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ENZYMIC DEGRADATION OF APPLE PECTINS

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ABSTRACT

Purified apple pectins extracted under mild conditions were degraded by purified pectin lyase (EC 4.2.2.10) and pectate lyase (EC 4.2.2.2). The degraded pectins were fractionated by gel permeation chromatography and the degree of esterification and the sugar composition determined for each fraction. More than 90% of the uronic acid residues can be isolated as homogalacturonan chains. The neutral sugar residues can be detected in the column eluates as high molecular weight fragments. Models of the apple pectin molecules are presented. In the models the neutral sugars are present as side chains, arranged in blocks (in so-called 'hairy regions'). The galacturonate residues in the hairy regions are esterified with methanol.

INTRODUCTION

In research on the structure of pectins, enzymes have been used either on isolated pectins or on pectins *in situ* (cell wall preparations). To elucidate the structure of 'proto-pectin' (the pectin as it is in the cell wall) both ways must be employed.

Important work on pectins *in situ* has been done by Albersheim and co-workers (1967). Talmadge *et al.* (1973) used endo-polygalacturonase from *Colletotrichum lindemuthianum* to isolate defined cell wall fragments thereby releasing 75% of the uronic acid residues from sycamore cell walls. Using the same endo-polygalacturonase Darvill *et al.* (1978) were able to isolate a very complicated pectic polysaccharide ('rhamnogalacturonan 2'). Knee *et al.* (1975) showed that two endo-polygalacturonase iso-enzymes released different amounts of neutral sugar and galacturonic acid residues from an apple cell wall preparation. Voragen *et al.* (1979) found that the combined action of endo-polygalacturonase and cellulase showed a synergistic effect on the

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solubilisation of pectic polysaccharides from apple cell walls. Ishii (1976) compared the action of endo-pectin lyase and of endo-polygalacturonase from *Aspergillus japonicus* and found that the two enzymes apparently differed in ability to release pectic substances from onion and radish tissue.

Less work has been done on the enzymic degradation of isolated pectins. Commercial enzyme preparations were used by Aspinall *et al.* (1967, 1968), Pfister (1977) and Bouveng (1965) to obtain oligosaccharides consisting of xylose and galacturonic acid residues. Kikuchi & Sugimoto (1976) incubated a soy sauce acidic polysaccharide after partial acid hydrolysis with an endo-polygalacturonase from *Aspergillus japonicus* while Hatanaka & Ozawa (1969) used partially purified endo-polygalacturonase on commercial *Citrus unshiu* pectins.

This paper deals with the action of purified pectin lyase and pectate lyase on purified apple pectin, extracted under mild conditions. The pectins were extracted and fractionated as described in a previous paper (de Vries *et al.*, 1981). The degraded pectins were fractionated by gel filtration and the degree of esterification of the galacturonic acid residues and the sugar composition (galacturonic acid, rhamnose, arabinose, xylose, galactose and glucose) were determined for each fraction.

METHODS

Gel filtration

A sample of enzyme-degraded pectin (10–50 mg in 2 ml of sodium phosphate or carbonate buffer) was applied to a Sephacryl S-300 column (80 x 2·5 cm) and eluted with water. The flow rate (0·3 ml min^{-1}) was controlled by an LKB peristaltic pump and the whole procedure was conducted at room temperature.

Analytical methods

The anhydro-uronic acid (AUA, M.W. 176) content of pectin fractions was determined by an automated carbazole-sulphuric acid assay (van Deventer-Schriemer and Pilnik, 1976). The neutral sugars were analysed gas chromatographically as their alditol-acetates (Albersheim *et al.*, 1967; Darvill *et al.*, 1975). The methoxyl content was determined by gas chromatographic analysis of the methanol released on alkaline de-esterification (1 h at room temperature; 0·1 M KOH). Methanol was converted to methyl nitrite and determined according to Versteeg (1979).

Enzymic degradation

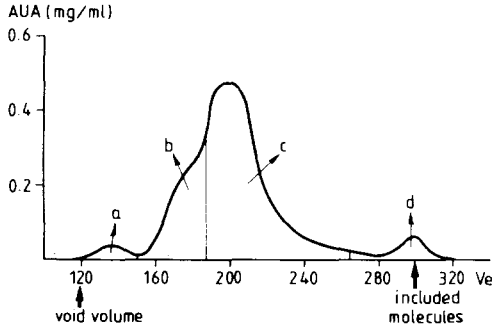
Highly purified pectin lyase ('type 2', van Houdenhoven, 1975) and pectate lyase (Rombouts *et al.*, 1978) were used. Enzyme reaction conditions were as follows: Pectin lyase (EC 4.2.2.10; poly-α-1,4-D-methyl-galacturonate lyase) – 0·2 mg ml^{-1} substrate and 0·02 units ml^{-1} enzyme (units as defined by van Houdenhoven, 1975) in 10 mM sodium citrate or phosphate buffer pH 5·2 at 30°C for 24 h. Pectate lyase

(EC 4.2.2.2; poly-α-1,4-D-galacturonate lyase) – 0·1 mg ml^{-1} substrate and 5 units ml^{-1} enzyme in 10 mM sodium carbonate buffer pH 7·0 at 30°C for 24 h.

The extent of degradation was determined spectrophotometrically at 235 nm, assuming $\epsilon_{235} = 4600\ M^{-1}\ cm^{-1}$ for the de-esterified unsaturated product and $\epsilon_{235} = 5500\ M^{-1}\ cm^{-1}$ for the esterified unsaturated product.

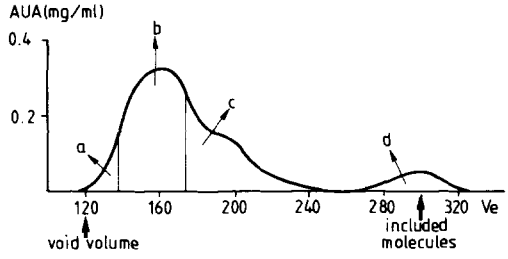
RESULTS AND DISCUSSION

Figures 1, 2 and 3 show typical gel filtration patterns of enzyme-degraded pectins. Figures 1 and 2 represent the results of pectate lyase degradation whereas Fig. 3 shows the result of pectin lyase degradation. In all cases the gel filtration patterns are similar. Patterns are as expected for an endo-enzyme. About 90% of the neutral sugar residues can be detected in the peak indicated in the figures as 'a'. This fraction 'a' contains about 5% of the uronic acid residues.



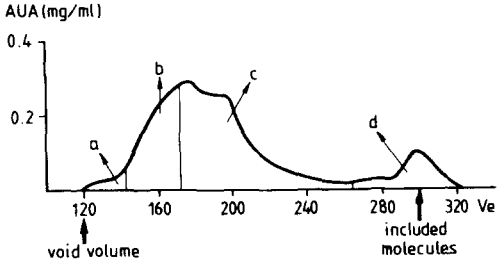
	a	b	c	d
Degree of esterification (%)	95	61	75	59
% of AUA	7	31	54	8
neutral sugar content (moles/mole of galacturonic acid residues)	1.33	.03	.02	.01
moles rhamnose/mole arabinose	.08	.06	.10	.05
moles xylose/mole arabinose	.09	.09	.10	.07
moles galactose/mole arabinose	.87	.71	1.0	6.1
moles glucose/mole arabinose	.10	.06	.10	.09

Fig. 1. Gel filtration of a pectate lyase degraded pectin. AUA = anhydro-uronic acid content. Ve = elution volume. The substrate was a DEAE-cellulose purified pectin from the cold buffer extract of alcohol insoluble solids (AIS) from ripe apples. Degradation with pectate lyase as described in the text. Extent of degradation was 4%. The eluent was water.



	a	b	c	d
% of AUA	11	53	33	3
neutral sugar content (moles/mole of galacturonic acid residues)	1.82	.09	.10	.01
moles rhamnose/mole arabinose	.06	.12	.14	.09
moles xylose/mole arabinose	.06	.08	.11	.10
moles galactose/mole arabinose	.28	.40	.60	.70
moles glucose/mole arabinose	.07	.09	.09	.10

Fig. 2. Gel filtration of a pectate lyase degraded pectin. AUA = anhydro-uronic acid content. Ve = elution volume. The substrate was a DEAE-cellulose purified pectin fraction from the oxalate extract of AIS from unripe apples. Degradation with pectate lyase as described in the text. Extent of degradation was 5%. The eluent was water.



	a	b	c	d
Degree of esterification (%)	94	58	63	89
% of AUA	6	35	47	12
Neutral sugar content (moles/mole of galacturonic acid residues)	2.64	.09	.05	.02
moles rhamnose/mole arabinose	.10	.09	.12	.12
moles xylose/mole of arabinose	.08	.10	.10	.10
moles galactose/mole of arabinose	.37	.51	.60	1.30
moles glucose/mole of arabinose	.09	.06	.10	.10

Fig. 3. Gel filtration of a pectin lyase degraded pectin. AUA = anhydro-uronic acid content. Ve = elution volume. The substrate was a DEAE-purified pectin from the oxalate extract of AIS from ripe apples. Degradation with pectin lyase as described in the text. Extent of degradation was 4%. The eluent was water.

From these results it can be concluded that the neutral sugar side chains are concentrated on 5%, or less than 5%, of the uronic acid residues as 'blocks' giving hairy regions. The larger parts of the pectin molecules are present as 'smooth regions' (homogalacturonan).

These findings are in full agreement with many other results in the literature (de Vries *et al.*, 1981). The composition of the neutral sugars in peak 'a' (and also in the other peaks) is the same as in the undegraded pectin. Even the sugar residues that are linked to shorter fragments ('b', 'c' and 'd' in Figs 1, 2 and 3) have the same composition, which indicates that within the neutral sugar side chain 'blocks', repeating units are present.

In Figs 1 and 2 degradation patterns of pectins that differ in their galactose/arabinose ratio can be compared. In both cases almost all of the galactose residues are found in the fraction indicated in the figures as 'a'. Although the galactose/arabinose ratio in the apple pectin fractions is not constant (as was found for the ratio of the other neutral sugar residues to arabinose residues), it must be concluded that the galactose residues are also situated in the hairy regions. Knee *et al.* (1975) were able to isolate low molecular weight pectin fragments consisting of galacturonic acid and galactose residues only (by degradation, with a polygalacturonase iso-enzyme, of material released from apple cell walls by a different polygalacturonase iso-enzyme). Ishii (1978) released mainly galacturonic acid and galactose residues from potato tissue by pectin lyase action. These data suggest that the distribution of galactose residues along the pectin molecule differs from the distribution of the other sugars. Our experiments did not confirm this idea. The apparent molecular weight of peak 'a' (Fig. 1) is high. Gel filtration with 0·1 M sodium phosphate buffer as eluent gives a lower apparent molecular weight for peak 'a' than gel filtration with water as eluent. Compared with dextrans the apparent molecular weight is about 25 000. Based on the assumption that the hairy regions contain 5% of the uronic acid residues, the molecular weight of the pectin molecules must be very high, several hundreds of thousands.

Gel filtration is not considered to be a reliable method of molecular weight determination for pectin (Masuda *et al.*, 1979). The molecules of peak 'a' contain mostly neutral sugar and esterified galacturonic acid residues; their gel filtration behaviour will look more like that of dextrans than that of the intact pectin molecules. However, dextran molecules are rod-like, whereas the 'hairy regions' may be more like spheres. Nevertheless, these data suggest that the pectin molecules are very large. Molecular weights over 200 000 D have been reported previously (Barret & Northcote, 1965; Kikuchi & Yokotsuka, 1972; Stein & Brown, 1975; Sakai & Okushima, 1978). Since the molecules of peak 'a' (Fig. 1) are very large, it can be concluded that all the neutral sugar residues are linked to relatively short segments of the galacturonan backbone. If the pectolytic enzymes could split galacturonosyl-galacturonide bonds between two adjacent side chains (as shown in Fig. 4) the molecular weight of the fragments containing the neutral sugar side chains would be much smaller (e.g. for a

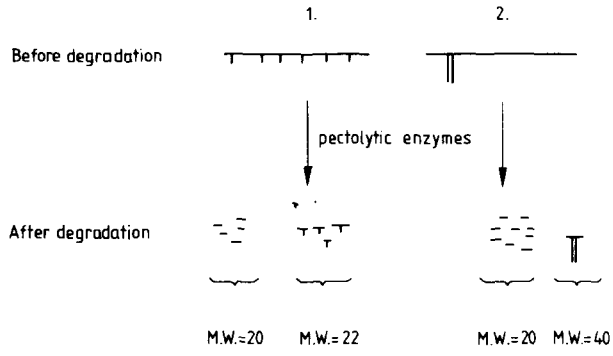


Fig. 4. Two simple models of pectin molecules with different distributions of the neutral sugar side chains along the molecule, before and after enzymic degradation of the backbone. Horizontal line: galacturonan backbone or galacturonan backbone fragments. Vertical line: neutral sugar side chains. Assumption: extent of degradation = 5%; neutral sugar content = 0·2 mole mole^{-1} galacturonate residues. 1. Degree of polymerisation of the side chains = 2. 2. Degree of polymerisation of the side chains = 10; the enzymes cannot split bonds between two adjacent side chains.

pectin molecule containing 10% of neutral sugars on 10 side chains, the molecular weight of the neutral sugar side chains should be 1% of the value for the original molecule).

Long neutral sugar side chains have been reported in the literature. Long side chains containing arabinose and galactose residues have been found by Barret & Northcote (1965), Talmadge *et al.* (1973), Toman *et al.* (1976) and Susheelama & Rao (1978). Keegstra *et al.* (1973) found xyloglucan side chains that could be stained by iodine and therefore must be long. Methylation analysis has shown that the side chains consisting of galactose and arabinose units and the side chains containing glucose and xylose residues are highly branched (Rees & Wight, 1969; Talmadge *et al.*, 1973; Kikuchi & Sugimoto, 1976; Siddiqui & Wood, 1976; Takovlev & Gorin, 1977; Simson & Timell, 1978). In the hairy regions, the neutral sugar content is two to three moles of neutral sugar residues per mole of galacturonate residues. Pectin lyase can degrade tetra-methyl-tetra-galacturonide and higher oligomers, but shows no activity on tri-methyl-tri-galacturonide and lower oligomers (Voragen, 1972). The assumption that there are four or five galacturonic acid units between two adjacent neutral sugar side chains, suggests that the side chains have an average degree of polymerisation of about 15. By combining the results of this paper and the previous paper (de Vries *et al.*, 1981) simple models of the pectin molecules A-E, present in the different extracts of apple alcohol insoluble solids, can be constructed (Fig. 5). By placing the neutral sugar side chain blocks at regular intervals and close to the chain ends the inverse relationship between the neutral sugar content and the apparent molecular weight of some pectin fractions can be explained (types C and D). Type B is the dominant pectin type in the extracts. As already pointed out in the previous paper type A and type E can be considered to be degraded pectins.

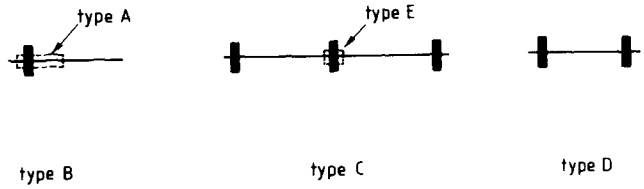
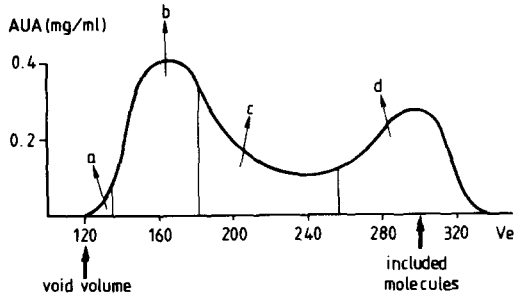


Fig. 5. Model of the pectins of type A-E. The pectin types were defined previously (de Vries *et al.*,1981). Horizontal lines: rhamnogalacturonan backbone of the pectin molecule. Black areas: blocks of neutral sugar side chains.

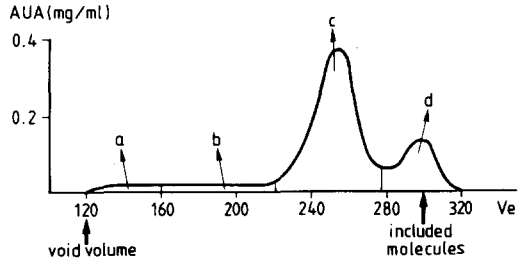
Our model suggests that the rhamnose units are very unevenly distributed along the pectin molecule. This might have implications for theories on pectin gelation in which rhamnose units play a role, because they introduce a kink in the galacturonan backbone (Rees & Wight, 1971).

Figures 6 and 7 show degradation patterns of pectins of type A and type E. These patterns point to a breakdown of the proto-pectin molecules during growth and ripening and during pectin extraction as indicated by the dotted lines in Fig. 5. Figures 1 and 3 provide evidence that the hairy regions have very high degrees of esterification, close to 100%. This can be found both by pectin lyase and pectate lyase action. The



	a	b	c	d
% of AUA	3	40	26	31
neutral sugar content (moles/mole of galacturonic acid residues)				
moles rhamnose/mole arabinose	.90	.03	.03	.06
moles xylose/mole arabinose	.08	.09	.12	.13
moles galactose/mole arabinose	1.00	.50	1.05	1.95
moles glucose/mole arabinose	.10	.06	.09	.12

Fig. 6. Gel filtration of a pectin lyase degraded pectin fraction that contained pectin of type A. AUA = anhydro-uronic acid content. Ve = elution volume. The substrate was a DEAE-purified pectin from the cold buffer extract of AIS from ripe apples. Degradation with pectin lyase as described in the text. Extent of degradation was 12%. The eluent was water.



	a	b	c	d
% of AUA	6	8	83	3
neutral sugar content (moles/mole of galacturonic acid residues)	3.3	2.7	.25	.00
moles rhamnose/mole arabinose	.10	.07	.20	-
moles xylose/mole arabinose	.09	.03	.07	-
moles galactose/mole arabinose	.26	.21	.26	-
moles glucose/mole arabinose	.07	.03	.00	-

Fig. 7. Gel filtration of a pectate lyase degraded pectin fraction that contained pectin of type E. AUA = anhydro-uronic acid content. Ve = elution volume. The substrate was a DEAE-cellulose purified pectin fraction from the acid extract of AIS from unripe apples. Degradation with pectate lyase as described in the text. Extent of degradation was 6%. The eluent was water.

distribution of the methoxyl groups along the molecule and the structure of the hairy regions will be the subject of our future research.

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