Apple Juice Pectic Substances

X. Rouau & J.-F. Thibault

Centre de Recherches Agro-Alimentaires, Laboratoire de Biochimie et Technologie des Glucides, 44072 Nantes Cédex, France

SUMMARY

Apple juice pectic substances are characterised by a high content of galacturonic acids (75·6%), a high degree of esterification (83), a low content of neutral sugars (0·109 moles/mole of galacturonate residues) and a high molecular weight ($\bar{M}_v = 95\,000$). Degradation by heat (β-elimination) and by endopolygalacturonase showed that the pectic macromolecules consist of long homogalacturonan regions (92% of total galacturonides) and of 'hairy' regions in which neutral sugars form long or short side-chains.

INTRODUCTION

Pectic substances are polyuronides composed mainly of 1,4-linked α-D-galacturonic acid (or its methyl ester) with associated neutral sugars, typically galactose and arabinose as neutral side-chains, and 2-linked rhamnose units which are present in the polygalacturonic backbone (Pilnik & Voragen, 1970; Darvill *et al.*, 1980). They are the major components of the primary cell walls and of the middle lamella of plant tissues, especially those of fruit tissues. Apple juice is rich in dissolved pectins which can suspend other materials in a stable colloidal system (Endo, 1965*a*). The resultant cloudy juice can be clarified by ultrafiltration (Heatherbell *et al.*, 1977) or, more usually, by an enzymic treatment (Rombouts & Pilnik, 1978). Thus, the clarification is

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achieved (i) by the degradation of juice pectins by the combined action of fungal pectinesterases and endopolygalacturonases (Endo, 1965*b*; Yamasaki *et al.*, 1967) or by the single action of pectin-lyase (Ishii & Yokotsuka, 1972) or (ii) by the formation of a calcium pectate gel resulting from the addition of fungal pectinesterase and calcium salts (Calvez *et al.*, 1978; Baron *et al.*, 1981). The action of these enzymes has been extensively studied. In contrast, the literature on apple juice pectic substances is very scarce. This paper deals with the chemical composition and some structural features of these pectic substances by means of chemical (alkaline) and endopolygalacturonase degradations.

MATERIALS AND METHODS

Isolation of pectins

Ripe apples (S III 5 cultivar) were harvested in 'Station de Recherches Cidricoles' (INRA, Rennes, France) in December 1980. In February 1981 they were cut and pressed with a pack press. The juice yield was 54·6 litres per 100 kg of apples. Pectins were precipitated with 80% boiling ethanol. After centrifugation, the precipitate was washed with 70% ethanol until the eluate was no longer coloured by the anthrone reagent. The precipitate was then solubilised in distilled water, centrifuged at 12 000 *g* for 15 min and freeze-dried.

Analyses

The anhydrogalacturonic acid (AGA) content was measured by the automated *m*-hydroxydiphenyl method (Thibault, 1979). Degrees of methylation (DM) were calculated from the AGA content and the methoxyl content determined colorimetrically (Wood & Siddiqui, 1971). AGA content and DM were also determined by titration (Schultz, 1965).

Individual neutral sugars were analysed by gas chromatography as their alditol-acetates (Sawardeker *et al.*, 1965). Samples (about 50 mg) were hydrolysed with 2 M trifluoroacetic acid at 120°C for 1 h (Albersheim *et al.*, 1967). Total neutral sugars were determined by the orcinol method (Tollier & Robin, 1979) and values were corrected for the

interference due to galacturonic acids and results are expressed in arabinose equivalents.

The intrinsic viscosity ($[\eta]$, dl g^{-1}) value was obtained at 25°C with an Ostwald viscometer (solvent flow time = 93·7 s). Pectin was dissolved in 0·155 M NaCl and 0·005 M EDTA. The viscosity-average molecular weight ($\bar{M}_v$) was calculated according to Owens *et al.* (1946).

De-esterification

Aqueous solutions of pectins (0·2%, w/v) were maintained at 2°C in an ice/salt bath and the pH was adjusted to 13 with 1 M NaOH at 2°C. After 1 h, the solution was brought to pH 4·5 with 1 M HCl. The solution was then dialysed against distilled water and freeze-dried.

β-elimination

A sample (0·5%, w/v) in phosphate buffer (0·1 M, pH 6·8) was treated at 80°C (Albersheim *et al.*, 1960). At appropriate intervals, 0·05 ml of the reaction mixture was diluted with 3 ml of distilled water and the UV absorbance at 235 nm was measured. The reaction was stopped by adjusting the pH to 4 with 0·1 M HCl and the solution was dialysed against distilled water. The percentage of $\alpha(1 \rightarrow 4)$ linkages split was calculated assuming that the molar extinction coefficient for the esterified 4,5-unsaturated galacturonic acid residue is 5500 M^{-1} cm^{-1} (Edstroem & Phaff, 1964).

Hydrolysis by endopolygalacturonase

A purified endopolygalacturonase (endoPG; EC 3.2.1.15) (Thibault & Mercier, 1977) was used for the degradation of pectin samples. Reaction mixtures, at 30°C, contained pectin samples (0·2%, w/v) in acetate buffer (0·05 M, pH 4·15) and enzyme (0·4 nkat ml^{-1}). Hydrolysis values were calculated from the increase of reducing power as determined by the method of Nelson (1944) using galacturonic acid as standard. The reaction was also followed by the decrease of the specific viscosity of the solution, measured with an Ostwald viscometer (solvent flow-time = 82·5 s).

Gel chromatography

Ion-exchange chromatography was performed on DEAE-Sepharose-CL-6B (Pharmacia) columns (7.5×1.6 cm) equilibrated with sodium acetate buffer (ionic strength $= 0.05$), pH 4.8. The pectin sample (4 mg in this buffer) was loaded on to the column and the gel was washed with 50 ml of the buffer at a flow-rate of 40 ml h^{-1}. The bound material was then eluted by a linear sodium acetate gradient at pH 4.8 (ionic strength $= 0.05-1$; 90 ml) and 3-ml fractions were collected.

Pectins were chromatographed on a Sepharose-CL-2B column (86×2.6 cm) and after degradation on a Sephadex G-100 column (80×2.2 cm) and on a Bio-gel P2 column. Elutions on Sepharose-CL-2B and on Sephadex G-100 were carried out in ascending direction with sodium acetate buffer (ionic strength $= 0.1$), pH 4.0, at a flow-rate of 15 ml h^{-1}. Samples of pectin (up to 100 mg) dissolved in the same buffer were applied to the column and 4-ml fraction, were collected. Results were 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 of the column and the total volume of the column, respectively.

Chromatograms were recalculated for 8 mg of injected AGA (Sepharose-CL-2B) and for 2 mg of injected AGA (Sephadex G-100). Chromatography on Bio-gel P2 was performed as described elsewhere (Thibault, 1980). About 1 mg of degraded pectins were injected on to the column and aliquots of the effluent (6 ml h^{-1}) were continuously analysed by the m-hydroxydiphenyl and the orcinol methods. Elution profiles represent the absorbance at 520 nm (m-hydroxydiphenyl detection) and at 425 nm (orcinol detection) versus elution volume without correction for the interference of AGA during orcinol detection.

RESULTS

Crude pectins

The AGA content of the apple juice was 1.2 mg ml^{-1}. The pectins were recovered by precipitation with 80% ethanol. Ion-exchange chromatography on DEAE-Sepharose-CL-6B (Fig. 1) showed that only 1% (w/w) of this material was not bound to the gel. The gradient in acetate released the pectic substances in a single peak containing both AGA and neutral sugars in a constant ratio throughout the peak.
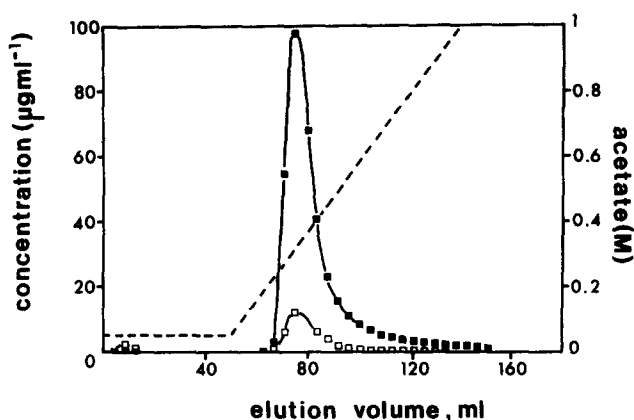
Fig. 1. Ion-exchange chromatography of apple juice pectins on DEAE-Sepharose-CL-6B. The column is first washed with sodium acetate buffer (ionic strength = 0·05), pH 4·8. Bound material was eluted by a linear sodium acetate gradient at pH 4·8 (ionic strength = 0·05-1; 90 ml). □, Neutral sugars; ■, galacturonic acids.

The chemical composition is shown in Table 1. The apple juice pectin is characterised by a high AGA content (75·6% by the *m*-hydroxydiphenyl method, 76·2% by titration) and a high DM (83·0 from the colorimetric determination of methoxyl groups, 85·2 by titration). The neutral sugar content is 0·109 moles/mole of galacturonate residues. Arabinose and galactose, in approximately equal amounts,

TABLE 1

Chemical Composition of Apple Juice Pectins
(Molar ratio of components to galacturonic acid)

Component	Crude pectin	De-esterified pectin	Pectin after β-elimination
Galacturonic acid	100	100	100
Rhamnose	0·9	0·7	0·7
Arabinose	5·2	4·3	5·0
Xylose	0·2	0·2	0·2
Galactose	4·3	2·6	3·4
Glucose	0·3	0·3	0·3
Methanol	83·0	<2·0	36·0

account for 78% (w/w) of total neutral sugars. Xylose and glucose appear as minor constituents while mannose has not been detected. These sugars are known to form side-chains linked to the main chain via C-2 or C-3 of galacturonic acid and presumably via the C-4 of rhamnose units (Pilnik & Voragen, 1970; Darvill *et al.*, 1980). From the rhamnose content, it can be calculated that there is one 'kink' (Rees & Wight, 1971) per 116 galacturonic acid residues. Viscosity measurements led to an intrinsic viscosity value of 6·6 dl g^{-1}, corresponding to a viscosity-average molecular weight of 95 000. The Huggins coefficient was 0·47.

The fractionation of apple juice pectins by gel-permeation chromatography on Sepharose-CL-2B (Fig. 2(a)) demonstrated that they have large hydrodynamic volumes and a wide molecular weight distribution. The pectic fraction eluted at the void volume of the column, accounting for 3% of the total material, is richer in neutral sugars (molar ratio AGA/neutral sugars, as arabinose = 1·5/1) than the main peak (AGA/neutral sugars = 7·6/1) eluted at K_{av} = 0·56. The fraction at the void
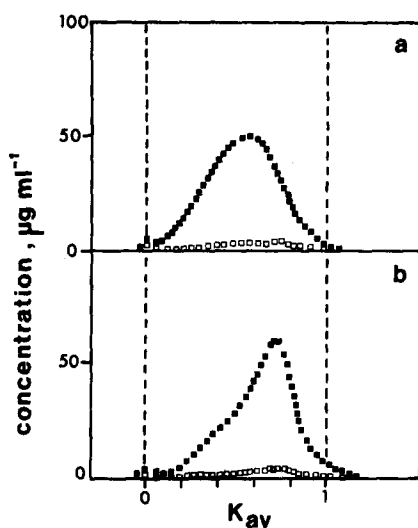

Fig. 2. Gel-permeation chromatography on Sepharose-CL-2B of apple juice pectins. Crude pectin (a) and alkali de-esterified pectin (b) were applied to a Sepharose-CL-2B column (V_0 = 180 ml; V_t = 480 ml) equilibrated with sodium acetate buffer (ionic strength = 0·1), pH 4·0. □, Neutral sugars; ■, galacturonic acids.

volume ($K_{av} < 0.1$), the pectic molecules with lowest molecular weight ($K_{av} > 0.5$) and the intermediate population ($0.1 < K_{av} < 0.5$) have DM values of 80, 90 and 75, respectively.

Enzymic degradation of the de-esterified crude pectin

Pectins were alkali de-esterified. Conditions were chosen in order to minimise chain cleavages by β-elimination (Albersheim *et al.*, 1960). Gel-permeation on Sepharose-CL-2B (Fig. 2(b)) of the de-esterified pectin shows that there is only a slight increase in the K_{av} of the main peak ($K_{av} = 0.70$). No intrinsic viscosity value could be obtained because of the insolubility of the pectate in the 0.155 M NaCl medium used for the measurements. The chemical composition of the de-esterified pectin is indicated in Table 1. The DM is less than 2 and the neutral sugar composition is close to that of the crude pectin, except for galactose, some of which is lost.

The resultant pectate was degraded by an endopolygalacturonase (endoPG). The course of degradation was monitored by the increase of reducing groups and by the decrease of specific viscosity of the solution. The reaction was stopped at 25 h. The percentage of hydrolysis and the decrease of specific viscosity were 30% and 94%, respectively (Fig. 3).

The reaction mixture was chromatographed on Sephadex G-100 and on Bio-gel P2. On Sephadex G-100 (Fig. 4(a)) the degraded pectate was
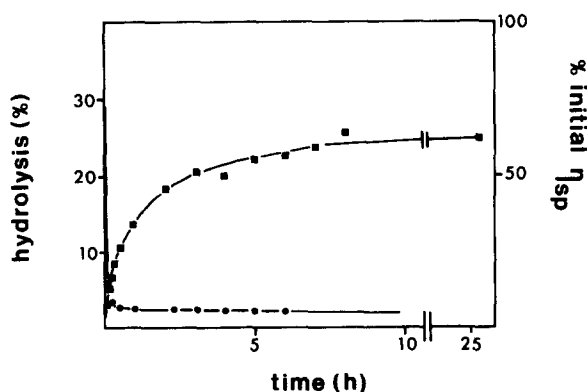

Fig. 3. Enzymatic degradation of de-esterified pectin. Percentage hydrolysis (■) by endopolygalacturonase was calculated from the increase in reducing groups; specific viscosity (●) was measured and expressed as % of initial specific viscosity.
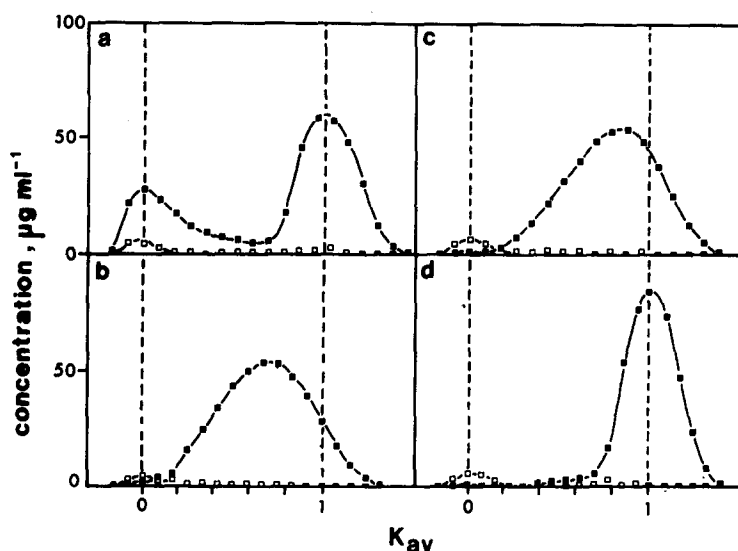
Fig. 4. Gel-permeation chromatography on Sephadex G-100 of degraded pectins. Samples were applied to a Sephadex G-100 column ($V_0 = 45$ ml; $V_t = 113$ ml) equilibrated by sodium acetate buffer (ionic strength $= 0\cdot1$), pH $4\cdot0$. □, Neutral sugars; ■, galacturonic acids. (a) Pectin after de-esterification and endopolygalacturonase degradation; (b) pectin after β-elimination; (c) pectin after β-elimination and endopolygalacturonase degradation; (d) pectin after β-elimination, de-esterification and endopolygalacturonase degradation.

eluted in two peaks: one at the void volume of the column accounting for 22% and 57% of total AGA and neutral sugars, respectively, and the other at the total volume representing 78% and 43% of total AGA and neutral sugars. On Bio-gel P2 (Fig. 5(a)), the same reaction mixture was separated in a series of peaks 1, 2, ..., 7 with the same elution volumes as oligogalacturonides of degree of polymerisation of 1, 2, ..., 7, and material excluded from the column.

Effluents were continuously detected for their AGA content (*m*-hydroxydiphenyl method) and for their neutral sugar or AGA content (orcinol method). For each peak, the ratio between the area obtained from the orcinol detection and the area obtained from the *m*-hydroxydiphenyl detection were calculated. The peaks 1–7 are characterised by a constant ratio equal to that obtained under the same conditions for a galacturonic acid standard, indicating that these peaks correspond to
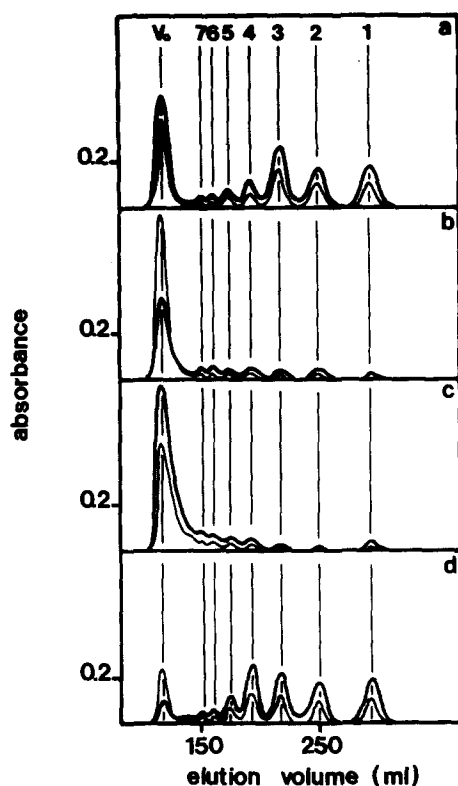
Fig. 5. Gel-permeation chromatography of degraded pectins on Bio-gel P2. Samples were loaded on to a Bio-gel P2 column (203 × 2 cm) equilibrated at 65°C with sodium acetate buffer (ionic strength = 0·1), pH 3·6. The eluate was continuously analysed: (━) absorbance at 520 nm (*m*-hydroxydiphenyl method) and (—) absorbance at 420 nm (orcinol method). V_0 = void volume; numbers 1–7 refer to degree of polymerisation of oligogalacturonides. (a) Pectin after de-esterification and endopolygalacturonase degradation; (b) products obtained after dialysis of β-eliminated pectin; (c) pectin after β-elimination and endopolygalacturonase degradation; (d) pectin after β-elimination, de-esterification and endopolygalacturonase degradation.

true oligogalacturonides, accounting for 68% of total AGA. In contrast, material eluted at the void volume contains AGA (32% of the total) and all the neutral sugars.

The material eluted at the void volume of the Sephadex G-100 column is necessarily excluded from the Bio-gel P2 column. Therefore

combined results of these two chromatograms show that products from pectin after de-esterification and endo-PG-degradation can be divided into three classes: oligogalacturonides, from the Bio-gel P2 elution pattern and material containing both AGA and neutral sugars but different by their hydrodynamic volumes from the Sephadex G-100 and Bio-gel P2 fractionations. The intermediate-molecular-weight product (10% of total AGA, 43% of total neutral sugars) contains 0·3 moles of neutral sugars/mole of AGA while the high-molecular-weight fragments (22% of total AGA, 57% of total neutral sugars) have 0·2 moles of neutral sugars/mole of AGA.

Enzymic degradations of β-eliminated pectins

Crude pectins were submitted to β-elimination by heat treatment. The resultant 4,5-unsaturated galacturonides were estimated by UV absorption (Fig. 6). After 4 h, the reaction was stopped and it was calculated that 11% of the α-(1 → 4) linkages were split.

The low-molecular-weight products (20% w/w of β-eliminated products) obtained after dialysis were chromatographed on Bio-gel P2 (Fig. 5(b)). Of the total AGA, 5·1% was eluted as pure oligogalacturonides. Non-dialysable material was applied to a Sephadex G-100 column (Fig. 4(b)) and separated into two populations: a high-molecular-weight fraction eluted at the void volume of the column (1·3% of total AGA, 50% of the total neutral sugars) with 5 moles of neutral sugars/mole of
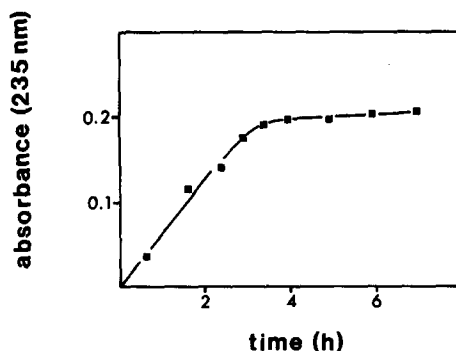

Fig. 6. Kinetics of β-elimination of pectins. Pectins were dissolved in 0·1 M phosphate buffer, pH 6·8, at 80°C. At appropriate intervals, 0·05 ml of the reaction mixture were diluted with 3 ml of distilled water and UV absorption at 235 nm was measured.

AGA and an intermediate-molecular-weight fraction eluted at $K_{av} = 0.7$ (93·6% of the total AGA, 50% of the total neutral sugars) containing 0·1 moles of neutral sugars/mole of AGA. The non-dialysable material was recovered and analysed (Table 1); the composition was quite similar to the crude pectin except for the DM which was reduced to 36. Chromatography on Sephadex G-100 showed that the distribution of the methoxyl groups was not regular since the material at the void volume ($K_{av} < 0.1$), the intermediate-molecular-weight fraction ($0.1 < K_{av} < 0.5$) and the low-molecular-weight products ($K_{av} > 0.5$) have DM values of 54·1, 37·7 and 27·2, respectively.

The β-eliminated pectin was submitted to endoPG degradation. Hydrolysis was very low ($<5\%$). As above, products were separated into three classes using Sephadex G-100 (Fig. 4(c)) and Bio-gel P2 (Fig. 5(c)) chromatographies: pure oligogalacturonides (22% of total AGA), intermediate-molecular-weight fragments (76·5% of total AGA, 43% of total neutral sugars) with a molar ratio neutral sugars/AGA less than 0·1 and high-molecular-weight fractions (1·5% of the total AGA, 57% of the total neutral sugars) containing 3·6 neutral sugars residues/AGA.

The β-eliminated pectin was then alkali de-esterified. Gel-permeation chromatography on Sephadex G-100 showed that the de-esterification led to very slight depolymerisation since the K_{av} is increased from 0·7 to only 0·8. The β-eliminated and de-esterified pectin has the same neutral sugars/AGA molar ratio as the parent pectin but the DM is 12·6. This material was further degraded by endoPG to 33% hydrolysis and products from pectin after β-elimination, de-esterification and endoPG-degradation were analysed by gel-permeation on Sephadex G-100 (Fig. 4(d)) and Bio-gel P2 (Fig. 5(d)). The products can be separated as above into three classes: pure oligogalacturonides accounting for 92% of total AGA, an intermediate-molecular-weight fraction (7·7% of total AGA, 50% of total neutral sugars) with 1·1 moles of neutral sugars/mole of AGA and high-molecular-weight fragments containing 0·3% of total AGA and 50% of total neutral sugars (molar ratio of 26·3 neutral sugars/AGA).

DISCUSSION

Pectins are the major component of the alcohol-insoluble material of apple juice since the DEAE-Sepharose-CL-6B column is able to bind as

much as 99% of this material. Apple juice pectins are very large mole-
cules with a high AGA content (75·6%), a low content of neutral sugars
(6·8%) with equal amounts of arabinose and galactose, and a high DM
(83). They differ from many other apple pectic fractions by their very
low content of neutral sugars. For example, the 'soluble-polymethyl-
galacturonate' (Knee, 1978), the acid-extracted (Barrett & Northcote,
1965) and the water-soluble (O'Beirne *et al.*, 1981) pectic fractions
have neutral sugar contents of 0·302, ≅0·2 and 0·139 moles/mole of
AGA, respectively. Apple juice pectins are quite similar to the EDTA-
soluble pectins, i.e. neutral sugars/AGA molar ratio of 0·06 (O'Beirne
et al., 1981). However, Knee (1973) pointed out that increasingly
insoluble pectins contain increasing amounts of neutral sugars, and
de Vries *et al.* (1981) reported that pectins with the lowest neutral
sugar content (neutral sugar/AGA = 0·167, w/w) and the highest DM
(80) were extracted at room temperature by a 0·05 M acetate buffer
(pH 5·2). A very high DM seems to be a characteristic for apple juice
pectins (Peynaud, 1951; Endo, 1965*a*).

The apple juice pectins have been degraded by heat (β-elimination)
and endoPG after de-esterification. β-elimination reaction occurs with
a simultaneous de-esterification since the DM was reduced to 36. These
two reactions are competitive: when too many methyl esters are
hydrolysed, the electronic environment hinders further β-elimination
Albersheim *et al.*, 1960; BeMiller & Kumari, 1972). This fact explains
why only 11% of the α-(1 → 4) linkages of the pectin were split after
β-elimination.

Results from gel-permeation chromatography of β-eliminated pectins
indicate a non-regularly repeating structure. Half of the neutral sugars
are concentrated on only 1·3% of total AGA and eluted at the void
volume of the Sephadex G-100 column. These results suggest the
presence of long side-chains in 'hairy' regions along with 'smooth'
regions consisting mainly of non-substituted galacturonan. Similar
results were found for β-eliminated pectic substances from apple
(Barrett & Northcote, 1965), from soy sauce (Kikuchi & Sugimoto,
1976), or from enzyme-degraded apple pectic substances (de Vries
et al., 1982).

In order to have more information on the 'hairy' and 'smooth'
regions, degradations with an endopolygalacturonase have been carried
out. This enzyme hydrolyses α-(1 → 4) linkages between anhydro-
galacturonic acids and therefore de-esterification was carried out on

crude pectin and on β-eliminated pectin. The residual DM was less than 2 for the crude de-esterified pectin and 12·6 for the β-eliminated de-esterified pectin. Whistler & BeMiller (1958) reported that a methyl ester close to a 4,5-double bond is more resistant to saponification. Alkali treatment leads to only a slight decrease in molecular weight and some loss in galactose content. This latter fact was not explained. The pectin after β-elimination and de-esterification was more readily degraded by the enzyme than the crude de-esterified pectin, as shown by the extent of hydrolysis and chromatographic separation. This fact could be explained by steric hindrance of endoPG action by side-chains. In contrast, the hydroxyl ions might be able to split linkages between galacturonic acids in the 'hairy' regions, leading to the production of new substrates for the enzyme. The products from β-eliminated, de-esterified and endoPG-degraded pectin are predominantly pure galacturonides (92% of total AGA). Neutral sugars form side-chains, concentrated on less than 8% of the AGA which can be divided into two classes according to the molar ratio of AGA/neutral sugars. Since endoPG is not able to hydrolyse trigalacturonic acid (Thibault & Mercier, 1978), it can be assumed that there are at least four continuous AGA residues between two side-chains. It can therefore be calculated that the mean degree of polymerisation of long side-chains is about 100 and that of short side-chains is about 4. From our results, it cannot be concluded that these two side-chains have a different chemical composition. Nevertheless, from the literature data, xylose is known to be present as single units or short side-chains (Bouveng, 1955; de Vries *et al.*, 1983; Kikuchi & Sugimoto, 1976), while longer side-chains are formed by branched arabinose residues and/or galactose residues (de Vries *et al.*, 1983; Knee *et al.*, 1975).

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