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ENZYMIC AND CHEMICAL DEGRADATION AND THE FINE STRUCTURE OF PECTINS FROM SUGAR-BEET PULP

FRANK M. ROMBOUTS* AND JEAN-FRANÇOIS THIBAULT
Institut National de la Recherche Agronomique, Laboratoire de Biochimie et Technologie des Glucides, Rue de la Géraudière, F-44072 Nantes (France)

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ABSTRACT

Pectins sequentially extracted from sugar-beet pulp with water (WSP), oxalate (OXP), hot acid (HP), and cold alkali (OHP) have been degraded variously by base-catalysed β-elimination, de-esterification, endopectin lyase, pectin methyl esterase, endopolygalacturonase, and endopectate lyase. The products were studied mainly by chromatography on Sephadex G-100. The pectins contain various amounts of degradation-resistant (hairy) fragments in which the molar ratios of neutral sugar residues to galacturonic acid residues were 4.8, 4.6, 3.8, and 3.7 for WSP, OXP, HP, and OHP, respectively. The molar ratios of rhamnose residues to galacturonic acid residues in these fragments were 0.15, 0.20, 0.38 and 0.35, respectively. The pectins also contained sequences of galacturonic acid residues with relatively little neutral sugar residues attached (smooth fragments). Methyl ester and acetyl groups were distributed fairly regularly along the smooth fragments. Evidence is presented for an association of oligogalacturonic acids with the hairy fragments under the conditions of gel chromatography. Feruloyl groups are located in the hairy fragments. Other phenolic compounds, associated with the purified pectins, appear not to be covalently linked.

INTRODUCTION

Pectins are generally described as rhamnogalacturonans in which (1→4)-linked α-D-galacturonan chains are interrupted at intervals with α-L-rhamnopyranosyl residues[1,2]. Other sugars, attached in side chains, include D-galactose, L-arabinose, D-xylose, and L-fucose[1,2]. It has been established by enzymic degradation that most of the side chains are located on relatively small proportions of the backbone[3–6] and this led De Vries *et al.*[5] to distinguish "hairy" and "smooth" regions in the pectin molecule. Various proportions of galactosyluronic acid residues carry methyl ester groups. We have sought to show that most of these

*Present address: Agricultural University, Department of Food Science, De Dreijen 12, 6703 BC Wageningen, The Netherlands.

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characteristics also apply to pectins from sugar beet which, in addition, contain a considerable proportion of acetyl groups[7,8] and a small proportion of feruloyl groups[8]. At least 80% of the acetyl groups of pectins isolated from sugar beet are attached to galactosyluronic acid residues[9].

In the preceding paper[8], the extraction, purification, and general composition of pectins from sugar-beet pulp were described. Four pectin fractions were obtained by sequential extraction with water, oxalate, hot dilute acid, and cold dilute alkali, and we now report on the chemical and enzymic degradation of these fractions.

EXPERIMENTAL

Pectins. — Water-soluble pectin (WSP), oxalate-soluble pectin (OXP), acid-soluble pectin (HP), and alkali-soluble pectin (OHP) were obtained by sequential extraction of sugar-beet pulp[8]. Portions (5 mL) of stock solutions (5 mg/mL) were stored at −20°.

Enzymes. — Endopolygalacturonase (EC 3.2.1.15) was purified from a preparation of *Aspergillus niger*[10,11], endopectate lyase (EC 4.2.2.2) from *Pseudomonas fluorescens*[12], endopectin lyase (type 2, EC 4.2.2.10)[13], and pectin methylesterase (EC 3.1.1.11)[14] from different preparations of *A. niger*. The activities of enzymes are expressed in nkat, 1 nkat being the amount of enzyme which produces 1 nmol of reducing groups or methanol (pectin esterase) per s, under standard conditions which were sometimes different from those used in this study.

Enzymic degradation. — Conditions for hydrolysis of pectins with endopolygalacturonase were 2 mg/mL of pectin, 0.05M sodium acetate buffer (pH 4.2), and 0.8 nkat/mL of enzyme (final concentrations) at 30°. Percentage hydrolyses were calculated from the increase in reducing groups and the content of "anhydrogalacturonic acid".

For endopectin lyase, the conditions were 2.5 mg/mL of pectin, 0.01M sodium phosphate buffer (pH 5.2), and 8.3 nkat/mL of enzyme. During incubation at 30° for 20 h, the reaction was monitored by measuring the A_{235} of aliquots diluted 30-fold with 0.1M hydrochloric acid. Percentages of degradation were calculated using a molar extinction coefficient[15] of 5,500.

With endopectate lyase, the mixtures contained (final concentrations) 2 mg/mL of pectin, 0.5mM calcium chloride, 0.1M Tris-HCl buffer (pH 7.0), and 45 nkat/mL of enzyme. During incubation at 30° for 20 h, the reaction was followed by measuring the A_{235} of aliquots diluted 30-fold with 0.01M Na_2EDTA. Percentages of degradation were calculated using a molar extinction coefficient[16] of 4,800. Although the optimum pH of this enzyme is 9.4[12], the reaction was done at pH 7 to avoid base-catalysed degradation and demethylation of the substrate.

Conditions for enzymic demethylation were 4 mg/mL of pectin, 0.1M sodium acetate buffer (pH 4.5), and 67 nkat/mL of pectin methylesterase, at 30°. The

reaction was monitored by measuring the increase in free methanol. After 20 h of incubation, the enzyme was inactivated at ~100° for 5 min. For subsequent degradation with pectate lyase, 2.5 mL of each solution of enzymically demethylated pectin was dialysed against 1 L of 0.1M Tris-HCl buffer (pH 7.0). Calcium chloride solution was added to a final concentration of 0.5mM and the volume was adjusted with buffer, to 5 mL by weighing.

Saponification. — Solutions of pectin (5 mg/mL) were dialysed against 200 vol. of 0.05M sodium hydroxide at 2° and subsequently against 200 vol. of the buffer of choice for enzymic depolymerisation. The volume of each solution was then adjusted to 2 mg/mL of pectin.

β-Elimination. — Solutions of pectin (2.5 mg/mL) in 0.1M sodium phosphate buffer (pH 6.8) were stored at 80° for 6 h. The reaction was followed by measuring the A_{235} of aliquots diluted 30-fold with distilled water. Percentages of degradation were calculated, using the molar extinction coefficient[15] of 5,500.

Column chromatography. — Columns (53 × 1.5 cm) of Sephadex G-100 or Sepharose CL-6B (Pharmacia) were loaded with 2-mL samples containing 4 mg of degraded pectin. Each column was eluted at 11 mL/h with 0.1M sodium acetate buffer (pH 4.0). The void (V_o) and the total (V_t) volumes were determined using commercial apple pectin and galacturonic acid, respectively. The K_{av} values of fractions were calculated as $(V_e - V_o)/(V_t - V_o)$, V_e being the elution volume of the fraction considered. Fractions (3.6 mL) were analysed for their contents of galacturonic acid and neutral sugars. Ion-exchange chromatography was done on DEAE-Sepharose CL-6B[8].

Analytical methods. — Galacturonic acid and neutral sugars (expressed as arabinose) were determined by the automated *m*-hydroxybiphenyl[17] and orcinol[18] methods, respectively, the latter being corrected for interfering galacturonic acid. Methanol liberated by pectin methylesterase and methyl ester groups were determined by the method of Wood and Siddiqui[19], and reducing sugars by the method of Nelson and Somogyi[20] (using galacturonic acid as standard) and by g.l.c.[8] of the alditol acetates. Samples were hydrolysed[21] with 2M trifluoroacetic acid at 120° for 1.5 h. Total phenols and feruloyl groups were determined as described previously[8].

RESULTS

In order to obtain information on the structural features of the four pectins extracted from sugar-beet pulp, each was degraded with endopectin lyase, endopolygalacturonase, and endopectate lyase (active against the galacturonan backbone), with pectin methylesterase (removal of methyl ester groups), and by β-elimination and saponification of the methyl ester and acetyl groups.

Degradation limits. — These are given in Table I as final values after prolonged incubation with excess of enzyme (see Figs. 1 and 2). For interpretation of the results in Table I, reference should be made to Tables II and III of the

TABLE I

DEGRADATION LIMITS OF SUGAR-BEET PECTINS BEFORE AND AFTER ENZYMIC DEMETHYLATION AND SAPONIFICATION

Pretreatment and mode of degradation[a]	*Pectins*			
	Water-soluble	*Oxalate-soluble*	*Acid-soluble*	*Alkali-soluble*
No pretreatment				
β-Elimination[b]	7.2[c]	4.9	4.6	—
Endopectin lyase	8.6	4.3	4.1	—
Endopolygalacturonase	1.2	3.7	1.7	26.9
Endopectate lyase	<1.0	4.8	1.3	23.7
After pectin esterase treatment				
Endopolygalacturonase	19.1	22.4	16.3	—
Endopectate lyase	11.8	13.0	9.9	—
After saponification				
Endopolygalacturonase	37.5	36.7	30.8	29.3
Endopectate lyase	33.4	34.5	31.0	26.4

[a]See Experimental for details of enzymic degradations. [b]In sodium phosphate buffer (pH 6.8) at 80°. [c]Percentages of galactosyluronic acid bonds broken.

previous paper[8], in which the compositions of the pectins are given. There was a close relationship between degrees of methyl esterification (75.5, 59.7, and 61.8% for WSP, OXP, and HP, respectively) and their β-elimination degradation limits (Table I). However, the percentages of degradation were low.

Alkali-soluble pectin, largely de-esterified during extraction (residual methyl ester and acetyl, 7.5 and 4%, respectively)[8], was extensively degraded by endo-

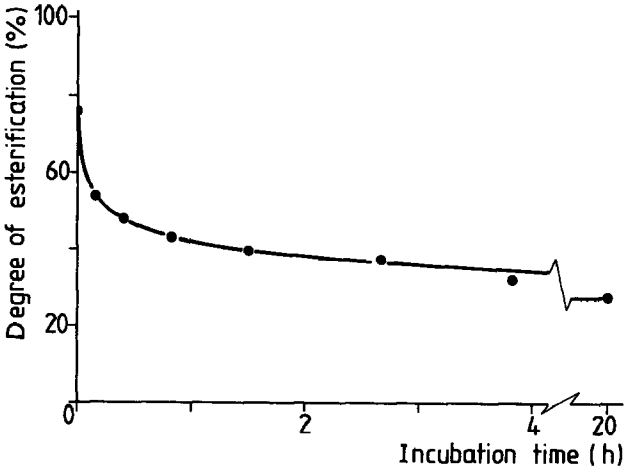

Fig. 1. Demethylation of water-soluble pectin (4 mg/mL, pH 4.5) by pectin methylesterase (67 nkat/mL) at 30° for 20 h.

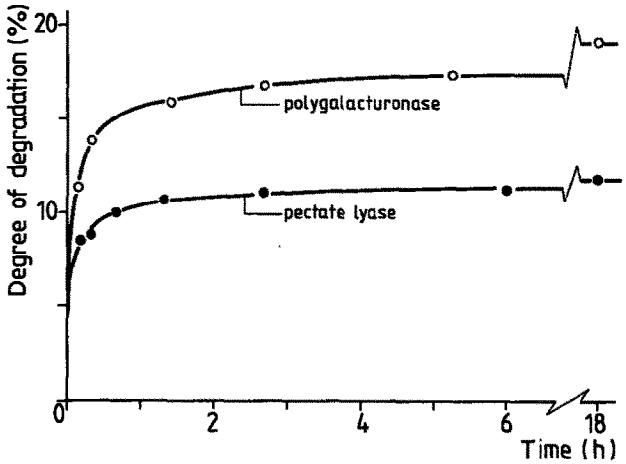

Fig. 2. Degradation of pectin esterase-demethylated, water-soluble pectin (2 mg/mL) with endo-polygalacturonase (0.8 nkat/mL) and endopectate lyase (45 nkat/mL) at 30° for 20 h.

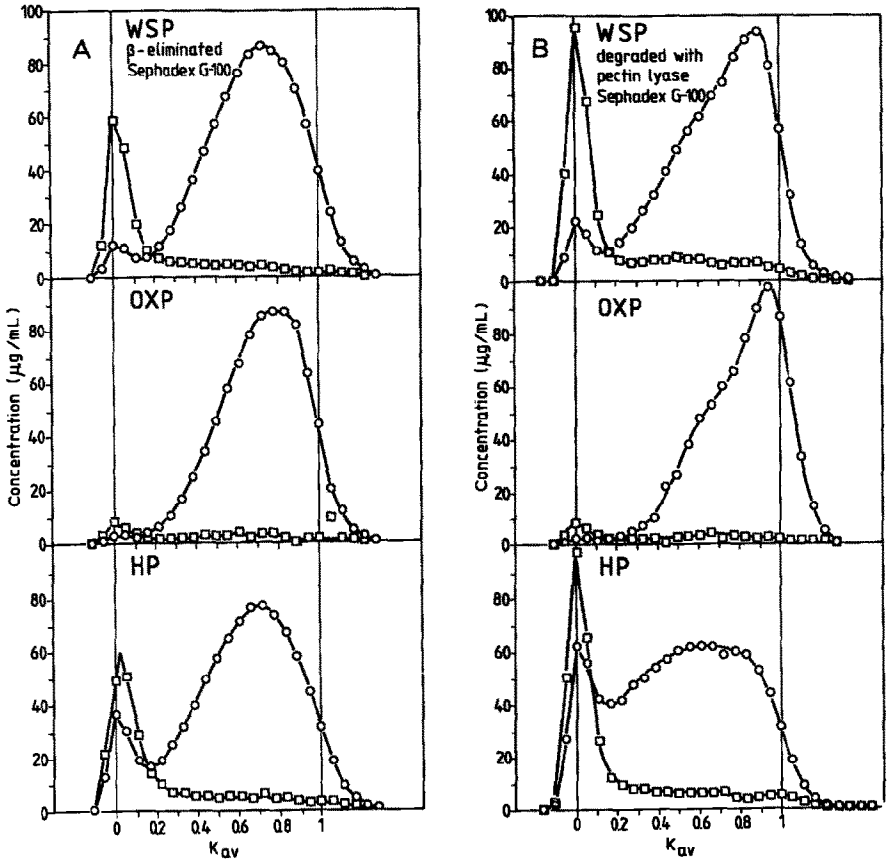

Fig. 3. Chromatography on Sephadex G-100 of pectins degraded by A, β-elimination to 7.2 (WSP), 4.9 (OXP), and 4.6% (HP); and B, endopectin lyase to 8.6 (WSP), 4.3 (OXP), and 4.1% (HP): —○—, galacturonic acid; —□—, neutral sugars.

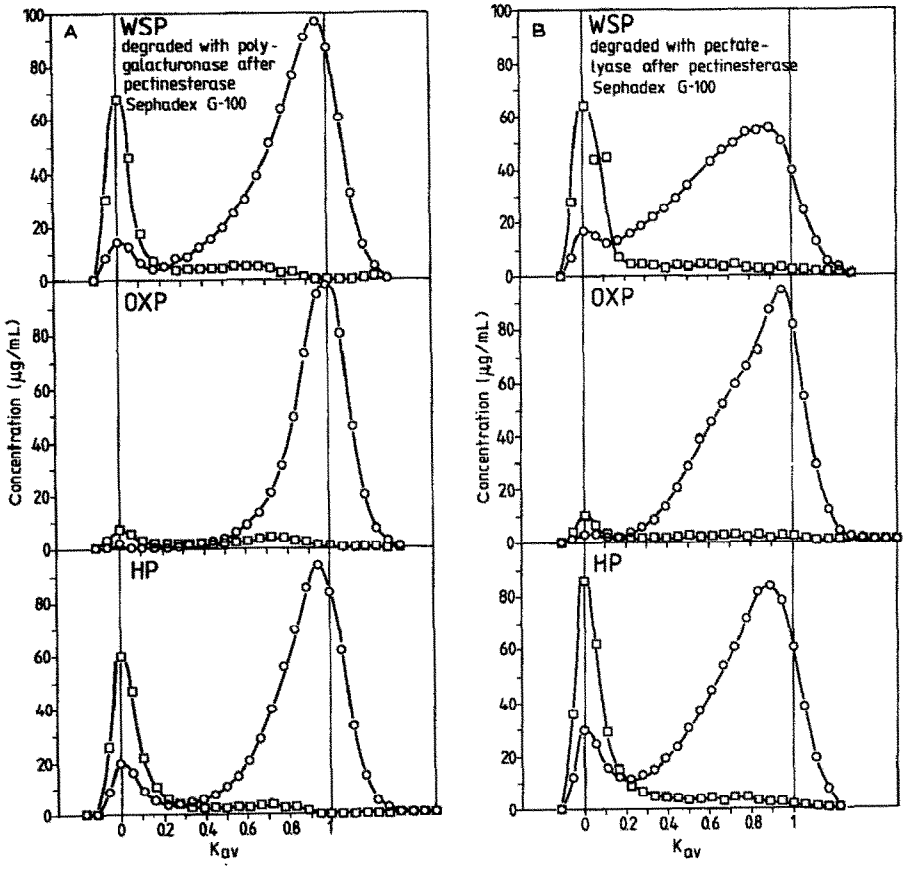

Fig. 4. Chromatography on Sephadex G-100 of pectins degraded, after demethylation with pectin esterase, with A, endopolygalacturonase to 19.1 (WSP), 22.4 (OXP), and 16.3% (HP); and B, endopectate lyase to 11.8 (WSP), 13.0 (OXP), and 9.9% (HP): —○—, galacturonic acid; —□—, neutral sugars.

polygalacturonase and endopectate lyase. These enzymes degraded <2% of the galactosyluronic acid bonds of WSP and HP, but 3.7 and 4.0%, respectively, of those in OXP. The more extensive degradation of OXP may be due to its lower contents of acetyl groups and neutral sugars and/or an irregular distribution of its methyl ester groups[8].

Treatment with pectin esterase did not remove all of the methyl ester groups (Fig. 1) and none of the acetyl groups, residual degrees of methyl esterification being 27.1, 17.9, and 26.2% for WSP, OXP, and HP, respectively. After this treatment, there was a substantial increase in the degradation limits, which was more pronounced for endopolygalacturonase than for endopectate lyase (Table I). After complete saponification of the methyl ester and acetyl groups, maximum percentages of degradation by the two enzymes were obtained (Table I). The de-esterification process (see Experimental) did not cause any loss of galacturonic acid or neutral sugars. The maximum values were 29.3–37.5 and 26.4–33.4% for endo-

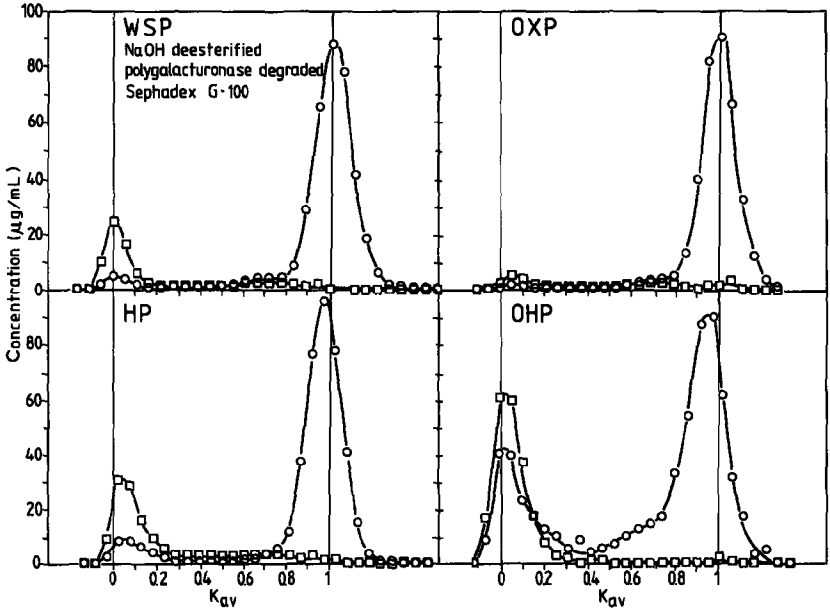

Fig. 5. Chromatography on Sephadex G-100 of pectins degraded, after saponification, with endopolygalacturonase to 37.5 (WSP), 36.7 (OXP), 30.8 (HP), and 29.3% (OHP): —○—, galacturonic acid; —□—, neutral sugars.

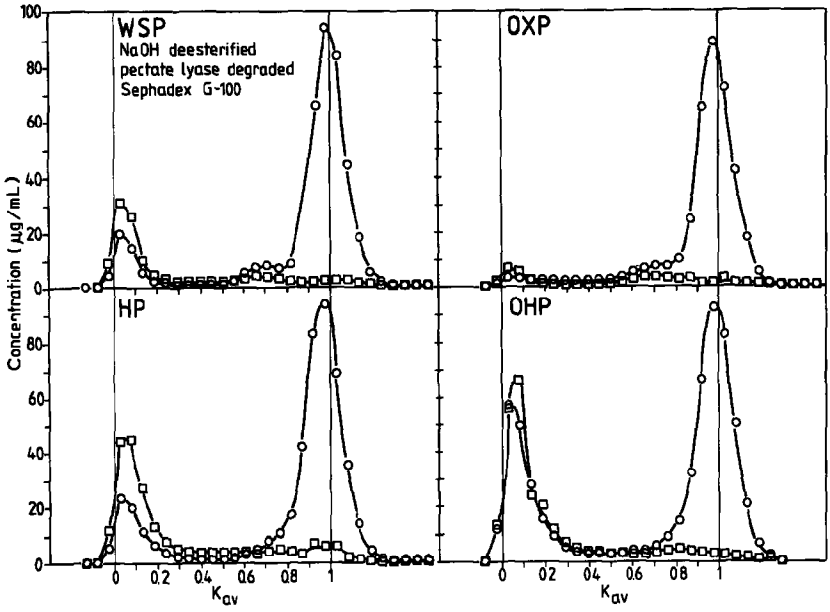

Fig. 6. Chromatography on Sephadex G-100 of pectins degraded, after saponification, with endopectate lyase to 33.4 (WSP), 34.5 (OXP), 31.0 (HP), and 26.4% (OHP): —○—, galacturonic acid; —□—, neutral sugars.

TABLE II

DISTRIBUTION OF GALACTURONIC ACID AND NEUTRAL SUGARS IN EXCLUDED AND INCLUDED FRACTIONS OF DEGRADED PECTINS AFTER CHROMATOGRAPHY ON SEPHADEX G-100

	Water-soluble				*Oxalate-soluble*				*Acid-soluble*				*Alkali-soluble*			
	Excluded[a] fractions		*Included[b] fractions*		*Excluded fractions*		*Included fractions*		*Excluded fractions*		*Included fractions*		*Excluded fractions*		*Included fractions*	
	Gal A[c] (%)	*NS[d] (%)*	*Gal A (%)*	*NS (%)*	*Gal A (%)*	*NS (%)*	*Gal A (%)*	*NS (%)*	*Gal A (%)*	*NS (%)*	*Gal A (%)*	*NS (%)*	*Gal A (%)*	*NS (%)*	*Gal A (%)*	*NS (%)*
No pretreatment																
β-Elimination	3.4	66.7	96.6	33.3	1.0	22.3	99.0	77.7	12.4	68.8	87.6	31.2	—	—	—	—
Endopectin lyase degradation	7.3	62.7	92.7	30.3	2.1	48.0	97.9	52.0	21.6	71.5	78.4	28.5	—	—	—	—
Endopolygalacturonase degradation	—	—	—	—	—	—	—	—	—	—	—	—	14.6	86.9	85.4	13.1
Endopectate lyase degradation	—	—	—	—	—	—	—	—	—	—	—	—	17.5	75.2	82.5	24.8
After enzymic demethylation																
Endopolygalacturonase degradation	4.8	83.4	95.2	16.6	1.9	31.5	98.1	68.5	10.2	87.2	89.8	12.8	—	—	—	—
Endopectate lyase degradation	7.4	67.8	92.6	32.8	1.4	46.5	98.6	53.5	12.5	81.2	87.5	18.8	—	—	—	—
After saponification																
Endopolygalacturonase degradation	6.0	89.3	94.0	10.7	2.3	48.9	97.7	51.1	8.7	81.2	91.3	18.8	20.7	92.5	79.3	7.5
Endopectate lyase degradation	10.2	60.9	89.8	39.1	2.5	32.8	97.5	67.2	12.8	58.3	87.2	41.7	25.1	62.9	74.9	37.1

[a]Material of Figs. 3–6 with $K_{av} \leq 0.15$. [b]Material of Figs. 3–6 with $K_{av} > 0.15$. [c]Percentage of total galacturonic acid (GalA). [d]Percentage of total neutral sugars.

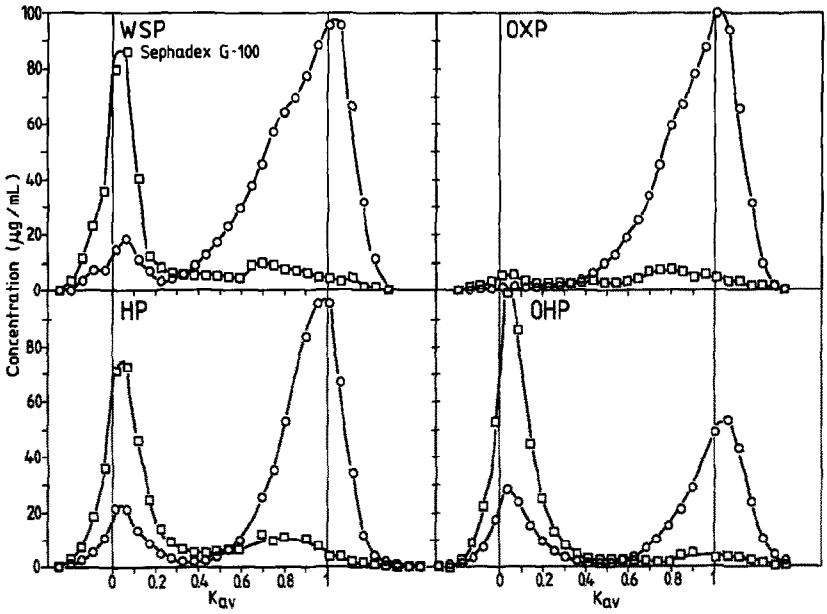

Fig. 7. Chromatography on Sephadex G-100 of pectins degraded, after saponification, with endopectate lyase 33.4 (WSP), 34.5 (OXP), 31.0 (HP), and 26.4% (OHP). The products were dialysed for 5 days against 4 changes of 10 vol. of distilled water prior to chromatography: —○—, galacturonic acid; —□—, neutral sugars.

TABLE III

SUGARS COMPOSITION OF SUGAR-BEET PECTINS AND THEIR "HAIRY" FRAGMENTS[a]

Sugar	*Pectins*				*Hairy fragments*			
	WSP	*OXP*	*HP*	*OHP*	*WSP*	*OXP*	*HP*	*OHP*
GalA	80[b]	92	77	72	17.2	17.7	20.8	21.1
Rha + Fuc	1.08	1.20	2.65	3.35	2.62	3.58	7.82	7.45
Ara	11.33	2.68	13.11	14.66	45.18	40.22	50.11	43.20
Xyl	0.20	0.26	0.23	0.27	0.64	1.65	0.48	0.86
Man	0.19	0.16	0.13	0	0.37	0.80	0.18	0
Gal	7.10	3.07	6.35	7.75	29.90	27.95	19.41	23.87
Glc	0.43	0.28	0.34	0.29	4.05	8.05	1.15	3.55

[a]Material with $K_{av} \leq 0.20$ from Fig. 7. [b]All numbers as mole percentages; values for initial pectins are calculated from data in ref. 8.

polygalacturonase and endopectate lyase, respectively. There were definite and systematic differences between the values for the two enzymes and for the four pectins.

Gel chromatography. — The pectin digests with degradation limits shown in Table I were chromatographed on Sephadex G-100 (Figs. 3–6). Even for preparations with relatively low extents of degradation, there was a general trend towards

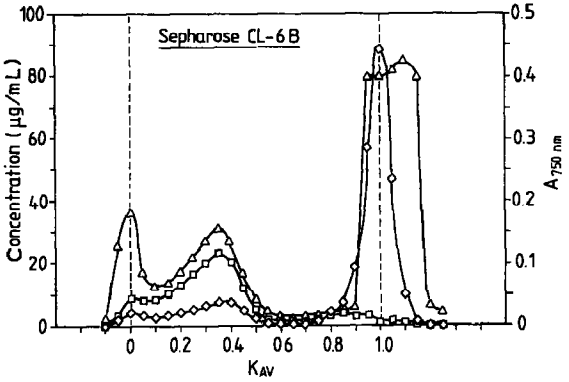

Fig. 8. Chromatography on Sepharose CL-6B of alkali-soluble pectin degraded to 26% with endopectate lyase at 30° for 20 h, by elution with 0.1M sodium acetate buffer (pH 4.0): —◇—, galacturonic acid; —□—, neutral sugars; —△—, phenolic compounds (A_{750} nm).

separation of the products into two large peaks. The peak at the void volume represented relatively large fragments rich in neutral sugars and rather poor in galacturonic acid. These fragments represent the "hairy regions" of the pectin molecules[5,6]. The broad peaks which were eluted subsequently contained small fragments composed of galacturonic acid residues, but with little or no neutral sugars, and represent the "smooth regions" of the pectin molecules[5,6], With increasing extents of degradation, there was little change in the peak at the void volume, but the subsequent peak was gradually broken down further and moved towards the total volume. This pattern was largely independent of the mode of degradation and suggests that the methyl ester and acetyl groups were distributed fairly regularly along these parts of the pectin molecules.

Table II contains the relative amounts of galacturonic acid and neutral sugars present in the excluded peaks ($K_{av} \leq 0.15$) and in the included peaks ($K_{av} > 0.15$). The excluded fractions, generated by base-catalysed β-elimination, contained less galacturonic acid than those generated by endopectin lyase. Although the degradation limits with the two methods are similar (Table I), it may be concluded that pectin lyase leaves almost twice as much galacturonic acid in the hairy fragments as does base-catalysed β-elimination.

The hairy fragments produced with endopolygalacturonase from pectins demethylated with pectin esterase have low contents of galacturonic acid (4.8, 1.9, and 10.2% for WSP, OXP, and HP, respectively), but have large contents of neutral sugars (83.4, 31.5, and 87.2% for WSP, OXP, and HP, respectively). The corresponding values for OHP degraded with endopolygalacturonase were 14.6 and 86.9%. These data show that the hairy fragments of the four pectins comprise different, though rather small, proportions of the rhamnogalacturonan backbone.

In other degradations, hairy fragments were obtained which were richer in galacturonic acid and poorer in neutral sugars. This was particularly true for pectate-lyase digests of pectins previously enzymically demethylated with pectin

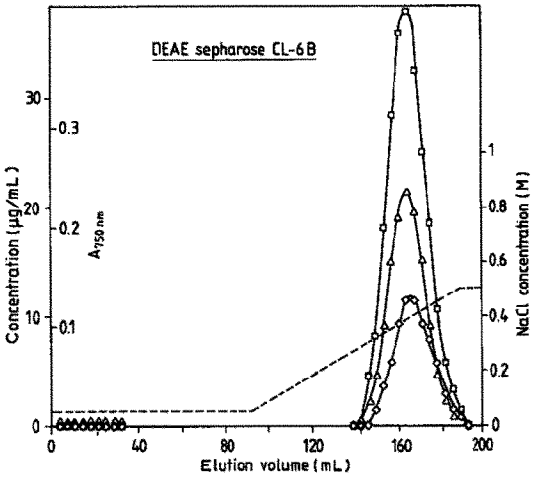

Fig. 9. Chromatography on DEAE-Sepharose CL-6B of the "hairy fragments" of alkali-soluble pectin. Fractions with $K_{av} \leq 0.5$ (Fig. 8) were dialysed against 0.05M sodium acetate buffer (pH 4.8), applied to the column, and eluted with a linear gradient 0→0.5M sodium chloride in the same buffer: —◇—, galacturonic acid; —□—, neutral sugars; —△—, phenolic compounds (A_{750} nm).

esterase or saponified. It was anticipated that, with increasing extents of degradation, the relative proportions of galacturonic acid and neutral sugars in the hairy fragments would decrease, or eventually remain constant. The increased content of galacturonic acid in the hairy fragments could point towards association of small oligogalacturonans with the neutral sugar side-chains of the hairy fragments, under the conditions of chromatography. This unexpected behaviour was confirmed with Figs. 6 and 7 which show chromatograms of saponified pectins degraded with pectate lyase, but the samples used in Fig. 7 were thoroughly dialysed before chromatography. In Fig. 6, the weight ratios of neutral sugars and galacturonic acid in the void peaks were 1.6, 1.8, 2.2, and 1.1 for WSP, OXP, HP, and OHP, respectively, whereas the corresponding ratios in Fig. 7 were 4.2, 4.0, 3.3, and 3.2.

Table III shows the sugar composition of the pectins in comparison to that of the hairy fragments obtained as described in Fig. 7. In the hairy fragments, the content of all neutral sugars, relative to the galacturonic acid, had increased markedly. There was also an enrichment of arabinose and glucose in the hairy fragments, at the expense of the other neutral sugars. The enrichment in glucose was an artefact, possibly due to "bleeding" from the Sephadex G-100. Preliminary studies of the material eluting towards the total volume of the column in Fig. 7 indicated that, amongst the neutral sugars, fucose, xylose, and mannose were present in greater proportions in the products of low molecular weight.

Location of feruloyl groups. — This was studied by degradation of the alkali-soluble pectin with endopectate lyase and chromatography of products on Sepharose CL-6B (Fig. 8). Phenolic compounds appeared to be associated with the products of both high and low molecular weight eluting near K_{av} 1. However, the phenolic compounds, as measured with the Folin reagent, at K_{av} 1 were not bound

to the oligogalacturonans. When the hairy fragments (K_{av} <0.5) were thoroughly dialysed and chromatographed on DEAE Sepharose CL-6B (Fig. 9), there was good overlap of peaks for phenolic compounds, neutral sugars, and galacturonic acid. A solution of this material in 0.1M glycine–sodium hydroxide buffer (pH 10) had λ_{max} at 375 nm, characteristic for feruloyl groups. The yield of feruloyl groups, calculated from this absorption value, was 82.3% of those originally present. Also, the feruloyl groups present in the peak in Fig. 9 accounted for all of the Folin-positive material.

The peak in Fig. 9 contained 260 μg of galacturonic acid, 850 μg of neutral sugars (measured as arabinose), and 30 μg of feruloyl residues. Determination of the reducing groups indicated a content, on average, of 11 galacturonic acid residues and 41 neutral sugar residues per hairy fragment. According to the data in Table III, the neutral sugar residues would be made up of ~4 rhamnose, ~23 arabinose, 0–1 xylose, 12–13 galactose, and, perhaps, 1 glucose residue. Furthermore, five such fragments would carry 6 feruloyl groups.

The above results justify the conclusion that feruloyl groups in sugar-beet pectins are located in the hairy fragments and are probably linked to the neutral sugar side-chains.

DISCUSSION

Degradability of the pectins. — Each of the enzymic and chemical degradation methods applied to the pectins from sugar-beet pulp had a different specificity. Base-catalysed β-elimination occurred in regions of the rhamnogalacturonan backbone where there was a relatively high density of methyl ester groups, *i.e.*, a low negative-charge density[22]. The reaction may be progressively inhibited by demethylation of the substrate. Because of the small size of the hydroxide ions, the attack will not be hindered much by side chains or other substituents[23]. Endopectin lyase requires a certain sequence of methyl galactosyluronate residues[24,25] and may be hindered or blocked by side chains or rhamnosyl substituents in the main chain. This difference in specificity explains why the hairy fragments produced with pectin lyase had more galactosyluronic acid residues attached than those produced by base-catalysed β-elimination.

Endopolygalacturonase requires completely demethylated pectins for maximum degree of hydrolysis, which may be as high as 38% for pectate from citrus pectin[26] or 56.7% for polygalacturonate[11]. Neutral sugar side-chains and rhamnosyl substituents in the main chain are obstacles which limit the degree of hydrolysis. Acetyl groups at positions 2 and/or 3 also decrease the hydrolysis limit by blocking the binding sites[26]. With *Pseudomonas* endopectate lyase, the maximum degradation limits are attained on pectins with low degrees of methyl esterification, 37% degradation being the limit for pectate from apple pectin[12]. *Aspergillus niger* pectin methylesterase removes methyl ester groups from its substrate in a random fashion[14] and is not active against acetyl groups. Both methyl

ester and acetyl groups are completely removed by saponification and, at the low temperature chosen for this treatment, β-elimination was virtually suppressed[1,22].

As shown by the data in Table I, there were few bonds in WSP, OXP, and HP that were susceptible to endopolygalacturonase and endopectate lyase. The number of degradable bonds was increased considerably by partial enzymic demethylation to 27.1, 17.9, and 26.2% of residual methyl ester groups in WSP, OXP, and HP, respectively. The percentages of bonds broken in these pectins, which were considerably higher for endopolygalacturonase than for endopectate lyase, indicated that acetyl groups limit the degradability not only for endopolygalacturonase, which is known[26], but also, and even more, for endopectate lyase. Non-acetylated apple pectins with degrees of methylation of 27, 18, and 26% were broken down to limits of 23, 35, and 24%, respectively[12]. Thus, HO-2 and/or HO-3 of the galactosyluronic acid residues are essential for the activity of endopectate lyase, although it has been reported[27] that this would not be so.

After complete removal of methyl ester and acetyl groups, the degradability by the two enzymes is still limited by the numbers of side chains and rhamnosyl substituents in the main galacturonan chain. The data in Table I indicate that, in this respect, the pectins may be divided into two groups, namely, WSP and OXP, and HP and OHP, the latter being the less degradable.

Composition and properties of the hairy fragments. — In gel chromatography, conditions were selected [0.1M sodium acetate buffer (pH 4.0)] in order to avoid ionic exclusion from the gel, as described for pectins[28] and oligogalacturonic acids[29]. However, Figs. 6 and 7 demonstrate that small, dialysable galacturonan products were associated with the hairy fragments with which they were co-eluted at the void volume. This effect was most pronounced with the products of endopectate lyase activity, especially with the saponified pectins. In the final stage of degradation, these small dialysable products were almost exclusively an unsaturated disaccharide (containing 4-deoxy-L-*threo*-hex-4-enopyranuronic acid as the non-reducing unit) and unsaturated trisaccharides[12,30]. The association was less pronounced with the final products of hydrolysis by endopolygalacturonase, namely, mono-, di-, and tri-galacturonic acid[11].

It is believed that "clean" hairy fragments are obtained by extensive dialysis of products before gel chromatography. This may be true, since subsequent chromatography on DEAE-Sepharose CL-6B does not change the ratio of neutral sugar residues to galacturonic acid residues of the hairy fragments (*cf.* Figs. 7 and 8); the ratios were then 4.2, 4.0, 3.3, and 3.2 for the hairy fragments of WSP, OXP, HP, and OHP, respectively, which are similar and rather small compared to those of cherry fruits[6] and apple pectins[5,23]. The molar ratios of rhamnose residues to galacturonic acid residues were 0.15, 0.20, 0.38, and 0.35 for WSP, OXP, HP, and OHP, respectively (*cf.* Table III), which means that there is about one rhamnose residue for every 6.6, 4.9, 2.7, and 2.8 galacturonic acid residues in the hairy fragments of the corresponding pectins.

The size of such fragments from OHP degraded by endopectate lyase, and

purified by gel-filtration chromatography, dialysis, and ion-exchange chromatography, was determined by measuring reducing groups. With 11 galacturonic acid residues and 41 neutral sugar residues, these fragments would have a molecular weight of ~8,000, similar to that of rhamnogalacturonan II described by Darvill *et al.*[31]. Oxalate-soluble pectin has[8] a viscosity-average molecular weight of 15,400. Its number-average molecular weight will be lower[32], perhaps also ~8,000. The average size of the hairy fragments of OHP would therefore be similar to those of the OXP molecules. Both were eluted at the V_0 of a Sephadex G-100 column, but OXP was eluted over the entire fractionation range of a Sepharose CL-6B column[8], whereas the hairy fragments of OHP were eluted at $K_{av} \leq 0.5$ (*cf.* Fig. 8). It is possible that, under the conditions of chromatography, these hairy fragments aggregate, for example, through their arabinan side-chains since (1→5)-α-arabinans have a strong tendency to aggregate in, and precipitate from, aqueous solutions[33].

Location of the feruloyl groups. — Sugar-beet pectins, even when purified by ion-exchange chromatography or copper precipitation, contain polyphenols. In the preceding paper[8], it was shown that part of these were feruloyl groups covalently linked to the pectins. Through degradation with endopectate lyase and chromatography of the products on Sepharose CL-6B (Fig. 8), it was shown that the major part of the polyphenols were not covalently linked to the pectin, although they were eluted with the products of low molecular weight but in incompletely overlapping peaks. On the Bio-Gel P-2, as described by Thibault[29], this mixture of oligogalacturonic acid products of low molecular weight and phenols could be completely resolved into peaks of the various unsaturated oligosaccharides and a single peak of phenols (results not shown). The reason why these phenols, although not covalently bound, remain associated with the pectins during purification and only separate upon extensive degradation is not known.

Another part of the phenols co-chromatographed with the hairy fragments on Sepharose CL-6B (Fig. 8) and DEAE-Sepharose CL-6B (Fig. 9). These were identified spectroscopically as the feruloyl groups described in the previous paper[8]. The feruloyl groups account for the total amount of polyphenols present in the hairy fragments and for 82% of the feruloyl groups in the undegraded pectin. The feruloyl groups are located in the hairy fragments, and they are presumably bound to the neutral sugar side-chains. These results are in agreement with those of Fry[34] who suggested that ferulic acid residues are bound to the non-reducing termini of arabinose- and/or galactose-containing domains of pectins from spinach.

The presence of feruloyl groups in sugar-beet pectin has led to the application of methods of cross-linking using peroxidase–hydrogen peroxide[35] and ammonium peroxysulphate described in the following paper[36].

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