0	21.1
Xyl				1.3	0.3							
Gal	34.0	74.6	100	90.4	88.2	16.7	35.6	81.0	93.8	98.4	93.9	17.8
Glc	0.6	3.7		1.6								
Feruloyl group	0.8	7.1	2.0	1.8		1.5	0.4	8.6	1.0			1.7

[a]1–5 corresponds to peaks 1–5. [b]Relative to the initial "hairy" fragments. [c]Relative weight percentages.

delayed elution probably resulted from interaction of the phenolic compounds and the polyacrylamide gel[4].

Peak 2 accounted for 3.1 and 1.9% of HP and OHP "hairy" fragments, respectively. Galactose occurred in higher amount than arabinose and some

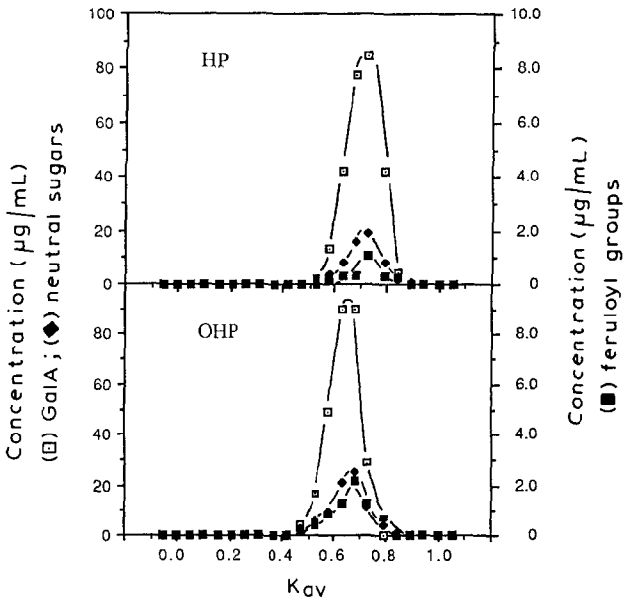

Fig. 6. Chromatography on Sepharose CL-6B of high mol.-wt.-fragments of HP and OHP "hairy" fragments degraded successively with Araf-ase (24 h) and 0.05M trifluoroacetic acid (100°, 1 h).

feruloyl groups were present (28.8 and 15.3% of the total feruloyl groups, respectively). Peaks 3 and 4 accounted for a small part of the initial "hairy" fragments and contained mainly galactose.

The material in the void volume was enriched in galacturonic acid at the expense of the neutral sugars in comparison to initial and enzyme-degraded "hairy" fragments (Table II). Successive hydrolysis by Ara*f*-ase and 0.05M trifluoroacetic acid released 87.5 and 78.2% of the initial arabinose, 62.4 and 72.8% of the initial galactose, and 45% of the initial feruloyl groups from HP and OHP "hairy" fragments, respectively.

When the residues from HP and OHP were chromatographed on Sepharose CL 6-B (Fig. 6), galacturonic acid, neutral sugars, and feruloyl groups were eluted in one sharp peak characterised by a constant ratio of neutral sugars to galacturonic acid (0.27 and 0.31 for the HP and the OHP "hairy" fragments, respectively), suggesting a homogenous distribution of the neutral sugars. Compared to initial "hairy" fragments, the elution volumes moved towards the total volume, which suggested a breakdown of the backbone.

DISCUSSION

The enzymes were chosen according to the data[4] from the methylation analysis of the "hairy" fragments and were used alone, in sequence, in combination, or prior to hydrolysis with 0.05M trifluoroacetic acid. Ara*f*-ase is known[5,6] to split (1→3)-, (1→2)-, and (1→5)-α-L-arabinosyl linkages and to be active preferentially on branched arabinans and on *p*-nitrophenyl α-L-arabinofuranoside. The high proportion of arabinose released by the Ara*f*-ase indicated that the linkages were α and that arabinose residues were in a highly branched structure. This inference was confirmed by the kinetic study. A similar pattern of hydrolysis was observed when Ara*f*-ase was incubated with commercial beet arabinan[5,6,14–17]; in the first stage, an essentially (1→5)-linked α-L-arabinan was obtained, indicating preferential splitting of α-(1→3) linkages. The maximum degree of hydrolysis reaches 90% on beet arabinan as substrate[14]. The lower value (55%) now reported may be ascribed to the presence of arabinopyranosyl units, feruloyl groups, and D-galactosyl units. The endo-Ara-ase showed a low activity, thus confirming the high degree of branching of the arabinans. Debranching by the Ara*f*-ase enhanced its activity and the maximum extent of degradation reached 60–90%. The existence of some galactosyl units attached to the arabinose side-chains was suggested by the presence of galactose in the endo-Ara-ase digests. The catalytic constants of Ara*f*-ase and endo-Ara-ase on the HP as well as on the OHP "hairy" fragments were in a similar range, suggesting similar structures for the arabinan fractions, which correspond to the structure usually described for neutral arabinans[18–22].

The action of Gal-ase, which was active on *p*-nitrophenyl β-D-galactopyranoside and on (1→4)-β-D-galacto-oligosaccharides, and the endo-Gal-ase, which was active on type I galactan, confirmed the (1→4)-linked galactose to be β.

Two main hypotheses may be formulated to explain why galactose was poorly liberated by these enzymes, namely, the galactose residues occur as short chains attached to the rhamnogalacturonan, or feruloyl groups or arabinosyl residues attached to the galactose side-chains, as is in apple pectins[23] or soya bean arabinogalactan[24], hindered the binding of the enzymes. The latter hypothesis was unlikely, because the removal of arabinose residues by hydrolysis with 0.05M trifluoroacetic acid did not change the relative proportions of branched, terminal, and (1→4)-linked galactosyl residues[4]. This inference was confirmed by enzymic hydrolysis, as the previous hydrolysis by the Araf-ase and the endo-Ara-ase did not increase the action of the endo-Gal-ase. This enzyme was more active on OHP than on HP "hairy" fragments, suggesting a higher d.p. and/or a more linear structure of the galactans in OHP than in HP "hairy" fragments. Furthermore, hydrolysis with 0.05M trifluoroacetic acid failed to remove all the galactose substituents. Therefore, it seems that some short side-chains of galactose residues are attached to galacturonic acid rather than to rhamnose residues.

The hydrodynamic values of the "hairy" fragments degraded by enzymes changed only slightly, whereas a substantial amount of sugar residues was removed. Therefore, the hydrodynamic properties mostly depend on the rigidity of the galacturonic backbone, and neutral sugars may occur as branched or short side-chains on the pectic backbone.

Up to 45 and 59%, respectively, of the initial feruloyl groups of HP and OHP "hairy" fragments were removed by hydrolysis with endo-enzymes, and 20–30% were removed on hydrolysis with the endo-Ara-ase from "hairy" fragments previously attacked by Araf-ase. These results accord with those of hydrolysis with 0.05M trifluoroacetic acid[4]. Possibly, feruloyl groups are located at the non-reducing

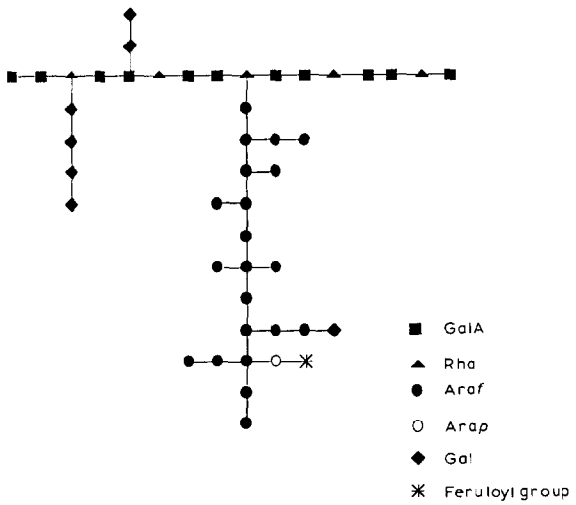

Fig. 7. Tentative structure for the "hairy" fragments of sugar-beet pectins.

arabinan termini as described for spinach pectins[25,26]. The arabinose–ferulic acid compounds were not characterised here because of their low amount. Of the remaining feruloyl groups, 10% was released together with galactose by the endo-Gal-ase, but a substantial amount (~30%) was obtained by a more severe treatment such as with 0.1M trifluoroacetic acid[4]. The remaining feruloyl groups were probably linked to residual galactosyl units. These results are important because they suggest that all the feruloyl groups are not in equally exposed domains of the pectins and, therefore, are not equally accessible to peroxidase[27] or persulfate ions[28] which could catalyse the oxidative coupling of the pectin molecules.

A tentative structure for the sugar-beet pectin "hairy" fragments is shown in Fig. 7.

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