Structural features of apple pectic substances

Karakteristiek van de struktuur van appelpektine

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Abstract.

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Pectic substances from unripe and ripe apples were extracted, purified and fractionated. The sugar composition (including types of glycosidic linkages) and the degree of esterification of the fractions were determined. Degradation with purified pectolytic enzymes and fractionation of the resulting pectin fragments were used to study the intra- and intermolecular distribution of the neutral sugar side chains and the methoxyl groups. Models of pectin molecules were proposed, in which the neutral sugar side chains are arranged in blocks ("hairy regions"). The distribution of the methoxyl groups probably is a random one.

"Thou art not for the fashion of
these times, where none will sweat
but for promotion".

Shakespeare (As you like it).

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CONTENTS

1. INTRODUCTION

Studies on pectic substances (in this case the chemical structure of pectic substances) can be found in various fields of science: food science, nutrition, plant physiology, biochemistry and carbohydrate chemistry. This fact not only causes the literature on the subject to be dispersed, it also brings about substantial differences in the choice of the object studied. Plant physiologists are mainly interested in the pectin as it is in the cell wall, whereas food technologists in their studies use pectin as it occurs in extracts of plant material.

Pectin research certainly can be called a long-term research program. Reviewers usually start the pectin history with the discoverer of pectic substances, Braconnot in 1825. In an article about ripening of fruit Frémy (1848) stated that in fruit an insoluble precursor of pectin is present. The term protopectin for this insoluble precursor was first used by Tschirch in 1907. In 1913 Von Fellenberg discovered that pectin was a methylester and in 1917 Ehrlich found out that pectin was composed of D-galacturonic acid. In 1934 pectin was recognized by Morell et al. as a linear polygalacturonic acid. The review of Hirst and Jones (1946) already mentioned the possible presence of rhamnose residues in the galacturonan chain. In the Netherlands a dissertation on pectic substances was published as early as 1928 (A.C. Sloep: "Onderzoekingen over pectinestoffen en hare enzymatische ontleding"). Two of her conclusions:

- Citrus pectin is a mixture of pectic substances of which the carboxyl groups are partly saturated with calcium and magnesium, or are esterified with methyl-alcohol; fully esterified pectin probably is a tetramethylester of an anhydro-mono-arabinose-tetra-galacturonic acid.
- Pectase is not a carbohydrase but an esterase.

Since 1945 the number of publications on pectic substances has been growing tremendously (Joslyn, 1962). New facts were discovered (e.g. the discovery of lyases and trans-eliminative degradation by Albersheim et al., 1960a, b), new methods were developed, laboratory techniques became

more efficient, but all the efforts have not yet resulted in a complete elucidation of the structure of pectic substances and of the structure of the cell wall. A better knowledge of pectin structure hopefully will lead to a better understanding of the role of native pectic substances in processed fruit and vegetables (cloud stability of turbid fruit juices, consistency of apple and tomato paste hardness of canned vegetables and potatoes), the rheological performance of extracted pectin used as thickening and gelling agent and the function of pectic substances as dietary fibre. My study was performed with pectic substances extracted under mild conditions from apples. Apples were chosen because of their use as food in fresh and processed form, technological importance of their pectic substances and because of the fact that the apple is a climacteric fruit (plant physiological relevance). Extraction was necessary because of the need of studying the results of enzymic degradation of isolated substrates (see the Introduction of Chapter 4). Mild extraction conditions were chosen as to prevent artefact formation.

In Chapter 2 the literature on pectin structure research is reviewed. The Chapters 3 - 8 represent the experimental part of this thesis. Chapter 3 describes the results of the extraction procedures. In Chapter 4 a model of apple pectin molecules, based on enzymic degradation experiments, is proposed. Chapter 5 gives some results concerning the structure of the neutral sugar side chains present in the pectin molecules. In Chapter 6 the intra- and intermolecular distribution of the methoxyl groups is described. The subject of Chapter 7 is the fate of the pectin molecules during the ripening of the apples. Chapter 8 is a comparison of the structures of apple and citrus pectic substances. The last Chapter (9) summarizes the Chapters 3 - 8; in this summary some attention is also paid to structure-function relations of pectin molecules

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2. Literature report

2.1. Pectic substances

2.1.1. Occurrence

Literature has been reviewed up to the end of 1981. The term "pectic substances" was defined by the American Chemical Society as a group designation for those complex, colloidal carbohydrate derivatives which occur in, or are prepared from, plants and contain a large proportion of anhydrogalacturonic acid units which are thought to exist in a chain-like combination. The carboxyl groups of poly-galacturonic acids may be partly esterified by methyl groups and partly or completely neutralized by one or more bases (Kertesz, 1951). Pectic substances occur in plant cell walls. The pectic substances of Angiosperms (both monocotyledons and dicotyledons) have been investigated. One Gymnosperm species has been the subject of pectin research, viz. "mountain pine" pollen (Bouveng, 1965). This pectin turned out to be a xylogalacturonan; in monocotyledonous cell walls, pectins of this type probably also occur in the genus Zostera (Ovodov, 1975).

An entire division of plant kingdom (Cryptogamae) has not been investigated at all. Most fungi do not possess cellulose and do not have pectic substances either. Studies on the pectic substances of Bryophytes, Pteridophytes and Gymnosperms could be of great value to cell wall research. Besides in cell walls, pectic substances may be present in the vacuoles of some plants, as has been claimed for the coloured flowers of Boraginaceae species (Hofmeister, 1941; Kenda & Weber, 1952; Bayer et al., 1966). Microscopic investigations showed that the cell wall can be divided into the three layers middle lamella,primary cell wall, secondary cell wall, and that the amount of pectin present decreases in this order (Northcote, 1958). In secondary walls, pectin may be virtually absent. A simplified model of the wall of cultured sycamore cells has been presented (Keegstra et al., 1973). Besides pectic substances, cell walls (can) contain cellulose, xyloglucan (dicotyledons), xylans, arabinoxylans (monocotyledons) arabinogalactan, galactan, glucans,(galacto)glucomannans (gymnosperms) and lignin

structures than their names suggest and may be covalently linked. Cell wall reviews have been written by Lamport (1970), Northcote (1972) and Darvill et al. (1978). Hardly anything is known about the structure of the middle lamella. Three methods can be used for middle lamella research (microscopic examination, macerating agents, comparison with extracellular polysaccharides in cell cultures) but none of these methods can be said to be very suitable. Microscopic examination has revealed that the middle lamella is a thin layer containing highly esterified pectin (Albersheim & Kilias, 1963). Electron-microscopy results suggest that cell wall pectin may have microcrystallinity (Roelofsen & Kreger, 1951). Electron-microscopic investigations combined with other techniques (e.g. X-ray analysis) support the idea of cell wall and middle lamella pectic substances having an oriented structure (Hayashi et al., 1981; Carr et al., 1980).

Carr et al. (1980) produced evidence that calcium is absent in middle lamellae. Frequently, however, Ca-ions are thought to be important in the middle lamellae (Ishii, 1976; Keijbets, 1974; Meurens, 1978). This idea originates from experiments with macerating agents or tissue weakening agents. The possibility that other parts of the cell wall interfere in these experiments cannot be excluded. This possibility is illustrated by the fact that pectin lyase as a macerating agent can, in some cases, solubilize almost all of the pectic substances present (Voragen et al., 1980). For this reason, the experiments of Ginzburg (1961) which suggest a function of protein in the middle lamella cannot be said to be conclusive. The presence of "extensin", a protein with a structural function (Lamport, 1970), in middle lamellae remains a matter of speculation (Bates and Ray, 1981; Winter et al., 1978). Extracellular polysaccharides (ECP), produced by cells in cell cultures, contain pectic substances. If we assume that ECPs contain material that is to be incorporated in the middle lamellae when the cell is part of a tissue, comparison may produce information about middle lamella pectin. This assumption has not been verified; it is interesting to know that regenerated cell walls (after cell wall lysis) differ from normal cell walls

(Asanizu and Nishi, 1980). In tobacco cell cultures ECP pectic substances seem to have a low neutral sugar content (Takeuchi & Komamine, 1978; Yamaoka & Sato, 1977).

2.1.2. Structural features of pectic substances.

The subject of the structural features of pectic substances will be treated briefly in this chapter, as it is dealt with in the introductions of the chapters 3 to 7.

Pectic substances are rhamnogalacturonans with side chains consisting of arabinose, galactose, xylose and glucose. "Minor sugars" of pectic substances are mannose, Me-xylose, apiose, fucose, Me-fucose, glucuronic acid and 4-Me-glucuronic acid. The presence of these sugar residues may be due to impurities. They may also be part of highly branched pectin fragments (Darvill et al., 1978). Recent reviews on the structure of pectic substances were written by Ovodov (1975) and McNeil et al. (1978).

One aspect of the structure has not been considered in the experimental part of this thesis, viz. the acetylation of pectic substances. Acetylation of residues is a general feature of polysaccharides. It is quite common in bacterial polysaccharides, but acetylated polysaccharides have been found in plants also (e.g. xylans). The following table shows some literature data.

Table 1: Acetylcontent of pectins

Pectin source	% w/w Acetylated residues	Literature source
apple, citrus, cherry	0.2	McCready (1970)
strawberry	1.4	Whistler (1969)
sunflower heads	2	Kim et al. (1978)
peach, pear	3 - 4	McCready (1970)
sugar-beet	2.5	Whistler (1969)
sugar-beet	3 - 4	McCready (1970)
sugar-beet	6	Lücker (1976).

The variation (evident from the case of sugar-beet) may represent the naturally occurring variation, but may also be due to factors related to the extraction method and the method of acetyl content determination. Methylation analysis can give information about the site(s) of attachment of the acetyl groups (Björndal et al., 1970). The impact on physico-chemical properties was discussed by Rees (1969).

An interesting study on the distribution of acetyl groups in bacterial alginates was made by Davidson et al. (1977). They concluded from experiments with polyguluronate lyase and polymannuronate lyase that the acetyl groups were unevenly distributed. Klebsiella "polysaccharide 54", however, was thought to have a regular distribution. In bacterial polysaccharide biosynthesis, lipid intermediates carrying oligomers play a role. Therefore, the regularity observed in the latter polysaccharide is to be expected. In higher plants, however, lipid intermediates are probably absent (see Chapter 2.3).

Table 1 shows that apple pectic substances have few acetyl groups, if any. A lot of factors may have an impact on the structure of pectic substances. These factors can be divided into "intrinsic" and extrinsic" factors. In practice, it is impossible to consider only one factor. Intrinsic factors are species, variety, type of tissue and the physiological state of the plant material. Extrinsic are those factors related to the treatment of the plant material in the laboratory: pretreatment, storage, extraction method (see Chapter 2.2.1).

Species. Kawabata (1977) compared the pectins of 39 fruit species and found some differences regarding the neutral sugar content: 4-16 w/w %. All pectins contained arabinose, galactose and rhamnose, but some species (e.g. grape fruit) did not show xylose and/or glucose. In the introduction to Chapter 3 some pectins lacking arabinose and galactose are mentioned.

Aspinall & Jiang (1974) did not observe differences between citrus and rapeseed pectic substances. Darvill et al. (1978) concluded that the cell walls of all dicotyledonous plants are structurally related, but that monocotyledonous plants differ from dicotyledonous plants in this respect.

Stoddart et al. (1967) investigated sycamore pectic substances and found three components: a neutral one, a weakly acidic one and a strongly acidic one. The weakly acidic component was similar to that of apples regarding the neutral sugar content and the electrophoretic behaviour. In apple, however, a strongly acidic component does not exist.

Zitko & Bishop (1965) compared sunflower head, sugar-beet, apple and citrus pectin; they stated that two acidic components are present, one of which has a high neutral sugar content.

Variety. Stein & Brown (1975) found that different tomato pectins showed similar electrophoretic behaviour, but Wallner & Bloom (1977) observed differences in the action of PG on pectins of different tomato varieties. Mango varieties do not show differences in MW or DE of their pectic substances (Srirangarajan & Shrikhande, 1979).

Type of tissue. Aspinall et al. (1968) did not observe differences between pectic substances of stems and leaves of lucerne. The same result was found by Stoddart et al. (1967) for sycamore callus and cambium pectin. In roots, pectic substances may be involved in ion-exchange processes (Ramamoorthy & Leppard, 1977); it is possible that such pectic substances have an "adapted" structure. It is known that the endocarp and the epicarp of apples have higher pectin contents than the mesocarp (Doesburg, 1965).

Physiological state of the tissue: ripening, rate of growth, age. The events during ripening will be discussed in Chapter 5. In the pectin of fast growing (auxin treated) plants, more side chains may be present (Rees, 1967).

In root caps, more glucose (radio-active labelled) is found to be incorporated in galacturonic acid, galactose and arabinose compared with older tissue (Northcote, 1974). In the stage of secondary wall formation, the degree of branching diminishes and the Ca-sensitive fraction of the pectic substances increases (Gould et al., 1965). In this stage, a decrease in the activities of pectin-synthesizing enzymes and an increase in these activities of hemicellulose-synthesizing enzymes can be observed (Dalessandro and Northcote, 1977).

2.1.3. Natural functions.

As a cell wall component, pectin may have a "lubricating" function in growing tissue, and a "cementing" function in older tissue (Rees and Wight, 1969). According to Northcote (1972), cell walls can be compared with glass-fiber-reinforced plastics. Cellulose plays the part of glass fibres and pectic substances together with hemicelluloses play the role of matrix. This comparison emphasizes the importance of pectin-hemicellulose-cellulose interactions. In fact the strength of pectin gels increases on addition of cellulose (Walter et al., 1978). Pectic substances may also interact with cellulose via xyloglucans (Keegstra et al., 1973).

Up to the present, a clear picture of the function of pectic substances in cell walls has not yet been obtained.

The function of pectic substances during ripening will be discussed in Chapter 7.

In phytopathogenesis, the cell wall serves as a barrier and/or as a substrate for the attacking organisms. There is no direct relation between pectolytic enzyme activity and pathogenicity of micro-organisms. Pathogens without pectolytic enzymes have been found (Torzilla and Andrykovitch, 1980). On the other hand, some organisms having these enzymes are harmless. Micro-organisms frequently have multiple forms of pectolytic enzymes; this may enhance their chance for survival as suggested by Naumann (1978). Soft rot is the only plant disease certainly caused by pectolytic enzymes (Wood, 1978).

During pathogenesis, complex interaction between plant and pathogen takes place. Kenning & Hanchey (1980) observed that during infection plant cells at a certain distance from the attacked site can react.

Fragments of pectic substances play a role as "elicitors" (Albersheim et al., 1981). The Colorado beetle can induce plants to release a Protein Inhibitor Inducing Factor (PIIF). In soy-bean cotyledons, an "endogenous elicitor" (EE) stimulates the production of phyto-alexins (antibiotics) during pathogenesis. Both PIIF and EE probably are fragments of pectic substances having very complex structures.

In the reviews of Rexova-Benkova and Markovitch (1975) and of Rombouts and Pilnik (1980) phytopathogenesis was discussed briefly. More detailed reviews are from Naumann (1978) and Wood (1978).

The ion-exchange capacity of pectic substances is used by the roots of plants to absorb calcium ions from the soil (Deuel and Stutz, 1958; Oades, 1978; Ramamoorthy and Leppard, 1977).

2.1.4. Applications.

In food industry, pectins are widely applied as gelling, thickening or stabilizing agents (Pederson, 1980; Pilnik and Voragen, 1980). Two main types of pectin gels exist: calcium pectate gels containing Low Methoxyl pectins (Weiss, 1979), and sugar-acid-pectin gels containing High Methoxyl pectins. Calcium binding properties and theories on gelation will be discussed in Chapter 6. In fruit juice technology, pectin and pectolytic enzymes play an important role (Krop, 1974; Rombouts and Pilnik, 1978).

Pectin has a nutritional function as dietary fiber component (Bock and Krause, 1978; Cummings et al., 1979; Jenkins, 1980; Stasse-Wolthuis, 1981).

Pectin also finds a limited medical application as a detoxifying agent, haemostatic agent and general intestinal regulator (Deuel and Stutz, 1958; Verstraete, 1979).

2.2. Structure research of pectin and other heteropolysaccharides.

Structure research of naturally occurring polysaccharides should include the following steps:

a) extraction from the neutral environment
b) isolation and fractionation
c) analysis of the whole molecules
d) analysis of degradation products of the molecules.

Complete elucidation of the structure implies that knowledge is available about: sugar composition, type of glycosidic linkages, anomeric and absolute configuration, sequence of the residues, substituents, possible linkages to other polysaccharides e.g. in a cell wall, polydispersity (molecular weight), distribution of parameters. In this chapter the four steps are discussed with special attention to the problems in pectin structure research.

2.2.1. Extraction from the natural environment.

This first step in the whole procedure represents the first problem, certainly in the case of pectin. Most pectin molecules are firmly bound in the cell wall in a way not yet understood. Keegstra et al. (1973) suggested that pectin is covalently linked to xyloglucans which are non-covalently linked to cellulose and there are other mechanisms involved as well. A possible role of Ca-bridges has often been mentioned (Doesburg, 1957; Keybets, 1974; Knee, 1978). Most of the hypotheses of the review of Joslyn in 1962 have not been confirmed or rejected since.

Consequently, it is not possible to extract pectin completely without degradation, as already stated by Ehrlich in 1932. In fact, even with degradative reagents it proved to be difficult to extract 100% of the pectin (e.g. Joslyn & Deuel, 1963). Degradation due to native enzymes, acid or alkali, occurs during the extraction. Anderson et al. (1961) showed that some decarboxylation occurs in boiling water. To make extraction easier, the pretreatment of the plant tissue is an important factor: freezing, drying, heating, alcohol extraction, etc. strongly influence the extractability of the pectin (Joslyn & Deuel, 1963). Joslyn and Deuel (1963) compared many different extractives like salts, acids, alkalis and sequestrants; one of their conclusions was that Ca-bridges are unlikely to be responsible for the insolubility of pectin. Ions enhance the extent of β-elimination during extraction (Keijbets, 1974). After extraction cell wall polysaccharides and (glyco)-proteins may aggregate (Knee, 1973). Many investigators use a serial extraction comparable with the one described in Chapter 3, of which the last step usually is an alkaline extraction (Raunhardt & Neukom, 1964).

2.2.2. Isolation and fractionation.

From the crude extract thus obtained the polysaccharide has to be isolated. Isolation and fractionation can be performed simultaneously. In order to have insight into the distribution of parameters like MW, fractionation is inevitable.

Enzymes can be very helpful to the isolation of polysaccharides from crude extracts: proteinases and amylases have been used repeatedly.

In general, a combination of fractionation techniques is needed to have sufficient insight into the distribution of parameters. The following methods have been used for the fractionation of pectin:

1. Ion exchange chromatography has been used in this study (Chapter 2). This method has been used by almost all pectin structure investigators. DEAE-cellulose or DEAE-dextrans are mostly used, although there are several other methods (Antal & Tomab, 1976). Mansoor Baig et al. (1980) separated

radio-active labelled pectin into 8 peaks by ion exchange chromatography. Neutral polysaccharides can be fractionated by DEAE-cellulose as borate-complexes (Linssen, 1981).

2. Gelpermeation chromatography (gelfiltration or exclusion chromatography) provides information about the distribution of the MW; it cannot be considered a reliable method of MW determination as comparison is needed with standards of known MW. Masuda et al. (1979) showed that the ionic strength is an important factor in gelfiltration of pectin: the hydrodynamic volume of charged molecules is, of course, expected to depend on factors like pH and salt concentration. In pectin studies, this method has been used, among others, by Barret and Northcote (1965), Anderson and Stoddart (1966) and O'Beirne (1980). Stein and Brown used gelfiltration to show that differences in MW among tomato pectins that had been extracted in different ways, were small. Sakai and Okushima (1978) showed by gelpermeation chromatography that a certain endo-PG released high molecular weight pectin fragments. Eda and Kato (1980) and Nishitani and Masuda (1980) applied this method to fractionate azuki bean and tobacco pectin respectively into 2 fractions, one of which was rich in neutral sugars (the fraction with the highest MW). Gelfiltration can also be performed in a HPLC-equipment (Barth and Regnier, 1979; Barth, 1980).

3. Adsorption and partition chromatography. Cellulose, Ca-phosphate and Celite columns can be used to fractionate polysaccharides (Bowness, 1965; Gardell, 1965).

4. Fractionation by graded precipitations. Polysaccharides can be fractionated by means of graded precipitation, and this has been done especially with pectins because of their charge. Aspinall et al. (1970) and Zitko & Bishop (1965) used sodium-acetate solutions of different concentrations and Anderson & Stoddart (1966) chose sodium sulphate solutions for pectin fractionation. The sensitivity of pectin to divalent ions allowed Barret and Northcote (1965) to precipitate pectin fractions with calcium salts. The fractionation obtained by graded precipitation can be characterized as quite "crude". Alcohol, BaOH, Cu^{2+}, Al^{3+} and quaternary ammonium salts can also be applied.

5. Electrophoresis. Charged polysaccharides can be fractionated by electrophoresis. This method was applied to gums by Anderson and Bell (1976) and to alginates by Bucke (1974). Electrophoretograms of apple pectin before and after chemical transelimination were published by Barret and Northcote (1965). Brookhart (1965) showed pectin factionation by starch gelelectrophoresis. Pectins isolated from onions, tomatoes and plum fruits have been fractionated with electrophoretic methods by Mankarios et al. (1980), Stein and Brown (1975) and Boothby (1980) respectively. They observed that broad zones appear in the electropherograms and that there is usually an immobile fraction. Plant cell wall components have been separated this way by Jarvis et al. (1977).

6. Lectin affinity chromatography. This method was used by Gleeson and Clarke (1980) to fractionate arabinogalactans. Pectins (mostly carrying arabinogalactan side chains) may be fractionated this way.

7. Hydrophobic binding. Bussey et al. (1975) fractionated glycoproteins and neutral polysaccharides by chromatography on substituted dextran columns; they assumed that "hydrophobic binding" was the binding mechanism involved.

2.2.3. Analysis of the whole molecules.

The third step in the procedure provides an answer to the following questions:

- Which sugar residues does the polysaccharide consist of?
- What are the types of glycosidic bonds present?
- What are the anomeric and absolute configurations and conformations of the sugar residues?
- What is the ring size of the sugar residues?
- What is the number of residues present (degree of polymerization)?

However, all this information is not sufficient to propose a structure: the sequence of the sugar residues is still unknown. Of course, comparison of the data with those of known polysaccharides can give indications; especially the method

mentioned hereafter under 2, 3 and 4 could give valuable information on the sequence.

In the analysis of the whole molecules, the following methods must or may be used:

1. Determination of the sugar residues present. Apparently in conflict with the idea of analyzing whole molecules, this must be done by means of complete hydrolysis. Hydrolysis, however, is always accompanied by decomposition; fructose, for instance, is readily decomposed and gulose is known to be transformed into its 1,6-anhydro form during hydrolysis (Aspinall, 1970). Pectin Hydrolyzates can contain methanol, acetic acid, furfural, reductic acid, arabinose, alginetin and 3-hydroxy-pyridine (Deuel and Stutz, 1958). Complete hydrolysis is hard to achieve for uronic acid polymers, aldobiuronic acids and cellulose. An optimum between too much degradation and too little hydrolysis must be chosen.

The monomers thus obtained can be separated by PC, TLC, HPLC and GLC. Despite the derivatization needed, GLC is most frequently used because of the sensitivity, the accuracy and the possibility of identification by combined GLC-mass spectrometry. Neutral sugar residues can be separated on GLC-columns as trimethylsilyl-esters, oxim-derivatives, aldononitriles, methylglycosides or alditol-acetates. The sensitivity was enhanced further by introducing radio-active labels (Prehm and Schei, 1978). The derivatization sometimes has as a side-effect the introduction of plasticizers into the system (Bacon, 1980).

Uronic acids cannot be analyzed as alditol-acetates; they can be separated gas-chromatographically as methylglycoside-methylesters (Kakuta and Misaki, 1979). Alternatively, they can be reduced to the corresponding neutral sugars. Several reducing agents have been used for this purpose (den Uijl, 1982). The method of Taylor and Conrad (1972) and reduction with LiAlH after methylation are frequently used. Complete reduction, however, can hardly be achieved. Uronic acids can also be separated on HPLC (Voragen et al., 1981).

2. Methylation analysis has been applied extensively to polysaccharides during the past decade. Methylation, hydrolysis, reduction and acetylation result in partially methylated alditol-acetates that can be separated, quantified and identified with GC-MS (Björndal et al., 1970). Acetylgroups indicate the presence of glycosidic linkages. In this way, binding types and ring sizes are determined simultaneously, although the ring sizes cannot be determined in all cases. During the stage of hydrolysis step, some demethylation cannot be avoided; for this reason, minor sugar derivatives should not be taken into account in the analysis of the results. The interpretation of the results is based upon the assumption of complete methylation; this can be achieved by the method of Hakomori (1964) using methyliodide in DMSO (and $DMSO^-$-ion as a catalyst). Methylated oligosaccharides can be analyzed without hydrolysis by GC-MS or HPLC-MS (McNeil et al., 1981).

3. Periodate oxydation and subsequent degradation give information about the degree of branching and the distribution of the branch-points. For the last few years the method has been replaced to some extent by methylation analysis. Periodate attack requires vicinal hydroxyl groups which are absent in most branchpoint sugar residues. In the sixties, the Smith degradation (periodate oxydation, reduction and sequential degradation by mild hydrolysis; Goldstein et al., 1965) replaced the Barry degradation, which used phenylhydrazine and diluted acetic acid after periodate oxydation. These methods have been described in several reviews (Aspinall, 1970; Lindberg et al., 1975). In the case of acidic polysaccharides, complete periodate oxydation is not readily achieved, as was shown for alginates by Larsen and Painter (1969). Side reactions occur; six member cyclic hemiacetals between neighbouring residues are formed. Despite this problem, several investigators applied this method to pectins (Anderson and Stoddart, 1966; Yakovlev and Gorin, 1977) and uronic acid containing gums (Aspinall and Nasir-ud-din, 1965).

4. Immunochemical reaction (Aspinall, 1970). The cross-reaction of a polysaccharide with the anti-serum of a specific poly-saccharide may prove structural similarity. E.g. beef lung galactan precipitates with anti-Pneumococcus type XIV serum; galactans with the same behaviour certainly are structurally

related to beef lung galactan.

5. Physical methods. Infrared spectrometry can show the presence of special functional groups like carboxyl- or sulphate groups. In some cases IR and NMR give information about the anomeric configuration. Degrees of polymerization can be determined by viscosimetry, light scattering, osmometry and endgroup methods. Ultracentrifugation may provide an indication about the homogeneity of the sample.

2.2.4. Degradation of the molecules.

In this last stage the sequence of the sugar residues is to be elucidated. It must be emphasized that in order to arrive at a correct interpretation of the results of this step, the results of step b (see page 8) must be available. Otherwise, one cannot distinguish between intermolecular and intramolecular distribution. In most cases the results of more than one type of degradation and of different degrees of degradation are required. Degradation methods can be divided into specific, selective and aselective methods. Another possibility is to divide them into endo and exo methods. In polysaccharide chemistry, exo-methods are hardly available: exo-enzymes and alkaline degradation from the reducing end have not yet proved their validity. Another problem with exo-methods is that, unlike proteins, the samples usually are non-homogeneous. Hence, in this chapter, specific, selective and aselective methods will be discussed. Again, methods especially suitable for pectin research will be emphasized, especially where specific methods (using the properties of a certain functional group) are concerned.

Specific methods (Lindberg et al., 1975; Aspinall, 1977).
1. Alkaline degradation of 4-O substituted hexuronate residues. At pH over 6 pectins undergo transeliminative degradation resulting in a double bond between C4 and C5 (Albersheim et al.,1960). This reaction can be used for structure research (Fielding, 1975), but complicating reactions occur as well (Henfrey, 1975; McCleary et al., 1967). Aspinall and Rosell (1977) applied this reaction to methylated polysaccharides. Especially in water, high degrees of degradation are difficult to obtain,

because saponification occurs (Albersheim et al., 1960). This difficulty can be overcome by avoiding water, as was tried by Curvall et al. (1975) and Lindberg et al. (1973). These authors prepared (1-methoxy)-ethylesters and took methyl-sulfinylmethanide as a base and DMSO as a solvent. Lindberg et al. (1978) used triethylamine as a base, acetic acid anhydride as a solvent and converted the carboxylgroups into mixed anhydrides by acetic acid anhydride and pyridine. But also in these cases, the results are poor. Apparently, these reactions are useful when low degrees of degradation are sufficient.

2. The Hofmann rearrangement is specific to esterified uronic acid residues. After amidation the substrate is oxidized by hypobromite resulting in 5-aminopentose (via isocyanate). At these points the chain can be split by very mild hydrolysis (Kotchetkov et al., 1970). The Weermann degradation (a modification of the Hofmann degradation) has been applied to birch xylan: the glucuronate residues could be removed this way, which yielded a linear polysaccharide (Gorin and Spencer, 1970).

3. The Lossen rearrangement also results in 5-amino-pentoses, but in this case the hydroxamic acid ester is oxidized by carbodiimides (Saier J r et al., 1968). Both the Lossen and the Hofmann rearrangements and subsequent degradations fail to proceed to completeness, certainly in the cases of pectin (Fielding, 1975).

4. Alkaline degradation from carbonylgroups. OH-groups can be oxidized to carbonylgroups by ruthenium tetraoxide and this makes the polysaccharide susceptible to alkaline degradation (sodium ethoxide in ethanol/dichloremethane). This so-called Svensson-degradation has been applied to a lipopolysaccharide from Salmonella typhimurium. The method is only useful when some specific OH-groups are present, as in the case of methylated polysaccharides after mild hydrolysis.

5. Alkaline degradation from 6-C-sulphonylgroups. A polysaccharide is susceptible to alkaline degradation when the primary alcohol is sulphonated. This fact has been used to investigate the distribution of the side chains in galactomannans from guar and locust bean gum (Baker and Whistler, 1975).

6. Alkaline degradation from the reducing end. This reaction, wellknown from cellulose research as the so-called "peeling"-reaction, is never complete. Side reactions occur. It might be applicable, however, to oligosaccharides (Aspinall, 1977).
7. Enzymic degradation is the most important method in my study. For this reason, this subject is discussed in a separate section (2.2.5).

Selective methods (Aspinall, 1977; Lindberg et al., 1975).
1. Partial acid hydrolysis is no doubt the degradation method used most frequently. Advantage is taken of the fact that different glycosidic bonds differ in rate of hydrolysis (BeMiller, 1967). Bonds in a polysaccharide may differ in this respect from bonds in disaccharides (BeMiller, 1967). Numerous investigators have used the difference in acid-sensitivity between hexoses and pentoses. The amount of information obtained can be enhanced by applying the method to methylated polysaccharides, but in this case the reduced acid-sensitivity of methylated polysaccharides must be taken into account. Very small amounts of oligosaccharides detected in the hydrolysates might be due to transglycosidation.

The selectivity can be influenced by reducing acidic residues to neutral residues, or by oxidizing neutral residues to acidic residues. A limited degree of oxidation can be achieved by O_2/Pt and this has been applied to bacterial dextrans, arabinogalactans and arabinoxylans. Another possibility is the introduction of acid-labile bonds, by e.g. hex-5-enopyranoside residues or 3,6-anhydroderivatives. The Smith degradation is another example of influencing the selectivity. Lindberg et al. (1975) suggested that acid-labile bonds are naturally occurring in all polysaccharides. They assumed that the weak linkages in cellulose may be hexodialdo-1,5-pyranosides.
2. Acetolysis, methanolysis, mercaptolysis and formolysis: hydrolyses in environments other than acidic water. These degradation methods result in other "cracking patterns" than acid hydrolysis does. A well-known fact in pectin research is the stability of the pseudoaldobiuronic acid bond between rhamnose and galacturonic acid during acetolysis.

3. Oxidative degradation. Oxidation with chromium trioxide is a method to determine the anomeric configuration of the residues present. Radicals can act as oxidizing agents; they can be introduced into the system by radiation (X-rays, γ-rays, UV) or by xanthine oxydase (Kon and Schwimmer, 1977). Aci-reductons like ascorbic acid in the presence of oxygen (Deuel and Stutz, 1958) and chlorine or bromine (Anderson et al., 1965) can be used as an oxidizing agent. High degrees of degradation cannot be achieved.

Aselective methods.
Methods of proved random attack are not available. However, in the methods mentioned below stochastic attack may be approached in some cases.
1. Mechanical treatment (mechanolysis), e.g. "dry ball milling" is known to degrade polysaccharides (Deuel and Stutz, 1958), but only very low degrees of degradation are to be expected (Dongowski and Bock, 1973).
2. Acid hydrolysis, oxidative degradation, acetolysis etc. can in some cases be assumed to be virtually aselective, e.g. when bonds of equal strength are present and limited degrees of degradation are considered.

The polysaccharide fragments obtained after degradation must be separated and identified. As mentioned before, methylated oligosaccharides can be separated and identified in one step by GC-MS or HPLC-MS (McNeill et al., 1981). But in most cases, more than one step is needed to elucidate the structure of a polysaccharide.

2.2.5. Enzymic degradation of the molecules.

Enzymes are used in structure research because of their specificity or selectivity. When only crude enzyme preparations are available, conditions must be chosen that favour the reaction desired. Complete selectivity can hardly ever be obtained; of course, in the case of homopolysaccharides complete selectivity is not a prerequisite. Polysaccharide-degrading enzymes are highly specific; in heteropolysaccharides they also show selectivity: bonds in the vicinity of side chains mostly are degraded at a much lower rate. In pectin,

the distribution of the methoxylgroups is another complicating factor: the selectivity of the different pectolytic enzymes towards different types of bonds in the pectin molecules is hardly known (see figure 2.1). The binding of enzymes and polysaccharides probably involves several sugar units.

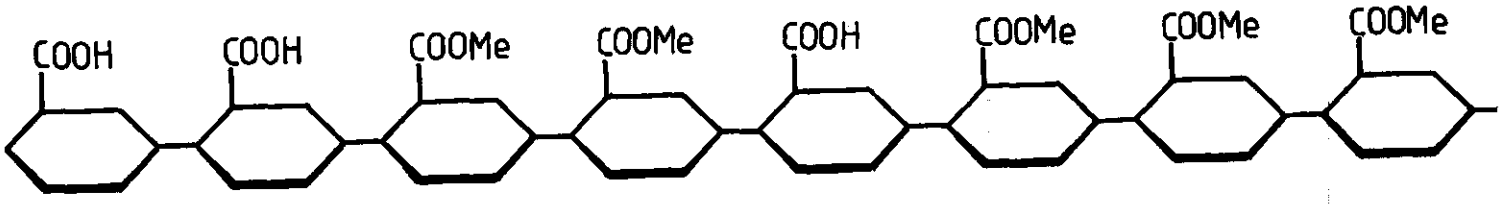


Fig.2.1 : The glycosidic bonds in a pectin molecule differ from each other, because they have a different "environment".

The enzymes can be used in two ways: on complex polysaccharide systems like cell walls or on isolated polysaccharides. A peculiar application is the "sequential enzyme induction" by which the structure of a glycoprotein from Klebsiella aerogenes was studied (Barker et al., 1963). Detailed studies on isolated polysaccharides require homogeneous substrates. In most heteropolysaccharides both an intermolecular and an intramolecular distribution of parameters is present. Many structural investigations result in a proposal for an intramolecular distribution of a parameter without thoroughly checking the homogeneity of the substrate used. Examples of applications of enzymes to isolated polysaccharides are xyloglucans (Bauer et al., 1973) and galactomannans (McCleary, 1979). The case of galactomannan illustrates the difficulties in interpretation of the results of enzymic degradation. The results of chemical and physical methods point to a regular distribution in guar galactomannans (Palmer and Ballantyne, 1950). McCleary (1979) claims a random distribution of the galactose single unit side chains, whereas Courtois and LeDizet (1970) propose a partial block structure in the case of carob galactomannan. In both cases β-mannanase was used (from different sources).

There are many reasons why one must be careful to draw conclusions from enzymic degradation; figure 2.2 illustrates this.

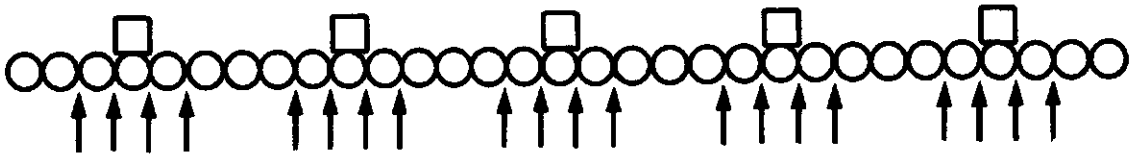
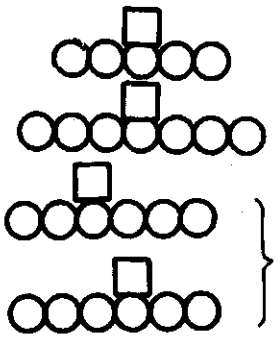


Fig.2.2. Complete enzymic degradation of a regular branched heteropolysaccharide. The enzyme cannot attack the bonds indicated by arrows; its end-products are dimers and all bonds are split at the same rate. Expected products:



25 (mole %)

25

50

The figure gives a hypothetical example of an enzymic degradation of a regularly branched polysaccharide of infinite length. If a random distribution of side chains had been present, the degradation would have resulted in oligomeric main chain fragments (dimers, trimers, etc.) and in highly branched fragments that cannot be degraded by the enzyme, and also in fragments similar to those in the figure. In this way regular distributions and random distributions can be distinguished; in practice, however, the results can be distorted by the presence of impurities and, as emphasized above, by inter-molecular distribution, incomplete degradation, product-inhibition, side reactions and "end-fragments" (fragments originating from the ends of the chain). In the case of pectin, side reactions are saponification and chemical transelimination. Pectin preparations sometimes contain impurities that may be bound to the pectin molecules such as proteins and polyphenols.

The "secondary" and "tertiary" structure of the substrate may influence the activity of an enzyme. Perhaps, a certain bond can be broken only in a certain configuration. One can imagine that in this way bonds far from the binding region influence the activity of the enzyme. A tetramer in a chain has another configuration than in solution. Of course, when a more complex regularity exists (e.g. a mixture of two types), the results can easily be misinterpreted.

The interpretation of the results of enzymic degradation of polysaccharides is complicated by numerous factors. Beside the factors related to the substrate, factors related to the mode of action of polysaccharide-degrading enzymes must be considered. Many investigations have been performed with amylases and this work has resulted in the insight that considerable differences among the amylases exist, and that the catalysis is a rather complicated event.

An important variable is the degree of multiple attack. "Single chain" and "multiple chain" mechanisms may be considered to be the extremes of a range of degrees of multiple attack (Bourne and Finch, 1970). French and Robyt (1967) compared three α-amylases and found degrees of multiple attack of 7.0, 3.0 and 2.9. For sulphuric acid they found 1.9 and not 1.0 as may be expected for purely random attack. Labelled oligosaccharides were chosen as substrates; there may, however, be a difference in degree of multiple attack between oligomeric and polymeric substrates. Another complicating factor is the occurrence of "multimolecular reactions": condensation, transglycosylation and "shifted binding" (Allen, 1980). Shifted binding is the binding of a second substrate molecule in a non-productive mode resulting in the cleavage of a different bond in the substrate molecule. Asp.oryzae α-amylase shows significant transglycosylase activity (Allen and Thoma, 1978), and recently it has been suggested that transglycosylase activity is normal for polysaccharide degrading enzymes (Kitahata et al., 1981). The possibility that the enzyme preparation used contains multiple forms of the enzyme must also be kept in mind. Many polysaccharide-degrading enzymes have multiple forms possibly differing in mode of action, and it is not always easy to separate them.

Some polysaccharide-degrading enzymes themselves contain sugars; especially extracellular enzymes seem to be glycoproteins: the saccharide part may be involved in excretion (Van Houdenhoven, 1975). However, the quantities of saccharides introduced into the system this way mostly are negligible. Bacterial extracellular enzymes do not contain sugar residues.

Differences in the mode of action of polysaccharide-degrading enzymes can be explained by using the subsite model. The binding of substrate and enzyme involves several sugar units. The "binding region" contains binding subsites and a catalytic subsite. Two methods have been developed to determine the length of the binding site: the "kinetic method" (Hiromi et al., 1973) and the "product analysis method" (Thoma et al., 1970). Both methods use oligomeric substrates; again, it must be emphasized that differences between oligomeric and polymeric substrates cannot be excluded.

The subsite model facilitates calculations concerning the extent of degradation of pectin. Different assumptions about the structure of the binding site result in different theoretical degrees of degradation. The figure shows an example for pectin lyase

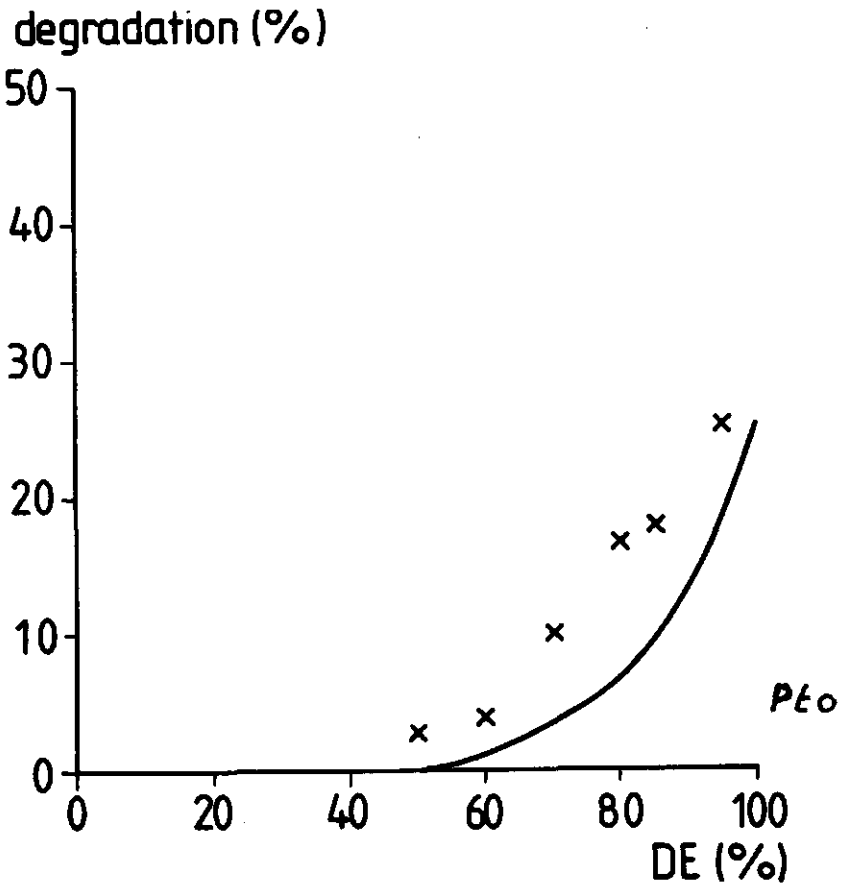


Fig.2.3.: Extent of degradation of pectin by pectin lyase.

✗ experimental value (Voragen, 1972).

——— theoretical values are based on the assumption that the subsite involves 8 galacturonide units one of which may be de-esterified (Van Houdenhoven, 1975). The pectins were esterified with methanol/H_2SO_4 and subsequently de-esterified with alkali; a random distribution of the methoxylgroups was assumed (see chapter 6).

"Subsite mapping" has shown in some cases that the subsite next to the catalytic site has an unfavourable interaction energy with monomeric substrate. In exo-enzymes this effect may be responsible for the mode of action of these enzymes: this barrier prevents binding at one site of the catalytic subsite (Allen, 1980). In the review of Rexova-Benkova and Markovic (1976) some subsite models for pectolytic enzymes are discussed.

Pectin-degrading enzymes are classified according to their mode of action (lyases or hydrolases, exo or endo), and their preference for either pectate or high methoxyl pectin. The classification scheme of Demain and Phaff (1957) was replaced in 1963 by that of Neukom after the discovery of lyases. Neukoms scheme in turn was in fact replaced by a new one (Rexova-Benkova and Markovic, 1976): polymethylgalacturonase (acting hydrolytically) probably does not exist (Voragen, 1972), pectate lyases show some activity on high methoxyl pectins (Rombouts, 1972), pectin lyases sometimes act on low methoxyl pectin (Sherwood, 1966; Bateman, 1966), and some exo-enzymes turned out to be oligogalacturonide hydrolases or lyases.

It has recently been suggested that there is an enzyme (in Erwinia species) acting as a lyase at high pH and as a hydrolase at low pH (Stack et al., 1980): another complicating factor, if it is true.

In this chapter some groups of pectolytic enzymes are discussed briefly: more detailed reviews on this subject have been published recently (Rombouts and Pilnik, 1980; Rexova-Benkova and Markovic, 1976). Therefore, this chapter has a somewhat eclectic nature.

Pectinesterase (PE, EC 3.1.1.11) was hardly used in my investigations, but it is discussed because of its possible implications for the distribution -in vivo- of the methoxylgroups along the pectin molecule(s). Plant PE acts according to a single chain mechanism (Heri et al., 1961; Kohn et al., 1968), which results in blocks of saponified galacturonide units. Differences can be observed among e.g. PE from tomatoes, Fusarium, Clostridium and Aspergillus (Rexova-Benkova and Markovic, 1976; Baron et al., 1980). PE is specific for pectin, but it can also slowly saponify ethyl- and propyl-esters of polygalacturonic acid. Plant PE is bound to the cell wall.

End product inhibition occurs, and PE never saponifies the pectin completely. The lower the initial DE, the lower the final DE. Neutral sugar side chains probably block PE-action (Versteeg, 1979). For an optimal enzyme-substrate complex, citrus PE possibly needs two saponified galacturonide units at a certain distance (Versteeg, 1978).

Of the pectin-degrading enzymes polygalacturonase (PG, EC 3.2.1.15 and EC 3.2.1.40-exo)has been studied mostly. Endo-PGs have been described with different numbers of subsites: 3, 4 or 5 (Rexova-Benkova and Markovic, 1976). The mode of action of different PGs is not the same. They have different degrees of multiple attack and the endproducts are different. Coniothyrum diplodiella produces 3 endo-PGs with 3, 4 and 10% of the bonds broken at 50% viscosity-reduction. The PG of Colletotrichum lindemuthianum has a high degree of multiple attack.

It is very well possible that an enzyme works at random at the first stage of the reaction, but not at a later stage; this has been suggested for soy-bean amylase (Greenwood et al., 1965); in such cases the degree of multiple attack changes during the reaction. The hydroxyl groups at C2 and C3 of the galacturonide units are not involved in the enzyme-substrate complex of Aspergillus niger PG (Koller and Neukom, 1969): can hydrolyse acetylated substrate. However, apiogalacturonan with 40% apiose cannot be degraded (Hart and Kindel, 1970). No PG can degrade

the substrate completely. Rhizopus arrhizus PG can degrade pectate up to 95% (Liu and Luh, 1980); 5% remains high-molecular, probably because it carries neutral sugar side chains.

The activity depends on the degree of polymerization (DP) of the substrate (Dongowsky et al., 1980). Liu and Luh (1980) observed for the degradation limit an optimum DP of 25. The same optimum DP was found for an exo-PG (Sato and Kaji, 1979), for which also an optimum DE was found (about 20%).

It is interesting to mention that Dongowski et al. (1980) found that Aspergillus niger PG was inhibited by a plant extract, whereas tomato PG was not. Data concerning the relation between degradation limit and DE were collected by Luh and Phaff (1954) for yeast PG, and by Koller and Neukom (1969) for Aspergillus PG. For yeast PG an inverse direct proportionality was found, and for Aspergillus PG a somewhat more complex curve. In both cases, the substrates were pectins of different DE prepared by first esterifying them and subsequentially saponifying them. If one assumes that the enzyme needs a certain sequence of esterified and saponified units, one should expect the curve to be exponential as in the figure 2.3. This assumption is too simple: the enzyme has a certain degree of freedom.

Exo-PG or exo-PAL is presumably very useful in determining the place of the neutral sugar side chains in the pectin molecule, but it has not yet been used for this purpose. Apples have an exo-PG (Bartley, 1978). Gelfiltration experiments produced evidence that peach exo-PG uses a multi-chain mechanism (Pressey and Avants, 1973).

Knee et al. (1975) used two PGs for cell wall structure research, and suggested that the difference in MW's of the enzymes explains the difference in their mode of action on cell walls.

Pectate-lyase (PAL, EC 4.2.2.1 or EC 4.2.2.2 - exo) has a high pH-optimum. Therefore, degradation with PAL can be accompanied by saponification and chemical trans-elimination. PAL shows some activity on high-methoxyl pectins; an optimum DE between 10% and 50% sometimes exists (Rombouts, 1972). Also the degradation limit sometimes has an optimum in this DE-range (Rombouts et al., 1978). As in the case of PG, no simple exponential relation between DE and the degradation limit exists. PAL of two Bacillus species were able to degrade Bacillus Vi antigen, which contains substituted galacturonate residues (McNicol and Baker, 1970): thus, the hydroxyl groups of the C2 and C3 atoms of the galacturonide units are not essential in the enzyme-substrate complex. Tragacanth gum and karaya gum were not attacked by Pseudomonas fluorescens PAL, which is an indication that neutral sugar side chains or the presence of rhamnose in the chain block PAL action. The percentage of bonds broken at 50% viscosity reduction varies among the PALs: the "degree of random behaviour" is not the same. Arthrobacter PAL and Bacillus polymyxa PAL have degrees of multiple attack that are 4.7 and 1.5 times respectively as high as the degree of multiple attack of Pseudomonas fluorescens GK5 PAL (Rombouts, 1972). Rexova-Benkova and Markovic (1976) divided PALs in two groups based on the degradation patterns of oligomers.

Clostridium multifermentans produces a complex of exo-PAL and PE (Miller and Macmillan, 1970). The complex (M.W. 400.000) attacks its substrate (either pectin or pectate) from the reducing end.

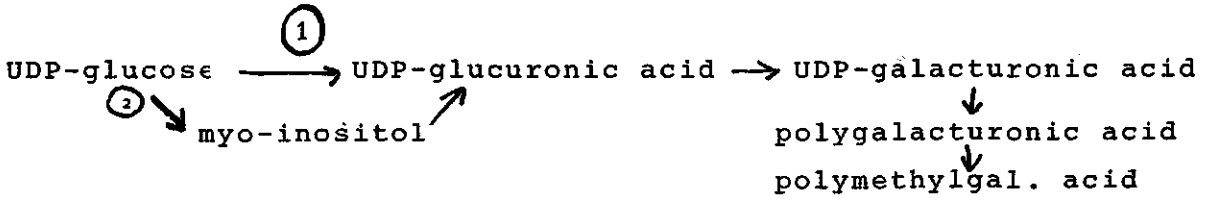
Pectin lyase (PL, EC 4.2.2.3) causes a rapid drop in the viscosity of substrate solutions (high methoxyl pectins). Voragen (1972) found 2% bonds broken at 50% reduction of the viscosity, whereas for chemical transelimination he found 2.3%. The activity is higher on pectins de-esterified with pectinesterase than on pectins de-esterified to the same extent with cold alkali (Voragen, 1972). The curve degradation limit - DE is approximately exponential (Fig. 2.3.).

For these reasons it can be assumed that PL acts exclusively on chain fragments consisting of only esterified galacturonide residues: it may need 3 adjacent esterified units (chapter 6).

PL is subjected to product inhibition (Voragen, 1972); Albersheim and Kilias, 1962). Van Houdenhoven (1975) determined the number of subsites of two Aspergillus niger PLs, and found 8 for PL type 1, and 9 or 10 for PL type 2.
In SDS-gelelectrophoretograms PL type 2 showed two bands which probably represent an active and an inactive form (equilibrium). PLs have been isolated that also act on low methoxyl pectin (Sherwood, 1966; Bateman, 1966). Aspergillus niger PL and Aspergillus fonsecaeus PL do not split the second bond from the reducing end, and the first bond from the non-reducing end (Voragen, 1972; Edstrom and Phaff, 1964). The glycylester of pectate and pectic acid amide are not degraded. Ca^{2+}-ions enhance PL activity, their activating effect depending on pH and the DE of the substrate (Voragen, 1972).

2.3. Biosynthesis of pectin.

The biosynthesis of pectin takes place in the membrane system of the cell: the Golgi apparatus and the endoplasmatic reticulum (Northcote, 1974). Vesicles carrying polysaccharides move to the cell wall and incorporation occurs with glucose as the starting point, two possible pathways to glucuronic acid can be followed (Kauss, 1974):



Pathway (2) is probably of minor importance (Kauss, 1974), but it received much attention in earlier literature. Loewus (1964) proposed a pathway for pectin biosynthesis in which methylation takes place before polymerization:

UDP-glucose → myo-inositol → bornesitol → UDP-methyl-glucuronic acid → UDP-galacturonic acid → polymethylgalacturonic acid

Methylation, however, probably takes place at the macromolecular level (Kauss, 1974):

- it is possible to synthesize pectin in vitro by incubating pectate and S-adenosyl-methionine (SAM, the compound from which the methylgroup is transferred to galacturonic acid) with a crude plant enzyme preparation.
- UDP-methyl-galacturonic acid is not a precursor of polymethyl-galacturonic acid, whereas with UDP-galacturonic acid it is possible to synthesize polygalacturonic acid in vitro (with an enzyme preparation).
- addition of SAM and PDE (phosphodiesterase destroys UDP-galacturonic acid) to an incubate of UDP-galacturonic acid and the enzyme preparation does not inhibit the methylation of the polygalacturonic acid, although the polymerization does not go on.

In conclusion, pectin is formed as polygalacturonic acid, but it is methylated immediately after synthesis. PE has no access to the pectin in statu nascendi; the pectin is present in a "structural compartment". Only after transport to the cell wall or after treatment with a detergent can saponification with PE occur (Kauss, 1974).

In the synthesis of bacterial cell wall polysaccharides "intermediate lipids" (glycosyl carrier lipids) play a role: chain fragments are transported to the site of incorporation by phosphorylated polyprenols. In this way, the cell can introduce a certain periodicity in the polysaccharides. The involvement of such lipid intermediates in plant cell wall biosynthesis, however, is uncertain (Darvill et al., 1979; Delmer, 1977).

In glycogen synthesis the mechanism of branching has been studied: at a certain minimal chain length (dp=13) a branch-point is introduced (by amylo 1,4→1,6 transglucosylase); after that glycogensynthetase can proceed. Also in the case of the O-antigen polysaccharide of Salmonella typhimurium a single unit side chain of abequose is introduced prior to polymerization (Osborn and Weiner, 1968). For pectin, however, nothing is known about the mechanism of branching.

Northcote (1972) states that polysaccharides are essentially polydisperse: protein synthesis implies a direct DNA transcription, whereas in polysaccharide synthesis a "biochemical variation" exists. E.g. in blood glycoproteins the protein part has a constant composition, while the saccharide part is variable in its composition.

Rye flour arabinoxylans and some bacterial dextrans may have side chains arranged at random (Abbott et al., 1966; Aspinall and Ross, 1963). This was concluded from the results of Smith degradation (see Chapter 2.2); it must be said that these results can also be explained by assuming a complex periodicity or a mixture of periodicities (heterogeneity).
Regular branching probably occurs in guar seed galactomannans (Palmer and Ballantyne, 1950 ,although McCleary (1979) claims irregular branching) and in the O-antigen polysaccharide mentioned above. The main chain of polysaccharides mostly shows regularity: a regular alternation of different monomers is present in carrageenan, hyaluronic acid, pullulan and chondroitin sulphate; a homopolysaccharide is present in xyloglucans, arabinoxylans etc. In the main chain of glucomannans, however, an irregular alternation of glucose and mannose may exist (Aspinall, 1970). In alginates blocks of mannuronan, blocks of guluronan and "mixed blocks" are present (e.g. Larsen et al., 1970). Pectin may resemble alginate in this respect: blocks of homogalacturonan and blocks of heteroglycanogalacturonan. In the main chain of the heteroglycanogalacturonan, an irregular alternation of galacturonic acid and rhamnose may occur: the oligosaccharide galacturonic acid-rhamnose-rhamnose has been found repeatedly. A very interesting pectin in this respect is "zosterine", in which blocks of homogalacturonan, heteroglycanoglycan and apiogalacturonan may be present (Ovodov,1975).

In any case a certain regularity is likely to exist: the levels of the enzymes involved are probably regulated in some way or other. Moreover, it is known that during growth the nature of the pectin deposited alters (Northcote, 1978). It may be concluded that the present knowledge of the biosynthesis of pectin does not contain information concerning the distribution of the neutral sugars or the methoxy groups. A comparison of the structures of related polysaccharides (containing galacturonic acid) may point to some common features of their structures.

2.4. Structural features of related polysaccharides.

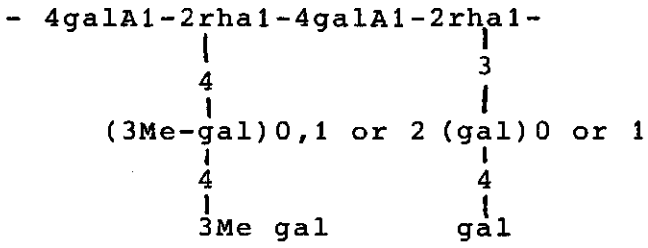
According to Aspinall (1969) related polysaccharides (i.e. polysaccharides containing galacturonic acid) can be divided into four groups:
a. the main chain consists of galacturonic acid: xylogalacturonan, apiogalacturonan, tragacanthic acid.
b. the main chain is a rhamnogalacturonan: Sterculia gum (including gum karaya), slippery elm mucilage, cress seed mucilage.
c. the main chain contains blocks of homogalacturonan and blocks of rhamnogalacturonan: Khaya gum, zosterine, opium poppy capsules mucilage.
d. galacturonic acid is only present in the side chains: jeol gum, cholla gum.

A xylogalacturonan of mountain pine pollen has been investigated by Bouveng (1965). Commercial pectinase slowly degrades this polysaccharide. Tragacanthic acid is a xylogalacturonan; in this case some fucose and galactose residues are attached to the single unit side chains of xylose. In tragacanthic acid small amounts of the aldobiuronic acids galacturonic acid-rhamnose, glucuronic acid-fucose and glucuronic acid-galactose could be detected (Aspinall et al., 1967). Both polysaccharides are accompanied by a galacturonic acid containing arabinogalactan. Gum tragacanth or demethylated gum tragacanth cannot be degraded by Pseudomonas pectate lyase (Rombouts et al., 1978).

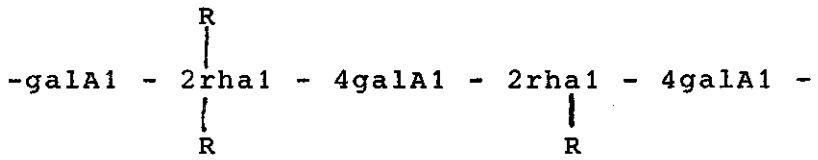
From Lemna minor an apiogalacturonan has been described (Hart and Kindel, 1970). The apiose residues are present in disaccharide side chains; some xylose can be found. Fractions containing 10 - 40% apiose can be obtained; fractions low in apiose can be degraded by pectolytic enzymes.

Sterculia gum (belonging to group b) is a very complicated polysaccharide or mixture of polysaccharides (Aspinall, 1969). The rhamnogalacturonan has side chains of glucuronic acid and is partially acetylated. Acetolysis produces the trisaccharide galactose-1,2-galacturonic acid-1,4-galactose, suggesting that the main chain also contains galactose units. Gum karaya or de-acetylated gum karaya cannot be degraded by Pseudomonas pectate lyase (Rombouts et al., 1978).

Slippery elm mucilage contains methylgalactose (Beveridge et al., 1971):



The same is true for the acidic polysaccharide from cress seed mucilage (Aspinall, 1970):



R: rha1-|3Megal1-4gal1 - |
gal1-| Xyl1-4gal1 -

Khaya gum hydrolysates contain galacturonan: therefore this gum belongs to group c. Single unit chains of galactose are attached to rhamnose; side chains of galactose and methyl-glucuronic acid also occur:

-gal1 4 - 1glucA4Me

These side chains have also been detected in the mucilage of opium poppy capsules. Zosterine is the pectin from Zosteraceae plants (marine plants): it probably contains blocks of homogalacturonan, apiogalacturonan and rhamno-galacturonan (Ovodov, 1975). The side chains of the rhamno-galacturonan are branched 1-4 xylans (with terminal xylose and arabinose bound to C3), and galactans of the 1-3, 1-6 type. The side chains are bound both to the rhamnose and the galacturonic acid units. Jeol gum and cholla gum (group d) have main chains of galactan (of the 1-3,1-6 type); galacturonic acid residues are present as terminal or near-terminal groups (Aspinall, 1969).

Cell wall polysaccharides and gums are structurally related, but gums appear to be more complicated. Based on this fact, the theory has been proposed that gums are modified cell wall polysaccharides; a theory, however, which is probably false (Aspinall, 1969). The fact that the same types of binding universally occur may be induced by conformational restrictions (Rees and Scott, 1971). Tentatively, all galacturonic acid-containing polysaccharides may be thought of as consisting of blocks of:

a. homogalacturonan
b. apiogalacturonan
c. xylogalacturonan
d. rhamnogalacturonan (with side chains of (arabino) galactans and xylans or xyloglucans)
e. galactogalacturonan.

Zosterine, for instance, can be thought of as containing blocks of a, b and d (Ovodov, 1975); khaya gum of a and d and Sterculia gum of d and e. However, every polysaccharide

also has its own specific features. In the case of plant pectins, it has been suggested that degraded pectin molecules play a role in plant-microbe interactions (Albersheim et al., 1981); in this function specific sugar unit sequences may be required.

2.5. Distribution of parameters in macromolecular systems.

Insight into the distribution of parameters is essential for structure analysis. Difficulties in assessing the distribution have been discussed in Chapter 2.2.5 in the case of enzymic degradation. This chapter briefly discusses the factors that determine the distribution and the terminology used for the descriptions of parameter distributions.

A problem in parameter distribution research of biopolysaccharides is that to the natural distribution a second distribution is added during extraction. The natural distribution may be complex as a result of different levels of synthesizing or degrading enzyme activities in different parts of the plant and the cells (i.c. lateral wall and end wall), or at different stages (e.g. of cell wall formation). An intrinsic distribution may be the result of variations in the biochemical process of synthesis. Unlike proteins, polysaccharides are "secondary gene products" and not "primary" ones (Northcote, 1972). As a result of all these processes, differences in MW and chemical composition of polysaccharide molecules are introduced.

The terminology involved has been discussed by Anderson and Stoddart (1966), Aspinall et al. (1970) and Gibbons (1963).

Polydisperse is a distribution when all the parameters have continuous variations.

Homogenenous refers to a distribution that is polydisperse, but with small variations (or, of course, no variation at all).

Heterogeneous applies to systems with one or more parameters having a discontinuous variation.

Polymolecular is a term sometimes used for systems having a continuously varying MW.

In my opinion, the term heteropolymolecular introduced by Anderson and Stoddart (1966) is not a useful extension of the terminology used. The terms are constructed to describe intermolecular distribution, but they can be applied to intramolecular distributions as well (mutatis mutandi). They can also be applied to one parameter. Determining the type of distribution present is far from simple. The resolution of the existing separation methods (Chapter 2.2.2) is rather low. Repeated fractionation and fractionation with two or more techniques is necessary: a time-consuming method. Hardly any method is specific for one parameter (ion-exchange chromatography is influenced by MW ; gel permeation chromatography is sometimes hindered by adsorption or charge effects).

An additional problem is that usually only a fraction of the polysaccharide population can be considered (on account of difficulties in complete extraction). Also in pectin research, attention has been paid to the problem of parameter distribution. For example the distribution of rhamnose residues has been described as intermolecular heterogeneous (Bhattacharjee and Timell, 1965; Neukom et al., 1980; Zitko and Bishop, 1965), as intramolecular heterogeneous (Barret and Northcote, 1965), as fitting in a polydisperse system (Aspinall et al., 1970) and as homogeneous (Powell et al., 1980).

In this thesis, both the intermolecular and the intramolecular distribution of rhamnose residues will be characterized as heterogeneous (Chapter 4). However, the possibility that the natural intermolecular distribution is homogeneous, remains open.

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EXTRACTION AND PURIFICATION OF PECTINS FROM ALCOHOL INSOLUBLE SOLIDS FROM RIPE AND UNRIPE APPLES

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ABSTRACT

Pectins are extracted from Alcohol Insoluble Solids of ripe and unripe apples and fractionated by ion exchange chromatography and gelfiltration. In the extracts mainly pectins with neutral sugar contents of 0·15, 0·24 and 0·53 mol neutral sugar residues/mole galacturonate residues are present. The pectin molecules contain rhamnose, arabinose, xylose, galactose, glucose and galacturonic acid residues. No mannose could be detected. The neutral sugar composition of the glycans bound to the galacturonan was found to be constant, except for the relative amount of galactose. During ripening the neutral sugar composition of the extractable pectin does not change.

INTRODUCTION

The chemical structure of pectin has been the subject of many scientific reports for more than 50 years. Elucidation of this structure is important because of the function of pectin in the cell wall as a 'lubricating' or 'cementing' agent (Rees & Wight, 1969), its role during ripening of fruits (Knee, 1978a, b), its role in food processing (Rombouts & Pilnik, 1978; van Buren, 1979) and its role as nutritional fibre.

The pectin molecule consists mainly of galacturonic acid, rhamnose, arabinose, xylose, galactose and glucose. In some pectins other sugars can be detected as minor constituents (e.g. in the 'RG 2' of Darvill *et al.*, 1978). The molecules have a galacturonan backbone, in which rhamnosyl residues are present (Aspinall & Fanshawe, 1961; Barret & Northcote, 1965; Aspinall *et al.*, 1967a, b; Foglietti & Percheron, 1968; Aspinall *et al.*, 1969; Aspinall *et al.*, 1970; Aspinall & Cottrell, 1970; Talmadge

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et al., 1973; Kato & Noguchi, 1976; Toman *et al.*, 1976; Pfister, 1977; Simson & Timell, 1978). The neutral sugar side chains are linked to the main chain via both galacturonic acid and rhamnose units. Covalent bonds between galactose and galacturonic acid and between xylose and galacturonic acid are often found (Bouveng, 1965; Aspinall *et al.*, 1967a, b; Stoddart *et al.*, 1967; Aspinall *et al.*, 1968; Foglietti & Percheron, 1968; Kikuchi & Sugimoto, 1976).

Covalent bonds of galactose and galacturonic acid and of xylose and galacturonic acid probably also occur in apple pectin (Barret & Northcote, 1965). The pseudo-aldobiuronic acid of galacturonic acid and arabinose (L-arabinofuranosyl-(1-3)-D-galacturonic acid) has also been detected in carnation root pectin and in lucerne pectin (Aspinall *et al.*, 1968; Foglietti & Percheron, 1968).

Methylation analysis reveals that rhamnose is present as chain unit, branch point unit and terminal unit (Talmadge *et al.*, 1973; Siddiqui & Wood, 1976; Simson & Timell, 1978).

Although the pectins of different species are structurally related (McNeil *et al.*, 1978), it cannot be said that the structure of pectin is independent of the species; an apiogalacturonan has been extracted from duck weed (*Lemna minor*) and a xylogalacturonan from mountain pine pollen (*Pinus mugo* var. Turra) (Mascaro & Kindel, 1977; Bouveng, 1965). The structure of pectin depends not only on the species, but also on the physiological state of the material studied (Gould *et al.*, 1965; Dalessandro & Northcote, 1977; Matheson & Saini, 1977).

In this paper the extraction and purification of pectins from Golden Delicious apple Alcohol Insoluble Solids (AIS) are described. The pectins were fractionated by ion exchange chromatography and gelpermeation chromatography and the amounts of the different neutral sugars in the pectin fractions were determined. Apples were chosen as the pectin source, not only because of the industrial use of apple marcs as a pectin source and the important contribution of apples to the diet of many people, but also because the apple is a climacteric fruit. Microscopic investigations show that after the climacteric, the middle lamella in apple fruit tissue has changed (Ben-Arie *et al.*, 1979). Pectin may be involved in this process as an important factor in fruit softening. We chose a sequential extraction with cold and hot acetate buffer, with oxalate/EDTA solutions and with dilute hydrochloric acid, realising that both the preparation of the AIS and the extractions have an impact on the chemical features of the pectins (Joslyn & Deuel, 1963). The temperature was kept below 70°C, in order to minimise the rate of hydrolysis of acid-labile glycosidic bonds. Ion exchange columns (i.e. DEAE-cellulose) have been successfully applied by various investigators to fractionate pectins (Kikuchi & Yokotsuka, 1972; Knee, 1973, 1978a, b; Berth *et al.*, 1977; Anger *et al.*, 1977). Ion exchange can be used to fractionate pectins according to their degree of esterification (van Deventer-Schriemer & Pilnik, 1976). However, it is likely that covalently linked neutral sugars and the molecular weight of the pectin also affect the elution profile (Anger *et al.*, 1977). Gelfiltration of pectin is also difficult to interpret because of the negative charge of the molecules; the molecular

weights (or rather the hydrodynamic volumes) found by calibration with dextrans must be considered as being too high as a result of the repulsion between sample and column material although this effect is probably reduced in the presence of buffers (Thibault, 1979).

METHODS

Preparation of AIS

Golden Delicious apples were obtained from 'de Boutenburg', an experimental apple orchard at Lienden, De Betuwe, The Netherlands. They were gathered on 12 October 1978, and stored in the open till 17 October 1978. Some of the apples were then allowed to ripen at 20°C for 4 weeks, the first week in an impermeable plastic bag. Both ripe and unripe apples were peeled, cored and soaked in a 0·2% solution of ascorbic acid to prevent browning before being mashed in a Kenwood Chef meat mincer. Immediately after mashing, portions of 1 kg were extracted three times with 2·5 litres 96% alcohol at 70°C. The AIS-preparation was air-dried overnight after solvent drying with acetone, ground in a hammer mill with a 10 μm sieve and stored at −40°C. The results of this procedure have been summarised in Table 1.

TABLE 1
Preparation of AIS from Apples

	Unripe	*Ripe*
Weight % of peels and cores	27·9	30·3
Weight % of pulp	72·1	69·7
Weight % of AIS compared with pulp	2·05	2·08
Anhydro-uronic acid content of AIS (mg/g)	279	284
Degree of esterification %	65	70

Extractions

Ten grams of AIS were extracted three times during 30 min (while stirred) with 300 ml 0·05 M Na-acetate buffer (pH = 5·2) at room temperature and the whole procedure was repeated at 70°C. The material was then further extracted with 0·05 M EDTA and 0·05 M NH_4-oxalate in 0·05 M Na-acetate buffer (again three times in 30 min at 70°C). After washing with water this extraction was followed by an extraction with dilute hydrochloric acid (three times, 30 min, 70°C, pH 2·5). The extracts were filtered and the pectins were precipitated with ethanol at 70% concentration. In the text these four extracts are referred to as the cold buffer, hot buffer, oxalate and acid extracts.

Ion Exchange

One-gram quantities of pectin were dissolved in 5 mM Na-phosphate buffer, pH = 5·1, and applied to a 30 x 1 cm column of DEAE-cellulose (Whatman DE 52). The pectins were eluted from the column (after washing thoroughly) with a linear gradient of 5–500 mM Na-phosphate buffers of pH = 5·1 (500 ml). Experiments were performed at room temperature.

Gelfiltration

Ten to fifty milligrams of pectin were dissolved in about 2 ml buffer and applied to a 80 x 2·5 cm column of Sephacryl S-300 (Pharmacia). The eluent was 0·1 M Na-phosphate, pH = 5·1. The flow rate was 0·3 ml/min, controlled by an LKB peristaltic pump. Experiments were performed at room temperature.

Analytical Methods

The anhydro-uronic acid (AUA, MW 176) content of pectin fractions was determined by an automated carbazole-sulphuric acid assay (van Deventer-Schriemer & Pilnik, 1976). The amount of AUA in the AIS preparations was determined according to Ahmed & Labavitch (1977) with meta-hydroxy-diphenyl. The neutral sugars were analysed gas chromatographically as their alditol-acetates (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).

RESULTS AND DISCUSSION

Extractions

The mild extraction processes applied in these investigations release less than 50% of the uronic acid residues present in the apple AIS (Table 2). The pectins which remain in the AIS are probably tightly bound to other cell wall components, but the possibility cannot be ruled out that the preparation of the AIS (especially drying) may cause some of the pectin molecules to become insoluble. More severe conditions of extraction give higher yields, but the products obtained are certainly degraded. The only way to elucidate the structure of the non-extractable pectins is to release them with purified enzymes (Darvill *et al.*, 1978; Voragen *et al.*, 1979). After ripening more galacturonan can be extracted. The average sugar contents in both cases are about the same, but a marked difference exists in the distribution of the neutral sugars among the extracts; the oxalate extract of the AIS from ripe apples has a remarkably low neutral sugar content. The larger part of the sugar residues in the extracts is covalently linked to the galacturonan (Table 2); after ripening the amount of unbound neutral sugars (free glycan) increases. This increase is almost completely due to xylose and glucose (results not shown in the table); about equal amounts of xylose and

TABLE 2
Properties of Pectins Extracted from AIS from Unripe and Ripe Apples

	Anhydro-uronic acid (mg/g AIS)	*DE (%)*	*Bound glycan sugars[a] (mg/g AIS)*	*Free glycan sugars[b] (mg/g AIS)*
Unripe apples				
Cold buffer	29	76	3	1
Hot buffer	28	71	6	2
Oxalate	22	77	11	0
Acid	19	68	19	5
Total	98[c]		39	8
Ripe apples				
Cold buffer	30	80	5	3
Hot buffer	30	76	7	3
Oxalate	31	78	5	1
Acid	40	63	42	9
Total	131[d]		59	16

Conditions during the extractions described in the text.
[a] Inseparable from galacturonan by DEAE-cellulose ion exchange chromatography.
[b] Separable from galacturonan by DEAE-cellulose ion exchange chromatography.
[c] The total amount of anhydrogalacturonate material extracted represents 35% of the amount present in the AIS.
[d] The total amount of anhydrogalacturonate material extracted represents 46% of the amount present in the AIS.
DE = degree of esterification.

glucose are released, indicating that a xyloglucan is released from the cell wall material during ripening.

The overall degree of esterification is almost the same in ripe apples as in unripe apples. It is interesting to note that the pectin that cannot be extracted from the AIS by the extraction methods used in this study has a high degree of esterification. The pectin in the oxalate extracts also has a high degree of esterification.

Fractionations

The fractionation of pectin on DEAE-cellulose ion exchange chromatography was used in this study to establish the distribution of the neutral sugars among the pectin molecules. The pectins from the four extracts elute from the DEAE-cellulose columns in one tailing peak, which was collected in many fractions. About 10% of the pectin does not bind to the column and must be fractionated after partial cold alkali saponification. A small percentage must be eluted with 0·01 N NaOH. The pectin fractions from each extract were combined to obtain 10 'pools' with equal amounts of uronic acid residues, as shown in Fig. 1 for the cold buffer extract of ripe apple AIS. In this way 50 pools were obtained from the four extracts, including 10 pools for the pectin fraction that does not bind to the column and the pectin fraction that must be eluted with 0·01 N NaOH. For each of these 50 pools the neutral sugar content was deter-

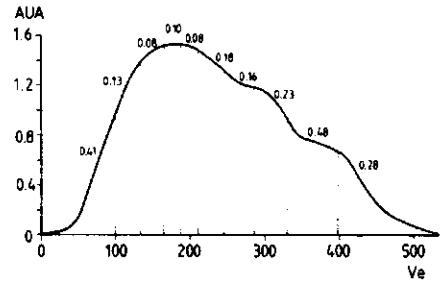

Fig. 1. Fractionation of the pectin from the cold buffer extract of ripe apple AIS on DEAE-cellulose. AUA, anhydro-uronic acid, mg/ml; Ve, elution volume, ml. The DEAE-cellulose column was eluted by a gradient of 5–500 mM Na-phosphate buffer pH = 5·1. The numbers in the figure indicate the neutral sugar residue content of the fractions, expressed as moles neutral sugar residues/mole of galacturonate residues.

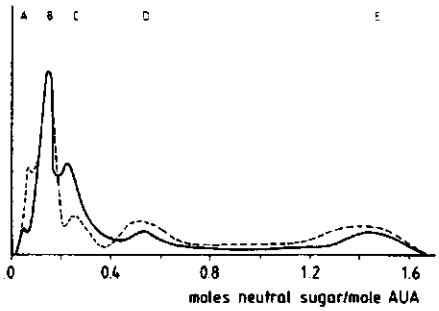

Fig. 2. Neutral sugar distribution curves of pectins extracted from ripe and unripe apple AIS. Construction described in the text. Abscissa: neutral sugar content as moles neutral sugar per mole anhydrogalacturonic acid. The total area represents 100% of the AUA present in the four extracts. —— Unripe apples; – – – ripe apples.

mined and the neutral sugar residues were analysed. The neutral sugar content varied from 0·04 to 1·7 mol neutral sugar residues per mole of galacturonic acid residues. However, the composition of the neutral sugars appeared to be constant, except for the relative amount of galactose. The galactose/arabinose ratio decreases with increasing neutral sugar content. In each of the four extracts the same neutral sugar composition was found. No mannose could be detected. Table 3 gives the neutral sugar composition of the fractions of Fig. 1, expressed as moles of neutral sugar residues per mole of galacturonic acid residues.

TABLE 3
Neutral Sugar Composition of the Pectin Pools of Fig. 1 Expressed as Moles Neutral Sugar Residues/Mole of Galacturonate Residues

Fraction number	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Rhamnose	0·024	0·005	0·004	0·004	0·005	0·007	0·008	0·010	0·026	0·018
Arabinose	0·231	0·060	0·035	0·042	0·035	0·098	0·091	0·128	0·296	0·158
Xylose	0·018	0·004	0·003	0·003	0·003	0·004	0·006	0·009	0·021	0·013
Galactose	0·120	0·058	0·034	0·053	0·032	0·059	0·064	0·077	0·104	0·079
Glucose	0·024	0·005	0·003	0·005	0·003	0·010	0·010	0·009	0·029	0·013
Total	0·42	0·13	0·08	0·10	0·08	0·18	0·18	0·23	0·48	0·28

More information about the distribution of the neutral sugars among the pectin molecules was obtained by constructing neutral sugar distribution curves. These curves were constructed as follows. The 50 pools from ripe apple pectin extracts and the 50

pools from unripe apple pectin extracts were arranged in ascending order of neutral sugar content. From these data two cumulative neutral sugar distribution curves were constructed. Numerical differentiation of these two curves results in the neutral sugar distribution curves of Fig. 2. This figure suggests that in pectin a discontinuous, rather than a continuous, distribution of the neutral sugars is present. The cold buffer extracts contain mainly pectins indicated in Fig. 2 as A, B and C. The cold buffer extract of ripe apple AIS also contains some pectin of type E. The hot buffer extracts contain B, C and D pectins while the oxalate extracts consist of pectins of types A, B, C and D. In the acid extracts C, D and E are the dominating types. The pectins of types A and E elute from the DEAE-cellulose column at lower ionic strength than the other types. Types C and D elute at the end of the gradient. Chromatography on DEAE-Sephadex A 25 gives the same results as those obtained on DEAE-cellulose. Table 4 shows (with the exception of galactose) the fairly constant composition of the neutral sugars. The values for the galactose/arabinose, xylose/arabinose ratios, etc., are average values for pectin fractions of about the same neutral sugar content from different extracts. Pectins of the same type (A, B, C, D or E) but from different extracts have the same composition.

TABLE 4
Neutral Sugar Composition of Pectins Extracted from Ripe and Unripe Apples (AIS)

	A[a]	*B*	*C*	*D*	*E*	*On average*
Ripe apples						
Rhamnose	0·15	0·07	0·08	0·07	0·09	0·09
Xylose	0·09	0·10	0·09	0·08	0·08	0·09
Galactose	1·42	0·80	0·69	0·38	0·29	
Glucose	0·17	0·13	0·12	0·12	0·03	0·10
Sugar content (moles/mole gal. A)	0·08	0·15	0·24	0·54	1·42	
Unripe apples						
Rhamnose	0·08	0·05	0·07	0·06	0·07	0·07
Xylose	0·07	0·07	0·07	0·08	0·09	0·07
Galactose	1·90	0·83	0·60	0·21	0·21	
Glucose	0·08	0·09	0·08	0·11	0·07	0·08
Sugar content (moles/mol gal. A)	0·04	0·14	0·24	0·53	1·48	

[a] A–E refer to Fig. 2. The neutral sugar composition is expressed as moles sugar per mole arabinose. The values are averages of those for pectins with about the same neutral sugar content in the four extracts. Mannose was absent in all cases.

It was realised that because of the interference of the degree of esterification, the molecular weight and the neutral sugar content in the fractionation on DEAE-cellulose columns, it is necessary to produce more evidence to support the postulated neutral sugar distribution. The existence of the different types of pectin molecules is evident

from gelfiltration and from rechromatography on DEAE-cellulose. Examples of rechromatography and gelfiltration are given in Fig. 3 and Fig. 4. In fractions of low neutral sugar content a high molecular weight coincides with a relatively high amount of neutral sugars; in fractions of higher neutral sugar content the situation is more complicated. The pectin with a high neutral sugar content in the acid extracts (type E) has a lower molecular weight than the other pectins extracted.

The presence of the pectin molecules of types A–D, and especially the presence of pectin molecules with a very high neutral sugar content (type E), is in agreement with the conception of pectin as consisting of 'smooth' and 'hairy' regions. Much of the evidence produced (Barret & Northcote, 1965; Bhattacharjee & Timell, 1965; Zitko & Bishop, 1965; Stoddart *et al.*, 1967; Siddiqui & Wood, 1976; Berth *et al.*, 1977; Pfister, 1977; Knee, 1978a,b; Shibuya & Iwasaki, 1978; McNeil *et al.*, 1978) shows that pectin contains regions of 'homogalacturonan' and regions rich in neutral sugars,

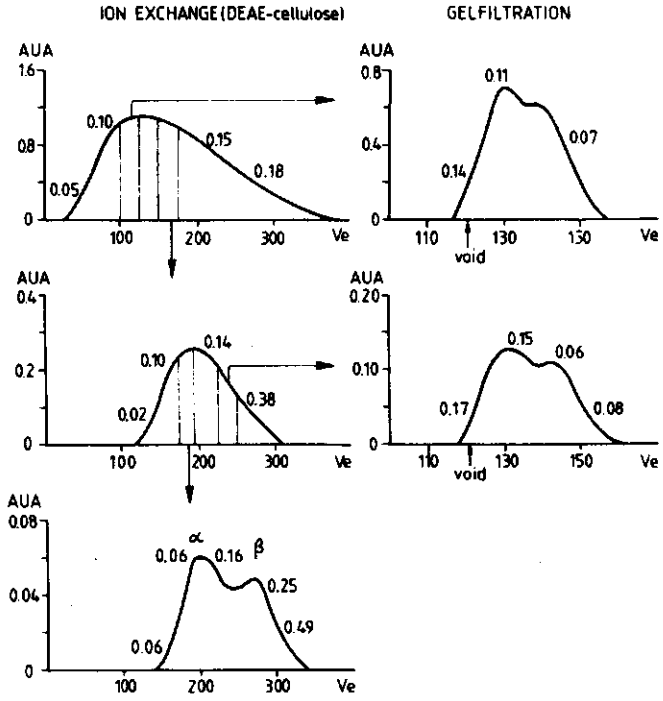

Fig. 3. Purification and fractionation of pectin from the oxalate extract of AIS from ripe apples. Ion exchange (left-hand side figures) and gelfiltration (right-hand side figures) as described in the text. AUA, anhydro-uronic acid, mg/ml; Ve, elution volume, ml. The numbers in the figures indicate the neutral sugar residue content of the fractions, expressed as moles neutral sugar residues/mole of galacturonate residues. The neutral sugar composition of the fractions is similar to the compositions given in Table 4, except for fraction α (Table 5). The arrows indicate fractions that have been rechromatographed on ion exchange or on gelfiltration columns.

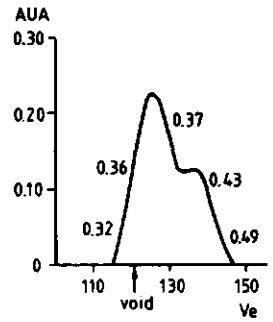

Fig. 4. Gelfiltration of a DEAE-cellulose purified pectin fraction from the acid extract of AIS from unripe apples. AUA, anhydro-uronic acid, mg/ml; Ve, elution volume, ml. The numbers in the figure indicate the neutral sugar residue content of the fractions, expressed as moles neutral sugar residues/mole of galacturonate residues.

'smooth' and 'hairy' regions. Our experiments do not support the suggestion of Aspinall *et al.* (1970) that pectin is a 'chemically homogeneous polydisperse system'; a polydisperse system implies a continuous distribution of the neutral sugar content.

In the cell wall model of Keegstra *et al.* (1973) chains of xyloglucan are linked to arabinogalactans which are, in turn, linked to the galacturonan backbone. Indeed, oligosaccharides consisting of galactose and xylose have been isolated (Aspinall *et al.*, 1967a, b; Kikuchi & Sugimoto, 1976). However, some experiments suggest that not only arabinogalactans but also xylans or xyloglucans are linked to the galacturonan backbone; the pseudo-aldobiuronic acid of xylose and galacturonic acid has repeatedly been isolated (see Introduction). Some of our pectin fractions have relatively high amounts of xylose and glucose (e.g. fraction α, Table 5). This might indicate the presence of separate xyloglucan side chains. However, this variation is only present in pectins of type A. This can be explained by assuming that pectins of type A are pectins with degraded hairy regions. After ripening more pectin of this type is present. The neutral sugar contents of the pectins of types B, C and D have ratios of 1 : 1·7 : 3·7 (Table 4). It might well be that these pectins correspond with molecules with 1, 2 and 3 or 4 hairy regions. Pectin E has 10 times the neutral sugar content as pectin B.

TABLE 5
Average Neutral Sugar Composition of Peaks α and β of Fig. 3, Expressed as Moles Neutral Sugar Residues/Mole of Arabinose Residues

	α	β	A^a
Rhamnose	0·28	0·11	0·15
Xylose	0·23	0·07	0·09
Galactose	1·04	0·40	1·42
Glucose	0·25	0·07	0·17

[a] Average neutral sugar composition of the pectins of type A (Table 4).

Pectins of type E occur dominantly in the acid extracts; they have a low molecular weight compared to the pectins of the other types and are probably the products of degradation.

Ripening

During ripening the neutral sugar content of the extracted pectins, the composition of the neutral sugar side chains and the overall degree of esterification do not change (Tables 1, 2 and 4). More pectin can be extracted after ripening (Table 2). Many authors found an increase in the amount of easily extractable pectin during ripening (e.g. Knee, 1973 and 1978a; Seipp, 1978). The softening of apple fruits, however, starts before the increase of water-soluble pectin (Doesburg, 1957). Some authors found differences between ripening on and off the tree, in respect to ease of pectin extraction (Esau *et al.*, 1962; Knee, 1973). This leads to the conclusion that the softening is not necessarily caused by pectin solubilisation.

Some degradation of the pectin occurs during ripening; as a result more pectin of type A and type E is present (Fig. 2) and the average apparent molecular weight of the acid extractable pectin has decreased (gelfiltration experiments). The latter can be explained by the increased amount of type E pectin in the acid extract as pectin molecules of type E are relatively small. The increased amount of type A pectin is in agreement with the results of Knee (1978a) who found that the neutral sugar content of DEAE-cellulose purified soluble pectin from apples decreased during ripening. As mentioned before, more xylose and glucose residues that are not bound to galacturonan appear in the buffer extracts. We have also extracted pectin from unripe apple AIS after treatment with partial purified cellulase (Voragen *et al.*, 1979). Cellulase treatment appears to have the same effect as ripening; the neutral sugar distribution curve looks like the curve for ripe apples, more pectin can be extracted and more unbound xylose and glucose residues are released from the AIS. The release of xylose and glucose residues (xyloglucan?) may indicate that the softening of fruits during ripening and, similarly, the processes of elongation growth (Albersheim, 1974) are due to events in the deeper parts of the cell wall.

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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 × 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

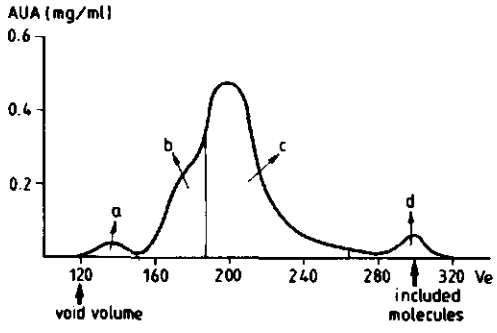
Highly purified pectin lyase ('type 2', van Houdenhoven, 1975) and pectate lyase (Rombouts *et al.*, 1978) were used. Enzyme reaction conditions were as follows: Pectin lyase (EC 4.2.2.10; poly-α-1,4-D-methyl-galacturonate lyase) – 0·2 mg ml^{-1} substrate and 0·02 units ml^{-1} enzyme (units as defined 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.

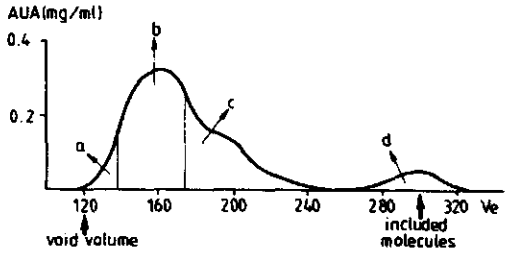
RESULTS AND DISCUSSION

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.



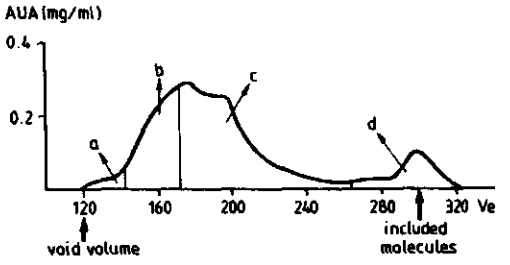
	a	b	c	d
Degree of esterification (%)	95	81	75	50
% of AUA	7	31	54	8
neutral sugar content (moles/mole of galacturonic acid residues)	1.33	.03	.02	.01
moles rhamnose/mole arabinose	.08	.06	.10	.05
moles xylose/mole arabinose	.09	.09	.10	.07
moles galactose/mole arabinose	.87	.71	3.9	6.1
moles glucose/mole 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	.13

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	59
% of AUA	6	35	47	12
Neutral sugar content (moles/mole of galacturonic acid residues)	2.64	.09	.05	.04
moles rhamnose/mole arabinose	.10	.09	.12	.12
moles xylose/mole of arabinose	.08	.10	.10	.10
moles galactose/mole of arabinose	.37	.51	.66	1.20
moles glucose/mole of arabinose	.09	.06	.10	.15

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

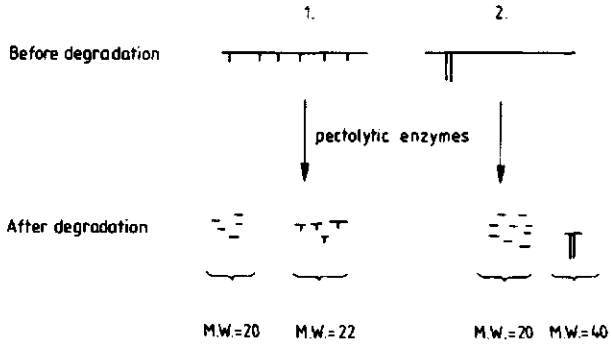


Fig. 4. Two simple models of pectin molecules with different distributions of the neutral sugar side chains along the molecule, before and after enzymic degradation of the backbone. Horizontal line: galacturonan backbone or galacturonan backbone fragments. Vertical line: neutral sugar side chains. Assumption: 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 trimethyl-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.

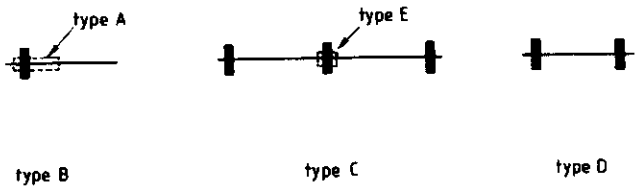
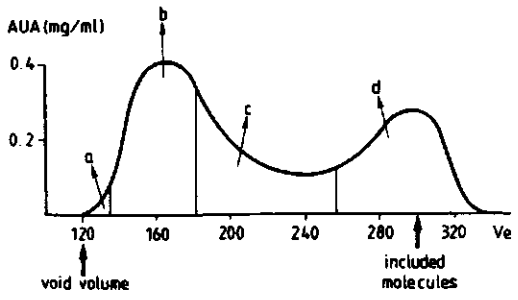


Fig. 5. Model of the pectins of type A–E. The pectin types were defined previously (de Vries *et al.*,1981). Horizontal lines: rhamnogalacturonan backbone of the pectin molecule. Black areas: blocks of neutral sugar side chains.

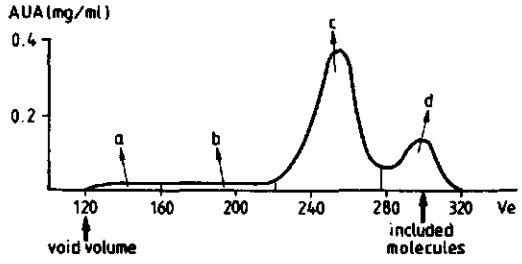
Our model suggests that the rhamnose units are very unevenly distributed along the pectin molecule. This might have implications for theories on pectin gelation in which rhamnose units play a role, because they introduce a kink in the galacturonan backbone (Rees & Wight, 1971).

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)	.90	.03	.03	.06
moles rhamnose/mole arabinose	.08	.09	.12	.13
moles xylose/mole arabinose				
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	.09	-

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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Structural Features of the Neutral Sugar Side Chains of Apple Pectic Substances

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SUMMARY

The 'hairy regions' of apple pectic substances (fragments of the rhamnogalacturonan chains carrying the neutral sugar side chains) have been subjected to enzymic, acidic and alkaline degradation. The results show that the hairy regions consist of rhamnogalacturonan fragments carrying arabinogalactans and galacturonan fragments carrying single unit xylose side chains. A separate population of molecules is present consisting of galacturonan main chains and side chains of 1,3/1,6-linked galactans. The structural relations of pectic substances from different plant species are discussed.

INTRODUCTION

In two preceding papers (de Vries *et al.*, 1981, 1982), the extraction and purification of pectic substances from apple AIS (Alcohol Insoluble Solids) have been described. The neutral sugar composition of these fractions and fractions obtained by enzymic degradation was determined. From these results a model of apple pectin molecules was proposed describing the intra- and inter-molecular distribution of the neutral sugar side chains. The molecules were thought to consist of 'smooth regions' (blocks of homogalacturonan) and 'hairy regions' (blocks of rhamnogalacturonan carrying side chains composed of arabinose, galactose, xylose and glucose).

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In this paper the structure of the hairy regions is discussed. The results of specific, selective as well as non-selective methods of degradation are reported. β-(1,4)-galactanase (Labavitch *et al.*, 1976) has been employed as a specific degradative agent. Mild acid hydrolysis was used as a selective method. Degradation of the fully esterified hairy regions by chemical β-elimination can be characterised as non-selective. The fragments obtained were fractionated by gel-permeation chromatography and the sugar composition of the fractions was determined. In addition, methylation analysis of some pectin fractions was performed.

MATERIALS AND METHODS

The preparation of purified pectin fractions and purified hairy regions
This was performed as described in the preceding papers (de Vries *et al.*, 1981, 1982).

Gel-permeation chromatography
A sample of (degraded) pectin 10–50 mg in 2 ml of buffer or water) was applied to a Sephacryl S-300 (Pharmacia) column (80 × 2·5 cm) or a Biogel P-2 (Biorad) column (70 × 1·5 cm) and eluted with water or 0·01 M sodium phosphate buffer of pH 5·5. The flow rate (0·3 ml min^{-1}) was controlled by an LKB peristaltic pump and the whole procedure was conducted at room temperature (S-300) or at 50°C (P-2).

Analytical methods
The anhydrouronic acid (AUA, M.W. 176) content of the pectin fractions was determined by an automated carbazole-sulphuric acid assay (van Deventer-Schriemer & Pilnik, 1976). The neutral sugars were analysed gas-chromatographically as their alditol-acetates (Albersheim *et al.*, 1967; Darvill *et al.*, 1975). Neutral sugars in column eluates were monitored by the anthrone assay.

Enzymic degradation
β-(1,4)-galactanase from *Bacillus subtilis* was purified on a DEAE-cellulose column (Labavitch *et al.*, 1976) and on an AH Sepharose-galactan column (Centen, 1979). No glycosidase activity or activity on arabans, xylans, cellulose, carboxy-methylcellulose or pectic acid could be detected. Reaction conditions: 150 mg of substrate and 0·02 units

of enzyme in 50 mM sodium acetate buffer of pH 6·0 (50 ml), 24 h reaction at 30°C.

Partial acid hydrolysis
This was performed with 0·01, 0·05 and 0·09 N TFA (trifluoroacetic acid) solutions for 1 h at 100°C in sealed test tubes containing about 200 mg of substrate in 5 ml of TFA.

β-Elimination
Pectin samples were heated in a 0·05 M sodium phosphate buffer, pH 6·0, for 2 h at 100°C.

Methylation analysis
The method of Hakomori (1964) as described by Talmadge *et al.* (1973) was used.

Carboxyl reduction
This was performed according to Taylor & Conrad (1972) using a Metrohm pH-stat. or by refluxing with $LiAlH_4$ (after methylation) in tetrahydrofuran for 8 h (Lindberg, 1972).

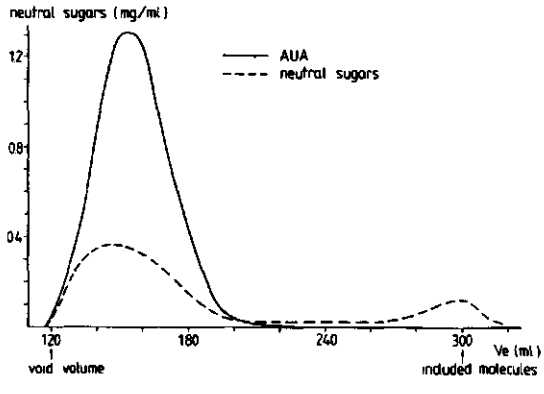

Fig. 1. Gel filtration (Sephacryl S-300) of a β-1,4-galactanase-degraded pectin fraction. AUA, anhydrouronic acid content. Ve, elution volume. The substrate was a DEAE-cellulose-purified pectin from the oxalate extract of AIS from ripe apples. Degradation with β-1,4-galactanase as described in the text. The eluent was water.

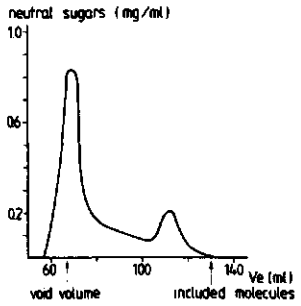

Fig. 2. Gel filtration (Biogel P-2) of the low molecular weight glycan fragments released by β-1,4-galactanase from apple pectin. Ve, elution volume. Substrate, the 'included molecules' shown in Fig. 1. The eluent was water. The second peak elutes from the column in the same elution volume as stachyose (tetramer).

RESULTS AND DISCUSSION

β(1,4)-galactanase action

In Figs 1 and 2 and in Tables 1 and 2, the results of β(1,4)-galactanase action on a pectin preparation are presented. The enzyme removes arabinose and galactose residues from the molecules. No xylose, glucose or rhamnose residues are released, which shows that blocks of arabinogalactans are present in the side chains. The arabinogalactan fragments released are mainly composed of arabinose (Table 1). From their elution volume it can be estimated that the araban side chains of the arabinogalactans have a degree of polymerisation of about 25. Preliminary experiments with a partially purified 1,5-arabanase indicated the presence of 1,5-linked arabans; this was confirmed by methylation analysis.

Methylation analysis and carboxyl reduction

Table 3 shows that about 1/3 of the arabinose residues have branch points. A large part of the arabinose residues are terminal; therefore, the arabans must be highly branched. Methylation analysis of carboxyl-reduced pectin preparations showed that some of the galacturonate

TABLE 1
Sugar Composition of the β-1,4-Galactanase-Degraded Pectin Fractions Shown in Fig. 1 (mg)

Sugar	*Elution volume (ml)*			*% Released**	*%Recovery*
	115–180	*180–280*	*280–320*		
Galacturonic acid	130	trace	trace		84
Rhamnose	1·9	trace	trace	2	71
Arabinose	11·2	7·3	1·4	44	93
Xylose	1·8	trace	trace	3	87
Galactose	2·7	2·4	0·9	55	88

* Present in elution volume 180–320 ml.

TABLE 2
Sugar Composition of the Low Molecular Weight Fraction Released from Apple Pectin by β-1,4-Galactanase and Fractionated as Shown in Fig. 2 (μg)

Sugar	*Elution volume (ml)*	
	60–100	*100–150*
Arabinose	1 220	160
Galactose	225	710

residues carry side chains. Table 3 shows that after reduction by the method of Taylor & Conrad (1972) small amounts of 2,6-dimethylgalactitol and 3,6-dimethylgalactitol appear in the chromatograms, indicating that some galacturonate residues are branched through their C-2 and C-3 atoms. Small quantities of partially methylated alditol acetates, however, can easily result from undermethylation. A complete reduction of the uronic acid residues, however, could not be achieved. The method of Taylor & Conrad (1972) resulted after repeated reaction in about 40% reduction and a separation of products and reagents was

TABLE 3

Methylation Analysis of Apple Pectins. The Substrates were 'Hairy Regions' Obtained by Gel-permeation Chromatography of Pectate Lyase Degraded Pectin Fractions (de Vries *et al.*, 1982)

Sugar residue	*Partially methylated alditol acetate*	*Mole %*		
		*a**	*b**	*c**
Arabinose	2,3,5-tri-O-Me	34·1	26·2	23·8
	2,5-di-O-Me	2·7	4·6	4·1
	2,3-di-O-Me	22·9	8·9	18·3
	2-mono-O-Me	14·0	6·4	11·8
	3-mono-O-Me	1·6	0·5	2·7
	0-O-Me	9·8	7·6	8·3
Galactose	2,3,4,6-tetra-O-Me	2·0	4·3	4·8
	2,3,6-tri-O-Me	7·8	4·1	10·7
	2,4,6-tri-O-Me	–	3·0	–
	2,3,4-tri-O-Me	–	0·7	0·6
	2,4-di-O-Me	–	14·4	3·8
	2,6-di-O-Me	–	–	0·6‡
	3,6-di-O-Me	–	–	0·7‡
	2-mono-O-Me	–	0·9†	–
	2,3-di-O-Me	–	3·1†	
Glucose	2,3,6-tri-O-Me	–	3·9	0·8
Xylose	2,3,4-tri-O-Me	1·7	4·0	2·1
	2,3-di-O-Me	2·3	4·9	–
Deoxyhexose	3,4-di-O-Me	0·3	1·3	3·1
	2,4-di-O-Me	–	1·3	2·7
	3-mono-O-Me	0·7	1·6	2·3

* a, Substrate from the acid extract of apple AIS; b, substrate from the cold buffer extract of apple AIS (de Vries *et al.*, 1981); c, substrate from the hot buffer extract of apple AIS.

† Deuteride label at C-6 atom (present only after reduction with LiAlD).

‡ Present only after carboxyl reduction (by the method of Taylor & Conrad, 1972).

not readily achieved. Reduction of the methylated pectin preparations with lithium aluminium hydride in tetrahydrofuran (Lindberg, 1972) also did not result in complete reduction. For this reason, reduction with lithium aluminium deuteride was applied. This showed that some galacturonate residues are branched through their C-3 atoms (Table 3). Table 3 also shows that galactans are present in two types: 1,4-linked (as confirmed by galactanase attack) and 1,3/1,6-linked galactans. Both types have frequently been found in plants (Clarke *et al.*, 1979). Zosterine (see below), panaxan (Solov'eva *et al.*, 1969) and jeol gum and cholla gum (Aspinall, 1969) contain 1,3/1,6-linked galactan. Talmadge *et al.* (1973) showed the presence of both types of galactans in cell wall preparations. In a previous paper (de Vries *et al.*, 1981) it was shown that the relative amounts of neutral sugars in pectin fractions from different extracts were constant with the exception of galactose. Methylation analysis revealed that in pectin fractions containing relatively high amounts of galactose, the galactans were predominantly 1,3/1,6-linked. These pectin fractions occur predominantly in the cold buffer extracts of the apple AIS (de Vries *et al.*, 1981). It can be concluded from these facts that a pectin fraction exists which consists of galacturonan residues which carry only 1,3/1,6-linked galactan side chains and which have a very high degree of esterification and, which are water-extractable. These pectin fractions are hardly degraded by the β(1,4)-galactanase. About 5% of the pectic substances present in the extracts of apple AIS belong to this fraction. A similar pectin fraction may be present in potato, onion and radish (Ishii, 1976, 1978). It is possible that this pectin fraction is part of the middle lamella pectin.

Xylose residues are mainly present as terminal residues, but also 1,4-linked xylose residues have been observed in methylation analysis. More information about the position of the xylose residues have been obtained by β-elimination experiments.

β-elimination

Figure 3 and Table 4 give the results of a typical experiment; β-elimination performed in an aqueous solution is accompanied by saponification and is very incomplete (Albersheim *et al.*, 1960; Fielding, 1975). In this case, the incompleteness of the reaction is an advantage, because only partial degradation can provide information about the distribution of

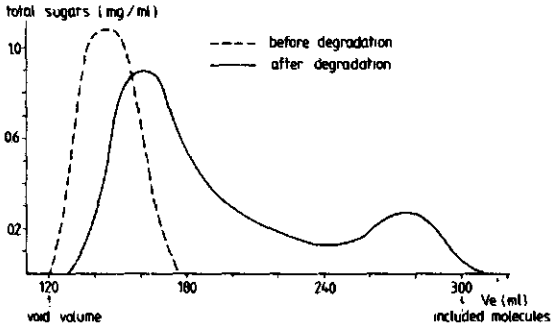

Fig. 3. Gel filtration (Sephacryl S-300) of apple pectin 'hairy regions' after β-elimination. Ve, elution volume. Substrate, hairy regions were prepared by pectate lyase degradation of pectins from the oxalate extract of AIS from ripe apples (de Vries *et al.*, 1982). Total sugars, addition of the values from the anthrone and the carbazole assays. The eluent was water.

the side chains in the hairy regions. The hairy regions cannot be degraded by *Aspergillus* pectin lyase or (after saponification) by *Pseudomonas* pectate lyase.

Figure 3 and Table 4 provide evidence in favour of the presence of xylogalacturonan regions, in which the xylose residues are directly linked to the galacturonate residues: a high proportion of xylose residues is present in the lower molecular weight fractions. In the xylogalacturonan regions, xylose is present as single unit side chains, but may also be present (1,4-linked) in very short side chains. The pseudo-

TABLE 4
Sugar Composition (Percentage of Total Amount of the Sugar Residue Present) of Alkaline-Degraded Pectin Fractions (Fractionated as Shown in Fig. 3)

Sugar	*Elution volume (ml)*		
	125–180	*180–250*	*250–300*
Galacturonic acid	64	16	20
Rhamnose	82	11	7
Arabinose	72	17	11
Xylose	42	26	32
Galactose	85	8	7

aldobiuronic acid xylosylgalacturonic acid has been detected several times in pectic substances (Bouveng, 1965; Aspinall *et al.*, 1967*a*,*b*; Stoddart *et al.*, 1967; Aspinall *et al.*, 1968; Foglietti & Percheron, 1968). It may also be present in apple pectic substances (Barret & Northcote, 1965; Pfister, 1977). In soya sauce polysaccharides, single unit side chains of xylose linked to galacturonan have been observed by Kikuchi & Sugimoto (1976). A pure xylogalacturonan was isolated from Mountain pine pollen by Bouveng (1965). Tragacanthic acid is a xylogalacturonan with some fucose and galactose residues linked to the single unit xylose side chains (Aspinall, 1969).

Structural features of galacturonic acid-containing polysaccharides

Ovodov and coworkers have been investigating the pectic substances of marine plants; for the pectic substance of plants of the genus *Zostera*, zosterine, a model was constructed (Ovodov, 1975). In this model, zosterine consists of several homogalacturonan- and apiogalacturonan-like regions. Aspinall (1969) divided the galacturonic acid-containing polysaccharides into three groups, one of which has blocks of homogalacturonan and blocks of rhamnogalacturonan. Although the methods applied in our studies do not give the exact sequence of the sugar residues, it can be concluded from our results that apple pectic substances can be thought to consist of homogalacturonan, xylogalacturonan and rhamnogalacturonan regions. All these results suggest that all galacturonic acid-containing polysaccharides are constructed from a limited number of building stones. In apple pectic substances, homogalacturonan ('smooth region') is the most important building stone, but in tragacanthic acid xylogalacturonan is the basic building stone.

In this scheme, zosterine represents a polysaccharide with several building stones. It should be realised, however, that each polysaccharide may also contain some very specific fragments of a complex structure. Such fragments may play a role in the host–parasite relation of plants and fungi or insects as 'elicitor' (Albersheim *et al.*, 1981).

Partial acid hydrolysis

The results as shown in Figs 1, 2 and 3 and in the corresponding Tables have been confirmed by partial acid hydrolysis experiments. Figure 4

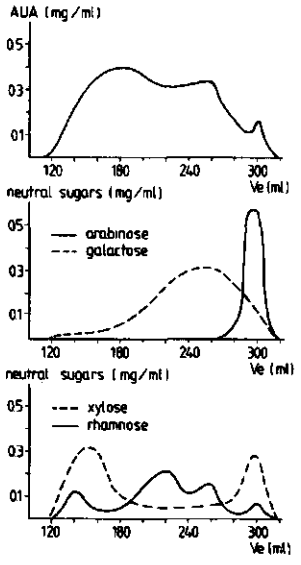

Fig. 4. Gel filtration (Sephacryl S-300) of partially hydrolysed pectin fractions. Ve, elution volume. Substrate, see Fig. 1. AUA, anhydrouronic acid content. Neutral sugars determined by GLC as described in the text. Degradation with 0·05 N TFA for 1 h at 100°C. The eluent was water.

represents an example of such experiments. The pattern of xylose is typical of short side chains, while the pattern of galactose indicates the presence of rather long galactan chains. On hydrolysis in 0·01 N TFA, almost all of the arabinose residues occur as dimers or trimers, while all the other residues still present are high-molecular (results not shown); it can, therefore, be concluded that arabans represent the outer branch of the arabinogalactan side chains. As can be seen in Fig. 4, galacturonic acid and rhamnose show similar patterns. Comparison of the patterns of the different sugar residues, provides evidence that the xylose residues are not bound to the arabinogalactan side chains. Pure arabinogalactans are present as side chains. The role of glucose residues could not be elucidated in these experiments; one of the reasons is that the columns of DEAE-cellulose and Sephacryl S-300 were 'glucose-bleeding'.

The data published in this paper about methylation analysis are in agreement with literature data (Rees & Wight, 1969; Talmadge *et al.*, 1973; Kikuchi & Sugimoto, 1976; Siddiqui & Wood, 1976; Yakovlev

& Gorin, 1977; Simson & Timell, 1978). In the cell wall model of Keegstra *et al.* (1973), xyloglucans are covalently linked to arabinogalactans which, in turn, are covalently linked to rhamnogalacturonans. In our experiments, xyloglucan fragments could not be detected.

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Distribution of Methoxyl Groups in Apple Pectic Substances

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ABSTRACT

The distribution of methoxyl groups in apple pectic substances was investigated by means of fractionation on ion-exchange and gel-filtration columns and by means of degradation of pectin fractions by pectin lyase and pectate lyase. Pectin fragments thus obtained were fractionated by gel-permeation chromatography and high-pressure liquid chromatography. It was concluded that a heterogeneous intermolecular distribution of the methoxyl groups exists with peaks at degrees of esterification of about 50%, 70% and 95%. The intramolecular distribution of the methoxyl groups cannot be distinguished from a random distribution. Since plant pectin esterases cause a blockwise de-esterification, it is unlikely that the biosynthesis of apple pectic substances passes through a stage of 100% esterification after which partial de-esterification by pectin esterase occurs.

INTRODUCTION

In the preceding papers (de Vries *et al.*, 1981, 1982, 1983) a model of apple pectin molecules was presented. According to this model the molecules consist of homogalacturonan regions ('smooth' regions) and heterogalacturonan regions ('hairy' regions). The degree of esterification (DE) of the heterogalacturonan regions is almost 100%. The long homogalacturonan regions (representing over 90% of the galacturonate residues) have an average degree of esterification of about 70%.

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This paper deals with the distribution of the methoxyl groups in the homogalacturonan regions. Extensive fractionation by different methods was performed to study the intermolecular distribution. The intramolecular distribution was studied by extensive degradation of pectin fractions with purified pectolytic enzymes. Because the side chains are located in a few short regions (de Vries *et al.*, 1981), it can be concluded that the effect of the side chains on the enzyme activities can be neglected in these studies. Pectin fractions extracted from apple were compared with pectins prepared by either alkaline or enzymic saponification of fully esterified pectin. Degraded pectin fractions were fractionated by high-pressure liquid or by gel-permeation chromatography.

MATERIALS AND METHODS

Materials

Pectic substances were extracted from apple alcohol insoluble solids (AIS) as described by de Vries *et al.* (1981). Commercial apple pectin was obtained from Obipectin Ltd, Bischofszell, Switzerland (Brown Ribbon pectin). Esterification of this pectin was performed in cold acidified absolute methanol (2°C) for two weeks as described by Heri *et al.* (1961); in the text this esterified pectin is referred to as 'pectin (DE = 95%)'. Saponification (de-esterification) to a final DE of 71% of this fully esterified apple pectin was performed in cold aqueous solutions (0°C) for 48 h by addition of a calculated amount of 0·1 N KOH. In the text the pectin thus obtained is referred to as 'trans-esterified' pectin. Complete de-esterification of Brown Ribbon pectin was performed by repeated addition of 0·1 N KOH.

Analytical methods

The anhydrouronic acid (AUA, $MW = 176$) content of pectin fractions was determined by an automated carbazole sulphuric acid assay (van Deventer-Schriemer & Pilnik, 1976). The neutral sugars were analysed by gas chromatography 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 N KOH). Methanol was converted to methyl nitrite and determined according to the method of Versteeg (1979).

Gel filtration

A sample of (degraded) pectin (10–50 mg in 2 ml of buffer) was applied to a Sephacryl S-300 column (80 × 2·5 cm) and eluted with 0·05 M sodium phosphate buffer, pH = 5·6 (unless otherwise stated). The flow rate (0·3 ml min^{-1}) was controlled by an LKB peristaltic pump and the whole procedure was conducted at room temperature. The void volume (Blue Dextran) of the column was 120 ml; the included molecules (glucose) appeared at an elution volume of 300 ml.

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(methoxygalacturonide) lyase): 0·2 mg ml^{-1} substrate and 0·01 (limited degradation) or 2 (extensive degradation) units ml^{-1} in 10 mM sodium citrate or phosphate buffer pH = 5·2 at 30°C for 4–10 h.

Endo-pectate lyase (EC 4.2.2.2; poly(1,4-α-D-galacturonide) lyase: 0·1 mg ml^{-1} substrate and 5–15 units ml^{-1} enzyme in 10 mM sodium carbonate buffer pH = 6·9 at 30°C for 4 h.

The extent of degradation (% bonds broken) was determined spectrophotometrically at 234 nm, assuming $\epsilon_{235} = 4800$ M^{-1} cm^{-1} (MacMillan & Vaughn, 1964) for the de-esterified unsaturated product and $\epsilon_{235} = 5500$ M^{-1} cm^{-1} (Edstrom & Phaff, 1964) for the esterified unsaturated product. In the case of extensive degradation, it was supposed that the degradation was complete when additional portions of enzyme did not result in an increase in the absorbance at 235 nm. The absorbance values of substrate blanks did not increase under the conditions mentioned above.

Ion-exchange

10–100 mg of pectin sample were dissolved in 5 mM sodium phosphate buffer, pH = 5·1 (unless otherwise stated) and applied to a 10 × 0·4 cm

column of DEAE-cellulose (Whatman DE 52). The pectins were eluted from the column with a linear gradient of 5–300 mM sodium phosphate buffer of pH = 5·1 (200 ml) (unless otherwise stated). Experiments were performed at room temperature.

High-pressure liquid chromatography (HPLC)

A Spectra Physics liquid chromatograph (SP 8000) equipped with a Schoeffel 770 variable wavelength detector was used. A 250 × 4·6 mm internal diameter LiChrosorb 10 NH_2 (Merck) with a Vydac 501 SC quard column (100 × 2·1 mm internal diameter, 37–44 μ, Chrompack) was eluted with sodium acetate buffers of varying pH and concentration (Voragen *et al.*, 1982). The exact conditions depended on the age and the condition of the column.

Calculations

Given a pectin with a known DE and assuming a random distribution of the methoxyl groups along the chain molecules the frequencies of certain sequences of galacturonate residues or esterified galacturonate residues (e.g. a sequence of exactly four esterified galacturonate residues) can be calculated using the statistics developed by Leegwater (1972) for the distribution of the functional groups in modified starches. The fraction of esterified residues present in sequences of exactly n esterified residues is:

$$(1 - \mathrm{DE})\,\mathrm{DE}^n$$

where for DE an infinite chain length is assumed. With these statistics we can also calculate the theoretical amounts of pectin fragments with certain sequences released from pectin polymers by enzymic degradation taking into account the mode of action of the enzyme. This may be illustrated with the example of pectin lyase degraded pectin. HPLC analysis of the digest shows peaks for oligomers with 0, 1, 2 or more non-esterified residues (Fig. 5(a)). Peak c represents all oligomers which have two non-esterified residues.

Assuming that pectin lyase can only split bonds in regions where there are sequences of at least four esterified galacturonate residues pectin fragments containing the following four sequences can occur in peak c:

A-A

A-E-A

A-E-E-A

A-E-E-E-A

in which E = esterified galacturonate residue and A = non-esterified galacturonate residue. The quantities of each of these four sequences can be calculated using the formula mentioned above. However, not all pectin fragments containing these sequences occur in peak c; other fragments containing the same sequence but having more than two non-esterified residues occur in peak d (Table 2). For instance from the fragments containing the sequence A-E-A fragments containing the following sequences occur in peak d:

A-A-E-A-A

A-E-A-E-A-E-A

A-E-A-E-A-E-A-E-A-E-A

A-E-E-A-E-A-E-A-E-A-E-E-A

A-E-A-E-A-E-A-E-A-E-E-A, etc.

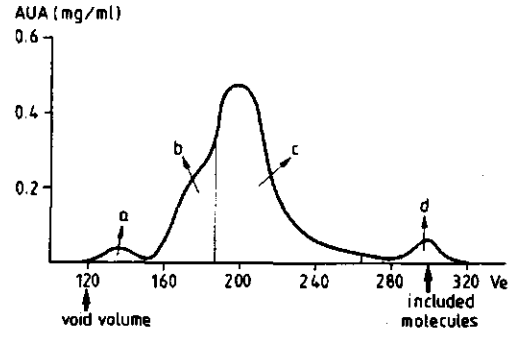
With the following formula the frequencies of the oligomers in peak c can be calculated:

$$b^{-1}\{(b^2a - 2(ab + a^2b + a^3b)\,b^2a\} + a^{-1}\{(ab - 2(b^2a + a^2b + a^3b)\,ab\}$$
$$+ a^{-1}\{(a^2b - 2(b^2a + ab + a^3b)\,a^2b\} + a^{-1}\{(a^3b - 2(b^2a + ab + a^2b)\,a^3b\}$$

where a = DE and $b = 1 - \text{DE}$ and in which the terms $-2(ab + \ldots)$, etc., correct for those fragments which have more than two non-esterified residues. The factors $a^{-1} + b^{-1}$ make the results apply to the total amount of galacturonate residues and not to the amount of esterified or non-esterified residues only.

RESULTS AND DISCUSSION

Figure 1 shows the typical gel-filtration pattern of a pectate lyase degraded pectin fraction. Three peaks can be observed, the first peak containing the hairy regions (de Vries *et al.*, 1982) and the two others being galacturonan chain fragments. This typical pattern can be ex-



	a	b	c	d
Degree of esterification (%)	95	81	75	50
% 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	3.0	6.1
moles glucose/mole arabinose	.10	.06	.10	.09

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

plained in three ways. The first possibility is that the pectolytic enzymes have a certain degree of multiple attack (French & Robyt, 1967). The fact that the gel-filtration pattern of pectate lyase degraded 'trans-esterified' pectin (see under 'Materials') shows a single peak (Fig. 2) rules out this possibility. It can also be concluded from Fig. 2 that the native distribution of the methoxyl and carboxyl groups differs from the distribution in trans-esterified pectin.

The other two possible explanations are that the pattern shown in Fig. 1 is the result of an intermolecular or an intramolecular distribution of the methoxyl groups.

Intermolecular distribution

Data about the intermolecular distribution of the methoxyl groups can only be obtained by extensive fractionation as represented in Fig. 3 and

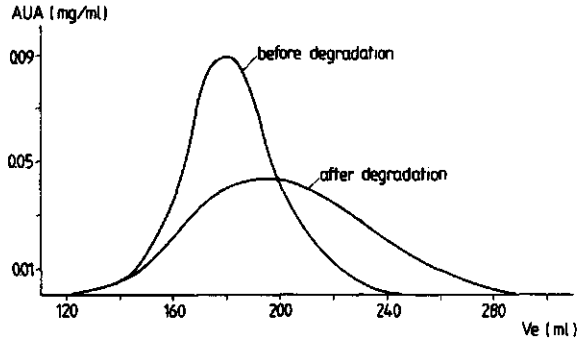


Fig. 2. Gel filtration of trans-esterified pectin before and after pectate lyase degradation. Ve = elution volume. The substrate was a commercial apple pectin which was esterified and subsequently alkali saponified to DE = 71% as described in the text. Degradation with pectate lyase as described in the text. Extent of degradation was 3%. The eluent was 0·05 M sodium phosphate buffer, pH = 5·6.

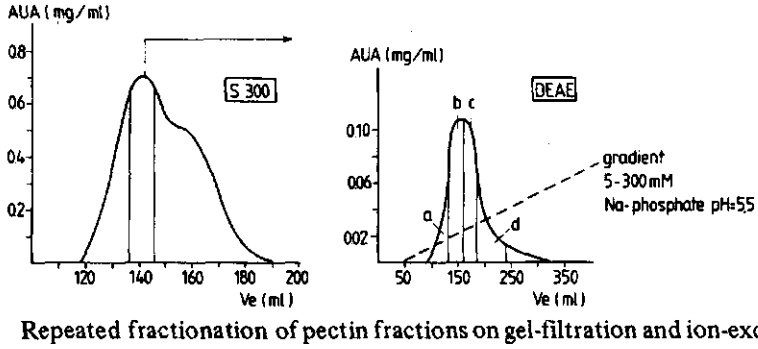


Fig. 3. Repeated fractionation of pectin fractions on gel-filtration and ion-exchange columns. Ve = elution volume. The substrate was a DEAE purified pectin fraction from the hot buffer extract of AIS from ripe apples. Sephacryl S-300: the eluent was 0·05 M sodium phosphate buffer, pH = 5·6.

Table 1. It was observed that an intermolecular distribution is present. Almost all the pectin fractions studied (de Vries *et al.*, 1981) have a DE of 70–80%. From extensive fractionation, however, it can be concluded that all the pectin fractions contain a small number of molecules with a DE of about 50% as well as a small number with a DE of about 95%. The construction of a distribution curve for the intermolecular distribution of the methoxyl groups, however, would demand a series of time-consuming fractionation experiments.

TABLE 1
DE of Pectin Fractions after Repeated Fractionation, as Shown in Fig. 3

Fraction	*AUA (%)*	*DE (%)*
a	19	86
b	44	78
c	29	71
d	7	65

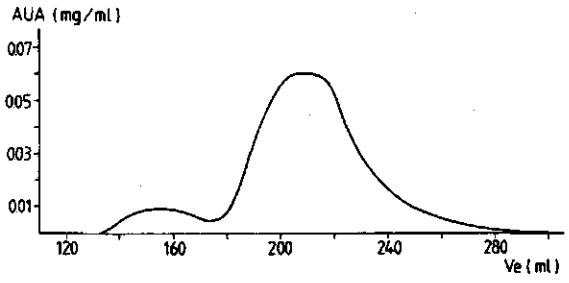


Fig. 4. Gel filtration of pectate lyase degraded pectin fraction c (see Table 1 and Fig. 3). Ve = elution volume. Degradation with pectate lyase as described in the text. The extent of degradation was 3%. The eluent was 0·05 M sodium phosphate buffer, pH = 5·6.

As shown in a previous paper (de Vries *et al.*, 1983), the fraction with DE = 95 has a neutral sugar side chain constitution different from that of the other fractions. It can be concluded from the extensive fractionation experiments that the typical pattern of Fig. 1 (three peaks) is caused by an intermolecular distribution present. Indeed, an extensively purified pectin fraction does not show this behaviour (Fig. 4).

Intramolecular distribution

The next question to be answered is: what is the intramolecular distribution? Extensive degradation by pectate lyase and pectin lyase and

subsequent fractionation of the resulting oligomeric partially esterified galacturonides on HPLC resulted in information about the intramolecular distribution. In the fractionation on HPLC an ion-exchange mechanism is involved (Voragen *et al.*, 1982). The chromatograms of partially esterified tri-galacturonic acid (Tjan *et al.*, 1974) show four peaks, namely of trimers with zero, one, two and three free carboxyl groups. It may therefore be concluded that partially esterified oligogalacturonides are separated according to the number of free carboxyl groups and not according to chain length. Some chromatograms are given in Fig. 5. The peaks indicated as a, b and c represent oligogalacturonides with zero, one and two free carboxyl groups. It can be seen in Fig. 6 that these peaks contain several oligogalacturonides. In this figure a comparison can be made between pectin with a native distribution of methoxyl groups and pectin with a modified distribution (trans-esterified pectin). Only small differences between the two cases are observed, suggesting that the native distribution and the modified distribution are similar. This hypothesis has been confirmed by an analysis of the results of enzymic degradation as represented in Table 2. In this table experimental results as found by HPLC are compared with theoretical results. The theoretical results are based on assumptions concerning the mode of action of the enzyme and on assumptions

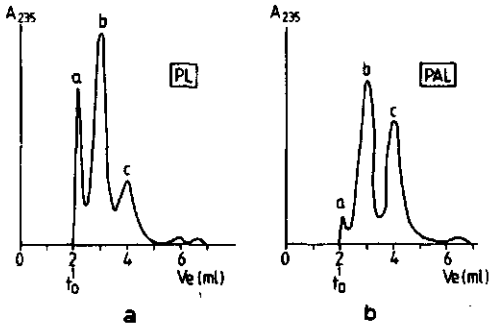


Fig. 5. High-pressure liquid chromatograms of pectin fractions degraded with (a) pectin lyase and (b) pectate lyase to degradation limits. A_{235} = absorbance at 235 nm; Ve = elution volume. HPLC conditions as described in the text. The substrate was a pectin fraction from the oxalate extract of AIS from ripe apples (de Vries *et al.*, 1981). Enzymic degradation as described in the text. Extent of degradation: (a) 18% and (b) 7%.

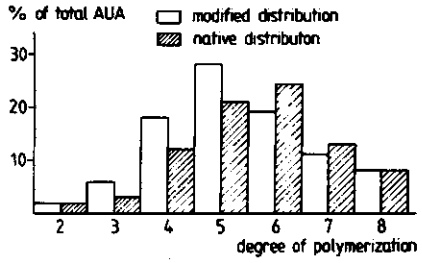


Fig. 6. Distribution of the degree of polymerization of pectin oligomers in peak b (as shown in Fig. 5(a)) in two different cases. The peaks b of several runs were collected and completely saponified (in alkali); the degree of polymerization of oligogalacturonides present was determined by HPLC as described by Voragen *et al.* (1982). Modified distribution: the substrate was trans-esterified pectin (see text). Native distribution: substrate as in Fig. 5.

concerning the intramolecular distribution of methoxyl groups. With the help of the statistics as developed by Leegwater (1972) for the distribution of functional groups in modified starches, frequencies of occurrence of certain sequences (e.g. fully esterified tetramers) can be calculated. Infinite chain length and a random distribution of methoxyl groups are assumed. The problem is that in order to draw conclusions about the distribution of methoxyl groups, assumptions must be made about the mode of action of the enzymes and vice versa. In Table 2 two sets of assumptions have been worked out. The calculations have been illustrated under 'Materials and Methods'. From Table 2 some conclusions can be drawn. The differences between extracted pectins and trans-esterified pectins are relatively small. Although the trans-esterified pectin was not fractionated extensively, intermolecular differences are not likely to exist because the de-esterification reaction proceeds randomly (although not completely – see below). It can be concluded that the intramolecular distribution of the methoxyl groups in both cases (extracted pectins and trans-esterified pectin) may very well be similar. However, a note of caution should be sounded. The degree of polymerization of extracted pectins and of trans-esterified pectins differ substantially (as was concluded from their gel-filtration patterns) and this certainly results in differences in the relative amounts of oligomers produced (boundary effects). Another problem is the instability of esterified oligomers: even at pH 5·5 a pectin lyase degraded

TABLE 2
Percentage of Total AUA Present in the Peaks of the High-Pressure Liquid Chromatograms of Pectin Lyase Degraded Substrate

Peak	*'4'*	*'3'*	*Extracted pectin*[a]	*AIS*[b]	*Trans-esterified pectin*[c]
a	6	6	13	19	7
b	22	31	48	42	35
c	17	38	30	24	39
d	55	25	9	15	8

[a] Pectin fraction from the oxalate extract of AIS from ripe apples (de Vries *et al.*, 1981). Figure 5(a) gives the corresponding high-pressure liquid chromatogram.
[b] Alcohol insoluble solids from ripe apples (de Vries *et al.*, 1981).
[c] See text under 'Materials'.
a, b and c, Pectin fragments with zero, one or two non-esterified galacturonate residues, respectively (Fig. 5).
d, Pectin fragments with three or more non-esterified galacturonate residues (theoretical values obtained using $d = 100 - (a + b + c)$.
'3' and '4', Theoretical values based on the assumption that pectin lyase can only split bonds in regions where there are sequences of at least three or four esterified galacturonate residues. See 'Materials and Methods'.
A random distribution of methoxyl groups is assumed and infinite chain length.
DE assumed = 70%.

pectin undergoes some de-esterification. A third problem is the occurrence of product inhibition (Voragen, 1972) which may be different in both cases. In the extraction procedure applied only 40–50% of the total AUA present in the AIS is extracted. Comparison of the columns 'extracted pectin' and 'AIS' in Table 2 indicates that the differences between extracted pectins and non-extractable pectins are small. In addition, the average DEs of the pectin extracted and of the residual pectin do not differ. Comparison of theoretical data (columns '3' and '4' in Table 2) with experimental data ('extracted pectin') suggests that pectin lyase can split wherever a sequence of at least three esterified

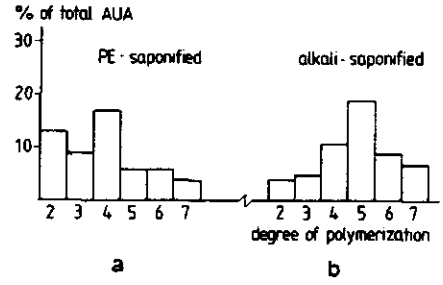


Fig. 7. Distribution of the degree of polymerization of pectate lyase degraded pectins with different distributions of the methoxyl groups. PE = pectin esterase. Extensive degradation with pectate lyase as described in the text. The pectate lyase digests were completely de-esterified (in alkali) and the degree of polymerization of oligogalacturonides was determined by HPLC as described by Voragen *et al.* (1982). (a) Pectin (DE = 95%) was de-esterified by citrus PE (Versteeg, 1979) to DE = 60%. (b) Pectin (DE = 95%) was de-esterified by alkali to DE = 60%. The resulting pectins were the substrates for the pectate lyase. Extent of degradation: (a) 12% and (b) 8%.

galacturonate residues occurs in a chain. It has been observed by Voragen (1972) that this pectin lyase cannot degrade fully esterified trimers. This shows that the mode of action of enzymes in the case of oligomers can differ substantially from that in the case of polymers.

In Fig. 7 it is shown that the action of pectin esterase (PE) can be detected by pectate lyase degradation and subsequent de-esterification of the substrate. In the case of PE action the amounts of dimers and trimers present are relatively high due to the preferential attack on the de-esterified regions by pectate lyase. In this way commercial citrus pectin was shown to be affected by PE. Apple pectins, however, appear not to be influenced by PE action. The pectins from the oxalate extracts did not differ from pectin fractions from the other extracts in this respect. Neither is the small fraction of molecules with a DE of about 50% affected by PE. This indicates that the role of plant PE is not influencing the distribution of methoxyl groups in the pectin of the plant. The role of PE *in vivo* is certainly not clearly understood (Versteeg, 1979). Figure 5 also provides information about the mode of action of pectate lyase. The chromatograms of pectate lyase degraded pectins show the presence of oligomers with only one non-esterified galacturonate residue. This implies that pectate lyase can split not only bonds between free (non-esterified) galacturonate residues, but also

other bonds, perhaps between free and esterified galacturonate residues. It can be concluded from Fig. 5 and Table 2 that the distribution of methoxyl groups may very well be a random one. It must be emphasized, however, that it is very hard to distinguish between a random distribution and a distribution characterized by short-range regularity. The distribution of methoxyl groups in the case of trans-esterified pectins is not a completely random one. The expected amounts of blocks of de-esterified residues could not be detected in the chromatograms, which indicates that the distribution of the methoxyl groups is more regular. This is not unexpected: the rate of de-esterification decreases with decreasing DE (Deuel & Stutz, 1958). Investigations of the mode of action of enzymes involved in the biosynthesis of highly esterified pectins may produce more information about this subject than can be obtained by enzymic degradation.

According to the literature (Joslyn, 1962; Darvill *et al.*, 1980) calcium bridges between pectin molecules play a role in the structure of plant cell walls. Calcium binding of pectins, however, may only occur when blocks of non-esterified galacturonate residues are present (Rees, 1969; Kohn, 1975). Blocks of more than three non-esterified galacturonate residues appeared to be practically absent in apple fruit cell walls, as was shown by the analysis of pectin lyase digests of apple AIS in this study.

Literature on the distribution of methoxyl groups in pectic substances is scarce. Boothby (1980) claimed a continuous distribution for plum fruit pectic substances; in electrophoretic separation, however, not only the DE but also the molecular weight and the neutral sugar content are important parameters. Fielding (1975) found some evidence in favour of a random distribution of methoxyl groups. Kohn (1975) showed that the calcium binding of extracted pectins could not be distinguished from trans-esterified pectins. Attempts are being made to obtain more information about the distribution of methoxyl groups in the oligogalacturonides by proton NMR studies according to the method of Tjan *et al.* (1974).

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Changes in the Structure of Apple Pectic Substances during Ripening and Storage

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ABSTRACT

During ripening, the degree of polymerization, the degree of esterification, the neutral sugar content and the neutral sugar composition of extractable apple pectin substances did not change. Some xylose and glucose containing polysaccharides can be extracted from the ripe cell walls suggesting that changes in the hemicelluloses take place. In senescent apples, significant changes in the structure of apple pectin substances could be observed. The degree of polymerization of both the galacturonan chains and the arabinogalactan side chains decreased. The amount of water-extractable pectin molecules carrying 1,3/1,6-linked galactans increased. The degree of esterification and the distribution of the methoxyl groups in the apple pectin substances did not change very much.

INTRODUCTION

Many studies on cell wall changes during fruit ripening have been published. In spite of all these efforts, a clear understanding of the phenomena observed has not yet emerged. Undoubtedly, an important factor in this respect is the impact of the ripening and storage conditions. Differences between ripening on and off the tree have been observed (Esau *et al.*, 1962; Knee, 1973). Another problem is that no distinct stages can be defined: some changes can already be found

before ethylene production starts (Platt-Aloia *et al.*, 1979). Microscopic investigations show that during ripening the middle lamella changes. This has been observed for apples (Ulrich & Hartmann, 1967; Ben-Arie *et al.*, 1979; Mohr, 1979), strawberries (Neal, 1965) and avocados (Platt-Aloia *et al.*, 1979). In the early ripening stages of pears, however, changes in the primary cell wall have also been reported (Ben-Arie *et al.*, 1979). Dorofeeva *et al.* (1973) found differences in apple fruit collenchym and parenchym tissue.

In this study, structural differences among apple pectic substances of different stages were investigated. Pectins were extracted, purified by ion-exchange chromatography and gel filtration and degraded by pectolytic enzymes. The results are discussed in relation to a model of apple pectin molecules previously described (de Vries *et al.*, 1981; 1982*a*, *b*, *c*).

METHODS

Analytical methods

The anhydrouronic acid (AUA; $MW = 176$) content of pectin fractions was determined by an automated carbazole-sulphuric acid assay (van Deventer-Schriemer & Pilnik, 1976). The amount of AUA in the alcohol insoluble solids (AIS) preparations was determined according to Ahmed & Labavitch (1977) with *meta*-hydroxydiphenyl. The neutral sugars were analyzed by gas chromatography as their alditol-acetates (Albersheim *et al.*, 1967; Darvill *et al.*, 1975). The methoxy 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).

Preparation of AIS

Golden Delicious apples were obtained from 'de Boutenburg', an experimental apple-orchard at Lienden, De Betuwe, The Netherlands. They were gathered in a pre-climacteric stage on 19 October 1980, and stored in the open until 23 October 1980 (*unripe apples*). Some of the apples were then allowed to ripen at 20°C, the first week in an

impermeable plastic bag in order to accelerate the ripening process (*ripe apples*). Some of the ripe apples were then stored at 20°C for two weeks and at 12°C for another two weeks (*senescent apples*). The temperature had to be lowered from 20°C to 12°C to avoid microbial spoilage. AISs were prepared from the unripe, ripe and senescent apples as described by de Vries *et al.* (1981).

Extractions

10 g of AISs were extracted on three occasions during 30 min (while stirred) with 300 ml 0·05 M sodium acetate buffer (pH = 5·2) at room temperature and the whole procedure was repeated at 70°C. Extraction with 0·05 M EDTA and 0·05 M ammonium oxalate in 0·05 M sodium acetate buffer was then applied (again three occasions, 30 min, 70°C). After washing with water this extraction was followed by an extraction with dilute hydrochloric acid (three occasions, 30 min, 70°C, pH = 2·5). The extracts were filtered and the pectins precipitated with ethanol at 70% concentration. In the text these four extracts are referred to as the cold buffer, hot buffer, oxalate and acid extracts.

The extraction scheme chosen was that previously used by the authors in studies on the structure of pectin substances (de Vries *et al.*, 1981: 1982*a*, *b*, *c*).

Enzymic degradation, ion exchange and gel permeation chromatography, pectin de-esterification and HPLC-analyses were performed as described by de Vries *et al.* (1982*c*).

RESULTS AND DISCUSSION

Table 1 shows the extractability of the pectin substances from the unripe, ripe and senescent apples used in out experiment. During ripening, a larger fraction of the pectic substances can be extracted due to loosening of the cell walls. This increase in pectin solubility has been found frequently, although not in every case (Esau *et al.*, 1962).

Many authors (e.g. Knee, 1978) have observed an increase in cold-water-soluble pectin during ripening, but in our case hardly any increase in cold-buffer-soluble pectin was found (Table 1).

Figure 1 and Table 2 give the results of the fractionation on DEAE-cellulose of the cold buffer extractable pectin from AIS from over ripe

TABLE 1

The extractability and DE of Pectin Substances from Unripe, Ripe and Senescent Apples. Condition During the Extractions Described in the Text. Percentage of Total AUA: the Total Amount of Anhydrogalacturonate Material Extracted as Percentage of the Amount Present in the AIS

	AUA (mg/g AIS)			*DE (%)*		
	unripe	*ripe*	*senescent*	*unripe*	*ripe*	*senescent*
Cold buffer extract	28	30	30	74	79	82
Hot buffer extract	25	31	22	71	77	74
Oxalate extract	25	31	24	77	72	76
Acid extract	20	39	47	68	63	69
% of total AUA	38	48	42			

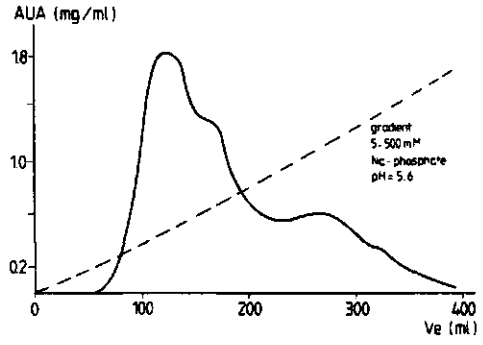

Fig. 1. Fractionation of the pectin from the cold buffer extract of senescent apple AIS on DEAE-cellulose. Ve = elution volume. The SEAE-cellulose column was eluted by a gradient of 5–400 mM sodium phosphate buffer pH = 5·6.

apples. In previous papers (de Vries *et al.*, 1982*b*,*c*), the occurrence of a pectin fraction with a high degree of esterification (DE) and containing only 1,3/1,6-linked galactan side chains has been described. Neutral sugar analysis of DEAE-cellulose purified pectins of various extracts (cold buffer, hot buffer and oxalate extracts of unripe, ripe and senescent apples) showed that this pectin fraction increases during ripening and storage from approximately 5% in the unripe stage to

TABLE 2
Neutral Sugar Composition of Pectin Fractions from the Cold Buffer Extract of AIS from Senescent Apples Fractionated as Shown in Fig. 1

	Elution volume (ml)			
	50–125	*125–200*	*200–300*	*300–400*
AUA, % (anhydrouronic acid)	13	44	30	13
Neutral sugars (mol/mol galacturonic acid residues)	0·20	0·15	0·28	0·36
Mol galactose/mol arabinose	0·79	0·74	0·68	0·96
Mol rhamnose/mol arabinose	0·08	0·18	0·10	0·15
Mol xylose/mol arabinose	0·02	0·06	0·09	0·14
Mol glucose/mol arabinose	0·03	0·03	0·10	0·19

about 12% in the senescent stage. Takeuchi & Komamine (1980) observed changes in cell wall 1,3/1,6-linked galactans during the growth of tobacco. Some authors concluded from the results of their experiments that during ripening a continuous synthesis and degradation of pectic substances occurs (Knegt *et al.*, 1974; Knee, 1978). According to Knee (1978), the newly synthesized material has a high DE. Due to the incompleteness of the extractions applied in these experiments, however, we cannot draw conclusions about the origin of the increased quantity of pectin extracted from senescent apple AIS. The molecular weight (*MW*) of the pectins does not change during ripening, but during storage after ripening a decrease can be observed (Fig. 2).

Gel filtration cannot be considered to be a reliable method of *MW* determination, but the conclusion that during storage after ripening a decrease in *MW* occurs is hardly questionable. Because the neutral sugar content of the extracted pectins is low (about 10%) and does not change very much, it is likely that the galacturonan chains are degraded during senescence.

Figure 2 shows the differences in the gel filtration pattern of the cold buffer extracts, but similar changes can be observed in the pectins of the other extracts. In literature on ripening, much attention is paid to changes in the activities of pectolytic enzymes. In tomatoes, polygalacturonase (PG) activity increases during ripening, but the increase

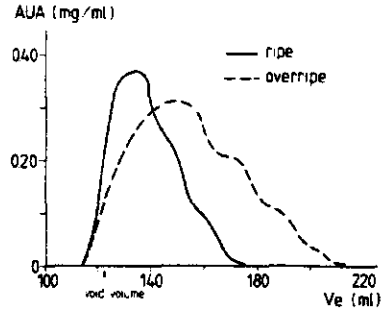

Fig. 2. Gel permeation chromatography on Sephacryl S-300 of the pectin from the cold buffer extract of ripe and senescent apple AIS. Ve = elution volume. The eluent was 0·1 M sodium phosphate buffer pH = 5·1.

in the water-extractable pectin fraction has already started before the rise in PG activity begins (Sawamura *et al.*, 1978). In tomatoes, PG is certainly not the only factor involved in fruit softening (Wallner & Bloom, 1977). Apples seem to have only exo-PG activity (Ben-Arie *et al.*, 1979). The interpretation of the results of experiments, in which enzyme activities are monitored during ripening, is difficult since a higher enzyme activity can result from better extractability, *de novo* synthesis or activation by changes in inhibitor-concentrations. Interestingly, Ben-Arie *et al.* (1979) observed that senescent apple tissue looks like the tissue of unripe apples treated with tomato endo-PG. It is possible (and in agreement with the data of Fig. 2) that in apples the pectic substances are affected by PG in senescence. However, not only the galacturonan chains are degraded during senescence but also the arabinogalactan side chains. The 'hairy' regions (the pectin main chain segments carrying the neutral sugar side chains; see de Vries *et al.*, 1981*b*) appear in higher elution volumes (Fig. 3, Table 3) than those of unripe or ripe apple pectin (de Vries *et al.*, 1982*c*). Pectin molecules that can be characterized as degraded hairy regions (molecules of 'type E', de Vries *et al.*, 1982*a*) can also be found in unripe and ripe apples. But especially in the period of storage after ripening, the amount of 'type E' pectin increases (as can be concluded from the increased neutral sugar content and the decreased molecular weight of the acid-extractable pectin) and it is striking that this pectin mainly occurs in the acid extracts (de Vries *et al.*, 1982*a*). This suggests that pectin molecules contain acid-labile bonds.

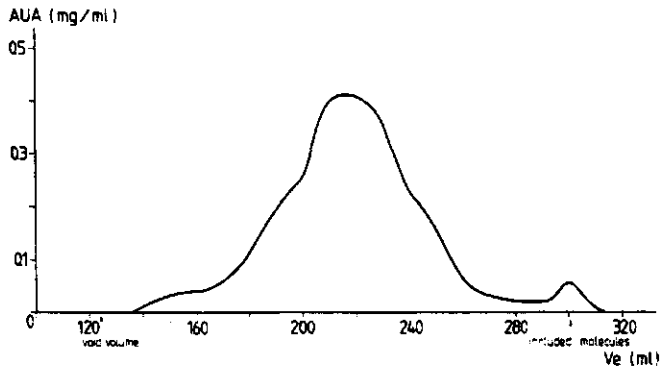

Fig. 3. Gel permeation chromatography on Sephacryl S-300 of a pectate lyase degraded pectin. Ve = elution volume. The substrate was a DEAE-cellulose purified pectin from the oxalate extract of AIS from senescent apples. Degradation with pectate lyase as described by de Vries *et al.* (1982*b*). Extent of degradation was 4%. The eluant was water.

TABLE 3
Neutral Sugar Composition of Pectate-Lyase-Degraded Pectin Fractions Fractionated as Shown in Fig. 3

	Elution volume (ml)			
	130–180	*180–210*	*210–250*	*250–320*
Anhydrouronic acid, %	8	28	38	6
Neutral sugars (mol/mol galacturonate residues)	1·01	0·30	0·08	0·05
Mol rhamnose/mol arabinose	0·13	0·06	0·11	0·04
Mol galactose/mol arabinose	1·20	0·80	0·70	0·90
Mol xylose/mol arabinose	0·04	0·06	0·13	0·08
Mol glucose/mol arabinose	0·06	0·07	0·16	0·08

In the theory of elongation growth of Cleland (1971), a role for acid-labile bonds has been postulated. The neutral sugar composition of the acid extractable pectin does not differ from that of the buffer- and oxalate-extractable pectin, suggesting that the acid-labile bonds broken during acid extraction are not arabinose glycosidic linkages.

TABLE 4

Free Glycan Sugars Present in the Oxalate Extracts of AIS from Apples of Different Stages of Ripeness. Free Glycan Sugars: Separable from Galacturonan by SEAE-Cellulose Ion Exchange Chromatography (mg/g AIS)

Sugar residue	*Unripe*	*Unripe cellulase*[a]	*Ripe*	*Senescent*
Rhamnose/fucose	0	0	0	0
Arabinose	0	0·02	0·04	0·10
Xylose	0·07	0·09	0·15	0·10
Galactose	0·03	0·03	0·08	0·13
Glucose	0·09	0·11	0·15	0·21
Total	0·19	0·25	0·42	0·55

[a] AIS from unripe apples treated with cellulase as described by Voragen *et al.* (1979).

What happens during acid-extraction remains unclear. The events occurring may have a physical rather than a chemical nature. The amounts of neutral polysaccharide residues, which are not covalently linked to galacturonans, present in the oxalate extract, are given in Table 4. During ripening, polysaccharides containing xylose and glucose residues can be extracted, suggesting that during ripening changes in the hemicelluloses take place. Furthermore the other extracts contain some free xylose and glucose containing polysaccharides. The larger part (about 75%) of the glycan residues in the extracts, however, is covalently bound to galacturonan residues. After ripening, the amounts of free galactose and arabinose residues also increase. This enhanced extractability probably results from wall loosening. Release of xyloglucans and glucans during growth has been reported (Johnson, 1979; Yamaota *et al.*, 1980). Ripening and growth are made possible through the same processes: in both cases, cell wall loosening occurs.

As reported in a previous paper (de Vries *et al.*, 1981), ripening of apples can to some extent be simulated by cellulase action. However, cellulase activity is probably absent in apples (Ben-Arie *et al.*, 1979). During growth, the activity of 1,3-glucanases increases (Goldberg, 1980). This may also happen during the ripening of apples.

Table 5
Distribution of the DP of Oligogalacturonides Present in the Pectate Lyase Digests of AIS from Unripe, Ripe and Senescent Apples Represented as a Percentage of the Total Amount of Anhydrouronic Acid Present in the AIS. The Pectate Lyase Digests were Alkali-Saponified and the DP of Oligomers Present was Determined by HPLC (de Vries *et al.*, 1982*b*)

DP	*Unripe*	*Ripe*	*Senescent*
2	0·05	0·02	0·31
3	–	–	0·09
4	–	–	–
5	–	–	–
6	1·2	0·9	1·2
7	5·6	5·1	6·0
8	7·0	8·0	8·1
9	8·5	13·1	12·6

As reported in a previous paper, the impact of pectin esterase (PE) on the distribution of the methoxyl groups in pectic substances can be investigated by HPLC-analysis of the pectins after degradation with pectate lyase and subsequent de-esterification (de Vries *et al.*, 1982*c*). After PE-action, an increased amount of di- and tri-galacturonic acid can be found in the chromatograms, due to the preferential attack of pectate lyase on blocks of de-esterified residues. Table 5 shows the results of pectate lyase degradation of apple AIS of different stages. In the senescent stage, some PE activity appears to be present. It cannot be excluded, however, that this PE activity can only be observed in senescence because of a better accessability of the pectate lyase to the substrate. It has been shown that it is unlikely that the biosynthesis of apple pectic substances passes through a stage of 100% esterification after which partial de-esterification by pectin esterase occurs. The overall DE is fairly constant during ripening (Table 1): the same has been reported in literature for apples (Knee, 1978), pears (Esau *et al.*, 1962) and strawberries (Neal, 1965). In avocado ripening, the DE decreases (Dolendo *et al.*, 1966; Eaks & Sinclair, 1978). No general

relation between PE level and ripening can be deduced from literature data. In apples, PE activity increases during ripening (Lee, 1969). Our results suggest that PE activity is not an important factor in the ripening process. However, when a combined PE/polygalacturonase action occurred the resulting pectin fragments were probably not detected.

In conclusion, our results suggest that the pectin molecules are hardly affected by ripening. In senescence degradation occurs. O'Beirne *et al.* (1981) observed a substantial decrease in DE during storage of apples at 0°C for 11 months. It is possible, that our conclusions do not apply to ripening and storage under other conditions and to other apple varieties.

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Comparison of the Structural Features of Apple and Citrus Pectic Substances

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ABSTRACT

Pectic substances were extracted from Alcohol Insoluble Solids from lemon peel (albedo) and fractionated by ion exchange chromatography and gelfiltration. The pectin molecules contained rhamnose, arabinose, galactose, glucose and galacturonic acid residues; xylose residues were almost absent. Degradation with purified pectolytic enzymes and subsequent gelfiltration of the resulting pectin fragments showed that the neutral sugar side chains were present in 'hairy regions' (blocks of neutral sugar side chains). The distribution of the methoxyl groups was studied by HPLC analysis of enzyme-degraded pectins. Some influence of native pectinesterase on the distribution of the methoxyl groups was found. The results are compared with those of similarly extracted and purified apple pectic substances.

INTRODUCTION

In previous papers, investigations of the structure of apple pectic substances have been reported (de Vries *et al.*, 1981, 1982, 1983*a*, *b*, 1984). The extraction and purification of pectins from apple Alcohol Insoluble Solids (AIS) was described (de Vries *et al.*, 1981) and a model of apple pectin molecules was presented (de Vries *et al.*, 1982). In this model, the molecules consist of 'hairy regions' (rhamnogalacturonan segments carrying the neutral sugar side chains) and 'smooth regions' (homogalacturonan segments). The distribution of the methoxyl groups was

studied (de Vries *et al.*, 1983*b*) and it was found by high-pressure liquid chromatography (HPLC) of enzyme-degraded pectins that the distribution of the methoxyl groups could be a random one. The extractability and the molecular weight (MW) of the apple pectic substances were found to depend on storage time (de Vries *et al.*, 1983*b*).

In this paper, structural studies on lemon peel pectic substances are reported. The methods developed in the above mentioned studies were applied to lemon peel pectic substances; in this way, apple and citrus pectic substances extracted and purified according to the same methods could be compared.

METHODS

Analytical methods

The anhydro-uronic acid (AUA, MW 176) content of pectin fractions was determined by an automated *m*-hydroxydiphenyl/sulphuric acid assay (Thibault, 1979). The amount of AUA in the AIS-preparations was determined according to Ahmed & Labavitch (1977). The neutral sugars were analysed gas-chromatographically as their alditol acetates (Albersheim *et al.*, 1967; Darvill *et al.*, 1975) after hydrolysation in 2 N TFA for 1 h. 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 the method of Versteeg (1979).

Extractions

AIS (10 g) was extracted three times for 30 min (while being continuously stirred) with 300 ml of 0·05 M Na-acetate buffer (pH = 5·2) at room temperature and the whole procedure was repeated at 70°C; extraction with 0·05 M EDTA and 0·05 M NH_4-oxalate in 0·05 M Na-acetate buffer followed (again three times, 70°C, 30 min). The extracts were filtered and the pectins were precipitated with ethanol at a concentration of 70%. These three extracts are referred to as the cold buffer, hot buffer and oxalate extracts.

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(methoxygalacturonide)lyase): 0·2 mg ml^{-1} substrate and 0·002 units ml^{-1} (limited degradation) or 3 units ml^{-1} (extensive degradation) in 10 mM sodium citrate buffer, pH = 5·2, at 30°C for 4–10 h. The extent of degradation was determined spectrophotometrically at 235 nm, assuming $\epsilon_{235} = 5500\ M^{-1}\ cm^{-1}$ for the esterified unsaturated product.

Pectate lyase (EC 4.2.2.2; poly(1,4-α-D-galacturonide)lyase): 1 mg ml^{-1} substrate and 15 units ml^{-1} enzyme in 25 mM sodium carbonate buffer, pH = 6·9, at 30°C for 4 h.

The extent of degradation (percentage of bonds broken) was determined spectrophotometrically at 235 nm, while $\epsilon_{235} = 4800\ M^{-1}\ cm^{-1}$ (McMillan & Vaughn, 1964) was assumed for the de-esterified unsaturated product and $\epsilon_{235} = 5500\ M^{-1}\ cm^{-1}$ (Edstrom & Phaff, 1964) for the esterified unsaturated product.

Gel filtration

An amount of 10–50 mg of (degraded) pectin was dissolved in about 2 ml water and applied to a 80 × 2·5 cm column of Sephacryl S-300 (Pharmacia). The void volume (Blue Dextran) of the column was 120 ml: the included molecules (glucose) appeared at an elution volume of 300 ml. The eluent was 0·1 M Na-phosphate, pH = 5·1, or water. The flow rate was 0·3 ml min^{-1}, controlled by an LKB peristaltic pump. Experiments were performed at room temperature.

High-pressure liquid chromatography (HPLC)

A Spectra Physics Liquid Chromatograph (SP 8000) equipped with a Schoeffel 770 variable wavelength detector was used. Oligomeric pectin fragments were separated on a 250 × 4·6 mm I.D. LiChrosorb $10NH_2$ (Merck) column with a Vydac 501 SC quard column (100 × 2·1 mm I.D., 37–44 μ, Chrompack). Na-acetate buffers of varying pH and concentration were used as eluents. The exact conditions depended on the age and the condition of the column (Voragen *et al.*, 1982).

Ion-exchange chromatography

About 100 mg of pectin sample was dissolved in 5 mM Na-phosphate buffer, pH = 5·1, and applied to a 10 × 0·4 cm column of DEAE-cellulose (Whatman DE 52). After washing thoroughly with the starting buffer, the pectins were eluted from the column with a linear gradient of 5–300 mM Na-phosphate buffer of pH = 5·1 (200 ml). Experiments were performed at room temperature.

TABLE 1
Characteristics of Alcohol Insoluble Solids (AIS) from Lemon Albedo and Ripe Apple Cortex

	AIS (% of fresh wt)	*AUA (mg/g AIS)*	*AUA (mg/g fresh wt)*	*DE (%)*
Lemon albedo	7·3	207	15·1	71
Ripe apple cortex	2·1	284	6·0	70

Note. AUA, anhydro-uronic acid; DE, degree of esterification. The preparation of the ripe apple AIS was described by de Vries *et al.* (1981).

Preparation of AIS

Lemons (*Citrus limon*) from Italy were purchased from a local greengrocer. The fruits were peeled (removal of the flavedo), after which the albedos were removed. The albedos were then mashed in a mincer and portions of 1 kg were extracted three times with 2·5 litres of 96% alcohol at 70°C. The AIS-preparation was air-dried overnight after solvent drying with acetone, ground in a hammer mill with a 10 μm sieve and stored at −40°C. Table 1 gives some characteristics of the AIS obtained by this procedure.

RESULTS AND DISCUSSION

Table 1 shows that there are differences between AIS from lemon peels and from apples. Lemon peels contain more pectin than apples, but the content in the AIS is lower. These differences can be explained by the difference in cell size: the cells of apple cortical tissue are large and the cell walls are relatively thin. The AUA content of lemon peel AIS (207 mg/g) is about the same as reported by Sinclair (1961) for orange albedo AIS (196 mg/g). The results of the pectin extractions are given in Table 2. Also in this table, a comparison can be made between apple AIS and lemon peel AIS.

The most important difference is that between the oxalate extracts in both cases: from lemon peel AIS more pectin can be extracted by oxalate. But also in this case, the degree of esterification (DE) is high

TABLE 2
Pectin Fractions Obtained from AIS from Ripe Apple Cortical Tissue and from Lemon Albedo by Fractional Extraction

Extract	*Amount extracted*				*Degree of esterification, %*	
	AUA (mg)/AIS (g)		*%*			
	apple cortex	*lemon albedo*	*apple cortex*	*lemon albedo*	*apple cortex*	*lemon albedo*
Cold buffer	30	17	11	8	80	74
Hot buffer	30	37	11	18	76	70
Oxalate	31	61	11	30	78	70
Total	91	115	33	56		

Note. AUA, anhydro-uronic acid. Conditions during the extractions are described in the text.

(70%). It must be said, however, that we did not investigate this 'oxalate effect' any further. Therefore the question whether this effect is specific for oxalate or not, remains open. Hence, it cannot be concluded that, in lemon peel cell walls, more pectin has been rendered insoluble by Ca^{2+}-binding than in apple cell walls. The purification of the pectin from the oxalate extract of lemon albedo AIS on DEAE-cellulose is shown in Fig. 1 and Table 3.

The neutral sugar composition of the pectin pools is expressed as moles sugar residues/mole arabinose residues to make comparison with apple pectin fractions easier (Table 4).

The pectin molecules contained rhamnose, arabinose, galactose and glucose residues. Only trace amounts of xylose residues were present. This is the most striking difference between the pectins from the oxalate extracts of apple and citrus AIS (Table 4). In a crude pectin extract from grapefruits, Kawabata (1977) also reported the absence of xylose residues.

Aspinall *et al.* (1968) found only traces of xylose residues in DEAE-cellulose purified lemon peel pectin. In the crude cold water extract of lemon peels, however, they found (after partial hydrolysis) the disaccharide xylose-(1,3)-galacturonic acid. This suggests that in citrus

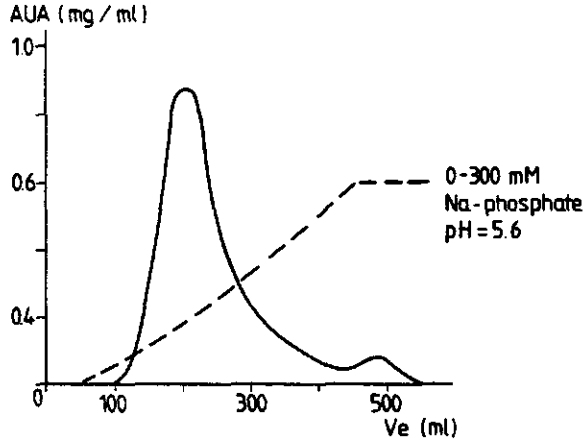

Fig. 1. Fractionation of the pectin from the oxalate extract of AIS from lemon peels on DEAE-cellulose. AUA, Anhydro-uronic acid; Ve, elution volume. The DEAE-cellulose column was eluted by a linear gradient of 5–300 mM Na-phosphate buffer, pH = 5·1 (400 ml).

TABLE 3
Neutral Sugar Composition of Citrus Pectin Pools Shown in Fig. 1

Sugar composition	*Elution volume (ml)*			
	100–190	190–250	250–400	400–500
Neutral sugar content (moles/mole of galacturonate residues)	0·09	0·12	0·15	0·0
Moles rhamnose/mole arabinose	0·09	0·05	0·09	0·1
Moles galactose/mole arabinose	0·55	0·59	0·26	0·3
Moles glucose/mole arabinose	0·10	0·05	0·25	0·2
Percentage of total anhydro-uronic acid	28	30	37	

pectin some xylogalacturonan regions are present, as has been shown to be the case for apple pectic substances (de Vries *et al.*, 1982). It may be that in citrus xylogalacturonans occur only in some parts of the cell wall. It is also possible that these pectin fragments containing xylose play a role as 'elicitor' in host–parasite relations of the plant (Albersheim *et al.*, 1981).

TABLE 4
Neutral Sugar Composition of the DEAE-Cellulose Purified Pectin from the Oxalate Extracts of AIS from Ripe Apple Cortex and Lemon Albedo

Sugar composition[a]	*Ripe apple cortex*	*Lemon albedo*
Rhamnose/arabinose	0·09	0·10
Xylose/arabinose	0·09	0·00
Glucose/arabinose	0·10	0·20
Galactose/arabinose	0·35	0·43

[a] Moles of neutral sugar residues/moles of arabinose residues.

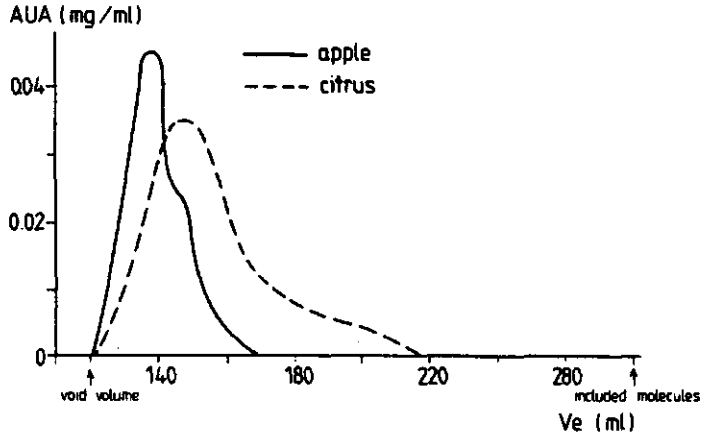

Fig. 2. (Gel filtration of DEAE-cellulose purified pectin fractions from the oxalate extracts of AIS from lemon peels and from ripe apples. AUA, Anhydro-uronic acid; Ve, elution volume. The eluent was 0·1 M Na-phosphate buffer, pH = 5·1.

Both in citrus and apple pectic substances the ratio of galactose/arabinose in the neutral sugar side chains is not constant (Table 3 and de Vries *et al.*, 1981). In both cases this inconstancy is caused by the presence of two types of arabinogalactans: 1,3/1,6-linked and 1,4-linked galactans (Aspinall & Cottrell, 1970; de Vries *et al.*, 1982).

Figure 2 suggests that the molecular weight (the hydrodynamic volumes) of citrus pectin is lower than that of apple pectin. The molecular weight of apple pectic substances, however, decreases during

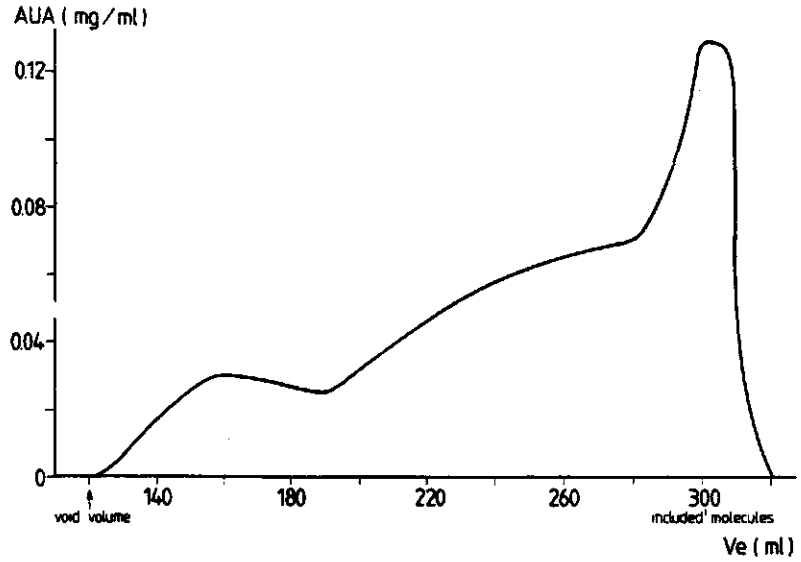

Fig. 3. Gel filtration of a pectin lyase degraded citrus pectin. AUA, anhydrouronic acid; Ve, elution volume. The substrate was a DEAE-cellulose purified pectin from the oxalate extract of AIS from lemon peels. Limited degradation with pectin lyase as described in the Results and Discussion section (extent of degradation was 7%); the eluent was water.

storage of the apples (de Vries *et al.*, 1983*b*). The interpretation of the data in Fig. 2 is also hampered by the fact that the oxalate fractions probably represent different sub-fractions of the pectic substances present (see Table 2).

Figure 3 shows the result of limited enzymic degradation and subsequent gelfiltration of a citrus pectin fraction from the oxalate extract. Nearly all the neutral sugar residues appear in elution volumes, where high-molecular-weight fragments can be expected (Table 5). It can be concluded that in citrus pectin the neutral sugar side chains are concentrated in regions that cannot be degraded by pectin lyase, the so-called 'hairy regions', exactly as was shown for apple pectic substances (de Vries *et al.*, 1982). Aspinall & Cottrell (1970) found small amounts of molecules rich in neutral sugars in their lemon peel extracts. Zitko & Bishop (1965) reported the presence of two types of molecules in commercial citrus pectin: one type was rich in neutral sugars and the other was poor in neutral sugars. Our results show that these two types

TABLE 5
Neutral Sugar Content of Citrus Pectin Pools Shown in Fig. 3

Sugar	*Elution volume (ml)*		
	120–180	180–280	280–320
Percentage of total AUA	7	53	40
Neutral sugar content (moles neutral sugar residues/mole of galacturonate residues)	1·31	0·01	0·00

are fragments of the same molecules and that low degrees of degradation result in the appearance of the two types of molecule reported by Zitko & Bishop (1965). Aspinall *et al.* (1970) and Aspinall & Cottrell (1970) studied the distribution of the methoxyl groups of lemon peel pectic substances. It can be concluded from their work that an intermolecular distribution exists which may be similar to the one for apple pectic substances (de Vries *et al.*, 1983*a*).

We studied the intramolecular distribution of the methoxyl groups by extensive degradation of pectin with pectin lyase and subsequent fractionation of the partially esterified oligogalacturonides by HPLC as shown in Fig. 4. Using the HPLC system, the partially esterified oligogalacturonides are separated according to their number of non-esterified galacturonic acid residues (de Vries *et al.*, 1983*a*). In Fig. 4, *a*, *b* and *c* indicate pectin fragments with 0, 1 and 2 free (non-esterified) galacturonate residues.

In Table 6 a comparison is made between the HPLC patterns of pectin fractions from apple and lemon peel. The differences appear to be small, which may indicate that no substantial differences between the intramolecular distributions of the methoxyl groups of apple and lemon peel pectin exist.

It was shown in a previous paper (de Vries *et al.*, 1983*b*) that the HPLC patterns of apple pectins do not differ much from those of 'trans-esterified' pectins (pectins esterified to 95% and subsequently de-esterified to 70% in cold alkali). This suggests that apple and citrus pectins have a random distribution of methoxyl groups.

When the whole AIS from both apple and lemon peel is degraded by pectate lyase and the resultant pectin fragments are analysed by HPLC

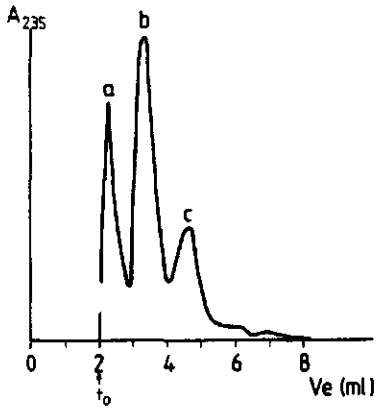

Fig. 4. High-pressure liquid chromatograms (HPLC) of citrus pectin degraded with pectin lyase. A_{235}, Absorbance at 235 nm; Ve, elution volume. HPLC conditions are as described in the text. The substrate was a pectin fraction from the oxalate extract of AIS from lemon peels. The extensive enzymic degradation is described in the Results and Discussion section.

TABLE 6
Percentage of Total AUA Present in the Peaks of the High-Pressure Liquid Chromatograms of Pectin lyase Degraded Substrates

Pectin fragment	*AUC, in extract, %*	
	Apple	*Lemon*
a	13	17
b	48	40
c	30	28
'*d*'	9	15

Note. The substrates were DEAE-cellulose purified pectin fractions from the oxalate extracts of AIS from ripe apples (de Vries *et al.*, 1981) and from lemon albedo. Figure 4 gives the high-pressure liquid chromatogram for the pectin from lemon albedo; *a*, *b* and *c* are pectin fragments with 0, 1 or 2 non-esterified galacturonate residues (see Fig. 4 and de Vries *et al.*, 1982*c*) '*d*' = 100 − (*a* + *b* + *c*).

TABLE 7
Amounts of Di- and Trigalacturonic Acid Present in De-esterified Pectate Lyase Digests of AIS from Ripe Apples and from Lemon Albedo, Expressed as Percentage of the Total Amount of Uronic Acid Residues Present in the AIS

	Ripe apple AIS	*Lemon albedo AIS*
Digalacturonate	0·02	0·79
Trigalacturonate	0·00	0·26

Note. The pectate lyase digests (for conditions see text) were alkali-saponified and the degree of polymerisation of oligomers present was determined by HPLC (de Vries *et al.*, 1982*c*).

(after de-esterification), differences between apple and lemon peel can be detected (Table 7). In the case of lemon peel AIS, small amounts of di-galacturonic acid and tri-galacturonic acid were found. In a previous paper (de Vries *et al.*, 1983*a*), it was shown that this occurrence of di- and tri-galacturonic acid is typical of pectins de-esterified by (citrus) pectinesterase. It can be concluded from Table 7 that citrus pectin-esterase influences the distribution of the methoxyl groups in the citrus fruits. This influence, however, is very small: the resultant decrease of the overall DE is only 1 or 2% (Table 7).

CONCLUSIONS

In conclusion our results show that apple and citrus pectin molecules are very similar. In both cases the neutral sugar side chains are located in 'hairy regions'. The distribution of the methoxyl groups is probably the same in both cases, namely a random one. A difference is that citrus pectin contains few xylose residues.

It must be realised, however, that pectin structural parameters are influenced by factors like fruit storage conditions (O'Beirne *et al.*, 1981), and conditions during extraction. Differences among batches of commercial pectins may result from these factors.

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9. Summary and conclusions

Pectins were extracted (under mild conditions) from Alcohol Insoluble Solids of ripe and unripe apples and fractionated by ion exchange chromatography and gelfiltration. The distribution of the neutral sugar residues among the molecules was found to be a discontinuous one: in the extracts mainly pectins with neutral sugar contents of 0.15, 0.24 and 0.53 moles neutral sugar residues/mole galacturonate residues are present. The pectin molecules contain rhamnose, arabinose, xylose, galactose, glucose and galacturonic acid residues. No mannose could be detected. The neutral sugar composition of the glycans bound to the galacturonan was found to be constant, except for the relative amount of galactose.

The purified pectins 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 gelpermeation chromatography and the degree of esterification and the sugar composition determined for each fraction. More than 90% of the uronic acid residues could be isolated as homogalacturonan chains. The neutral sugar residues could be detected in the column eluates as high-molecular-weight fragments. Models of the apple pectin molecules were presented in Chapter 4. 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.

The hairy regions have been subjected to enzymic, acidic and alkaline degradation. The results showed that the hairy regions consist of rhamnogalacturonan fragments carrying arabinogalactans and galacturonan fragments carrying single unit xylose side chains (Chapter 5). A separate population of molecules is present consisting of galacturonan main chains and side chains of 1,3/1,6-linked galactans. The structural relations of pectic substances from different plant species are briefly discussed in Chapter 5.

In the well-known cell wall model of Keegstra et al. (1973) rhamnogalacturonan, arabinogalactan and xyloglucan are covalently linked. Xyloglucan molecules are non-covalently linked to cellulose fibrils. The pectin fragment released from sycamore cell walls certainly contain xyloglucans (Talmadge et al., 1973), but my investigations show that the extractable pectin molecules (over 50%) do not contain xyloglucan fragments. This suggests that pectin molecules contribute to the firmness of the cell wall in another way than by covalent linkages to other cell wall components. Our conclusion is hampered by the fact that about 50% of the pectic substances could not be extracted by non-degradative extractants. However, it is unlikely that all of the extracted pectin molecules originate from the middle lamellae: under the microscope the middle lamella turns out to be a thin layer (Nultsch, 1963). Another possibility is that pectin molecules in the cell wall build a gel comparable with pectin-sugar-acid gels. Some experiments suggest that pectin molecules aggregate in dilute solutions (Davis et al., 1980; Jordan & Brant, 1978), but it is unlikely that this phenomenon results in strong gels in the cell walls. In the cell walls the role of sugar in pectin-sugar-acid gels may be taken over by arabinogalactans: it is known that they are remarkably hydrophilic (Rees & Scott, 1971; Susheelama & Rao, 1978). However, the amount of arabinogalactans present as side chains is too small to account for this role. Rees & Wight (1969) suggested a "lubri cating" function for pectin molecules with many side chains and a "cementing" function for pectin molecules without side chains. In the extracted pectin molecules the distance between neighbouring neutral sugar side chain blocks may be constant (Chapter 4). Also the fact that the side chains are present in blocks is evidence against this lubri cating function of the side chains. The function of the side chains remains unclear, but they are probably not involved in gelation mechanisms.

The distribution of the methoxyl groups in apple pectic substances (Chapter 6) was investigated by means of fractionation on ion-exchange and gelfiltration columns and by means of degradation of pectin fractions by pectin lyase and pectate lyase. Pectin fragments thus obtained were fractionated by gelpermeation chromatography and high-pressure-liquid chromatography. It was concluded that a heterogeneous intermolecular distribution exists with peaks in the degrees of esterification of about 50%, 70% and 95%. The intramolecular distribution of the methoxyl groups could not be distinguished from a random distribution. Since plant pectinesterases cause a blockwise de-esterification, it is unlikely that the biosynthesis of apple pectic substances passes through a stage of 100% esterification after which partial de-esterification by pectinesterase occurs.

Pectin molecules possibly contribute to the firmness of the cell wall through the formation of Ca^{2+}-pectate gels. A role for Ca^{2+}-pectate gels is often mentioned (Joslyn, 1962; Knee, 1978), but conclusive evidence has not yet been produced. Addition of $CaCl_2$ to plant tissues partially prevent the extraction of pectic substances (Bates & Ray, 1981; O'Beirne et al., 1980). Joslyn & Deuel (1963) concluded from pectin extraction experiments that "the data do not support the hypotheses that polyvalent ions such as Ca^{2+} are responsible for the insolubility of apple pectin". Carr et al. (1980) also found evidence against a role for Ca-ions in the middle lamellae of leaf parenchymic tissue. The results of my investigations as presented in Chapter 6 are in agreement with the conclusion of Joslyn & Deuel. It is known that only pectins with a low degree of esterification show a strong Ca-binding (Kohn, 1975). My results suggest that there are no pectin molecules or even segments with a low degree of esterification. This was found to be the case with both extractable and non-extractable pectin fractions.

Also in theories about gelation the distribution of the methoxyl groups is an important parameter. A gel is a metastable state (Rees, 1969). This can be concluded from the occurrence of syneresis, which shows that no equilibrium exists. It can, therefore, be concluded that a gel is not hold together by dynamic forces. Mechanisms like ionic bridging cannot explain the occurrence of a gel state for this reason. Rees' theory on gelation (1969) states that polymers in gels interact in junction zones. In carrageenan gels the junction zones are double helix structures. In the case of pectin the junction zones are "mycrocrystallite" according to Rees. This is thought to be the case in both types of pectin gels: Ca-pectate gels and pectin-sugar-acid gels. In Ca-pectate gels a well-known model of the mycrocrystallites is the egg-box model of Rees. It has recently been shown that in Ca-pectate gels not only egg-boxes, but also mycrocrystallites of the "pectin-sugar-acid"-type play a role (Gidley et al., 1980). It is generally assumed that the Ca-binding of pectin differs from Ca-binding of other poly-electrolytes, even from the Ca-binding in Ca-carboxymethylcellulose gels (Kohn, 1975; Powell et al., 1980; Rees, 1969). However, the existence of an essential difference can be doubted, as the Ca-binding of pectate is in agreement with Manning's poly-electrolyte behaviour theory (Ravanat & Rinaudo, 1980; de Vries, 1977). In any case, in the egg-box model the junction zones consist of low-esterified galacturonan segments consisting of more than 7 galacturonate residues (Powell et al., 1981). My investigation of the distribution of the methoxyl groups (Chapter 6) show that such galacturonan segments are virtually absent in apple and citrus pectic substances.

A proposal for the structure of the junction zones in pectin-sugar-acid gels has not yet been made. The fact that the distance between the neutral sugar side chain blocks is long (Chapter 4) suggests that neutral sugar side chains are not an important factor in gelation. This conclusion is supported by the fact that in commercial pectin samples no relation between neutral sugar content or composition and gel strength exists (Otterbach, 1981). Our structure

of junction zones. A similar conclusion was drawn by McCleary (1979) from structural investigations of galactomannans: mannan blocks in galactomannans do not serve as junction zones. As for exudate gums, there is no relation between physical properties and sugar composition (Phillips et al., 1981). It is possible and probable that specific chain segments suitable for functioning as junction zones are absent in pectin, as they are in carboxymethylcellulose.

During ripening, the degree of polymerization, the degree of esterification, the neutral sugar content and the neutral sugar composition of extractable apple pectic substances did not change (Chapter 7). The amount of water-soluble pectin increased and this pectin had a high degree of esterification and contained 1,3/1,6-linked galactans. Some xylose and glucose containing polysaccharides can be extracted from the ripe cell walls. In senescent apples, significant changes in the structure of apple pectic substances could be observed. The degree of polymerization of both the galacturonan chains and the arabinogalactan side chains decreased. The amount of water-extractable pectin molecules carrying 1,3/1,6-linked galactans increased further. The degree of esterification and the distribution of the methoxyl groups in the apple pectic substances did not change much.

The pectic substances extracted from AIS from lemon peel (albedo) were compared with those from apple AIS (Chapter 8). The citrus pectin molecules contain rhamnose, arabinose, galactose, glucose and galacturonic acid residues. Xylose residues are virtually absent. Degradation with purified pectolytic enzymes and subsequent gelfiltration of the resulting pectin fragments showed that the neutral sugar side chains are present in hairy regions. The distribution of the methoxyl groups in citrus pectin does not differ much from that in apple pectin, but in citrus pectin some influence of native pectinesterase was found.

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10. Samenvatting en discussie

Uit de in alcohol onoplosbare bestanddelen ("AIS") van rijpe en onrijpe appels heb ik pectine geëxtraheerd zoals beschreven in hoofdstuk 3. De pectine werd gefractioneerd met behulp van ionenwisselingschromatografie en gelfiltratie. Daaruit bleek, dat de verdeling van de neutrale-suiker-eenheden over de moleculen geen continue verdeling is: er komen in de extracten vooral pectines voor met neutrale-suiker-gehaltes van 0,15, 0,24 en 0,53 mol neutrale-suiker-residuen per mol galacturonzuur-residuen. De pectine-moleculen bevatten de volgende neutrale suikers: rhamnose, arabinose, xylose, galactose en glucose. Mannose, dat vaak wordt aangetroffen in ongezuiverde pectine-preparaten, bleek niet aanwezig te zijn. De samenstelling van de neutrale suikers was in alle fracties dezelfde, uitgezonderd de relatieve hoeveelheid galactose.

De gezuiverde pectines werden behandeld met de gezuiverde enzymen pectine lyase (EC 4.2.2.10) en pectaat lyase (EC 4.2.2.2). De verkregen brokstukken werden gefractioneerd met behulp van gelfiltratie; van elke fractie werd de veresteringsgraad en de suikersamenstelling bepaald. Na beperkte afbraak door de enzymen kon meer dan 90% van de galacturonzuur-eenheden worden gefsoleerd als homo-galacturonaan-ketens. De neutrale-suiker-eenheden verschenen in de brokstukken, die nog een hoog molekuulgewicht hadden. In hoofdstuk 4 staan enkele modellen van pectine-moleculen, die zijn gebaseerd op deze gegevens. De neutrale suikers zijn in de modellen gesitueerd in zijketens, die in groepjes voorkomen (de zg. "hairy regions"). De galacturonzuur-eenheden in de "hairy regions" zijn allemaal veresterd met methanol; in de homo-galacturonaan-stukken zijn ze voor ongeveer 70% veresterd met methanol. De "hairy regions" heb ik behandeld met enzymen, met zuur en met loog (hoofdstuk 5). De resultaten konden worden verklaard door aan te nemen, dat de "hairy regions" bestaan uit 2 componenten, n.l. rhamnogalacturonaanstukken met arabinogalactanen als zijketens en galacturonaan-stukken met korte xylaan zijketens. Er bestaat een aparte populatie van moleculen, die bestaan uit galacturonaan hoofdketens en 1,3/1,6-gebonden galactaan als zijketen. In hoofdstuk 5

staat een korte discussie over de verbanden tussen de structuren van pectines van verschillende planten.

In het veel geciteerde celwandmodel van Keegstra et al. (1973) zijn rhamnogalacturonaan, arabinogalactaan en xyloglucaan covalent aan elkaar gebonden. De xyloglucaan molekulen zijn niet-covalent aan de cellulose fibrillen gebonden. Pectine-fragmenten, die uit esdoorn celwanden werden vrijgemaakt bevatten inderdaad xyloglucaan fragmenten (Talmadge et al., 1973), maar uit mijn onderzoek blijkt, dat de extraheerbare pectine-molekulen geen xyloglucaan-fragmenten bezitten. Waarschijnlijk dragen pectine-molekulen op een andere manier dan via covalente bindingen bij tot de stevigheid van de celwand. Deze conclusie kan ik niet voor alle pectine-molekulen trekken, omdat ongeveer de helft niet geëxtraheerd kon worden met middelen, die de molekulen intact laten. Dat alle geextraheerde molekulen uit de middenlamel afkomstig zijn, lijkt overigens onwaarschijnlijk: onder microscoop verschijnt de middenlamel als een dun laagje. Het zou kunnen zijn, dat pectine-molekulen in de celwand een gel vormen van het suiker-zuur-pectine type. Er zijn berichten over aggregatie van pectine-molekulen in verdunde oplossingen (Davis et al., 1980; Jordan & Brant, 1978), maar het is onwaarschijnlijk, dat dit verschijnsel resulteert in een stevig gel in de celwand. De rol van suiker in suiker-zuur-pectine-gelen zou in de celwand kunnen zijn overgenomen door arabinogalactanen: die staan bekend als opmerkelijk hydrofiel (Rees & Scott, 1971; Susheelama & Rao, 1978). De hoeveelheid arabinogalactaan, dat als zijketen aanwezig is, is echter te klein om deze functie te kunnen vervullen. Rees & Wight (1969) veronderstelden, dat pectine-molekulen met veel zijketens een functie als "glijmiddel" vervullen en pectine-molekulen met weinig zijketens een "lijmfunctie" hebben. In de geëxtraheerde molekulen echter is de afstand tussen de zijketens waarschijnlijk constant (hoofdstuk 4). Ook het feit, dat de zijketens in groepjes voorkomen pleit niet voor deze glijmiddel-functie van de zijketens. De functie van de zijketens blijft onduidelijk, maar ze vervullen waarschijnlijk geen functie bij de gelvorming.

De verdeling van de methanol-ester-groepen in appelpectine (behandeld in hoofdstuk 6) heb ik onderzocht door verregaande fractionering van de geëxtraheerde pectines toe te passen met behulp van ionenwisselingschromatografie en gelfiltratie. Er bleek een heterogene intermoleculaire verdeling te bestaan met pieken bij veresteringsgraden van 50%, 70% en 95%. De piek bij 70% is verreweg het grootst. De intramoleculaire verdeling heb ik onderzocht door ver-gezuiverde pectinefracties af te breken met pectine lyase en pectaat lyase en de verkregen brokstukken vervolgens te analyseren met behulp van gelfiltratie en hogedruk-vloeistof-chromatografie. De intramoleculaire verdeling van de methanolester-groepen is zeker geen bloksgewijze en kon in mijn onderzoek niet worden onderscheiden van een verdeling, die volgens de "wetten van het toeval" tot stand gekomen was. De in planten aanwezige pectine-esterase brengt een bloksgewijze ontestering tot stand. Het lijkt daarom uitgesloten, dat appelpectine in de biosynthese een stadium van volledige verestering doormaakt, waarna pectineesterase voor een gedeeltelijke verzeping zorgt.

Pectine molekulen dragen misschien bij tot de stevigheid van de celwand door de vorming van Ca^{2+}-pectaat gelen. In de desbetreffende literatuur (bijv. Joslyn, 1962; Knee, 1978) wordt vaak op deze mogelijkheid gewezen, maar overtuigend bewijsmateriaal is er nog niet. Toevoeging van $CaCl_2$ aan planteweefsel vermindert de oplosbaarheid van pectine (Bates & Ray, 1981; O'Beirne et al., 1980). Joslyn & Deuel (1961) concludeerden uit extractie-proeven, dat de "gegevens de hypotheses, dat meerwaardige ionen zoals Ca^{2+} verantwoordelijk zijn voor de onoplosbaarheid van pectines niet steunen". Ook Carr et al. (1980) vonden aanwijzingen, dat Ca^{2+}-ionen geen rol spelen in de middenlamellen van blad parenchymweefsel. De resultaten van mijn onderzoekingen (hoofdstuk 6) zijn in overeenstemming met de conclusie van Joslyn & Deuel. Het is bekend, dat alleen pectines met een lage veresteringsgraad een sterke Ca-binding vertonen (Kohn, 1975). Uit mijn resultaten blijkt, dat er haast geen pectine-molekulen of segmenten zijn met een lage veresteringsgraad. Dit geldt voor zowel

de extraheerbare molekulen als de niet-extraheerbare. De verdeling van de methanolgroepen is ook in theoriën over gelvorming een belangrijke parameter. Een gel is een meta-stabiele toestand (Rees, 1969). Dat blijkt uit het optreden van synerese, waaruit geconcludeerd kan worden, dat een gel geen evenwichtstoestand is. Daarom kan het niet zo zijn, dat een gel in stand wordt gehouden door dynamische krachten en dit sluit mechanismes zoals zoutbrugvorming uit. De theorie van Rees (1969) over gelvorming zegt, dat in gelen interacties bestaan tussen bepaalde stukken van de polymeren, de z.g. "junction zones". Zo zijn de junction zones in carrageen-gelen dubbele helix structure. Volgens Rees zijn de junction zones in pectine gelen "micro-kristallijne" zones; dit zou niet alleen voor Ca-pectaat gelen gelden, maar ook voor suiker-zuur-pectine-gelen. Rees eierdoos-model is een bekend model van de micro-kristallijne zones in Ca-pectaat gelen. In Ca-pectaat gelen schijnen ook nog micro-kristallijne zones van het suiker-zuur-pectine type een rol te spelen (Gidley et al., 1980). Men neemt aan, dat de Ca-binding van pectine verschilt van de Ca-binding van andere poly-electrolyten, zelfs van de Ca-binding in Ca-carboxymethyl-cellulose gelen (Kohn, 1975; Powell et al., 1980; Rees, 1969). Of er een essentieel verschil is kan echter worden betwijfeld: de Ca-binding van pectaat is in overeenstemming met de poly-electrolyt-theorie van Manning (Ravanat & Rinaudo, 1980; de Vries, 1977). Hoe dit ook zij, de junction zones in het eierdoos-model bestaan uit laag-veresterde galacturonaan segmenten van meer dan 7 eenheden (Powell et al., 1980). Mijn onderzoek naar de verdeling van de methanol-ester-groepen (hoofdstuk 6) laat zien, dat zulke segmenten praktisch afwezig zijn in appel- en citrus-pectine. Er is nog geen model van de junction zones in suiker-zuur-pectine gelen. De afstand tussen de zijketen-groepjes is groot (hoofdstuk 4) en dat suggereert, dat de neutrale-suiker-eenheden geen belangrijke rol bij de gelvorming spelen. Die conclusie wordt ondersteund door het feit, dat er in commerciële pectine-monsters geen verband bestaat tussen het neutrale-suiker-gehalte (of de samenstelling) en de gelsterkte (Otterbach, 1981). Mijn structuur-onder-

Een dergelijke conclusie werd ook getrokken door McCleary (1979): in galactomannanen doen de aanwezige mannaan-blokken geen dienst als junction zone. Ook voor gums geldt, dat er geen eenduidig verband is tussen fysisch gedrag en suiker-samenstelling (Phillips et al., 1981).

Tijdens de rijping van appels veranderden de polymerisatiegraad, de veresteringsgraad, het neutrale-suikergehalte en de samenstelling van de neutrale suikers maar weinig (hoofdstuk 7). De hoeveelheid extraheerbare pectine nam toe. Een hoog-veresterde pectine-fractie, die 1,3/1,6-gebonden galactaan bevatte, nam toe in hoeveelheid. Uit de celwanden van rijpe appels kon wat glucose en xylose bevattend polysaccharide-materiaal worden geëxtraheerd. In overrijpe appels echter blijkt er wel verandering in de structuur te zijn aangebracht: de polymerisatiegraad van zowel de hoofdketens als de arabinogalactaan zijketens is lager geworden. De hoeveelheid oplosbare pectine met 1,3/1,6-gebonden galactaan nam verder toe. De veresteringsgraad en de verdeling van de methanol-ester groepen veranderde tijdens rijpen en bewaren niet.

Tenslotte heb ik appelpectine en citruspectine met elkaar vergeleken (hoofdstuk 8). De citruspectine-molekulen bleken geen xylose-eenheden te bevatten. Ook in citruspectine zijn de neutrale suikers gesitueerd in "hairy regions". De verdeling van de methanol-ester groepen in citruspectine verschilt niet veel van die in appelpectine. Wel kon in citruspectine enige invloed van de in citrus aanwezige pectine-esterase worden aangetoond.

Literatuur

Zie hoofdstuk 9.

CURRICULUM VITAE.

Jacob Ailko de Vries werd geboren te Baflo in 1953. Hij studeerde van 1971 tot 1977 aan de Landbouwhogeschool te Wageningen, in de richting levensmiddelentechnologie. Zijn hoofdvak tijdens de ingenieursstudie was levensmiddelenchemie, bijvakken waren levensmiddelenmicrobiologie en biochemie. Het promotie-onderzoek vond plaats van 1978 tot 1982 op het Laboratorium voor Levensmiddelenchemie en -microbiologie van de Landbouwhogeschool in het kader van een zg. promotie-assistentschap.

10. Bij het construeren van het celwandmodel van Keegstra et al(1973) heeft de esthetiek een te belangrijke plaats ingenomen.

    Keegstra,K.,Talmadge,K.W.,Bauer,W.D. en Albersheim,P.(1973),Plant Physiol. 51,188-196.

11. Gedetailleerde kennis van de samenstelling en opbouw van dieetvezels levert geen inzicht op in de rol van dieetvezels als voedingsbestanddeel zolang voedingskundige theoriëen op dit gebied ontbreken.

    Southgate,D.A.T.(1981),Food Technol.Australia 33,24-25.

12. Proefschriften horen niet alleen over de inhoud van een bepaalde vakwetenschap te gaan,maar ook over de vorm daarvan.

13. Bij de reductie van het laboratorium tot het "lab" zonder oratorium is de vrijgekomen ruimte helaas niet opgevuld.

14. Om hinderlijk samenvallen van de produktieve en de prokreatieve leeftijd te voorkomen zouden mannen meer gebruik moeten maken van de mogelijkheid zich pas op de leeftijd der zeer sterken voort te planten.

Proefschrift van J.A.de Vries
Structural features of apple pectic substances
Wageningen,11 november 1983.