Carbohydrate Research, 238 (1993) 271–286
Elsevier Science Publishers B.V., Amsterdam

Studies of the length of homogalacturonic regions in pectins by acid hydrolysis

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(Received March 10th, 1992; accepted May 28th, 1992)

ABSTRACT

The different susceptibilities to acid hydrolysis of the glycosidic linkages in a pectin backbone were used to isolate fractions corresponding to the "smooth", homo-D-galacturonic regions. Pectins from apple, beet, and citrus were de-esterified, and the resulting pectic acids were hydrolysed in 0.1 M HCl at 80°C for up to 72 h. The intrinsic viscosities of the hydrolysates decreased, and two stages could be distinguished. Up to 10 h, there was a fast decrease, corresponding to the cleavage of the more susceptible linkages between L-rhamnose and galacturonic acid residues, followed by a slower stage, corresponding to cleavage of the linkages between galacturonic acid residues. During the course of the reaction, some galacturonic acid and most of the neutral sugars were solubilised, giving two fractions on Sepharose CL-6B. A minor fraction, composed mostly of galacturonic acid and rhamnose, with rhamnose–galacturonic acid ratios of 1:1.5, 1:2.9, and 1:2.1 for apple, beet, and citrus, respectively, eluted at K_{av} 0.8, and a major fraction, composed essentially of L-arabinose and D-galactose, eluted at the total volume. The acid-insoluble materials represented 84, 66, and 90% of the original pectic acids for apple, beet, and citrus, respectively. They were progressively freed of neutral sugars; after hydrolysis for 72 h, almost pure polygalacturonates (more than 98 mol% galacturonic acid), representing the homogalacturonic regions, were obtained. The molecular weights of these 72-h acid-insoluble materials from apple, beet, and citrus were similar (respectively, 21000, 19000, and 24000), corresponding to lengths of the homgalacturonic regions estimated to be a minimum of 72–100 galacturonic acid residues.

INTRODUCTION

Pectins are a family of plant polysaccharides that are used for their gelling power. They are characterised by a backbone of α-(1 → 4)-linked galacturonic acid residues which are partly methyl-esterified[1]; in some commercial samples, some of the methyl ester groups may be amidated[2]. Pectins always contain some associated neutral sugars, typically, L-arabinose, D-galactose, L-rhamnose, D-xylose, and D-glu-

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cose[1]. The location and the distribution of these sugars have been the subject of numerous studies.

The polygalacturonic backbone may be interrupted by (1 → 2)-linked rhamnose[3,4] and the study on the frequency and the regularity of these interruptions has led to conflicting results. Powell et al. suggested[5], from results of acid hydrolysis, that the polygalacturonic backbone was formed of repetitive uronate sequences of constant length (≈ 25 residues) between rhamnose residues for citrus, apple, and sunflower pectins. In contrast, other work based mainly on enzymic degradations[6–9] led to the conclusion that apple, beet, and citrus pectins have long and regular uronic regions ("smooth") and rhamnose-rich regions ("hairy") carrying the neutral sugars as side-chains. The "hairy" regions have been isolated[7,10,11]; they have high molecular weights and contain only a few percent of the initial galacturonic acid residues.

The aim of this work was to study the length of the "smooth" regions. Detection of repeating sequences was dependent on the use of suitably specific methods for cleaving the polymer backbone at precisely defined sites. Polygalacturonases, pectin-lyases, or pectate-lyases cannot be used for this purpose because they degrade the smooth regions which are under study; "rhamnogalacturonase", a new pectolytic enzyme acting on the "hairy" regions of native pectins, has no action on commercial pectins[12]. A possibility is to use the differences in susceptibility to acid hydrolysis of the glycosidic linkages. At pH values < 2, the linkages between uronic acid residues are very stable. They are notably more stable than the linkages between a uronic acid and a neutral sugar[13]; e.g., for the pectins, the linkages corresponding to the fragments (GalA 1 → 2 Rha) or (Rha 1 → 4 GalA). This leads to preferential cleavage of the linkages between galacturonic acid and rhamnose, thus liberating part of the smooth regions. Beet, citrus, and apple pectins have been studied in this paper; they were de-esterified in order to have the same resistance to acid hydrolysis of all the bonds in the galacturonic regions.

MATERIALS AND METHODS

Pectins.—HM citrus (degree of methylation, dm, of 72) and apple (dm = 75) pectins were from Copenhagen Pektinfabrik (Denmark) and Obipektin (Switzerland), respectively; sugar-beet pectin (dm = 54) was a gift of Copenhagen Pektinfabrik (lot No. 64863-0).

Purification and de-esterification.—The apple and citrus pectins were purified by precipitation with cupric ions[14]; sugar-beet pectins were purified only by alcoholic precipitation. They were de-esterified in conditions designed to minimise[15] degradation: solutions of pectins (1.5%) in water were chilled to 4°C. Cold NaOH (0.1 M) was added slowly to the pectin solution until pH 12; this pH was maintained for 24 h by addition of NaOH to the solution kept at 4°C with mild stirring. After 24 h, the pH of the solution was brought to 5 by 0.1 M HCl and the de-esterified pectins were precipitated by 4 vol of cold EtOH (96%). The precipitate was washed with

65% EtOH until the total disappearance of alkali, and finally dried by solvent exchange.

Hydrolysis.—The de-esterified pectins (0.1%, w/v) were hydrolysed in 0.1 M HCl at 80°C for up to 72 h in sealed tubes.

The mixtures were neutralised by 0.1 M NaOH before chromatography, and neutralised and made to 0.155 M in NaCl before measuring the viscosity.

Acid-soluble and acid-insoluble fractions were separated after cooling by centrifugation of the reaction mixture at 31000*g* for 15 min. The insoluble fraction was resolubilised by neutralisation for chromatography. It was brought to pH 6 (with 5 mL of 0.1 M NaOH) and made to 0.155 M in NaCl before measuring the viscosity. For light-scattering measurements, it was neutralised to pH 7.5 with 0.02 M NaOH and left overnight at 4°C under mild stirring, then dialysed against 0.05 M $NaNO_3$, heated to 60°C, and finally filtered through a 0.22-μm filter prior to injection.

Chemical characterisation of the samples.—The galacturonic acid content was determined by the *m*-hydroxybiphenyl method[16]; neutral sugar and galacturonic acid contents were obtained after methanolysis and HPLC fractionation[17]. The degree of methylation (dm, mol of MeOH per 100 mol of galacturonic acid) and degree of acetylation (da, mol of AcOH per 100 mol of galacturonic acid) were calculated from the amount of MeOH and acetic acid, respectively, as determined[18] by HPLC after total de-esterification and from the amount of galacturonic acid.

Physicochemical characteristics.—Gel-permeation chromatography was performed at room temperature on Sepharose CL-6B columns. Samples (1 to 7 mg) were loaded on a column (51 cm × 1.6 cm) of Sepharose CL-6B and eluted (upward direction) with degassed NaOAc buffer (ionic strength 0.05 M; pH 4) at a flow rate of 18.6 mL/h. Fractions (3 mL) were assayed for galacturonic acids and total neutral sugars (as galactose) by the *m*-hydroxybiphenyl method[16] and the orcinol method[19] after correction for interfering galacturonic acid, respectively. The results are expressed as a function of $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e, V_0, and V_t are the elution volume of the fraction, the void volume, and the total volume of the column, respectively. V_0 and V_t were determined as the elution volume of blue dextran and galacturonic acid, respectively.

Intrinsic viscosities ($[\eta]$, in mL/g) were obtained at 25°C by measuring the flow times of solutions of pectins in 0.155 M NaCl (specific viscosity range, 0.2–1.5) in an automatic Ubbelhode viscometer (Amtec, France) and by using the double extrapolation to zero concentration based on the Huggins and Kraemer equations[20].

To evaluate the polymer distribution and average molecular weights, pectins and pectic acids were analysed by aqueous, high-performance size-exclusion chromatography (HPSEC) coupled on-line with multi-angle laser-light-scattering (MALLS) photometry.

The HPSEC system consisted of a Waters 6000 A solvent delivery system, a Valco C6W injector with a 50-μL injection loop, an Erma ERC-3312 degasser, a Touzart & Matignon pulse dampener, an Erma ERC-7510 differential refractive index detector (RI), and three Shodex OHpak KB-800 series columns (Showa Denko K.K., Tokyo, Japan). The three columns (300 × 8 mm each), listed in the order connected, included KB-806, KB-805, and KB-804 (estimated exclusion limit using polyethylene glycol: 2×10^7, 4×10^6, and 4×10^5, respectively), and were immersed in a home-constructed water jacket at 40°C. The water used was taken from a Milli-Q water purification system supplied by Millipore and the eluent was 0.05 M $NaNO_3$ containing NaN_3 (200 ppm) (as a bactericide), carefully degassed and filtered through Millipore GS (0.22 μm) before use. The mobile phase had a flow rate of 1 mL/min and was filtered, on-line before the injector, through Millipore VC (0.1 μm).

The MALLS detector, a Dawn-F fitted with a K5 flow cell (Wyatt Technology Corp, Santa Barbara, CA), was installed on-line between the columns and the refractometer. The number-average molecular weight ($\overline{M}_n$) and weight-average molecular weight ($\overline{M}_w$) were determined with ASTRA software (version 2.02). A differential index of refraction (dn/dc) of 0.145 mL/g was employed[21]. All samples (concentration < 5.6 mg/mL) were made dust free by filtering through Millipore GS (0.22 μm) for molecular weights below 10^5 and through Millipore HA (0.45 μm) for higher molecular weights. Pullulan and dextran standards were used to verify that light-scattering results were consistent with suppliers' values.

RESULTS

Initial samples.—Pectins from apple, citrus, and beet were de-esterified (demethylated and, for the beet pectin, deacetylated) in order to obtain the same resistance towards acid of every linkage between galacturonic acids. The characteristics of the de-esterified pectins (Table I) show that the de-esterification process was efficient, as the resulting pectic acids have residual dm or da values of < 5%. The chemical composition is also shown in this table. The galacturonic acid content is in the range 66% (beet) to 83.2% (citrus), and the neutral sugars 4.7% (citrus) to 18.6% (beet); the rhamnose content was ~ 5% for the beet pectic acid, ~ 2% for the apple pectic acid, and ~ 1% for the citrus pectic acid.

The molecular weights of the initial pectic acids were measured by HPSEC–MALLS. The beet pectic acid showed (Fig. 1) a broad distribution of molecular weights with material eluting at the void volume of the column, and had a high polydispersity index (almost 4). It had the highest average molecular weight, in contrast to what is commonly found[22,23], and in contrast to the viscometric data (cf. Table I). Apple and citrus pectic acids had narrower distributions and lower polydispersity indexes (about 2.4).

General characterisation of the hydrolysed pectic acids.—The initial pectic acids were insoluble under the conditions of the acid hydrolysis. After some degradation, some pectic material became soluble, while the greater part (Table II) remained insoluble. Very high yields of insoluble blocks were obtained; after hydrolysis for 72 h, more than 80% of the apple and citrus pectins and 66% of the

TABLE I

Chemical composition (% w/w) and some physicochemical properties of the samples

	Apple	Citrus	Beet
GalA	82.5	83.2	66.0
Rha	2.2	1.1	4.8
Ara	0.4	0.9	4.9
Xyl	1.4	tr	tr
Man	tr	tr	tr
Gal	2.5	2.7	8.9
Glc	2.5	tr	tr
Degree of methylation [a]	3	3	5
Degree of acetylation [a]	0	0	3
$[\eta]$ (mL/g in 0.155 M NaCl)	267	323	284
$\overline{M}_n$	38000	48300	78700
$\overline{M}_w$	89000	119600	266000

[a] The degree of methylation and degree of acetylation are expressed as mol of methanol and acetic acid, respectively, per 100 mol of galacturonic acid.

beet pectin were still insoluble. The reaction mixture was neutralised to solubilise the insoluble material prior to global characterisation.

Viscosity measurements can be used to follow the degradation of polysaccharides and give values related to the kinetic constants. For a sample of N_0 monomers (of mass m_0) forming $N_{(0)}$ chains, the number of glycosidic bonds $L_{(0)}$ is:

$$L_{(0)} = N_0 - N_{(0)}. \tag{1}$$

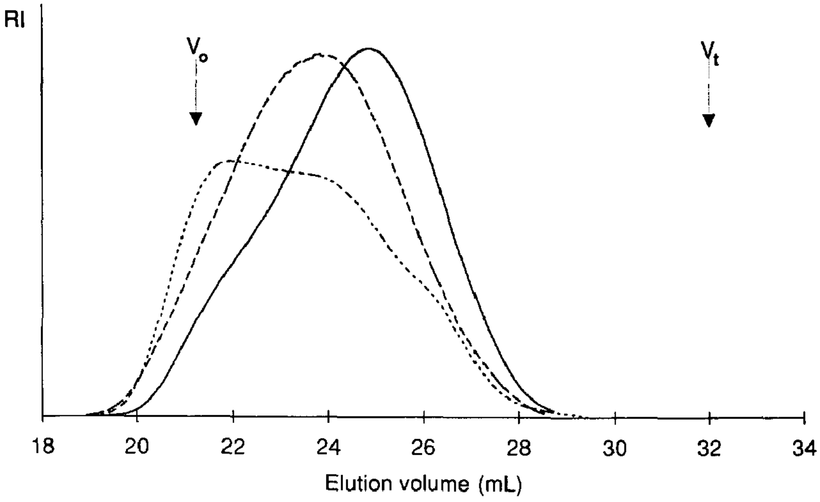

Fig. 1. HPSEC elution patterns of citrus (– –), apple (———), and beet (······) pectic acids.

TABLE II

Characterisation of the soluble and insoluble materials obtained after acid hydrolysis of pectic acids

	Fraction	Yield (% w)	Sugar composition (mol %)					
			Rha	Ara	Xyl	Gal	Glc	GalA
Apple								
5 h	Soluble	8.9	19.0	4.4	4.5	25.1	22.0	23.8
	Insoluble	91.1	1.2	0.1	1.2	1.0	0.5	96.1
24 h	Soluble	11.4	14.2	3.8	2.6	22.4	20.9	35.7
	Insoluble	88.6	0.7	0.1	1.2	1.0	0.5	96.1
72 h	Soluble	16.2	14.8	5.2	5.9	19.4	17.7	33.4
	Insoluble	83.8	0.3	0.0	1.1	0.1	0.2	98.1
Beet								
6 h	Soluble	18.4	15.1	28.2	0.0	32.5	1.0	23.3
	Insoluble	81.6	4.7	0.4	0.3	4.8	0.0	89.5
24 h	Soluble	28.0	17.4	20.5	0.0	32.7	0.0	26.7
	Insoluble	72.0	2.5	0.1	0.4	1.7	0.0	95.2
72 h	Soluble	33.6	17.5	16.8	0.0	30.4	1.0	34.3
	Insoluble	66.4	0.9	0.1	0.2	0.2	0.0	98.4
Citrus								
5 h	Soluble	5.0	14.4	26.0	0.0	31.7	1.4	23.4
	Insoluble	95.0	1.4	0.2	0.4	1.1	0.2	96.8
24 h	Soluble	7.1	18.2	16.6	1.0	31.1	1.6	28.4
	Insoluble	92.9	1.0	0.2	0.5	0.4	0.2	97.8
72 h	Soluble	10.5	14.5	15.8	0.5	24.5	1.5	40.0
	Insoluble	89.5	0.4	0.0	0.6	0.1	0.1	98.8

At the time t, the number of glycosidic bonds $L_{(t)}$ becomes

$$L_{(t)} = N_0 - N_{(t)}, \tag{2}$$

with $N_{(t)}$ chains.

If we assume that acid hydrolysis of polysaccharides is a first-order cleavage reaction, the relationship between $L_{(0)}$ and $L_{(t)}$ is:

$$L_{(t)} = L_{(0)}\,\mathrm{e}^{-kt}, \tag{3}$$

which can be transformed into

$$1 - N_{(t)}/N_0 = \left(1 - N_{(0)}/N_0\right)\mathrm{e}^{-kt}. \tag{4}$$

With $\overline{dp}_{n(t)}$ (average degree of polymerisation at time $t) = N_0/N_{(t)}$, eq (*4*) can be transformed into

$$1 - 1/\overline{dp}_{n(t)} = \left(1 - 1/\overline{dp}_{n(o)}\right)\mathrm{e}^{-kt}, \tag{5}$$

or, supposing that $\overline{dp}_{n(t)} \gg 1$ and $kt \ll 1$, eq (*5*) becomes

$$1/\overline{dp}_{n(t)} - 1/\overline{dp}_{n(0)} = kt, \text{ or} \tag{6}$$

$$1/\overline{M}_{n(t)} - 1/\overline{M}_{n(0)} = (k/m_0)t, \tag{7}$$

with $\overline{M}_{n(t)}$ = number-average molecular weight at time t.

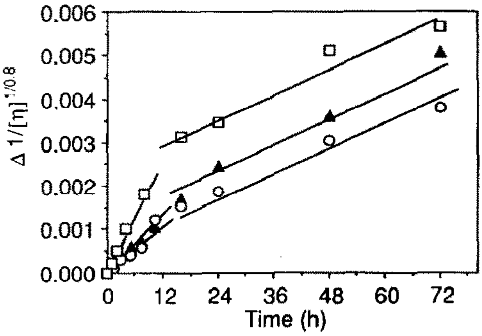

Fig. 2. Hydrolysis followed by intrinsic viscosity measurements of apple (-▲-), citrus (-○-), and beet (-□-) pectic acids by 0.1 M HCl at 80°C.

If the hydrolysis is random, the polydispersity of the sample should not be modified during hydrolysis. Taking $p=\overline{M}_n/\overline{M}_v$ ($\overline{M}_v$ = viscosity-average molecular weight) as a polydispersity index, and assuming that the polymer follows the Mark–Houwink relation ($[\eta]=K\overline{M}_v^a$) whatever its molecular weight, eq (*6*) becomes:

$$1/[\eta]_{(t)}^{1/a}-1/[\eta]_{(0)}^{1/a}=\left(K^{1/a}m_0/p\right)kt=k_{\mathrm{obs}}t.$$

The intrinsic viscosity of the reaction mixtures was studied after resolubilisation of the whole hydrolysates. The values decreased very rapidly and steadily, and reached close values, around 60–75 mL/g, after 72 h of hydrolysis.

Fig. 2 shows the values of intrinsic viscosities plotted as $1/[\eta]_{(t)}^{1/a}-1/[\eta]_{(0)}^{1/a}$, with[8] $a=0.8$. Two distinct stages can be observed, each one being linear, indicating that hydrolysis follows two consecutive pseudo-first-order kinetics. The first one is rapid (hydrolysis time < ~ 10h) and was interpreted as the hydrolysis of the weaker linkages between rhamnose and galacturonic acids. This would explain the differences in the values of the observed rate constant (2.2×10^{-4}, 1.1×10^{-4}, and 0.9×10^{-4} h^{-1} for beet, apple, and citrus pectic acids, respectively), which correspond to decreasing amounts of rhamnose. The second stage was characterised by a slower degradation, with similar values of the observed rate constant ($\sim 0.5\times10^{-4}$ h^{-1}) for all the pectic acids. It could correspond to the slow hydrolysis of the more resistant linkages between galacturonic acids.

After neutralisation, the hydrolysed pectic acids were chromatographed on Sepharose CL 6B. Some typical elution patterns of citrus and apple hydrolysed pectic acid are indicated in Fig. 3.

For all the substrates, the first (< 5 h) stages of the hydrolysis led to the appearance of products rich in neutral sugars which were eluted at the total volume of the column. The side chains, constituted of arabinans and arabinogalactans[9,23] and containing the more labile linkages, were rapidly hydrolysed to small

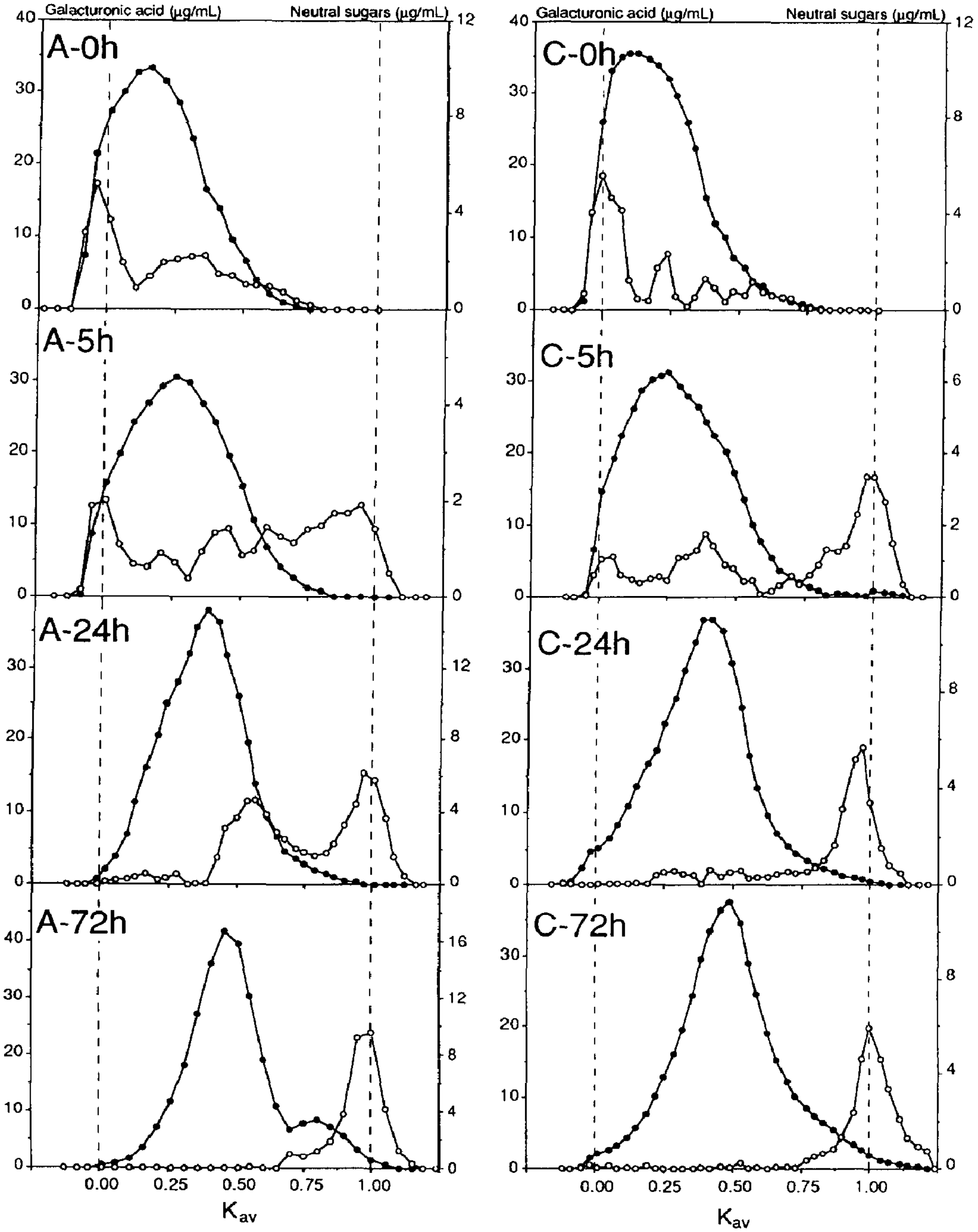

Fig. 3. Elution patterns of apple (A) and citrus (C) pectic acids on Sepharose CL-6B after 0, 5, 24, and 72 h of hydrolysis by 0.1 M HCl at 80°C (galacturonic acid, –●–; neutral sugars, –○–).

fragments. The galacturonic acid peaks showed comparatively little change: they shifted toward higher K_{av} values and became more symmetrical.

As the hydrolysis proceeded, the peaks constituted mainly of galacturonic acid residues moved progressively to higher K_{av} values, at the same time becoming more symmetrical and narrower. This shift was marked for hydrolysis up to 24 h but seemed much slower between 24 and 72 h. Finally, a peak enriched in galacturonic acid remained at a $K_{av} \sim 0.5$ for all the pectic acids. The proportion of neutral sugars in the low K_{av} fraction diminished during the hydrolysis, until they were almost all present at the total volume or, for a minor fraction, in a tail of the main galacturonic acid peak at about K_{av} 0.8.

The soluble and insoluble materials were separated by centrifugation and further studied separately.

Characterisation of the acid-soluble materials.—The amounts and composition of the acid-soluble products are given in Table II. They had some common features whatever the origin of the pectin. Arabinose residues were first and very rapidly released (~ 100% after 24 h); galactose residues were freed more slowly; 90% after hydrolysis for 24 h for all the substrates, and 99% after 72 h. The rhamnose units were also liberated by the hydrolysis; after 72 h, 91–92% of the rhamnose for the beet and apple pectic acids and 84% for the citrus sample were recovered in the acid-soluble fractions. Xylose, which was present in significant amounts only in apple pectic acids, is more resistant to acid hydrolysis since only 50% was recovered in the apple acid-soluble material; beet or citrus pectic acid contained only traces of xylose, which was virtually absent in their acid-soluble materials. The acid-soluble materials contained some galacturonic acids; 17, 7, and 5% of the initial galacturonic acids were recovered in these materials from beet, apple, and citrus pectic acids, respectively.

The acid-soluble materials were chromatographed on a Sepharose CL-6B column (Fig. 4). The elution patterns and their evolution during the hydrolysis are remarkably similar for all the substrates. Generally speaking, two peaks can be observed: a peak very rich in neutral sugars, always eluted at the total volume of the column, and a peak rich in galacturonic acid. The latter peak is eluted at K_{av} values that increase with the hydrolysis time: for all the pectic acids, it has a K_{av} of 0.7 for 24-h hydrolysis, which tends to 0.8 for 72-h hydrolysis. The material eluted at $K_{av} < 0.85$ contained associated neutral sugars, mostly rhamnose (Table III). After 72-h hydrolysis, most of the rhamnose of the acid-soluble materials (about 50% for the beet, 70–75% for the citrus and apple) was recovered in this peak, whereas most of the arabinose (80–100%) and galactose (95–86%) were recovered in the peak at the total volume. The rhamnose–galacturonic ratios in the first fraction were very high: 1:3 (beet), 1:1.5 (apple), and 1:2 (citrus), as compared, for example, to the 1:3 ratio reported[24] for whole apple hairy regions obtained by enzymic hydrolysis. The high proportions of glucose and mannose found in the apple and citrus samples might be due to contamination.

These results may be caused by the presence, in the acid-soluble products, of two types of pectin fragments: degraded side-chains mainly constituted of arabinans and arabinogalactans, at the total volume, and the rhamnogalacturonic backbone of the hairy regions in the peaks at K_{av} 0.8. During hydrolysis, the

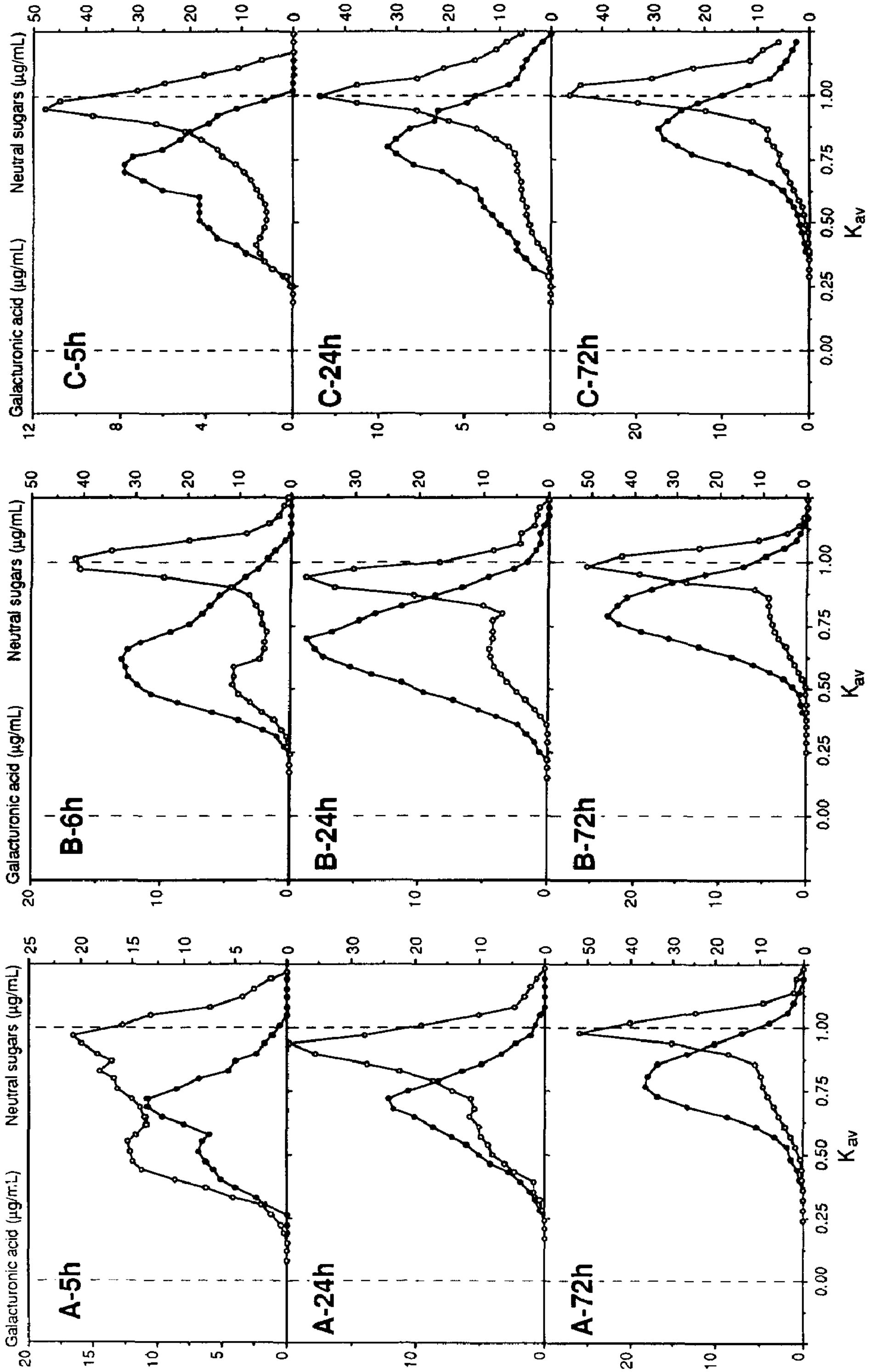

Fig. 4. Elution patterns of apple (A), beet (B), and citrus (C) acid-soluble pectic acids on Sepharose CL-6B after 0, 5, 24, and 72 h of hydrolysis by 0.1 M HCl at 80°C (galacturonic acid, -●-; neutral sugars, -○-).

TABLE III

Sugar composition of the galacturonic-rich fraction of the acid-soluble materials (eluting at $K_{av} < 0.85$)

Sugars (mol%)	Apple	Beet	Citrus
Rha	28.4	23.9	27.2
Ara	2.8		1.8
Xyl	2.8		
Man	0.8		0.5
Gal	8.5	5.2	8.1
Glc	14.8	0.6	6.2
GalA	42.0	70.3	56.2

neutral side chains are rapidly split off as small fragments, and the inner linkages of the "hairy region" backbone and the attached neutral sugars are progressively hydrolysed. The amounts of acid-soluble products and the relative proportions of the two populations differ with the origin of the sample, but their composition and size are very similar.

Characterisation of the acid-insoluble materials.—The insoluble materials represented most of the original pectic acid (Table II). After 72 h, 66, 83, and 90% of beet, apple, and citrus pectic acids, respectively, were recovered as acid-insoluble material.

The sugar analysis showed that these fractions, whatever their origin (beet, apple, or citrus), were progressively freed of arabinose, galactose, or glucose residues. The acid-insoluble materials obtained after 72-h hydrolysis were nearly exclusively built of galacturonic acid residues (more than 98%) and rhamnose residues. These fractions contained 92.7, 83.4, and 94.8% of the initial galacturonic acids and 9, 8, and 16% of the rhamnose residues of apple, beet, and citrus pectic acids, respectively. There was one rhamnose for 300, 114, and 267 galacturonic acid residues for apple, beet, and citrus pectic acid, respectively. Xylose resists hydrolysis better than arabinose and galactose, as noted earlier, and appears to be bound directly to the galacturonic acid residues, in contrast to arabinose and galactose.

The chromatography on Sepharose CL-6B of the acid-insoluble materials gave very similar elution patterns for all three samples (Fig. 5). After 5-h hydrolysis, they showed an asymmetrical polygalacturonic acid peak, with neutral sugars associated with the highest molecular weight fraction. Compared to the initial pectic acids (Fig. 3), there was already a diminution of the neutral sugars and a shift towards a higher K_{av} value. This trend continued during the hydrolysis, accompanied by an increase of the symmetry of the peak. After 72-h hydrolysis, the acid-insoluble material formed a narrow symmetrical peak of mostly pure polygalacturonic acid at K_{av} 0.5 for all three samples. These results suggest also that the molecular weights of these resistant fractions are similar.

The determination of the intrinsic viscosity as well as the molecular weights of the insoluble fraction obtained after 72-h hydrolysis (Table IV) confirm these

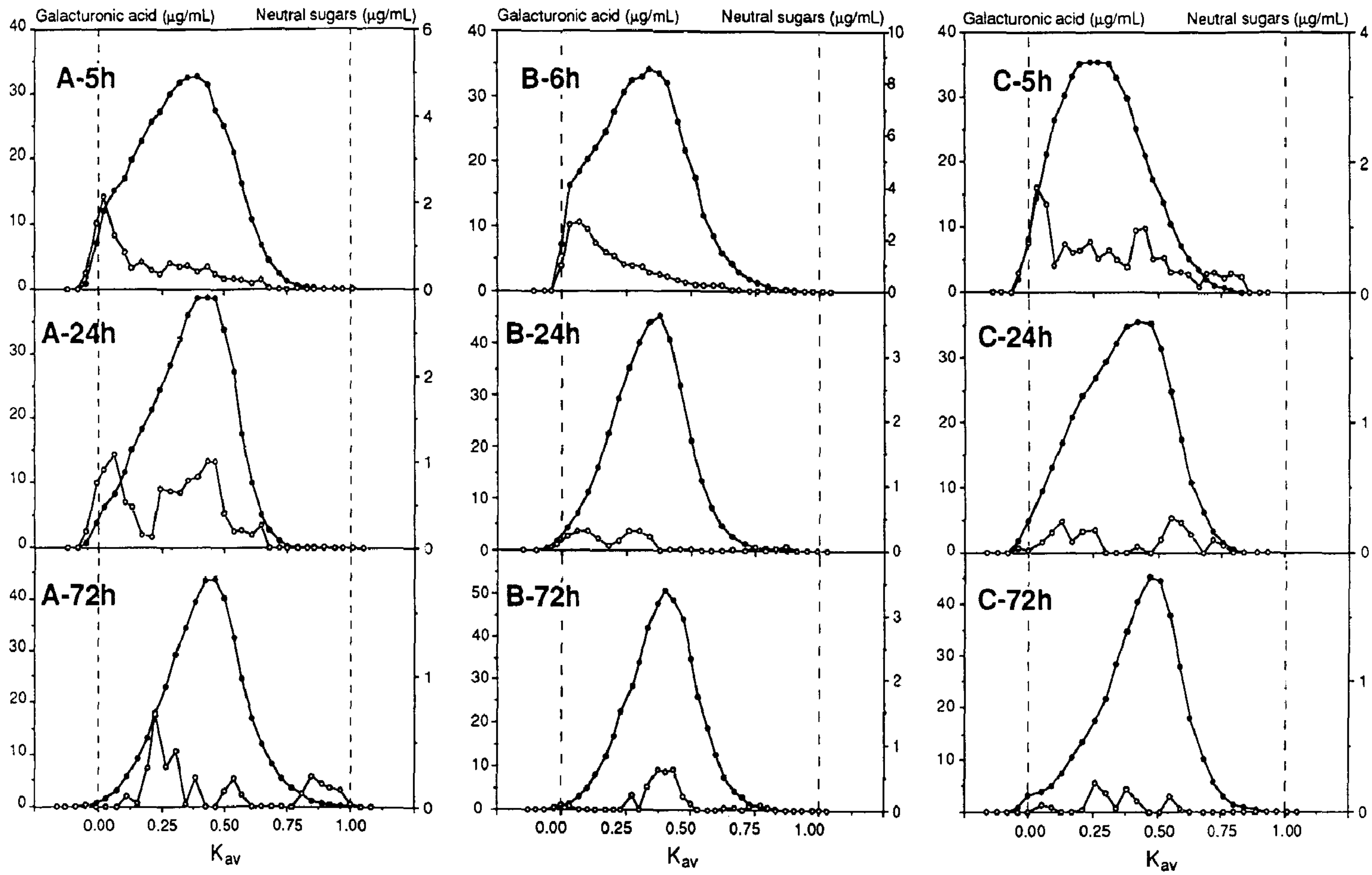

Fig. 5. Elution patterns of apple (A), beet (B), and citrus (C) acid-insoluble pectic acids on Sepharose CL-6B after 0, 5, 24, and 72 h of hydrolysis by 0.1 M HCl at 80°C (galacturonic acid, -●-; neutral sugars, -○-).

TABLE IV

Intrinsic viscosity (mL/g; in 0.155 M NaCl) and average-molecular weights of the insoluble material from acid hydrolysis of apple, beet, and citrus pectic acids

Substrate	Hydrolysis (h)	[η]	$\overline{M}_w$	$\overline{M}_n$
Apple	5	244		
	24	155		
	72	77	21200	12700
Beet	6	265		
	24	185		
	72	92	19000	16000
Citrus	5	312		
	24	193		
	72	86	24200	20000

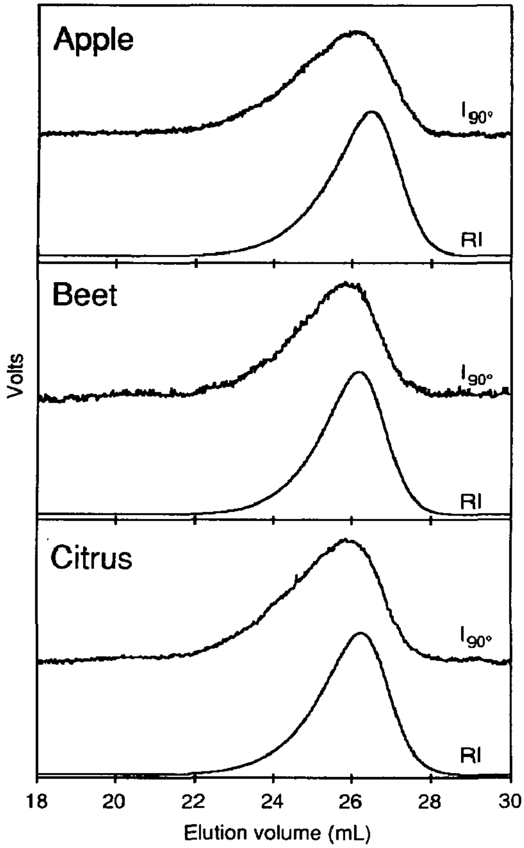

Fig. 6. HPSEC–MALLS of the acid-insoluble pectic acids obtained after 72 h of hydrolysis by 0.1 M HCl at 80°C.

results. The acid-insoluble materials had similar intrinsic viscosities, between 77 and 92 mL/g. They gave a single narrow and symmetrical peak by chromatography (Fig. 6), and here again the elution patterns were almost identical. The molecular weights determined by HPSEC–MALLS are given in Table IV. From these results, number- and weight-average degrees of polymerisation of 120–72, 108–91, and 138–114 could be calculated for apple, beet, and citrus, respectively.

DISCUSSION

Pectic acid is insoluble in an aqueous acid medium and the hydrolysis was therefore a heterogeneous reaction. The degradation under such conditions is a complex physical and chemical process involving acid diffusion into the polymer and the splitting of glycoside bonds[25]. From their studies of the hydrolysis of alginates, Smidsrød et al. concluded[26] that heterogeneous (acid medium) and homogeneous hydrolysis (pH 4) reactions followed pseudo-first-order kinetics. Our results showed that the hydrolysis of (insoluble) pectic acid followed two pseudo-first-order kinetics.

There is not, however, a succession of two reactions, but superposition of two mechanisms of different speed and probability. The viscosity data allow a rough estimation of the number of bonds broken in the rhamnogalacturonic backbone and thus of the pseudo-first-order rate constant. However, a complete theoretical treatment is not possible here because the polydispersity of the samples is too large and changes during hydrolysis, and because the conformation of the pectins and, therefore, the value of the constant *a* in the Mark–Houwink relationship, may be changed by splitting the rhamnose residues, which can act as flexible kinks[20].

However, we have isolated, after mild acid hydrolysis of pectins, almost pure polygalacturonates, which are representative of the resistant "smooth" regions. Resistant does not mean that the bonds cannot be broken, but that the rate of the splitting of the resistant linkages is much lower than that of the hydrolysable linkages in the "hairy" regions. Indeed, though faster cleavage at the rhamnose residues allowed isolation of long homogalacturonic acid sequences, the internal bonds between uronic acids are also susceptible to acid hydrolysis, so that the length of those homogalacturonans underestimates that of the "smooth" regions. The minimal length thus calculated is of 72–100 galacturonic acid residues, and it is similar for pectins from apple, beet, and citrus. Our results show that the minimal distance between rhamnose residues is much more than the 25 units reported[5] by Powell et al. However, they used harsher hydrolysis conditions (0.5 M HCl, 3 h, and 100°C), and isolated the uronic blocks on a Sepharose G-50 column eluted in water, i.e., in conditions where the separation of uronides bears little relation to their size[27,28]. They obtained mostly one single sharp peak, at the total volume. The average size of the hydrolysed material, which was not chemically characterised, was estimated by measuring the reducing power, using an arbitrary rhamnose calibration. The size of the uronic blocks thus calculated (25 units) implies a minimum rhamnose content of 4%. The amount of rhamnose, though it may be underestimated[17] by the methods generally used, is not constant within the pectins and rarely reaches 4%. Konno et al. obtained[29], under quite similar conditions (1 M HCl, 4 h, and 80–90°C), a whole range of hydrolysis products, including oligomers.

We have used milder hydrolysis conditions, starting from demethylated pectins, so that all the linkages between galacturonic acids had the same probability of being cleaved. Due to their low dm, these pectic acids were initially insoluble in the reaction mixture, and after some time part of the hydrolysed material became soluble. We have obtained three fractions, which can each be related to one of the main structural features of pectins. The neutral side chains, composed[6,10] mostly of arabinans and arabinogalactans, were rapidly split off and hydrolysed to low molecular weight fragments. The rhamnose-rich backbone of the "hairy" regions formed a separate fraction with an intermediate size and a high rhamnose–galacturonic acid ratio. Finally, the "smooth" regions gave galacturonans that remained insoluble.

Another possibility[26,30] could be that the easily hydrolysable parts occur as separate molecules. The data of intrinsic viscosity and of gel-permeation chromatography indicated, however, that the resistant fractions are formed by splitting of long chains. These regions are therefore distributed along the pectic chain and separated by the more easily hydrolysable part of the molecule.

Therefore, it seems justified to conclude from these results that the pectins from apple, beet, or citrus are composed of repeating units which are "smooth" (homogalacturonic) regions and "hairy" regions (part of the rhamnogalacturonan rich in side chains). The length of the "smooth" regions is independent of the origin of the pectins, but the various proportions of rhamnose (and other neutral sugars) show that this is not the case in the "hairy" regions. The beet pectins thus have a higher proportion of "hairy" regions than both the apple and citrus pectins. Another noticeable fact is that at least part of the xylose residues appear to be linked directly to the galacturonic acid residues of the "smooth" regions.

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