Carbohydrate Polymers **20** (1993) 195–205

Enzymic and chemical degradation of some industrial pectins

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Three industrial high-methoxyl pectins purified by copper precipitation have been degraded by acid, chemical β-elimination and endopolygalacturonase. Degraded pectins from β-elimination and enzyme treatments have been fractionated by preparative SEC and the resulting fractions have been analysed for their neutral-sugar, protein and phenolic contents. Distribution of neutral sugars indicates that the model of alternating 'hairy' and 'smooth' regions is also valid for industrial pectins. Most of the phenolics present in the copper-purified samples are probably not bound to the pectin molecules except for a small proportion that was found to be associated with the segments rich in neutral sugars. Proteins are associated with the hairy regions. Relative proportions of the oligomers with DP up to 17 produced by the action of endoPG and analysed by high-performance ion exchange chromatography were compared with a theoretical distribution model. The abundance of mono-, di- and trigalacturonides suggests that, in the smooth regions of industrial pectins, free carboxyl groups are present as blocks of non-esterified galacturonide units. HPSEC elution patterns of acid-degraded samples suggest that homogalacturonan blocks of average degree of polymerization of 40–60 are interspaced by rhamnose units. Degradations by β-elimination and endoPG indicate that most of the rhamnose units are concentrated in the regions carrying the side chains. This would mean that the pectin backbone consists of rhamnogalacturonan regions alternated with homogalacturonans regions, the homogalacturonan regions being interrupted at regular intervals by rhamnose units.

INTRODUCTION

In order to explain differences in the physical behaviour of three industrial high-methoxyl pectins, the nature of the constituent monosaccharides (Kravtchenko *et al.*, 1992*a*) as well as their distribution among the pectin molecules (inter-molecular distribution, Kravtchenko *et al.*, 1992*c*, 1992*d*) have been investigated. For instance, the inter-molecular distribution of the methyl esters was shown to be important in determining the reactivity of pectin molecules towards calcium ions (Kravtchenko *et al.*, 1992*c*). However, differences in physical behaviour may also depend upon the sequence in which the various structural units are arranged (intra-molecular distribution). The specific splitting of glycosidic bonds and the subsequent analysis of the reaction products is still one of the most efficient means to provide information for the sequences in which pectin constituents are linked (Aspinall, 1977).

Acid hydrolysis has been used for the selective degradation of pectic substances (Aspinall *et al.*, 1967*a*, 1967*b*; Morris *et al.*, 1980; Powell *et al.*, 1982; Durand *et al.*, 1990), using the fact that the different glycosidic linkages are hydrolysed at different rates. Glycosidic linkages at C-1 of neutral sugars, especially those of furanosides, are hydrolysed very readily, whereas glycosiduronic acid linkages are highly resistant to acidic conditions (Aspinall, 1970; Fry, 1988). Under appropriate conditions of hydrolysis it is therefore expected to degrade totally the neutral-sugar side chains as well as the pectin backbone at all insertions of a rhamnose unit without affecting the homopolygalacturonan sequences.

Neukom and Deuel (1958) discovered that linkages between galacturonic acid units are subjected to

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Carbohydrate Polymers 0144-8617/93/$06.00

depolymerization in hot alkaline or weakly acidic conditions following a reaction of β-elimination. In natural pectins, this reaction has been shown to depend upon the presence of an ester group on the C-6 next to the glycosidic bond (Albersheim, 1959) and may therefore be used for the specific depolymerization of the galacturonan backbone according to the distribution of their methyl ester groups.

Specific depolymerization of the polygalacturonic acid chain can also be achieved by using purified pectolytic enzymes. Polygalacturonase hydrolyses glycosidic linkages of low methoxylated pectins. Pectin lyase and pectate lyase split the glycosidic bonds of high-methoxyl and low-methoxyl pectins, respectively, by a mechanism of β-elimination. Properties as well as purification procedures of pectolytic enzymes have been particularly well reviewed (Rexova-Benkova & Markovic, 1976; Pilnik & Rombouts, 1979, 1981). Endopolygalacturonase (endoPG) has been used to extract defined pectic fragments from cell materials (Talmadge *et al.*, 1973; Knee *et al.*, 1975; Ishii, 1976; Renard *et al.*, 1991) and allowed Darvill *et al.* (1978) to isolate rhamnogalacturonan II, a very complicated pectic polysaccharide from suspension-cultured sycamore cell wall. EndoPG has also been used to degrade isolated pectins (Hatanaka & Ozawa, 1969; Kikuchi & Sugimoto, 1976; Thibault, 1983; Rombouts & Thibault, 1986; Saulnier & Thibault, 1987). The problem is that conclusions about the distribution of the bonds broken can only be drawn when the knowledge of the mode of action of the enzyme used is sufficient. In fact, the activity of pectolytic enzymes does not depend only upon the presence or the absence of methoxyl esters on the galacturonan backbone. However, the information we get from the study of enzymic degradation is not limited to that obtained from the characterization of the low molecular weight cleavage products. Significance must also be attached to the resistance of certain fragments (Aspinall, 1970).

This report deals with the characterization of the degradation products obtained from the specific depolymerization of three purified industrial pectins by acid hydrolysis, chemical β-elimination and enzymic degradation with endopolygalacturonase.

MATERIALS AND METHODS

Pectin samples

Three unstandardized industrial pectins were obtained from Sanofi Bio Industries (Paris, France): two from lemon peels (lemon A and B) and one from apple pomace extracted by the same hot-acid industrial process, in the same factory. They have been purified by copper precipitation as described previously (Kravtchenko *et al.*, 1992*a*). Their chemical composition has been extensively described in a previous paper (Kravtchenko *et al.*, 1992*a*).

Polygalacturonic acid was obtained from Fluka AG (Buchs, Switzerland).

Enzymic degradation

Highly purified endopolygalacturonase (endoPG, E.C. 3.2.1.15) was isolated from a preparation of *Kluyveromyces fragilis* as described by Versteeg (1979). Enzyme activity is expressed in units, one unit being the amount of enzyme which splits one μmol of glycosidic bond per min under defined conditions. Enzyme activity was assayed by the formation of reducing groups under the conditions used for the pectin degradation (see below). The enzyme preparation was devoid of activity of pectin lyase, pectate lyase and pectinesterase.

Two mg/ml pectin solutions in 0·05 M NH_3-acetate buffer at pH 4·2 were treated with 0·03 U/ml of endoPG at 30°C for 24 h. Extent of hydrolysis was evaluated by measuring the appearance of reducing end groups.

β-Elimination

β-Elimination was achieved by heating pectin solutions (5 mg/ml) in 0·2 M NH_3-carbonate/HCl buffer pH 6·8 at 80°C for 8 h.

Resulting 4,5-unsaturated galacturonide residues were determined spectrophotometrically at 235 nm, assuming a molar extinction coefficient of 5500 mol/cm for the unsaturated products (Kravtchenko *et al.*, 1992*b*).

Acid hydrolysis

Pectins were heated in 0·5 M TFA at 100°C for various times. After the treatment, TFA was evaporated under a stream of air and degradation products were redissolved in distilled water before HPSEC analysis.

Preparative size exclusion chromatography

Pectin degradation products (200 mg) were fractionated by size exclusion chromatography (SEC) on a column (100 cm × 2·5 cm) of Sephacryl S200 gel (Pharmacia, Uppsala, Sweden). The column was eluted at 40 ml/h with 0·2 M NH_3-acetate buffer pH 4·0. Retention times (RT) were expressed as a function of the partition coefficient K_{av}. The void (V_0) and the total (V_t) volumes were determined using undegraded lemon B pectin and galacturonic acid, respectively. Fractions (7·5 ml) were collected, assayed for their contents of galacturonic acid and total neutral sugars, and pooled. Pools were directly freeze-dried prior to further analysis.

Volatile buffers were used for pectin degradation and preparative fractionation in order to avoid dialysis.

High-performance size exclusion chromatography (HPSEC)

Degraded pectins or fractions from the Sephacryl S200 column were injected on a series of Biogel columns TSK 40 XL, 30 XL and 20 XL (Bio-Rads, Richmond, USA) (300 mm × 7·5 mm) being used in combination with a Biogel TSK XL guard column (75 mm × 7·5 mm). Elution was performed with 0·4 M Na-acetate buffer pH 3·0 at a flow rate of 0·8 ml/min at 30°C. Elution products were detected with a Shodex SE 61 refractive-index detector at 40°C.

High-performance ion exchange chromatography (HPIEC)

High-performance ion exchange chromatography of the degradation products was performed with a Dionex BioLC system (Dionex, Sunnyvale, CA) equipped with a Dionex Carbopac PA-1 column (250 mm × 4 mm). After sample injection, the column, pre-equilibrated with 0·1 M NaOH, was eluted with two successive linear gradients of NaAc in 0·1 M NaOH (0·35–0·7 M, 35 min and 0·7–1 M, 5 min), washed for 5 min with 1 M NaAc in 0·1 M NaOH and then re-equilibrated for 15 min with 0·1 M NaOH. The flow rate was 1 ml/min. Detection was made with a PAD II pulsed-amperometric detector (Dionex, Sunnyvale, CA) equipped with a gold working-electrode and an Ag/AgCl reference electrode. The following pulse potentials and durations were used: E_1 = 0·1 V, t_1 = 500 ms; E_2 = 0·6 V, t_2 = 100 ms; E_3 = −0·6 V, t_3 = 100 ms.

Analytical methods

Galacturonic acid and total neutral sugars (expressed as arabinose) were determined by the automated *m*-hydroxydiphenyl (*m*hdp, Thibault, 1979) and orcinol (Tollier & Robin, 1979) methods, respectively. Neutral-sugar content was corrected for interference of galacturonic acid with the orcinol assay.

Reducing end groups were determined by the Nelson–Somogyi method (Spiro, 1966) with galacturonic acid as standard.

Neutral sugars were determined by GLC as described previously (Kravtchenko *et al.*, 1992*a*).

Degree of methoxylation (DM) was determined by HPLC with the method of Voragen *et al.* (1986) modified by Kravtchenko *et al.* (1992*c*).

Total phenols were estimated with the Folin-Ciocalteu reagent, as described elsewhere (Kravtchenko *et al.*, 1992*c*).

Protein content was evaluated by the Sedmak & Grossberg assay (1977) using micro-titer plates as described by Rylatt & Parish (1982) and bovine serum albumin as standard.

RESULTS AND DISCUSSION

Acid hydrolysis

Acid hydrolysis of pectins was performed in 0·5 M trifluoroacetic acid (TFA) at 100°C in order to determine the distribution pattern of rhamnose units in the main chain. Under these conditions, the polyuronate chain sequences would be expected to remain intact whereas Rha-Gal.A linkages should be completely hydrolysed (Fry, 1988).

HPSEC analysis of the degradation products after different times of acid treatment (Fig. 1) shows the appearance of a peak with narrow distribution at a retention time of *c.* 27·5 min, which is particularly visible after 5–16 h of treatment. This peak can be ascribed to homopolygalacturonic acid sequences. Its narrowness suggests that rhamnose residues interrupt regularly polygalacturonic-acid chains of nearly constant length. This peak elutes at the same retention time and with the same distribution pattern as the polygalacturonic acid obtained from Fluka. Dortland (1972) used the method of Rombouts *et al.* (1970) to determine the number-average degree of polymerization (DP) of several commercial polygalacturonic-acid preparations. Since they are probably all prepared in the same way (i.e. acid hydrolysis of pectin), they were found to have an average DP in the range 40–60. We therefore assumed that the polygalacturonic acid that we used has an average DP of about 40–60. From similar acid degradations of other pectin samples, galacturonic acids were found to be arranged in sequences of 25 (Powell *et al.*, 1982) to 50 (Durand *et al.*, 1990) monomers.

The peak, presumed to consist of homogalacturonate segments, was slowly degraded into oligomeric fragments of very low molecular weight without formation of fragments of intermediate size. A similar phenomenon was observed during the treatment of polygalacturonic acid (not shown). This suggests that homo-

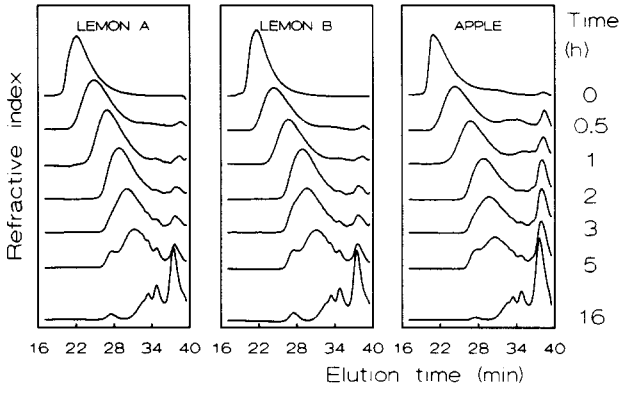

Fig. 1. Change in the HPSEC elution pattern of the three purified pectin samples under various times of heating at 100°C in 0·5 M TFA.

galacturonan segments are slowly degraded in acid conditions by a reaction affecting only chain extremities.

Chemical β-elimination

Cu-purified pectin samples were degraded by chemical β-elimination at pH 6·8 and 80°C in order to split the glycosiduronic bonds next to methoxylated galacturonic acid units. Figure 2 shows the rate of degradation of the three purified industrial pectins. The depolymerization was very fast in the first stage of the reaction. Then, the speed of the reaction decreased rapidly to stop completely after 6 h. After 8 h, the percentage of glycosiduronic bonds broken was only 10·0, 9·8 and 9·9% for lemon A, lemon B and apple pectins, respectively. Indeed, the reaction stops because of the removal of methoxyl groups by saponification (Albersheim *et al.*, 1960). Similar degrees of degradation have been obtained by other investigators for pectins of similar DM under similar conditions (Thibault, 1983; Rombouts & Thibault, 1986). Since, apple and lemon pectins differ greatly in neutral-sugar and acetyl-ester contents (Kravtchenko *et al.*, 1992*a*), these results confirm that chemical β-elimination is not affected by structural features other than the presence of a methyl ester next to the glycosidic bond to be split (Rouau & Thibault, 1984).

HPSEC analysis of the degradation products (Fig. 3) indicates that chemical β-elimination led to a reduction in molecular size of almost all the pectin molecules. Since hydroxide ions which induce the reaction are not limited by the presence of side chains or other substituents, and assuming a random distribution of methyl esters, the galacturonan backbone should be degraded in fragments with an average size of *c.* 10 units. However, especially in the case of the apple pectin sample, some molecules elute very early on HPSEC and thus appear to have a much larger molecular size. These high molecular weight fragments elute in two peaks at *c.* 22 and 24 min, respectively.

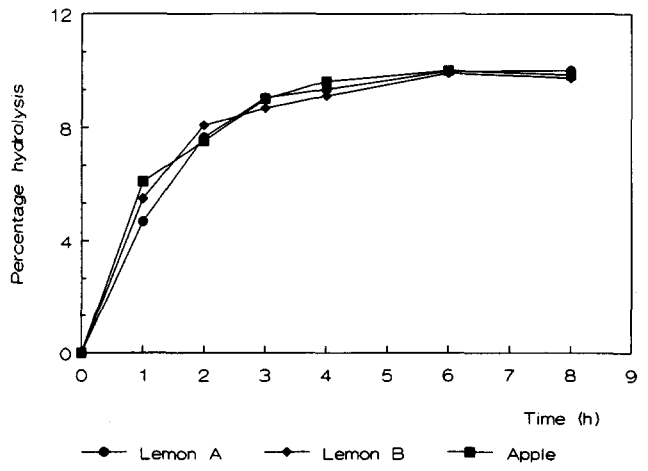

Fig. 2. Rate of degradation of the three purified pectin samples by chemical β-elimination.

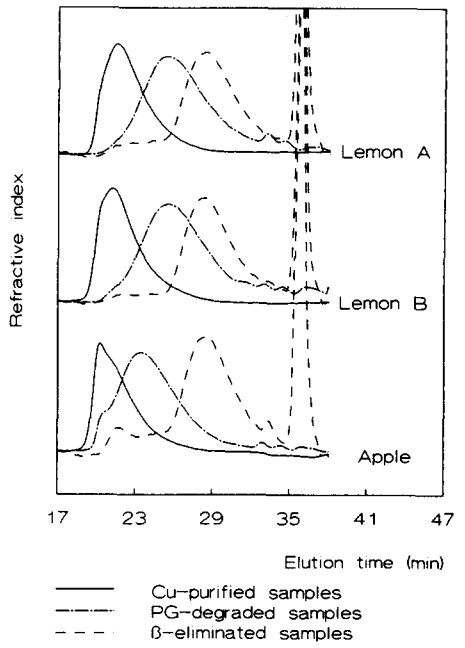

Fig. 3. HPSEC elution pattern of the three purified pectin samples degraded by chemical β-elimination and endoPG.

Degradation products were separated on Sephacryl S200 as shown in Fig. 4. The elution patterns obtained show two quite different polysaccharide populations. The peak excluded from the gel contains only a small fraction of the galacturonide, but most of the neutral-sugar units, whereas the peak eluting in the fractionation range of the column is composed mainly of galacturonic acid units. In addition, lemon pectins exhibit an incompletely resolved peak at K_{av} = 0·5 that corresponds to undegraded fragments of intermediate size. These fragments may correspond to blocks of non-esterified galacturonic acids. This peak appears to be more important in the degradation products from the lemon A pectin sample.

SEC fractions were grouped in 7 pools as shown in Fig. 4. Neutral sugars were determined in each pool as their alditol acetates. Table 1 clearly confirms that most of the neutral-sugar units are concentrated on large fragments that elute close to the void volume of the column. More than 70% of the neutral-sugar units are concentrated in fractions 1 and 2, which represent only about 7% of the galacturonic acid residues. This confirms that pectin molecules are made of a succession of branched and unbranched galacturonan segments (Barrett & Northcote, 1965; Kikuchi & Sugimoto, 1976; de Vries *et al.*, 1982; Thibault, 1983; Rombouts & Thibault, 1986) that de Vries *et al.* (1982) called 'hairy' and 'smooth' regions, respectively.

Since the galacturonan backbone can be expected to have been broken in fragments of equivalent sizes (see above), the large molecular size of the fragments rich in

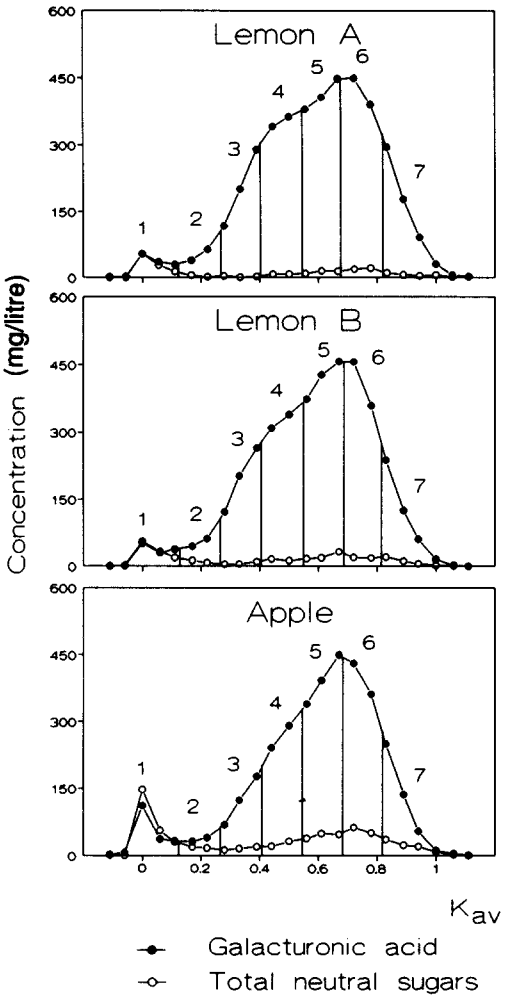

Fig. 4. SEC elution pattern on Sephacryl S200 of the three purified pectin samples degraded by chemical β-elimination. Fractions have been pooled as indicated by the vertical bars. Numbers indicate the pool number.

neutral sugars indicates that neutral-sugar units are arranged as long side chains. Talmadge *et al.* (1973) suggested the presence of long homo-arabinans in pectic substances from sycamore, while galactans of high DP have also been obtained (Aspinall & Jiang, 1974; Darvill *et al.*, 1980; Eda & Kato, 1980).

Rechromatography on HPSEC (not shown) indicated that the fraction eluting at the void volume of the Sephacryl S200 column contains the two high molecular weight fractions which were also observed by HPSEC analysis of the unfractionated degradation products (Fig. 3). These two separated peaks as well as fraction 2 from the preparative column may represent different fragment populations of fragments which probably differ in neutral-sugar content and therefore in the size of their side chains. The fractions eluting on HPSEC at RT 22 min probably correspond to fraction 'a' isolated from apple pectin by de Vries *et al.* (1982) which contained 90% of the neutral sugars and only 5% of the galacturonide of the sample. This is in agreement with Knee *et al.* (1975) and Thibault (1983) who observed that pectin fragments from apple contain two types of side chain distinguishable by their length.

In addition, the amount of neutral sugars is higher in fragments of low size from the apple pectin, indicating that some short side chains or single neutral-sugar units may also be present on the smooth regions of apple pectin molecules.

The molar ratios of rhamnose residues to galacturonic acids were found to be *c.* 0·9 in fractions 1, irrespective of the plant origin. This means that there is about one rhamnose residue for every galacturonic acid residue in hairy regions. In contrast, smooth regions (fractions 3–7) contain only about 1 rhamnose unit per 200 galacturonic acid units.

Table 1 also shows the distribution of phenolic and proteinaceous compounds over the SEC fractions obtained from the three β-eliminated samples. Most of the phenolic compounds elute with the low molecular weight fragments (fractions 6 and 7). However, some phenolic compounds coelute with the high molecular weight fragments, and therefore seem to be associated with hairy regions. In contrast, proteins appear to be mainly present in the fractions rich in neutral sugars, probably associated with neutral side chains (Lamport 1969; Lamport *et al.*, 1973).

Enzymic degradation

The three Cu-purified pectins were submitted to the action of a purified endoPG from *Kluyveromyces fragilis*. After maximum degradation with excess enzyme (addition of enzyme did not lead to any further degradation), the percentage hydrolysis of the glycosiduronic linkages were 4·1, 3·8 and 1·4% for lemon A, lemon B and apple pectin samples, respectively. Assuming the complete degradation of all glycosidic linkages next to free carboxylic acid, the extent of degradation of such pectins should be over 25%. However, to act on the pectin backbone, endoPG requires not only a single non-methoxylated galacturonic acid unit, but a sequence of non-methoxylated galacturonides (Rexova-Benkova & Markovic, 1976). The endoPG that we used has been incubated with polygalacturonic acid and the degradation products were chromatographed on the dionex PA-1 column (not shown). At maximum hydrolysis, mono- di- and trigalacturonic acids remained as end products, indicating that endoPG is not able to degrade di- and trigalacturonic acids. The probability of finding sequences of non-esterified galacturonides of a given length and therefore the potential extent of degradation by endoPG decreases very quickly with increasing DM (Pilnik *et al.*, 1973). Koller and Neukom (1969) found that the hydrolysis of pectin decreases with increasing DM: pectins with a DM above 75% were not hydrolysed. This may explain why the extent of degradation of the apple pectin is so

Table 1. Chemical composition of the SEC fractions obtained by β-elimination (expressed as percentage weight of AUA)

				Lemon A			
Fraction	1	2	3	4	5	6	7
AUA (%)[a]	1·9	3·4	12·1	20·8	24·5	24·5	12·7
Rhamnose	75·5	34·9	2·0	0·6	0·4	0·3	0·4
Arabinose	50·2	25·5	1·3	0·5	0·6	0·6	1·1
Xylose	6·3	1·6	0·2	0·2	0·1	0·2	0·5
Mannose	t	0·2	t	t	t	t	0·2
Galactose	133·0	32·2	1·9	1·1	1·1	1·0	1·6
Glucose	4·5	3·7	0·5	0·2	0·2	0·2	1·5
Total NS	269·5	98·1	5·9	2·6	2·4	2·3	5·3
Phenolics	0·4	0·2	0·2	0·1	0·2	0·2	1·2
Proteins	0·9	0·8	0·2	0·2	0·1	0·1	0·1
				Lemon B			
Fraction	1	2	3	4	5	6	7
AUA (%)[a]	2·0	3·7	12·3	20·2	26·3	24·8	9·5
Rhamnose	70·1	44·8	2·1	0·7	0·4	0·2	0·3
Arabinose	50·2	37·4	2·0	0·9	0·6	0·5	0·9
Xylose	4·9	2·2	0·3	0·2	0·1	0·1	0·4
Mannose	t	0·4	t	0·1	0·1	0·1	0·3
Galactose	151·1	49·8	4·5	2·5	1·3	0·8	0·8
Glucose	4·1	8·9	1·1	0·7	0·4	0·2	1·9
Total NS	280·4	143·5	10·0	5·1	2·9	1·9	4·6
Phenolics	0·2	0·1	0·1	0·1	0·1	0·3	1·7
Proteins	0·9	0·5	0·2	0·2	0·1	0·1	0·3
				Apple			
Fraction	1	2	3	4	5	6	7
AUA (%)[a]	4·8	2·7	8·5	19·1	27·2	26·8	10·9
Rhamnose	71·2	45·6	3·4	0·8	0·5	0·2	0·2
Arabinose	68·0	42·8	3·2	1·4	1·3	0·7	0·4
Xylose	38·4	22·0	3·6	0·9	0·4	0·2	0·2
Mannose	0·8	0·8	0·3	0·1	0·1	0·1	0·1
Galactose	112·0	44·0	4·2	1·7	1·3	0·6	0·4
Glucose	214·8	80·8	11·9	3·7	1·2	0·5	2·4
Total NS	505·2	236·0	26·6	8·6	4·8	2·3	3·7
Phenolics	0·7	1·0	1·1	0·1	0·2	0·2	4·1
Proteins	0·9	0·3	0·2	0·2	0·1	0·1	0·1

[a]Percentage fraction of the whole sample.
NS = neutral sugars.
t = trace.

low compared to that of the lemon pectins although its average DM is only slightly higher (Kravtchenko *et al.*, 1992*a*). However, other structural features may also explain the difference of extent of degradation between apple and lemon pectins. The action of endoPG is limited by the presence of neutral side chains by steric hindrance (de Vries *et al.*, 1982; Thibault, 1983; Pasculli *et al.*, 1991) or the presence of acetyl esters on C-2 or C-3 of the galacturonic acid units (Solms & Deuel, 1951; Rexova-Benkova *et al.*, 1977; Pasculli *et al.*, 1991). A blockwise distribution of the free carboxyl groups in the lemon pectins would increase the frequency of sequences of non-esterified galacturonides with the required length for endoPG attack. The low degradation limits observed in this study are in agreement with those obtained by other investigators with high-methoxyl pectins (Thibault & Mercier, 1978; Rombouts & Thibault, 1986).

Figure 3 shows the change in HPSEC elution pattern of the three pectin samples on treatment with endoPG. Despite low extents of degradation, there is a clear shift towards fragments of lower molecular size. These patterns are as expected for an endoenzyme. The lesser change in molecular size of the apple pectin confirms its lower extent of degradation compared to lemon pectins.

After heat inactivation of the enzyme, the reaction products were directly freeze-dried. Pectin fragments were separated by preparative SEC on Sephacryl S200 (Fig. 5). Elution patterns are similar to those obtained by de Vries *et al.* (1982) with apple pectins degraded by pectin and pectate lyases. However, the SEC column that we used had a more restricted fractionation range and did not allow the separation of the fraction 'a' isolated by de Vries *et al.* (1982).

Because of the lower extent of degradation, separation between smooth and hairy regions is not as clear as in the case of β-elimination. However, it is clear that most of the neutral-sugar units are located in the fractions of large molecular size. Table 2 shows the neutral-sugar composition of the S200 fractions. The distribution of neutral sugars over the degradation products obtained by treatment with endoPG is similar to that of the fragments obtained by treatment with chemical β-elimination. However, the neutral-sugar/galacturonic acid ratio is lower in the high molecular weight range because the same neutral-sugar side chains are carried by longer segments of galacturonic acid. On the other hand, it seems that treatment with endoPG has led to the formation of a larger proportion of fragments of much lower size than chemical β-elimination.

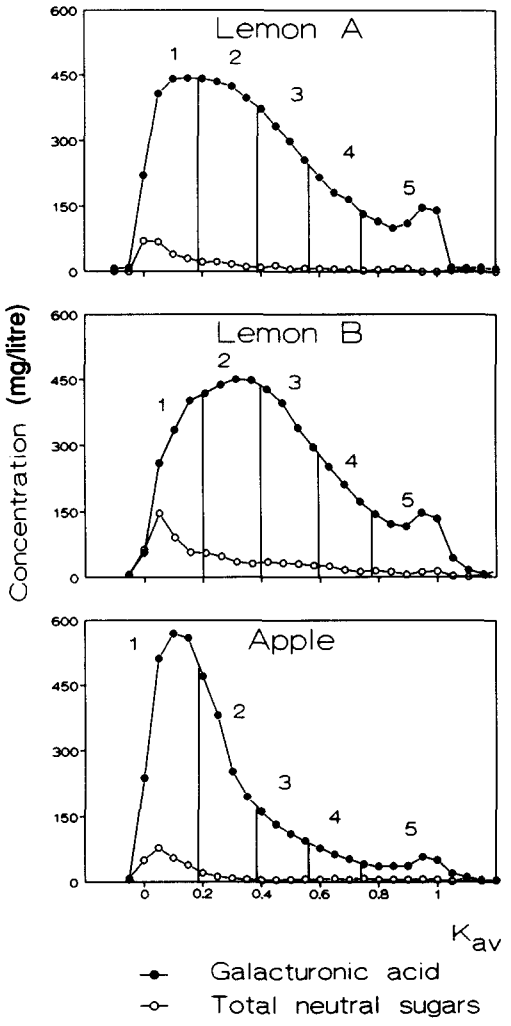

Fig. 5. SEC elution pattern on Sephacryl S200 of the three purified pectin samples degraded by endoPG.

Table 2. Chemical composition of the SEC fractions obtained by degradation with endoPG (expressed as percentage weight of AUA)

	Lemon A				
Fraction	1	2	3	4	5
AUA (%)[a]	28·2	29·2	19·1	11·3	12·2
Rhamnose	9·3	1·5	0·4	0·3	0·6
Arabinose	5·9	1·4	0·7	0·7	1·5
Xylose	0·7	0·1	t	t	0·3
Mannose	0·1	0·1	0·1	0·1	0·2
Galactose	13·2	1·7	1·4	1·5	1·3
Glucose	1·2	1·0	1·1	1·6	3·8
Total NS	30·4	5·8	3·7	4·2	7·7
Phenolics	0·1	0·1	0·2	0·3	1·9
	Lemon B				
Fraction	1	2	3	4	5
AUA (%)[a]	20·7	31·5	22·9	13·3	11·6
Rhamnose	5·4	0·6	0·1	0·1	0·2
Arabinose	4·2	0·7	0·4	0·3	0·8
Xylose	0·4	0·1	t	t	0·1
Mannose	0·1	t	t	0·1	t
Galactose	13·8	3·7	3·0	2·8	1·8
Glucose	0·8	0·4	0·4	0·5	1·0
Total NS	24·7	5·5	3·9	3·8	3·9
Phenolics	0·1	t	0·1	0·1	4·5
	Apple				
Fraction	1	2	3	4	5
AUA (%)[a]	46·0	32·2	10·1	5·2	6·5
Rhamnose	8·6	2·4	0·7	0·9	1·5
Arabinose	8·3	2·7	1·8	2·5	5·2
Xylose	5·2	1·2	0·7	0·6	0·5
Mannose	0·1	t	0·1	0·2	0·2
Galactose	10·7	3·2	3·9	5·2	4·4
Glucose	11·8	3·9	4·5	7·3	7·1
Total NS	44·7	13·4	12·6	16·7	18·9
Phenolics	0·4	0·2	0·4	0·7	3·0

[a]Percentage fraction of the whole sample.
NS = neutral sugars.
t = trace.

Apple pectin differs from the lemon pectins by the presence of large amounts of glucose and xylose units. This may indicate the presence of separate xyloglucan and/or single-unit xylose side chains. Indeed, covalent bonds between xylose and galacturonic acid have often been found (Barrett & Northcote, 1965; Aspinall *et al.*, 1967*a*, 1967*c*; Schols *et al.*, 1990).

The distribution of phenolic compounds over the endoPG degradation products (Table 2) is similar to that observed on pectins degraded by β-elimination.

Distribution of the oligomers

After freeze-drying, an aliquot of the degradation products obtained by endoPG was chromatographed on a Dionex PA-1 column. Figure 6 shows the chromatograms obtained by degradation of the three pectin samples. Oligogalacturonides of DP1–17 elute as well-separated peaks. Since the separation was performed at very high pH, all methoxyl and acetyl esters were probably hydrolysed very rapidly and thus did not affect the separation. The proportion of galacturonic acid present among the oligomers of DP up to 17 was calculated assuming that the response of the pulsed amperometric detector is proportional to the concentration of HCOH groups (Koizumi *et al.*, 1989).

With a computerized mathematical model, galacturonan chains with the DM of the industrial samples but with a random distribution of methyl esters were generated. From these model molecules, the theoretical distribution of the galacturonic acid units among segments of contiguous esterified or non-esterified residues with a given DP was established. Assuming the place of splitting by endoPG, we calculated the theoretical distribution of the oligomers formed. These theoretical results were compared with the experimental ones obtained by the treatment of the pectin samples with endoPG (Fig. 7).

The proportion of mono-, di- and trimers produced by treatment with endoPG is much higher than expected from the theoretical distribution of the methoxyl groups. A large proportion of oligomers of DP up to 3 indicates that large galacturonan regions, i.e. non-esterified regions, have been readily degraded by endoPG. This suggests that in these industrial pectins, free carboxylic acids are unevenly distributed.

EndoPG is known to require a certain sequence of unesterified galacturonic units. Partly methoxylated galacturonic acid chains, especially those with a high DM, would therefore be less degraded, and the release of oligomers of small size would be lower than with the simple mode of degradation that we have assumed in our model. Thus, taking into account a more realistic mode of action for endoPG, supports the view that free carboxyl groups appear in blocks.

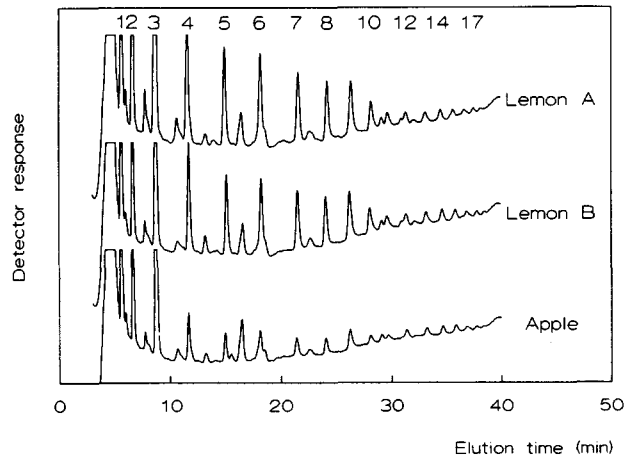

Fig. 6. High-performance ion exchange chromatography of the oligogalacturonides produced by the action of endoPG on the three pectin samples. Figures indicate the oligomer size.

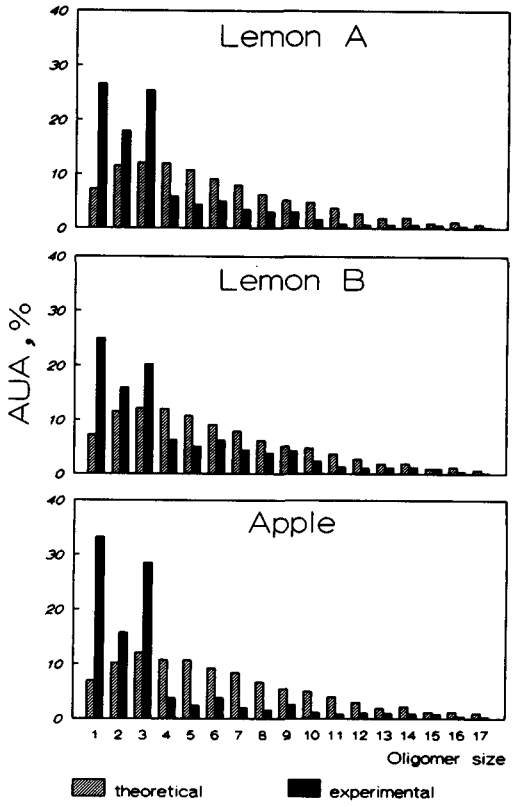

Fig. 7. Experimental *versus* theoretical random distribution of the oligomers produced by the action of endoPG on the three industrial pectin samples.

It must be kept in mind that endoPG can be expected to degrade the smooth regions more readily than the hairy regions. de Vries *et al.* (1982) found that the DM is much higher in hairy than in smooth regions. This means that homogalacturonan regions would be better substrates for endoPG than expected from the average DM of the whole samples. DMs have been measured in the high molecular weight fragments (fractions 1) produced by the action of endoPG on the three purified pectins. Values of 58, 57 and 24% were found for lemon A, lemon B and apple, respectively, indicating that in industrial pectins, smooth regions are more methoxylated than hairy regions. Assuming a random distribution of the free carboxyl groups, their degradation with endoPG should therefore produce less low molecular weight oligomers than expected from their average DM. Again, the high proportion of low molecular weight oligomers suggests a blockwise distribution of free carboxyl groups.

An argument against our conclusions may be found

in the fact that some oligogalacturonides are able to aggregate with high molecular weight fragments (Rombouts & Thibault, 1986). Such a phenomenon may hinder the chromatographic separation of the oligomers and therefore modify the observed distribution. However, no evidence was provided to assume that oligomers of DP higher than 3 are more prone to such an aggregation than lower oligomers.

Among the oligomers of DP lower than 17, produced by the action of endoPG on non-methoxylated regions of the molecules, compared to lemon pectins, the apple pectin contains a higher proportion of mono-, di- and trimers, indicating that acid blocks are probably larger than in the lemon pectins. However, Fig. 5 clearly shows that compared to the total pectin content, the production of low molecular weight fragments which elute close to the total volume of the column is much lower for the apple than for the lemon pectins. This indicates that in the apple, there are less suitable sites for the action of endoPG, i.e. less blocks of free carboxyl groups.

CONCLUSIONS

Each of the degradation methods applied to the three industrial pectin samples had a different specificity. Chemical β-elimination occurred all along the galacturonan backbone without hindrance by side chains or other substituents. EndoPG requires a certain sequence of non-methoxylated galacturonate residues and is hindered or blocked by side chains, rhamnosyl residues or acetyl groups at C-2 and/or C-3 of galacturonic acid units. Both methods showed that neutral sugars are unevenly distributed along the pectin molecules. It may thus be concluded that industrial pectins are also made of alternating smooth galacturonan and hairy rhamnogalacturonan regions. However, hairy regions of industrial pectins carry less neutral sugars than pectins extracted in mild conditions (de Vries *et al.*, 1982; Thibault, 1983; Rouau & Thibault, 1984; Rombouts & Thibault, 1986) because of the trimming reactions which occur during the industrial process of extraction (Kravtchenko *et al.*, 1992*a*). In addition to arabinogalactan side chains, apple pectin hairy regions may carry separate xylan and/or xyloglucan side chains.

Degradation with chemical β-elimination and endoPG showed that rhamnose units are concentrated in the segments of the galacturonan backbone rich in neutral side chains, leaving more than 90% of the galacturonan chain almost devoid of rhamnose insertions. This is in agreement with the fact that most side chains are linked to C-4 of the rhamnose units (Aspinall *et al.*, 1967*c*; Talmadge *et al.*, 1973; Eda & Kato, 1980; McNeil *et al.*, 1980; Sun *et al.*, 1987; Schols *et al.*, 1990). Acid hydrolysis suggests that at least one rhamnose unit is present between homogalacturonan regions of about 40–60 units. Smooth regions, however, were found to contain only 1 rhamnose unit per 200 galacturonic acid units. This would mean that smooth regions are almost completely devoid of rhamnose. This discrepancy may be explained by the fact that with the usual hydrolysis conditions, because of the high stability of glycosiduronic linkages, most rhamnose units remain attached to galacturonic acid units as aldobiuronic acids (Aspinall, 1970), whereas stronger acid conditions lead to a substantial destruction of rhamnose units. Therefore, rhamnose content may be considerably underestimated. An alternative method would be to reduce galacturonic acids to galactose residues so that acid-stable glycosiduronic bonds are converted into normal glycosidic linkages. Thus, with a higher rhamnose content than measured, rhamnose units may very well be present alternately with galacturonic acid units in hairy regions (Schols *et al.*, 1990) as well as at regular intervals with homogalacturonan chains in smooth regions (Powell *et al.*, 1982; Durand *et al.*, 1990).

After degradation by β-elimination or endoPG, most phenolic compounds co-eluted on SEC with the smallest pectin fragments. Rombouts and Thibault (1986) showed that most of the polyphenols from sugar-beet which also eluted with the smallest enzyme degradation products are not covalently linked to the pectin molecules. These are probably the same phenolic compounds which eluted with molecules of small size on preparative SEC of the undegraded pectin samples (Kravtchenko *et al.*, 1992*c*). The reason why these phenols remain associated with pectin during purification and only separate upon extensive degradation is not known. However, another part of the phenols, especially in the case of the apple pectin, co-elutes with the fragments rich in neutral sugars. Ferulic acids were found to be covalently associated with pectin side chains of spinach (Fry, 1983) and sugar-beet (Rombouts & Thibault, 1986). Industrial pectins from apple and lemon do not contain any phenolic acids (Kravtchenko *et al.*, 1992*a*) but some other phenolic compounds are probably bound to their neutral side chains.

DM was found to be lower for the fractions rich in neutral sugars than for the whole undegraded samples. This means that DM is lower in hairy regions than in smooth regions. This contradicts the findings of de Vries *et al.* (1982), but is in agreement with the work of Saulnier and Thibault (1987) who found that hairy regions of acid-extracted pectins require a high ionic strength to elute from the ion exchange chromatography (IEC) column. In apple hairy regions, Schols *et al.* (1990) also found an average DM of 40%. DM of hairy regions from the industrial apple pectin was found to be lower than that of the two lemon pectins. This explains the much lower neutral-sugar/galacturonic acid ratio of the fragments rich in neutral sugars from the β-eliminated apple compared to that of the lemon

pectins: apple hairy regions were probably less degraded by chemical β-elimination than those from lemon and, rhamnogalacturonan fragments carrying the side chains were therefore longer. Analysis of the oligomers produced by the action of endoPG suggests that in smooth regions, free carboxyl groups are arranged as blocks. This contradicts previous studies (Fielding, 1975; Kohn, 1975; de Vries *et al.*, 1983, 1986; Westerlund *et al.*, 1991) which indicated that in high methoxyl pectins, free carboxyl groups are distributed at random. This is probably true on average, although the presence of a few blocks of free carboxyl groups would have been difficult to detect. Blocks of free carboxyl groups probably result from the action of pectin methyl esterase (Taylor, 1982) on high methoxyl pectins, i.e. with DM in the range 70–75%. However, even after some action of the enzyme, the average DM remains higher than 70%, indicating that blocks only involve a very small proportion of the free carboxyl groups, the others being distributed at random. These blocks, which are of very high technological importance, are therefore very difficult to detect and quantify. EndoPG specifically degrades acid blocks and ignores isolated carboxylic acids randomly spread in between methoxyl esters. The extent of degradation is indicative of the amount of galacturonic-acid units involved in blocks, and the size distribution of the resulting oligomers indicates the size of the blocks. It should be noted that the degradation of pectin molecules and the appearance of small oligomers may also result from the action of endoPG on pectin molecules of low DM.

ACKNOWLEDGEMENT

The authors thank Sanofi Bio Industries (France) for providing pectin samples and financial support.

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