

# **Chemistry and Function of Pectins**



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# Chemistry and Function of Pectins

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# FOREWORD

The ACS SYMPOSIUM SERIES was founded in 1974 to provide a medium for publishing symposia quickly in book form. The format of the Series parallels that of the continuing ADVANCES IN CHEMISTRY SERIES except that, in order to save time, the papers are not typeset but are reproduced as they are submitted by the authors in camera-ready form. Papers are reviewed under the supervision of the Editors with the assistance of the Series Advisory Board and are selected to maintain the integrity of the symposia; however, verbatim reproductions of previously published papers are not accepted. Both reviews and reports of research are acceptable, because symposia may embrace both types of presentation.

# PREFACE

THE PHYSICAL, BIOCHEMICAL, AND FUNCTIONAL PROPERTIES of pectin are of great interest to a diverse cross section of scientists and technologists because pectin can be classified as a polyelectrolyte, a complex polysaccharide, an important food fiber, a major plant cell wall component, and a ubiquitous nutritional factor and gelling agent in foods. An explosion in pectin research is evidenced by the more than 200 references cited in 1983 and 1984. Nevertheless, a book devoted strictly to pectin research has not appeared in over 10 years. The intention in writing this volume is to assemble chapters from leading scientists and technologists in the pectin research field to produce a state-of-the-art multidisciplinary book that will advance pectin research through a cross fertilization of sound research ideas. In any book of this nature, some duplication as well as contradictory interpretations of the same phenomena will occur. These problems should provide the impetus for new and continued research leading to a better understanding of the chemical and functional properties of pectins.

Although we tried very hard to spread evenly the coverage of every aspect of pectin research in this book, unavoidably some areas are not covered adequately. We thank the authors for their excellent oral presentations at the symposium and their cooperation in completing the written manuscript in a timely manner. We also thank the reviewers for their incisive and constructive criticism. We sincerely hope the next book on pectin research does not have to wait 10 years to appear in print.

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## An Introduction to Pectins: Structure and Properties

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Pectin, a polysaccharide, is composed primarily of essentially linear polymers of D-galactopyranosyluronic acid units joined in  $\alpha$ -D-(1 $\rightarrow$ 4) glycosidic linkages; the polymer chains are esterified to various degrees with methanol. This regular structure is interrupted, however, with L-rhamnopyranosyl units and with side chains containing other neutral sugars. The polymer chains may also be partially acetylated. The most important physical property of pectin is its ability to form spreadable gels. Gel formation results when the polymer chains interact over a portion of their length to form a three-dimensional network. This aggregation of chains occurs through hydrogen bonding, divalent cation crossbridging, and/or hydrophobic interactions.

### Terminology

In 1944, definitions for pectins were established by the Committee for the Revision of the Nomenclature of Pectic Substances (1); but since then, terminology has changed somewhat, and modified definitions have been used. At this time, there are no universally agreed upon and accepted definitions. The definitions presented here are those generally in current commercial use (2).

Pectic acids are galacturonoglycans [poly( $\alpha$ -D-galactopyranosyluronic acids)] without, or with only a negligible content of, methyl ester groups. Pectic acids may have varying degrees of neutralization. Salts of pectic acids are called pectates.

Pectinic acids are galacturonoglycans with various, but greater than negligible, contents of methyl ester groups. Pectinic acids may have varying degrees of neutralization. Salts of pectinic acids are called pectinates.

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Pectins are mixtures of polysaccharides that originate from plants, contain pectinic acids as major components, are water soluble, and are able to form gels under suitable conditions (See section on Physical Properties).

In this chapter, as is frequently done, the term pectin will be used in a generic sense to designate those water-soluble galacturonoglycans of varying methyl ester content and degree of neutralization that are capable of forming gels under suitable conditions (See section on Physical Properties), i.e., other polysaccharides that may be present in commercial mixtures will be ignored.

Pectins are subdivided according to their degree of esterification (DE), a designation of the percent of carboxyl groups esterified with methanol. Pectins with DE > 50% are high-methoxyl pectins (HM-pectins); those with DE < 50% are low-methoxyl pectins (LM-pectins).

The degree of amidation (DA) indicates the percent of carboxyl groups in the amide form (See section on Chemical Properties).

### Structure

For a more detailed discussion of the chemical structure of pectins, see reference 3.

Pectin, a structural, cell-wall polysaccharide of all higher plants, like most other polysaccharides, is both polymolecular and polydisperse, i.e., it is heterogeneous with respect to both chemical structure and molecular weight (4). From molecule to molecule, in any sample of pectin, both the number and percentage of individual monomeric unit types will vary, and the average composition and distribution of molecular weights can vary with the source, the conditions used for isolation, and any subsequent treatments. Because both parameters determine physical properties, various functional types of pectin can be produced by controlling the source, isolation procedure, and subsequent treatment(s).

Pectin is primarily a polymer of D-galacturonic acid. The principal and key feature of all pectin molecules is a linear chain of (1→4)-linked α-D-galactopyranosyluronic acid units, making it an α-D-galacturonan [a poly(α-D-galactopyranosyluronic acid) or an α-D-galacturonoglycan].

In all natural pectins, some of the carboxyl groups are in the methyl ester form. Depending on the isolation conditions, the remaining free carboxylic acid groups may be partly or fully neutralized, i.e., partly or fully present as sodium, potassium or ammonium carboxylate groups. The ratio of esterified D-galacturonic acid units to total D-galacturonic acid units is called the degree of esterification (DE) and strongly influences the solubility, gel forming ability, conditions required for gelation, gelling temperature, and gel properties of the preparation.

In pectin from some sources, some of the units occur as 0-2 or 0-3 acetates. Such esterification hampers gelation, so much so that complete inhibition of gelation occurs when one out of eight

D-galactopyranosyluronic acid units are monoesterified with acetic acid at O-2 or O-3. The presence of acetyl groups, therefore, makes certain potential sources of commercial pectin, e.g., sugar beet (5), sunflower, and potato, less desirable.

Neutral sugars, primarily L-rhamnose, are also present. However, there is disagreement over their distribution in the linear chain. One group of workers (6) reported that, in citrus, apple, and sunflower pectins, L-rhamnopyranosyl units are more or less evenly inserted into the galacturonan chain in the following manner:  $O-\alpha-D-GalpA-(1\rightarrow2)-O-L-Rhap-(1\rightarrow4)-O-\alpha-D-GalpA$ . The configuration of the L-rhamnopyranosyl linkage is unknown, but calculations have shown that it should be beta in order to provide the necessary degree of kinking in the structure (6) (See section on Physical Properties).

Another group (7) reported that the L-rhamnopyranosyl units are quite unevenly distributed within the galacturonan backbone. These workers report (8) that apple pectin consists of "smooth" regions [poly( $\alpha$ -D-galactopyranosyluronic acid) regions] and "hairy" regions. According to them, the latter regions consist of rhamnogalacturonan sequences that contain highly branched arabinogalactan side chains and galacturonan sequences with short side chains composed of D-xylose. The same may also be true of sugar beet pectin (5).

The data of the former group (9) also indicates that, however the L-rhamnopyranosyl units occur in the chain, the length of the poly( $\alpha$ -D-galactopyranosyluronic acid) sequences between L-rhamnopyranose interruptions (whether single units or blocks of units) is rather constant and that the sequences are about 25 units long in each pectin (citrus, apple, sunflower) studied.

The total content of neutral sugars varies with the source, the extraction conditions, and subsequent treatments.

Ferulic acid is esterified to the neutral sugar side chains of pectin from spinach (10) and sugar beet (5,11).

### Conformation

Gels are formed when polymer molecules interact over a portion of their length to form a network that entraps solvent and solute molecules. The junction zones that result from these chain interactions must be of limited size. If they are too large, a precipitate, rather than a gel, results. The inserted L-rhamnopyranosyl units may provide the necessary irregularities (kinks) in the structure and limit the size of the junction zones. The presence of "hairy" regions may also be a factor that limits the extent of chain association. As will be discussed further in the section on Physical Properties, junction zones are formed between regular, unbranched pectin chains when the negative charges on the carboxylate groups are removed (addition of acid), hydration of the molecules is reduced (addition of a cosolute), and/or polymer chains are bridged by divalent cations (calcium ions).

Analysis of proton n.m.r. spectra and mathematical model building suggests that individual  $\alpha$ -D-galactopyranosyluronic acid units have the  ${}^4C_1$  conformation (6). Sodium and calcium pectates,

pectic acid, and pectinic acid all occur in the solid state (fibers) as right-handed ( $3_1$ ) helices with a three-fold screw axis (trisaccharide repeat) (12-15). In solid pectinic acid, the polymer molecules pack so that the chains are parallel to each other; the pectates pack as corrugated sheets of antiparallel chains (14,15).

It is further suggested that junction zones in pectinic acid (HM-pectin plus sucrose) gels are formed by a columnar stacking of methyl ester groups to form cylindrical hydrophobic areas parallel to the helix axes. Two models for the formation of junction zones in calcium pectate (LM-pectin) gels have been proposed. One suggests an aggregation of chains by a crosslinking of carboxylate anions with calcium ions to form a structure similar to that of the corrugated sheets of antiparallel helices (3-6 chains in an average junction zone) found in solid calcium pectate (15). The other is the "egg box" model used to describe the formation of calcium alginate gels (16,17). This model is proposed because of the close similarity between (1+4)-linked poly( $\alpha$ -D-galactopyranosyluronic acid) segments of pectic acid and  $\bar{1}$ (1+4)-linked poly( $\alpha$ -L-gulopyranosyluronic acid) segments of alginic acids, segments that are mirror images except for the configuration at C-3. From circular dichroism and equilibrium dialysis studies, it has been concluded that interchain association of hydrated pectinic acid molecules, in the presence of swamping levels of monovalent counterions, is limited to the formation of dimers of chains of  $2_1$  helical symmetry with specific site-binding of calcium ions along one face of each participating chain (18,19; see also reference 20). When  $\text{Ca}^{2+}$  is the sole or principal counterion, these dimers further aggregate without rearrangement, leading to an approximate doubling of the amount of  $\text{Ca}^{2+}$  bound cooperatively (18,19). Based on available information, the Unilever Research group (19) has concluded that drying of a calcium pectinate gel effects a polymorphic phase transition in which associated, regular, buckled chains with two-fold symmetry ("egg box") as found in L-gulonoglycan chain segments are converted into associated  $\bar{3}$  chains with three-fold symmetry as found in solid state calcium pectinate (12-15). It should be noted that the axial-axial linkages in a chain of aldohexopyranosyl units linked 1+4 gives a buckled conformation naturally (Figure 1) and that the gel structure(s) is(are) as yet not well understood.

### Physical Properties

Pectins are soluble in pure water, but they are insoluble in aqueous solutions in which they would gel at the same temperature if dissolved at a higher temperature. Monovalent cation (alkali metal) salts of pectinic and pectic acids are usually soluble in water; di- and trivalent cation salts are weakly soluble or insoluble.

Although pectins are not employed as thickening agents, pectin solutions exhibit the non-Newtonian, pseudoplastic behavior characteristic of most polysaccharides. As with solubility, the viscosity of a pectin solution is related to the molecular weight, DE, and concentration of the preparation and the pH and presence

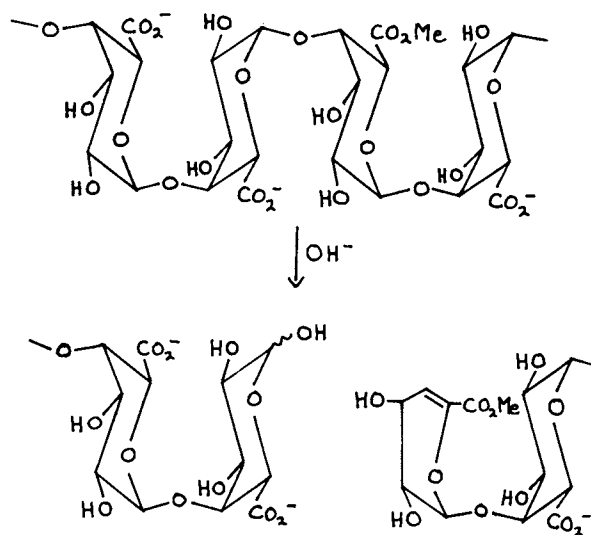


Figure 1. Alkaline depolymerization of a sequence of a pectinic acid of DE 25.

of counterions in the solution. For example, addition of monovalent cations effects a reduction in viscosity, the degree of which is greater with decreasing DE. Addition of salts of di- and trivalent cations has an opposite effect (20). In general, viscosity, solubility, and gelation are related, i.e., factors that increase gel strength, for example, will increase the tendency to gel, decrease solubility, and increase viscosity, and vice versa.

These physical properties of pectins are a function of their structure which is that of a linear polyanion (polycarboxylate). As such, monovalent cation salts of pectins are highly ionized in solution, and the distribution of ionic charges along the molecule tends to keep it in an extended form by reason of coulombic repulsion (21). Furthermore, this same coulombic repulsion between the carboxylate anions prevents aggregation of the polymer chains. (The number of negative charges is, of course, determined by the DE.) In addition, each polysaccharide chain, and especially each carboxylate group, will be highly hydrated. Solutions of monovalent salts of pectins exhibit stable viscosity because each polymer chain is hydrated, extended, and independent.

Because the commercial importance of pectin is predominately the result of its unique ability to form spreadable gels in the presence of a dehydrating agent (sugar) at a pH at or near 3 or in the presence of calcium ion (jams, jellies, and marmalades made from fruit juices or whole fruit), that is the property most often studied and focused upon. Factors that determine whether gelation can occur and that influence gel characteristics are pH, concentration of cosolutes (sugars), concentration and type of cations, temperature, and pectin concentration. The ways in which these factors influence gelation are dependent upon the following molecular properties of the specific pectin: molecular weight (4), degree of esterification (DE), degree of amidation (DA), presence of acetate esters, and heterogeneity. All these parameters are interdependent. In general, under similar conditions, the degree of gelation, the gelling temperature, and the gel strength are generally proportional to each other and each property is generally proportional to the molecular weight and inversely proportional to the DE.

As the pH is lowered, i.e., as the hydrogen ion concentration of the solution is increased, ionization of the carboxylate groups is repressed, i.e., the highly hydrated carboxylate groups are converted into only slightly hydrated carboxylic acid groups. As a result of losing some of their charge, the polysaccharide molecules no longer repel each other over their entire length; and as a result of losing some of the water of hydration, they can associate over a portion of their length to form a gel. Apparent pK values (pH at 50% dissociation) vary with the DE of the pectin (22); a 65% DE pectin has an apparent pK of 3.55, while a 0% DE pectic acid has an apparent pK of 4.10. However, pectins with increasingly greater degrees of methylation will gel at somewhat higher pH, undoubtedly because they have fewer carboxylate anions at any given pH (See later paragraph). pH affects gel texture more than gel strength.

It is generally regarded that gels form when pectin molecules, normally strongly hydrated by water molecules, lose some water of hydration owing to competitive hydration of cosolute molecules. Reduced hydration results in greater contact between pectin chains, forming junction zones (primarily by means of hydrogen bonding) and resulting in a network of polymer chains that entraps water and solute molecules (23). In most jams, jellies, and marmalades, the cosolute is sugar (sucrose). HM-pectins will gel only in the presence of large concentrations (at least 55% w/w) of sugar. LM-pectin will gel in the absence of sugar (if a divalent cation is present), but increasing the soluble solids will raise the gelling temperature and gel strength.

LM-pectin will gel only in the presence of divalent cations. Increasing the concentration of divalent cations (only calcium ion is used in food applications) increases the gelling temperature and gel strength. Divalent cations are unnecessary for the formation of a HM-pectin gel because of the low number of carboxylate groups that need to be bridged and because of the formation of hydrophobic areas parallel to the helix axes by a columnar stacking of methyl ester groups (15).

Any system containing pectin at potential gelling conditions (i.e., necessary concentration of an appropriate pectin, pH, concentration of cosolutes, and concentration of divalent cations) must be prepared at a temperature above the gelling temperature. Then, as the hot pectin solution is cooled, the thermal energy of the molecules decreases and their tendency to form junction zones upon collision increases. The temperature at which gelation occurs is the gelling temperature. Gels made with LM-pectin form rapidly; those made with HM-pectin form slowly. LM-pectin gels are thermoreversible; HM-pectin gels are not.

The concentration of pectin required for gel formation is inversely related to the concentration of soluble solids, for in general, increasing the concentration of cosolutes, i.e., decreasing the water activity, increases the size and number of junction zones. When other factors are held constant, increasing the concentration of pectin increases the gel strength because it increases the number of junction zones.

At constant pH, gel strength of HM-pectin gels increases with increasing DE, as does the rate of gelation. As the DE of a HM-pectin is lowered, a lower pH is required for gelation. Again, this can be explained by the fact that, as the DE is lowered, even though the pH required to produce a given percentage of carboxyl groups in the carboxylic acid form increases owing to an increase in apparent pK (22), the absolute number of contiguous carboxylate groups that must be converted into unionized carboxylic acid groups before chain association can occur also increases. Thus, as the DE of a HM-pectin is lowered, a greater and greater percentage of carboxylate groups must be converted into free carboxylic acid groups to effect gelation.

Among the commercial LM-pectins, those with the lowest DE values have the highest gelling temperatures and the greatest requirement for divalent cations (for crossbridging).

Amidation results in a higher gelling temperature and a decreased need for a divalent cation.

The distribution of carboxyl/carboxylate groups also affects gelation. Pectins with blocks of methyl ester and carboxyl groups (as opposed to a random distribution) generally produce weaker gels and have a greater requirement for divalent cations.

### Chemical Properties

Dissolved pectins undergo deesterification and depolymerization in aqueous systems. The pH of greatest stability is about 4. At pH values both above and below 4, deesterification and depolymerization occur concurrently, with the rate of deesterification being greater than the rate of depolymerization. The presence of solutes, which lowers water activity, reduces the rates of both reactions.

Deesterification occurs by normal acid- and base-catalyzed mechanisms of ester hydrolysis. Depolymerization at low pH values occurs by means of acid-catalyzed hydrolysis of glycosidic bonds (24). Acid-catalyzed hydrolysis occurs preferentially at the L-rhamnopyranosyl glycosidic bonds. Hydrolysis of these linkages produces galacturonoglycan chains with a degree of polymerization of about 25 (6,9). Side chains, particularly those containing L-arabinofuranosyl units, should also be preferentially removed by hydrolysis because of the inherent stability to acid-catalyzed hydrolysis of glycuronosyl glycosidic bonds and the inherent lability of furanosyl glycosidic bonds (24). However, if the side chains are attached to rhamnogalacturonan sequences (8), it should not be possible to convert "hairy" regions to "smooth" regions by treatment with acid because the lability of the L-rhamnopyranosyl bonds would result in concurrent depolymerization of the main chain.

At pH values of 5-6 pectin solutions are stable only at room temperature. As the temperature is raised, pectin chains cleave by a beta-elimination reaction (25-39) (Figure 1), a reaction which is stimulated by organic anions (40). Deesterification of pectin proceeds simultaneously with the beta-elimination depolymerization reaction, which occurs only at monosaccharide units that are esterified. At pH values above 6, deesterification and depolymerization are rapid reactions even at room temperature, the rate of each reaction increasing with increasing pH.

Hydroxyl-group reactions, such as etherification, acetalation (41), esterification (42-51), and oxidation, can be done in the same manner as they are on other polysaccharides. Esterifications (47) of carboxyl groups and interactions with cations, including polycations such as proteins below their isoelectric pH, occur as they do with other glycuronoglycans. Reduction of carboxyl groups to hydroxymethyl groups has been done with diborane (52, see also 53) and by borohydride treatment of methyl and hydroxyethyl esters (54). Reduction of carboxyl groups which have been activated with a water-soluble carbodiimide should be straightforward (55,56).

When ammonia (57-61) is used to prepare LM-pectin from HM-pectin, some of the methyl carboxylate groups are converted into carboxamide groups, producing "amidated pectin". The

presence of amide groups in an LM-pectin makes the molecules less hydrophilic, increasing their tendency to form gels, and less sensitive to calcium ions. In general, the gelling temperature increases with increasing DA. Pectin amides can also be made by reaction of pectins with primary and secondary alkylamines (62-64).

There are several types of enzymes that act on pectin molecules. Those enzymes produced by the higher plants themselves play a significant role in the processes resulting in textural changes in fruits and vegetables during ripening, storage, and processing (65-67). Fungal enzyme preparations are used by the fruit juice industry to improve the clarity of juices and the yield from pressing (66-67). Control of their action is important to the production of pectin.

Pectinesterases catalyze hydrolysis of the methyl ester group (68). Because many pectinesterases act preferentially on a methyl  $\alpha$ -D-galactopyranosyluronate unit adjacent to a nonesterified  $\alpha$ -D-galactopyranosyluronic acid unit, they produce pectins that contain blocks, rather than a random distribution, of carboxyl groups (68) and that are generally undesirable for commercial use. Some fungal pectinesterases, however, produce LM-pectins that are similar to pectins deesterified with acids or bases with respect to gelling ability (69) and sensitivity to calcium ions (70).

Several types of lyases or transeliminases are known (68). All catalyze depolymerization by a beta-elimination reaction like that which occurs during base-catalyzed depolymerization. Pectin lyases, all of which are endo-enzymes, catalyze beta-eliminations at esterified D-galacturonic acid units (See Figure 1) (68). Pectate lyases catalyze beta-eliminations at nonesterified D-galacturonic acid units. Both exo- and endo-pectate lyases are known (68).

Polygalacturonases depolymerize pectins by catalyzing hydrolysis of glycosidic bonds (68,71). The fact that the rate of depolymerization is inversely proportional to the DE suggests a requirement for a nonesterified D-galacturonic acid unit. Exo-polygalacturonases release mono- or disaccharides from nonreducing termini; endo-polygalacturonases attack randomly (68).

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## Analytical Methods for Determining Pectin Composition

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Chemical and modern chromatographic methods have been applied to the determination of pectin composition and structure. The dominant structural feature of pectin is a linear chain of galacturonic acid residues, some of which are esterified with methyl groups. Chemical methods (including reactions with carbazole and substituted phenols) and chromatographic methods (GLC and HPLC) are available for galacturonic acid determination. Methyl ester levels are determined either chemically (after oxidation to formaldehyde) or by GLC after pectin ester saponification. A variety of neutral sugars are present in pectin, mainly rhamnose, galactose, arabinose, and xylose. Effective GLC procedures are available for their determination, and applicable liquid chromatographic methods have been developed. Measurements of less the less-common substituents, such as O-acetyl and O-feruloyl esters have been achieved by colorimetric and titrimetric procedures. Developments in infrared and  $^{13}\text{C}$ -NMR spectroscopy have resulted in these being applied to structural analysis in pectin.

Pectin polysaccharides and the hemicelluloses are matrix components in the cell walls of higher plants. Traditionally, these classes of carbohydrates have been defined operationally by their presence in fractions obtained by sequential extraction of cell walls. The pectic substances are extracted with water, dilute acid, or with calcium chelating agents, such as EDTA, ammonium oxalate, or sodium hexametaphosphate. But classification of polysaccharides is best based on structural components rather than on the method used for its isolation. According to structure, the pectic substances would include galacturonans, rhamnogalacturonans, arabinans, galactans, and arabinogalactans which possess a linear  $\beta$ -1,4-D-galactan backbone.

A recent classification (1) describes the pectic polysaccharides as those polymers found in covalent association with galacturonosyl-containing polysaccharides. The hemicelluloses are those carbohydrate polymers which are noncovalently associated with cellulose. Diverse categories of pectic polysaccharides occur not only among plant sources, but among tissues in a given source.

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A comprehensive (2) review on the structure of pectin has recently been published. Pectin was described as consisting of a branched block, in which the main galacturonan chain is interrupted by rhamnose units. Many of these rhamnoses carry arabinan or galactan chains; the galactan chains are sometimes further substituted with arabinan segments. These heavily branched galacturonan chains alternate with unbranched blocks in which rhamnoses are rarely present. Methyl esters of galacturonic acid are also present as blocks, alternating with sequences of non-esterified galacturonic acid.

This review will describe the analytical methods available to determine the structural components of pectin. These features determine the important physical, chemical, and biological properties of pectin. Included will be discussion of galacturonic acid determinations, degree of esterification with methyl groups, the neutral-sugar composition, and analysis of some less-common entities, such as O-acetyl and O-feruloyl linkages.

#### Quantitative Analysis of Galacturonic Acid in Pectin

The dominant and unifying structural feature in pectins is a linear 1-4- $\alpha$ -linked D-galactopyranosyl-uronic acid chain.  $\alpha$ -L-Rhamnosyl residues are inserted at intervals in the chain, and variable proportions of the uronic acid residues are esterified with methanol. Neutral sugars other than rhamnose are present, and neutral sugar levels often total about 20%. The other neutral sugars are mainly D-galactose, L-arabinose, and D-xylose, and these are likely to be attached in branches to the rhamnose residues in the main chain. In a later section, various chromatographic approaches for determining the levels of individual neutral sugars will be described.

Chemical Methods. Determinations of galacturonic acid of pectin usually includes both the free and esterified forms, since strongly acidic media are employed in the colorimetric methods. Procedures which continue to be used widely are modifications of those described early by Dische. One is based upon reaction with cysteine (3) and the other with carbazole (4). One modification of the carbazole method, which gave a doubling in sensitivity, along with an increased stability in color and greater reproducibility, was reported in 1962 (5). Inclusion of borate into the assay medium was responsible for the enhancement of the method. In all the colorimetric methods, galacturonic acid is liberated during the assay by hydrolysis of polymeric pectin. An application of the carbazole method, after extraction of pectin from various fruits and vegetables, has been described (6), as has been an automated carbazole method for monitoring uronic acid levels in pectin fractions (7).

A more rapid and somewhat simpler procedure for uronic acid determination was described in 1973 (8). This method is advantageous for determining galacturonic acid in pectin, as interference by neutral sugars is reduced. This method is based on the color formation which accompanies the addition of *m*-hydroxybiphenyl to heated solutions of uronic acids in sulfuric acid/boric

acid. This assay has been applied to the determination of galacturonic acid in food pectins (9,10), and interference by neutral sugars was minimized (9). Another phenol, 3,5-dimethylphenol, has been found (11) to be more selective than *m*-hydroxydiphenyl when large amounts of neutral sugars are present in the sample. This method has recently been employed in studies of the degree of methylation of pectin in plant cell walls (12).

A procedure employing colloidal titration has been used for the determination of galacturonic acid in pectin, and indirectly, also for determining the degree of esterification (13). Samples are titrated with poly-*N,N*-dimethylallylammonium chloride, and a distinct flocculation occurs, the endpoint of which is determined by use of toluidine blue indicator. In a duplicate sample, ester methyl groups can be saponified, and total galacturonic acid determined; by difference, the degree of methyl esterification is calculated. The quantitation of this colloidal titration method is more precise with pectins of high degrees of polymerization. In another titrimetric method, total galacturonic acid and the degree of esterification is determined by copper-binding before and after saponification (14). The bound copper is determined by atomic absorption spectrometry. Application of this copper-binding approach to the analysis of cell-wall polysaccharides in many fruits and vegetables has been reported (15).

Decarboxylation with hydroiodic acid (16) was the basis for a procedure used in determining uronic acid levels in dietary fiber fractions (17). The carbon dioxide from decarboxylation was purified, trapped in a cell containing standard sodium hydroxide, and conductivity changes were measured using an Ingold electrode.

Studies comparing the distribution of free carboxyl groups in enzymatically and chemically de-esterified pectins are important because the gelling behavior of resulting products is a function of the method used. Enzymatically de-esterified pectins have a blockwise distribution of non-esterified galacturonic acid residues, and gel with calcium at higher degrees of esterification than do acid de-esterified pectins, which possess a more random distribution of free carboxyl groups. Free carboxyl distribution has been studied (18) by first esterifying by reaction with ethylene oxide (glycolation), and then treating the sample with a mixture of pectin enzymes. The glycolated fragments are unreactive toward these enzymes. Finally, the hydrolysis products are separated from the glycolated fragments by ion exchange chromatography, and after deglycolation, chain size is determined by gel filtration. An application of this approach in studies of orange-peel pectin has been reported (19).

Physical Methods. Infrared (IR), Raman, and nuclear magnetic resonance (NMR) spectroscopic methods have been applied to structural analysis of polysaccharides such as pectin. These applications have been reviewed (20), and reference IR spectra of pectic substances have been published (21). Quantitative IR has been used to estimate acid dissociation constants of polyuronides from the ratio of  $-CO_2H$  to  $-CO_2^-$  as a function of pH (22). Also, by

use of solution IR in  $D_2O$ , the ratio of  $-CO_2H$  to  $-CO_2CH_3$  (free to esterified) groups in pectins can be determined (23). The ester-carbonyl stretching band is observed at  $1740\text{ cm}^{-1}$  and a carboxylate stretching band at  $1650\text{ cm}^{-1}$ .  $^{13}C$ -NMR spectroscopy has also been useful in determining relative proportions of free and esterified carboxyl groups in pectin (24). In this approach, the ratio of peak areas at  $172.8\text{ ppm}$  ( $-CO_2H$ ) is determined relative to the areas either at  $171.3\text{ ppm}$  ( $-CO_2CH_3$ ) or  $53.7\text{ ppm}$  ( $-OCH_3$ ). In addition, pectin from sugar-beet has been examined by  $^{13}C$ -NMR, and O-acetyl, carbons-one of the minor constituents galactose and arabinose, and carbon-six of rhamnose can be discerned (25).

**Chromatographic Methods.** The high levels of galacturonic acid (free and esterified) in pectin has resulted in the development of chemical methods which are rather non-specific. With corrections applied for neutral sugars however, reasonable estimates of its levels can be made. The colorimetric procedures are conducted in strongly acidic media, which results in some loss of galacturonic acid by decarboxylation to L-arabinose. Also, all uronic acids respond to the various colorimetric tests, so for analyzing mixtures of uronic acids, gentler hydrolytic steps and far more selective assays are needed. To this end, several gas-liquid (GLC) and high-performance liquid chromatographic (HPLC) methods have been developed for determining galacturonic acid. Some have employed specific enzymes for depolymerization as an alternative to acid hydrolysis. Combinations of pectic enzymes and acid catalysis are required to quantitatively hydrolyze pectin. This section will describe applications of modern chromatographic methods for determining galacturonic acid in pectin. For excellent and comprehensive descriptions of sugar chromatography in general, review articles on GLC (26) and HPLC (27,28) have been published.

A sensitive GLC procedure for determining galacturonic acid in pectin has been developed (29) from an earlier described method for analyzing uronic acids (30,31). Pectins, after extraction from plant tissues with ammonium oxalate, are depolymerized with pectinase. The liberated galacturonic acid is then reduced by sodium borohydride to galactonic acid, which is converted to L-galactono-1,4-lactone. The trimethylsilyl derivative of the lactone gives a sharp peak on SE-30 stationary phase, and per-trimethylsilyl xylitol is used as an internal standard. Often, the decomposition of monomeric sugars which result from the resistance to acid hydrolysis of polysaccharides containing acidic groups such as pectin is overcome by first reducing the uronic acids to neutral sugars. This reduction method (32), which should be repeated at least twice for quantitative conversion, is widely used in polysaccharide structural analysis, and has been applied in a GLC procedure for galacturonic acid (33). After activation of pectin carboxyl functions with a water-soluble diimide, reduction with sodium borodeuteride converts the galacturonic acid residues to 6,6-dideutero-galactose derivatives. Then, after acid hydrolysis, standard gas chromatography-mass spectrometry

methods to resolve neutral sugars are applied. The galactose (dideutero) which had been generated from galacturonic acid in pectin is distinguished from galactose (diprotio) naturally present in pectin by mass spectrometry. These GLC methods are characteristically very sensitive and efficient.

Liquid chromatographic procedures have been developed more recently, and some are quite effective for determining galacturonic acid in pectin. An automated anion-exchange chromatographic system (34) allows the separation of individual uronic acids, including galacturonic acid. Column effluents were sensitively analyzed for uronic acids by post-column reaction with orcinol and monitoring at 420 nm. More rapid HPLC approaches have been described. By using strong anion-exchange columns, 0.7M acetic acid mobile phase, and refractive index detection, galacturonic acid was separated from mannuronic and glucuronic acids in less than 15 minutes (35). Similar conditions were employed to separate oligogalacturonic acids of up to DP-8, and Bio-Gel P-2 has been used for the same purpose (36). In another report (37), strong anion-exchange HPLC also was used, with a mobile-phase consisting of boric acid/potassium hydroxide buffer, and post-column fluorometric detection (2-cyanoacetamide), allowing the resolution and sensitive detection of four uronic acids, including galacturonic acid. Polygalacturonic acid has been subjected to methanolysis (reaction with methanolic hydrogen chloride); multiple peaks result in HPLC chromatograms, due to the presence of  $\alpha,\beta$ -mixtures of the methyl glycosides (38). Although the procedure is not ideal for quantitation, it is useful for qualitative analysis of uronic acid composition in polysaccharides. In a recent report (39) polygalacturonic acid was subjected to both acid and polygalacturonase catalyzed hydrolysis. The hydrolyzates were analyzed by HPLC on a cation-exchange column of HPX-87-H<sup>+</sup>, and galacturonic acid was eluted in 8.5 minutes. The advantages of enzyme over acid-catalyzed hydrolysis were apparent. The yield of monomer was greater, no monomer degradation products were present, and a far lesser quantity of oligogalacturonic acid chains were produced.

#### Determination of Methyl, Acetyl, and Feruloyl Substitution in Pectin

Pectin consists mainly of polygalacturonate chains, and the carboxyl groups are significant determinants of its chemical and biological properties. In plant cell walls, more than 50% of the carboxyl groups are often esterified with methanol. The degree of esterification largely determines the ion-exchange, water-binding, cross-linking, and hydrogen-bonding capacities of pectin. Similarly, properties of pectin in cell walls are sometimes modified by low levels of hydroxyl esterification with acetyl groups. The distribution of acetyl groups in pectin is unknown, but in sugar beet, pear, and apricot pectin, acetyl levels approach 4%. In addition, alkali-labile ferulic acid groups are found in ester linkage to pectin; they are believed to be carried by arabinose and/or galactose residues on neutral side chains. This section will describe recent methods to determine pectin substitution with methyl, acetyl, and feruloyl groups.

Methyl Esters In Pectin. A titration method has been reported (40), in which methyl ester levels are calculated from the number of equivalents of standard sodium hydroxide required to neutralize the pectin sample before and after saponification. The copper titration procedure described earlier for determination of galacturonic acid residues in pectin (15), is also used to determine methyl ester levels from the increase in copper-binding after hydrolysis of the esters. An accurate and sensitive colorimetric method (41) is rather time-consuming, but several samples can be run in parallel. Samples are saponified, the released methanol oxidized to formaldehyde, and the formaldehyde determined by spectrophometric assay (412nm) of its condensation product with pentane-2,4-dione.

GLC procedures are widely used for methyl ester determinations; after saponification with 0.5N base, methanol is measured by GLC on columns of Poropak Q at 120°C (42), or on Carbowax 1500 at 125°C (12). In the latter study, analyses were conducted on small samples of isolated plant cell wall preparations. In studies on the enzymatic incorporation of methyl groups from S-adenosyl-L-methionine into pectin (43), <sup>14</sup>C-methyl-labelled substrate was used. The <sup>14</sup>C-methanol, after release from pectin, was determined by GLC on Carbowax 300 using a radioactivity counting detector. The coupling of analytical pyrolysis to GLC has resulted in the detection of characteristic fragments from macromolecules, such as pectin. This topic has been reviewed (44), and correlations between degree of methyl esterification and intensity of some of the peaks have been made (45).

Acetyl and Feruloyl Esters in Pectin. A colorimetric method for determining degrees of acetylation in pectins from various sources (46), has been shown to be rapid and quite sensitive. Hydroxylamine is reactive toward both the methyl and acetyl esters in pectin, and ferric ion complexes with the resulting hydroxamic acids are red. The pectin complex is insoluble and removed by filtration; the intensity at 520nm in the soluble fraction, consisting of the ferric complex with acetohydroxamic acid, is a measure of acetyl content. The accuracy of the method was demonstrated in determinations of O-acetyl levels in standard per-acetylated polysaccharides. Another method (47) involves alkaline hydrolysis of the acetyl groups from pectin, followed by distillation of acetic acid and its titration with standard base.

In a study of the structure and functions of feruloylated pectins in primary cell walls in spinach, about one feruloyl group was found per sixty sugar residues (48). Ferulic acid was determined after alkaline hydrolysis by the Folin-Ciocalteu phenol reagent.

#### Neutral Sugar Composition of Pectin

The neutral sugars, with the exception of L-rhamnose, are attached exclusively in sidechains, and include D-galactose, L-arabinose, D-xylose, and less frequently, D-glucose, D-mannose, L-fucose, 2-O-methyl-D-xylose, 2-O-methyl-D-fucose, and D-apiose. Whether



the sugars are determined by GLC or HPLC methods, it is essential that the polysaccharide first be hydrolyzed to its monomeric sugars. Acid-catalyzed hydrolytic methods are most often used, but the various linkages have different susceptibilities, as do the various sugars when released upon hydrolysis. These problems have been discussed, along with a review of the sugar GLC literature prior to 1973 (49). It was stated that "no one method of hydrolysis will necessarily cleave every linkage and give each component in quantitative yield." The Saeman hydrolysis (50), which employs 72% sulfuric acid, or 2N trifluoroacetic acid (51) are used most often. When possible, enzymatic approaches in combination with acid hydrolysis are preferred for polysaccharide hydrolysis. After hydrolysis, the most widely used methods for sugar determination are based on GLC of suitably volatile derivatives. The derivatives in which the anomeric center is eliminated so that single peaks result are most effective.

Single-peak sugar derivatives which allow the resolution of sugar constituents in pectin include the trimethylsilylated methyloximes (52), acetylated aldonitriles (53), trimethylsilylated alditols (54), and acetylated alditols (51). A comprehensive review article on GLC of sugars has been published (26).

In studies of polysaccharides structure, the alditol acetate procedure remains the most widely used GLC procedure. The advent of high-resolution glass capillary columns has allowed very efficient separations. Recent applications of these columns to alditol acetate separations have been described (55-57). The alditol acetate procedure requires reduction of the sugars with sodium borohydride. After removal of boric acid, the sample is acetylated by conventional means. Various polar stationary phases have been used in GLC separation of alditol acetates, in both packed and capillary columns. A low-polarity phase was used in a report (54) which demonstrated the separation of trimethylsilylated alditols, and the neutral sugars in a hemicellulose sample were resolved.

A liquid chromatographic system has been applied in a study of monomer composition in cell-wall polysaccharide hydrolyzates (58). A strong-base anion-exchange column was eluted with a borate buffer step-gradient. Post-column reaction with orcinol allowed the sensitive determination of the sugars rhamnose, xylose, arabinose, glucose, galactose, and mannose. An improved two-step HPLC procedure for total resolution of the above neutral sugars has been published (59). On aminopropyl silica with acetonitrile-water as mobile phase, rhamnose is separated, but the pentoses xylose and arabinose are not well resolved, nor are the hexoses glucose, galactose, and mannose. These two multi-component peaks are collected and re-chromatographed on Aminex HPX-87P, a heavy metal cation-exchanger in the lead form. Total resolution of the five monosaccharides results.

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Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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## A Critical Reexamination of Molecular Weight and Dimensions for Citrus Pectins

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E-1000 and E-linear  $\mu$  Bondagel<sup>1</sup> high performance size exclusion chromatography (HPSEC) columns were calibrated in root-mean square (RMS) radii of gyration ( $R_g$ ) by using a combination of pullulan and dextran standards. By assuming that universal calibration applies, to pectins the number-average  $R_g$  in 0.05M and 0.1M NaCl for a series of pectins with varying degrees of esterification in the protonated and sodium forms were calculated from chromatograms. This procedure yields well-defined  $R_g$  averages that can be used readily to calculate molecular parameters for comparison with other methods. Such comparisons were not feasible when an earlier HPSEC method was used. Furthermore, by assuming rod-like structure for the pectins, number-average lengths ( $\bar{l}_n$ ) and degree of polymerization ( $\overline{DP}_n$ ) were calculated from  $\bar{R}_{gn}$ . Importantly,  $\overline{DP}_n$  and  $\bar{l}_n$  values from HPSEC fell between values from end group titration and membrane osmometry which is consistent with previous findings that pectin can undergo a concentration dependent disaggregation.

Over the last ten years evidence has accumulated that pectin can undergo self disaggregation (1-4). More recently, by size exclusion chromatography (SEC) and determination of number-average degrees of

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polymerization ( $\overline{DP}_n$ ) from end group titrations (EGT) (5), we have demonstrated self disaggregation over the pH range 3.7 - 7.3 for citrus pectins with degree of methyl esterification (DM) between 37 and 73%. Moreover, by membrane osmometry (O) and EGT we have shown that protonated and neutralized pectins form metastable aggregates which can be dissociated by heat activation and that these activated pectins undergo concentration dependent disaggregation (6,7). Furthermore, EGT gives the  $\overline{DP}_n$  of pectin monomer whereas osmometry gives the  $\overline{DP}_n$  of aggregated pectins. All but protonated pectins with low DM (35 or 37%) exhibited disaggregation with a steep concentration dependence. Thus, neutralized pectins and protonated pectin with medium and high DM gave van't Hoff plots which exhibited a minimum at about 0.1 g/dl. Furthermore, the osmotic data could be extrapolated to  $\pi/c$  values obtained from EGT (Figure 1). Similar behavior by proteins has been interpreted as that of a nonideal dissociating system (8). In the case of the pectins, van't Hoff plots appear linear and nonideal above 0.1 g/dl (i.e., they have a positive slope). In the past, the  $\lim_{c \rightarrow 0} (\pi/c)$  for the linear portion of the curve has been used to obtain  $\overline{DP}_n$  from membrane osmometry. Here we obtain number-average ( $\overline{R}_{gn}$ ), weight-average ( $\overline{R}_{gw}$ ) and z-average ( $\overline{R}_{gz}$ ) RMS of radii gyration from high performance size exclusion chromatography (HPSEC). By assuming rod-like structure we obtain  $\overline{DP}_n$  values from corresponding  $\overline{R}_{gn}$  values. These are compared with  $\overline{DP}_n$  values from end group analysis and osmometry.

### Experimental

Materials. Commercial citrus pectins with degree of methyl esterification (DM) 35, 58-60, and 70 were gifts from Bulmers Limited, Hereford, England. Two other citrus pectin samples DM 37 and 72-73 were manufactured by Bulmers but were gifts from Drs. E. R. Morris and M. J. Gidley at Unilever. The DM 57 citrus pectin was a gift

from Sunkist Growers, Corona, Ca. One pectin sample was extracted from fresh grapefruit peels, according to standard procedure (9). It had a degree of esterification of 73 and was labeled 73G. Characterization and preparation of samples were as reported previously (5) with minor modification. Samples to be neutralized with NaOH were dissolved in 0.01 M phosphate buffer (pH 6.1) containing 0.1 M EDTA, titrated to pH 7 with 0.1 M NaOH, dialysed against four changes of water over 48 hr., centrifuged for 1 hr. at 30,000 x g to remove insoluble matter and then lyophilized. Protonated samples were dissolved in deionized water (Continental Water Systems) followed by dialysis, centrifugation, and lyophilization weight cut off of 12,000. Dextran standards were from Pharmacia Chemical Co., Piscataway, N.J. The  $\bar{M}_w$  values of the dextran standards were as follows: T-500,  $5.32 \times 10^5$ ; T-250,  $2.53 \times 10^5$ ; T-110,  $1.06 \times 10^5$ ; T-70,  $7 \times 10^4$ ; T-40,  $4.44 \times 10^4$ ; T-20,  $2.23 \times 10^4$ ; T-10,  $9.3 \times 10^3$ . Pullulan standards were from Polymer Laboratories, Inc., Amherst, MA. The  $\bar{M}_w$  values of the pullulans were  $8.53 \times 10^5$ ,  $3.80 \times 10^5$ ,  $1.86 \times 10^5$ ,  $1.00 \times 10^5$ ,  $4.8 \times 10^4$ ,  $2.37 \times 10^4$ ,  $1.22 \times 10^4$  and  $5.8 \times 10^3$ . The pullulans had ratios of  $\bar{M}_w/\bar{M}_n$  of 1.14, 1.12, 1.13, 1.10, 1.09, 1.07, 1.06, and 1.07 respectively.

### HPSEC

Apparatus, sample preparation and chromatographic conditions were as reported previously (5) with the following modifications. High performance size exclusion chromatography was performed either on a Waters E-1000  $\mu$ -Bondagel column (30 x 0.39 cm I.D.) or an E-linear  $\mu$ -Bondagel column (30 x 0.39 cm I.D.). Twenty  $\mu$ l of a 0.3 mg/ml sample were injected. Mobile phase was either 0.05 or 0.1M NaCl. The solvent in the reservoir was stirred with a magnetic stirrer and the column was wrapped with a soft foam insulator. The chromatograph was kept in a constant temperature room at  $23 \pm 1^\circ\text{C}$ . Flow rates were measured by an air bubble injected into a calibrated measuring pipette connected to the exit line of the chromatograph (10). The pump was set at a nominal flow rate of 0.5 ml/min. Long term flow

rates were measured to be within  $\pm 2\%$  of the nominal value. Over any 8 hour period, flow rates were precise to  $\pm 0.3\%$ . Generally, peak maxima for 3 consecutive runs agreed within 2 seconds.

Peaks emerging from the size exclusion column were detected by refractive index and UV absorbance at 206 nm. Analog signals were digitized at a rate of 150 points per minute and in a remote location by a modcomp 7861 minicomputer, equipped with an analog input subsystem.

To obtain averaged root-mean-square radii of gyration, partition coefficients ( $K_{av}$ ) were transformed point by point to  $R_g$  values as appropriate integrals were summed. Integrations were by a trapezoidal algorithm. Transformations were obtained from the following calibration curves:

$$K_1 > K_{av} \quad \ln(Y) = a_0 + a_1 K_{av} \quad (1)$$

$$K_1 < K_{av} < K_2 \quad \ln(Y) = b_0 + b_1 K_{av} + b_2 K_{av}^2 + b_3 K_{av}^3 \quad (2)$$

$$K_{av} > K_2 \quad \ln(Y) = c_0 + c_0 K_{av} \quad (3)$$

Here,  $Y$  is  $R_g$ . With the aid of equations 1-3, the best regression line is fitted using values of  $Y$  and  $K_{av}$ . The constants  $a_0$ ,  $a_1$ ,  $c_1$  and  $c_0$  are constrained to make the calibration curve and its first derivative continuous at  $K_1$  and  $K_2$ . The boundaries  $K_1$ , and  $K_2$  are chosen to minimize the sum of the residuals squared. The constants,  $b_0$ ,  $b_1$ ,  $b_2$  and  $b_3$  governing that portion of the calibration curve with  $K_1 < K_{av} < K_2$  was obtained by non linear regression using the Gauss Newton algorithm. For E-1000 columns, the calibration curve is a cubic polynomial with straight lines at the ends, whereas E-linear calibration curves are cubic polynomials.

For the narrow pullulan molecular weight standards, partition coefficient values corresponding to the peak maximum of the differential refractive index trace ( $\Delta RI$ ) of the chromatogram were correlated with the z-average radii of gyration ( $\bar{R}_{gz}$ ) obtained from the literature (11). For each broad dextran standard, integral distribution curves supplied by the manufacturer gave weight percentage

values corresponding to  $\bar{M}_w$ , the weight-average molecular weight of the standard. Weight percentages were equated with area percentages from the  $\Delta RI$  trace of the corresponding dextran chromatogram, so that  $K_{av}$  on the chromatogram could be correlated with  $\bar{M}_w$ . By utilizing a combination of  $\bar{R}_{gz}$  values from light scattering and viscosity measurements (5),  $\bar{R}_{gz}$  values were obtained which correspond to the  $\bar{M}_w$  values for each dextran standard. Thus, for each dextran standard,  $K_{av}$  values could be correlated with  $\bar{R}_{gz}$  values through  $\bar{M}_w$ .

### Membrane Osmometry

Number average molecular weight ( $\bar{M}_n$ ) by osmotic pressure measurements has been described previously (12). The osmometer cell was thermostatted at  $35 \pm 0.1^\circ C$  and the solvent was 0.047 M in NaCl and 0.003 M in  $NaN_3$ . All samples were immersed in boiling water for 10 minutes, quenched and stored at  $35^\circ C$  for a minimum of 3 days prior to serial dilution for osmometry.

### End Group Titration

The  $\bar{M}_n$  of pectins was determined also by the reaction of sodium chlorite with aldehyde end groups. This method was developed for polysaccharides (13) and modified specifically for pectins (14). The chlorite reaction was allowed to proceed for a minimum of 16 hrs. in order to obtain results which were independent of whether end group standards were galacturonic acid or rhamnose.

### Results and Discussion

Calibration of Columns. Figure 2 demonstrates that branched dextrans which are relatively compact and linear pullulans which are relatively extended in solution (11) will co-elute on  $\mu$ -Bondagel columns if they have the same  $R_g$ . Furthermore, for the broad dextran samples, the partition coefficient at which  $\bar{M}_w$  elutes correlates well with the RMS radius of gyration from light scattering. Thus, we have demonstrated "Universal calibration" (15) for these



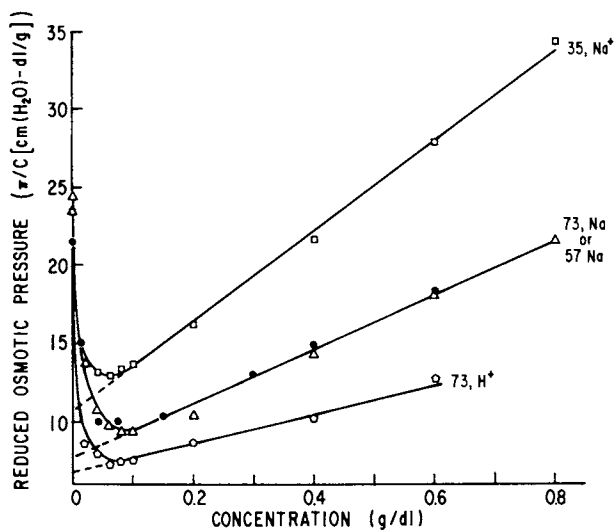


Figure 1. van't Hoff plots demonstrating concentration dependence of pectin dissociation. Values at intercept calculated from end group titrations.

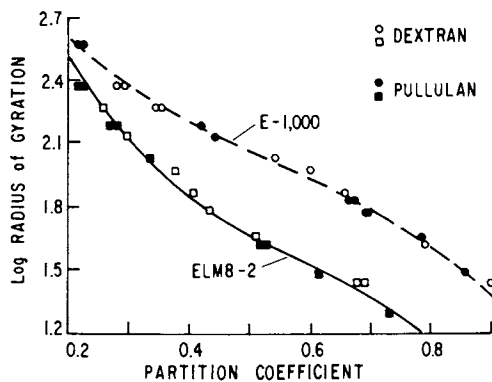


Figure 2. Universal calibration curves for mixture of broad and narrow distributions of polysaccharides in 0.1M NaCl mobile phase.

two classes of polysaccharides without fractionating the commercially available and relatively inexpensive dextran standards (11). On the basis of these results, we have assumed that pectins with  $R_g$  values identical to that of dextran or pullulan standards will co-elute.

Table I contains number-, ( $\bar{R}_{gn}$ ), weight-, ( $\bar{R}_{gw}$ ), and z-average, ( $\bar{R}_{gz}$ ), radii of gyration. These are defined by equations 4-6

$$\bar{R}_{gn} = \frac{\sum_i C_i}{\sum_i (C_i/R_{gi})} \quad (4)$$

$$\bar{R}_{gw} = \frac{\sum_i C_i R_{gi}}{\sum_i C_i} \quad (5)$$

$$\bar{R}_{gz} = \frac{\sum_i C_i R_{gi}^2}{\sum_i C_i R_{gi}} \quad (6)$$

Where  $R_{gi}$  is the radius of gyration of species  $i$  and  $C_i$  is its concentration.

For each DM,  $\bar{R}_{gn} < \bar{R}_{gw} < \bar{R}_{gz}$ , which is the expected order. Interestingly, for the radius of gyration at peak position ( $R_{gp}$ )<sub>0</sub> (maximum concentration of pectin),  $\bar{R}_{gn_0} < \bar{R}_{gp} < \bar{R}_{gw}$  for  $R_{gp} < 135$  Å whereas  $\bar{R}_{gw} < \bar{R}_{gp} < \bar{R}_{gz}$  for  $R_{gp} > 247$  Å. Typical chromatograms are shown in Fig. 3.

As indicated by Table I, pooling data without regard to column type, salt concentration in the mobile phase, or pectin form gave data with a standard deviation ranging from 3 - 15% of the mean for the number-, weight- or Z-average  $R_g$ . In case of the number average radius of gyration, the standard deviation ranged from 3.5 - 10%. Since, it was our intention to compare molecular weight and size values from SEC with those from end group titrations, and osmometry, (comparisons of  $\log \bar{R}_{gn}$  reduced the heterogeneity in variance (16)) values were analyzed for variance at the ( $p < 0.05$ ) confidence level. Such analysis was used to determine if  $R_g$  values were affected significantly by: (1) the concentration of salt in the mobile phase; (2) whether the form of the carboxylate ion was hydrogen or sodium; (3) the pore size distribution (psd) of the HPSEC columns. No global trends were identified because of interactions between combinations

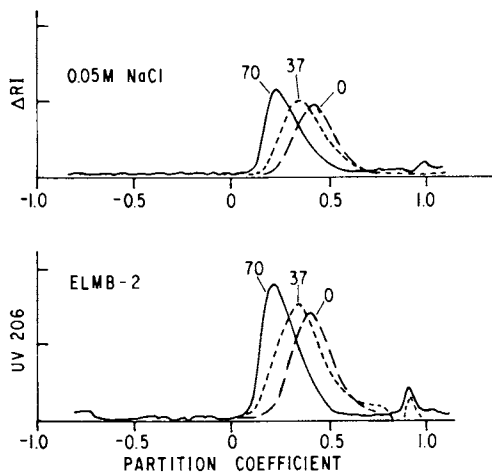


Figure 3. Typical chromatograms for pectins with 0, 37 and 70% methylation esterification.

TABLE I RADIUS OF GYRATION ( $R_g$ , Å) FOR PECTINS<sup>1</sup>

DM \ $R_g$	NUMBER-AVERAGE	WEIGHT-AVERAGE	Z-AVERAGE	PEAK POS.
0	51.7 ± 2.2	65.2 ± 4.1	84.5 ± 11	53.7 ± 4.2 <sup>2</sup>
35	99.6 ± 3.8	144 ± 3.0	212 ± 7.8	123 ± 11
37	70.5 ± 2.2	105 ± 2.9	165 ± 12	81.5 ± 8.2
57	131 ± 9.4	207 ± 17	309 ± 40	258 ± 28
58-60	124 ± 4.3	194 ± 4.5	288 ± 11	252 ± 33
70	126 ± 6.0	201 ± 10	301 ± 12	247 ± 35
72-73 <sup>3</sup>	123 ± 12	206 ± 11	314 ± 12	292 ± 35
73G	101 ± 3.3	149 ± 4.0	225 ± 15	135 ± 31

<sup>1</sup> DATA AVERAGED OVER MOBILE PHASE CONCENTRATION (0.05M & 0.1M NaCl), OVER FORM (ACID & NEUTRALIZED) AND OVER COLUMN (E-1000 AND E-LINEAR) 24 DETERMINATIONS.

<sup>2</sup> STANDARD DEVIATION OF POOLED DATA AS IN (1).

<sup>3</sup> DATA FOR E-1000 COLUMN ONLY (12 DETERMINATIONS).

of mobile phase concentration, carboxylate counter ion and column psd. In another approach, at constant degree of methylation,  $\bar{R}_{gn}$  values were separated at the ( $P \leq 0.05$ ) confidence level through differences in their logs by the Bonferroni LSD method (17). Thus within any horizontal row in Table II, means not followed by the same letter were significantly different at the ( $p < .05$ ) confidence level. At constant column type and salt concentration only 1/3 of the comparisons in Table II gave significant differences in  $\bar{R}_{gn}$  with carboxylate counter ion (e.g. Na against H). Comparisons revealed that differences due to carboxylate counter ion were equally divided between the two column types. Generally at each DM, values of  $\bar{R}_{gn}$  for the E-1000 columns tended to cluster around values  $\bar{R}_{gn}$  averaged over column type, salt concentration in the mobile phase and counter ion form (i.e. the value of  $\bar{R}_{gn}$  in Table I). Multiple letters indicate multiple overlap in means for the E-linear columns,  $\bar{R}_{gn}$  values at each DM tended to be higher than  $\bar{R}_{gn}$  values for E-1000 columns if salt mobile phase concentration was 0.05 M whereas  $\bar{R}_{gn}$  values tended to be lower than E-1000 values of  $\bar{R}_{gn}$  if NaCl in the mobile phase was 0.1 M. If one assumes a rod-like model for pectin it is readily shown (12) that the degree of polymerization DP is related to  $R_g$  by equation 7.

$$R_g = DP \times h / \sqrt{12} \quad (7)$$

Here h is the virtual bond length, the length of a monomer unit projected along the x axis, provided the pectin rod is parallel to that axis. If one replaced DP with the ratio of polymer molecular weight M to monomer residue weight,  $M_o$ , equation 8 is obtained.

$$R_g = M \times h / \sqrt{12} \times M_o \quad (8)$$

Substituting equation 8 into equation 4, yields equation 9.

$$\bar{R}_{gn} = (h / \sqrt{12} \times M_o) \left( \sum_i c_i / \sum_i (c_i / M_i) \right) \quad (9)$$

TABLE II PECTIN NUMBER AVERAGE RADIUS OF GYRATION ( $\bar{r}_g^0$ )<sup>1</sup>

C O L U M N	E L M B <sup>2</sup> - 2						E - 1 0 0 0					
	0.05M			0.1M			0.05M			0.1M		
	H	Na	SD. <sup>3</sup>	H	Na	SD. <sup>3</sup>	H	Na	SD. <sup>3</sup>	H	Na	SD. <sup>3</sup>
DM	0	55.9b <sup>4</sup>	49.6bc	44.6c	31.9d	54.8b	72.8a	51.8b	52.4b	2.2		
	35	109a	113a	93.8bc	80.4d	86.6cd	108a	101ab	105a	3.8		
	37	79.2a	81.9a	59.6c	57.1c	72.1b	69.9b	73.6b	70.9b	2.2		
	57	152ab	159a	108cd	89.8d	141ab	136ab	136ab	128bc	9.4		
	58-60	139ab	144a	115d	94.1e	110d	127c	132bc	128c	4.4		
	70	148ab	145abc	105d	84.8e	107d	133bc	126c	158a	6.0		
	72-73	- - -	- - -	121a	106b	133a	141a	133a	128a	12.0		
	73G	110ab	106ab	113a	84.9d	111ab	104b	106ab	95.3c	3.3		

<sup>1</sup> TRIPPLICATE DETERMINATION<sup>2</sup> E-linear  $\mu$ -Bondagel<sup>3</sup> POOLED STANDARD DEVIATION OF VALUES IN ROW<sup>4</sup> MEANS WITHIN ROW WITH DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT AT 95% CONFIDENCE LEVEL BY BONFERRONI LSD METHOD.

or

$$\bar{R}_{gn} = h \times \bar{M}_n / \sqrt{12} \times M_0 \quad (10)$$

Finally

$$\bar{R}_{gn} = h \times \bar{DP}_n / \sqrt{12} \quad (11)$$

Similar equations could be derived for weight- and Z-average molecular weights respectively. According to equation 11, molecules with  $\bar{M}_n$  have a radii of gyration equal to  $\bar{R}_{gn}$ . This result cannot be generalized to macromolecules which are not rod-like (18).

A test of equation 11 as the model for pectin is to measure the ratio  $\bar{R}_{gn}/\bar{DP}_n$ . According to equation 11, this characteristic parameter for rods should be dependent on h. If we take the literature value (19), of 5 Å for pectin, the theoretical characteristic parameters for pectins would be 1.44. In Table III, we have calculated characteristic parameters (CP) from the ratio of  $\bar{R}_{gn}$  from SEC and  $\bar{DP}_n$  from end group titration. Values for  $\bar{R}_{gn}$  were pooled data from the two type columns with 0.05 M NaCl in the mobile phase. Characteristic parameter values for protonated and Na forms of the carboxylate were not pooled. Interestingly in all cases but the grapefruit pectin, CP exceeds the theoretical value of 1.44. Values in Table III may exceed 1.44 because  $\bar{R}_{gn}$  from SEC is that of aggregated pectins whereas  $\bar{DP}_n$  from end group titrations is "monomeric" pectin. At constant DM, in all but one case in Table III, differences in characteristic parameter between the sodium and protonated forms are 10% or less.

From equation 11 and  $\bar{R}_{gn}$  from size exclusion chromatography (SEC), it is possible to calculate the  $\bar{DP}_n$  of the backbone residues in pectin. The results of these calculation appear in Table IV under the heading SEC-1. Since pectins contain about 22% neutral sugars side chains, dividing the  $\bar{DP}_n$  values under SEC-1 by 0.78 yields the  $\bar{DP}_n$  values for the entire pectin molecule. The results of those calculations appear under the heading SEC-2 in Table IV. Values of  $\bar{R}_{gn}$  were obtained by combining data from the two type of columns and the two pectin forms. Here, 0.05 M NaCl was the mobile phase. For

TABLE III CHARACTERISTIC PARAMETERS FROM NUMBER AVERAGE<sup>1</sup>

DM	H	NA <sup>+</sup>
0	1.8	2.0
35	1.9	2.1
37	2.3	2.3
57	2.2	2.2
58-60	1.9	2.1
70	1.9	2.1
72,73	1.8	2.3
73G	1.2	1.2

<sup>1</sup> POOLED DATA FROM ELMB & E-1000 COLUMNS, SOLVENT 0.05M NaClTABLE IV. NUMBER-AVERAGE DEGREE OF POLYMERIZATION ( $\overline{DP}_n$ ) OF PECTINS

METHOD DM	EGT <sup>1</sup>	SEC-1 <sup>2</sup>	SEC-2 <sup>2</sup>	OSMOMETRY
0	30	40	40	51 <sup>3</sup>
35	51	72	92	126 <sup>3</sup>
37	33	52	67	94 <sup>3</sup>
57	66	102	131	170
58-60	66	90	116	171
70	68	93	119	174
72,73	60	86	111	190
73G	85	71	91	- -

<sup>1</sup> END GROUP TITRATION.<sup>2</sup> SIZE EXCLUSION CHROMATOGRAPHY.<sup>3</sup> NEUTRALIZED PECTINS. REMAINING DATA IS A COMBINATION OF Na & H FORMS.

comparison, we have included values of  $\overline{DP}_n$  from end group titrations (EGT) and osmometry (O) for these same pectins. As in the cases of the SEC values, except where noted,  $\overline{DP}_n$  for Na and protonated forms were combined. The data of Table IV reveal that  $\overline{DP}_n$  (EGT) <  $\overline{DP}_n$  (SEC) <  $\overline{DP}_n$  (O). The order of  $\overline{DP}_n$  values could be predicted from Fig 1, since the concentration at which pectin was measured in SEC was intermediate between 0 and 0.1 g/dl. We note that the osmometry data was measured at 35°C whereas the SEC was obtained at 23°C. Thus, if we assume that pectin aggregates primarily through hydrogen bonds, we could expect that pectin  $\overline{DP}_n$  values from osmometry at 23°C would be even larger than those measured at 35°C.

The linearity of the osmotic pressure data above 0.1g/dl (see figure 1) may indicate that above this critical concentration, the "monomeric" pectin concentration is practically constant whereas it changes with total polysaccharide concentration below the critical concentration. Ionic detergents behave in this fashion.

Since the product of  $\overline{DP}_n$  and  $h$  is the number average length,  $\overline{l}_n$ , equation 11 permits a calculation of  $\overline{l}_n$  by dividing  $\overline{R}_{gn}$  from SEC by  $\sqrt{12}$ . In the case of osmometry, as indicated by figure 1, taking the  $\lim_{c \rightarrow 0} (\pi/c)$  of the linear portion of the curve gives the aggregated molecular weight of pectin. Thus, if one multiplies a corrected  $\overline{DP}_n$  by ( $h$ ), then a maximum value of  $\overline{l}_n$  (i.e.  $\overline{l}_n$  for end to end aggregation) will be obtained. Corrected  $\overline{DP}_n$  is obtained by multiplying  $\overline{DP}_n$  by 0.78, the mole fraction of galacturonate in the polysaccharide backbone. For  $\overline{DP}_n$  from end group titration, multiplying  $\overline{DP}_n$  by 0.78 will give the length of monomer residues in the backbone.

Comparison of number average lengths obtained from end group titration,  $\overline{l}_n$  (EGT), and from size exclusion chromatography  $\overline{l}_n$  (SEC) (Table V) revealed that pectin length decreased, with decreasing concentration presumably due to disaggregation. Furthermore, the maximum length by osmometry is consistent with the concentration-dependent disaggregation.

In summary, we have demonstrated that by utilizing SEC and a variation of the universal calibration principle, one can measure



TABLE V NUMBER AVERAGE LENGTH ( $\bar{A}$ ) FOR PECTINS

METHOD DM	ln (EGT)	ln (SEC)	ln (O)
0	150	200	255
35	199	360	491
37	129	260	367
57	258	510	663
58-60	258	450	667
70	265	465	679
72, 73	234	430	741
73G	332	355	-

quantitatively, the radius of gyration of pectin in solution. Furthermore, by assuming simple rod-like structure one can obtain molecular dimensions and weights. Statistical analysis of the data revealed that short term precision was about 3% whereas long term precision was about 10%. This discrepancy is probably due to small changes in retention time induced by mechanisms other than size exclusion. Within a precision of  $\pm 10\%$ , differences due to counterion, salt concentration in the mobile phase or pore size distribution of the column packing are marginal. In the long term, somewhat better precision is obtained with the E-1000 columns than with E-linear columns.

Comparison of molecular dimensions and weights for pectins from the various techniques revealed their magnitude to be in the order end group titration < size exclusion chromatography < osmometry. These results are consistent with the hypothesis that pectin can undergo a concentration-dependent disaggregation. Interestingly, an activation step such as briefly heating is necessary to observe disaggregation in the case of osmometry whereas in the case of size exclusion chromatography shear forces within the column appear to perform the same function.

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## Structural Studies of Apple Pectins with Pectolytic Enzymes

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Apple pectic substances consist of chains of partially esterified galacturonic acid residues with side chains composed of arabinose, galactose and xylose. Degradation with purified pectolytic enzymes shows that the side chains are present in blocks ("hairy regions"). Enzymic and chemical degradation of the hairy regions reveals that they consist of arabinogalactan side chains and short xylose side chains. It can be concluded that apple pectic substances are constructed of homogalacturonan, xylogalacturonan and rhamnogalacturonan regions with side chains of arabinogalactan. About 95% of the uronic acid residues is present in the homogalacturonan regions. The arabinogalactans are highly branched. HPLC analysis of enzymic degradation products suggests that the methoxyl groups of the uronic acid residues are randomly distributed.

There is consensus about the basic fine structure of pectin molecules. Everyone agrees with the idea of an  $\alpha$ -1,4-D-galacturonan with few  $\alpha$ -1,2 bound L-rhamnose residues in the main chain, the galacturonan being partly esterified with methanol and partly acetylated. The main chain further carries covalently attached neutral sugars as side groups and/or as side chains which may be substituted with acetyl and phenols, many of these side chains being bound to L-rhamnose. There is also agreement that a pectin preparation consists of a mixture of various molecules. Many of them are artifacts in the case of extraction with cell wall modifying reagents. Structural features also depend on origin and degree of ripeness of the plant source. These views have been obtained and substantiated over 30 years. Much work was done on degradation, work up of oligomeric fragments followed by determination of structure with classical methods and recently by GC and GC/MS after chemical modification (reduction) and derivation.

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At present, a number of questions remain open. There is the specific question on the structure of the often branched side chains and the more general question on whether structural features are intra- or intermolecular phenomena. Do the molecules of a pectin preparation have smooth and hairy regions or is there a mixture of pure galacturonan molecules and galacturonan molecules with many side chains? How is the inter- and intramolecular distribution of methoxylated and free carboxyl groups?

The availability of pure pectolytic enzymes with known specificity plus chromatography techniques provides the possibility for relatively simple experiments to answer some of these questions.

In recent years we have used these possibilities in our studies on the structural features of apple pectic substances (1-6). This work is reviewed here and complemented with results of other investigators. For detailed descriptions of experimental work the reader is referred to (1-4). The apple pectins studied were extracted from Golden Delicious apples as schematically shown in Fig. 1. In total less than 50% of the pectin present in the Alcohol Insoluble Solids (AIS) prepared from peeled and cored apples could be extracted under the mild conditions used. The amounts of pectin in the various extracts are indicated in Fig. 1.

#### Neutral Sugar Distribution

To establish the distribution of the neutral sugars among the pectin molecules we (1) fractionated the four pectin extracts on DEAE-cellulose. Fig. 2 shows a typical elution profile obtained with a linear gradient of 5-500 mM Na-phosphate buffers of pH 5.1 for the cold-buffer extract of ripe apple AIS. The pectic material was found to elute as one tailing peak. About 10% of the pectin did not bind to the column and could only be fractionated after partial cold alkali saponification. A small percentage was retained on the column and could only be eluted with 0.01 M NaOH. The pectin containing fractions from each extract were collected in 10 pools, each pool containing 10% of the anhydrogalacturonic acid (AUA) applied to the column (Fig. 2). In total 48 pools were obtained for the four extracts, this includes the pectin fractions which did not bind to the column and the ones that had to be eluted with alkali. The neutral sugar content in each pool was estimated and calculated as moles neutral sugar residues per mole of galacturonosyl residues. It was found to vary between 0.04 to 1.7 moles/mole AUA, pools with ratio's of 0.15, 0.24 and 0.53 were prevailing. By arranging the pools in ascending order of moles neutral sugars per mole AUA and plotting the AUA content of pools with a certain ratio in % of AUA present in all pools cumulatively against the ratio, a cumulative neutral sugar distribution curve was constructed. Numerical differentiation of this curve results in the neutral sugar distribution curve as shown in Fig. 3 for extracts of ripe and unripe apples. These curves suggest that in pectin a discontinuous rather than a continuous distribution of the neutral sugars is present. Five types of pectins (A to E) are indicated by the neutral sugar distribution curves. The neutral sugar contents of the pectins of types B, C, D and E have ratio's of 1:1.7:3.7:10. Cold buffer extracts were found to contain mainly pectins of type A, B and C, in ripe apples also type E was present.

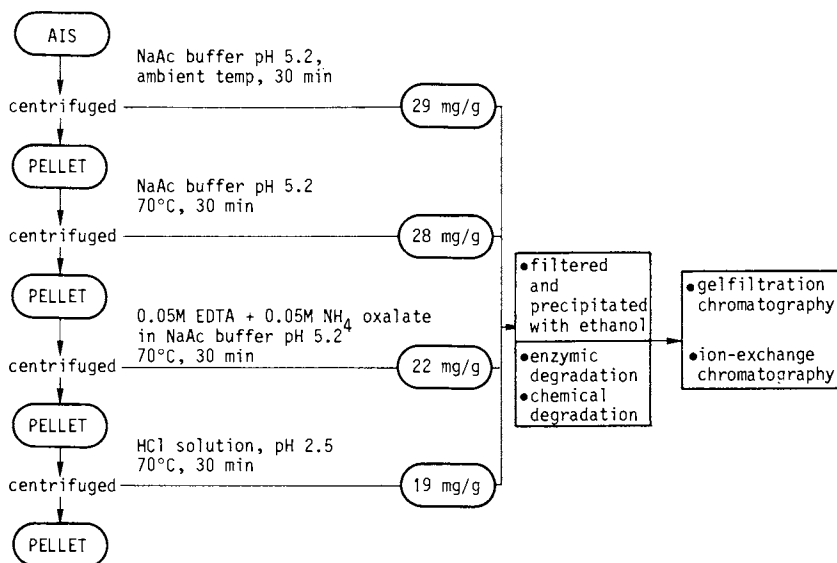


Figure 1. Extraction of pectin fractions of AIS of apples (peeled and cored).

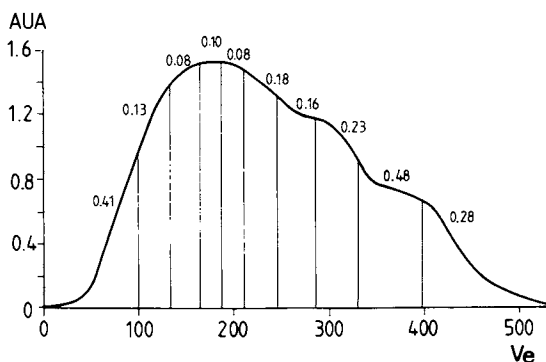


Figure 2. Fractionation of the pectin from the cold buffer extract of the ripe apple AIS on DEAE-cellulose. AUA, anhydrouronic acid, mg/ml;  $V_e$ , elution volume, ml. The numbers in the figure indicate the neutral sugar residue content of the fractions, expressed as moles of neutral sugar residues/mole of galacturonate residues. (Reproduced with permission from reference 1. Copyright 1981 Carbohydrate Polymer).

Hot buffer extracts contained mainly types B, C and D, oxalate extracts types A, B, C and D and in acid extracts types C, D and E dominated.

Ion-exchange chromatography has been successfully applied by various investigators to fractionate pectins (7, 8, 9). The fractionation is mainly based on the degree of esterification of pectins (10). However, it is likely that covalently linked neutral sugars and the molecular weight of the pectin also affect the elution profile (11). To verify, if the possible interactions between degree of esterification, molecular weight and neutral sugar content interfered in the DEAE-cellulose fractionation, pools were further fractionated by gel filtration and rechromatographed on DEAE-cellulose. The existence of the different types of pectin molecules was confirmed.

Important additional information on the fine structure was obtained by subjecting isolated pectins to enzymic degradation by pure pectin lyase or pectate lyase. The digests were then fractionated by gel filtration chromatography on Sephacryl S-300. A typical profile for a pectin fraction isolated from cold buffer extract of AIS from ripe apples with 4% of the glycosidic linkages split by pectate lyase is shown in Fig. 4. Fractions were collected in 4 pools as indicated, the degree of esterification and neutral sugar composition were established and are given in the figure. Pool  $\alpha$  was found to contain only 5% of the galacturonic acid residues but contained 90% of the neutral sugar residues. The pectin in this pool was highly esterified. Fractions  $\beta$ ,  $\gamma$  and  $\delta$  had a high AUA content and a low level of neutral sugars. The degree of esterification of the pectin fragments in these pools varied between 50 and 81%. Similar observations were made for other isolated pectins degraded with pectin lyase or pectate lyase. From these results it was concluded that almost all neutral sugar residues are linked to relatively short segments of the rhamnogalacturonan backbone ("hairy" regions) leaving large parts of the rhamnogalacturonan backbone unsubstituted ("smooth" regions). Evidence for the conception of pectin as consisting of "smooth" and "hairy" regions has also been produced by other investigators (7, 12, 13) and this conception is also in agreement with the existence of five types of pectins indicated by the neutral sugar distribution curve. This can be visualized by the simple model of pectins of type A to E shown in Fig. 5. Type B was found to be the dominant pectin type in the extracts, type A and E can be considered to be degraded pectins.

Table I shows the neutral sugar composition as estimated for pectin types A to E from ripe apples expressed as moles sugar per mole arabinose. For the pectins from unripe apples similar results were obtained. It can be seen that the neutral sugar composition for all pectin types is fairly constant with the exception of galactose. The same is true for pools  $\alpha$  to  $\delta$  of Fig. 4; here galactose is practically only present in pool  $\alpha$ . From these results the conclusion was derived that within the neutral sugar side chain blocks a repeating pattern is present.

Isolated "hairy" region fractions were found to be rather resistant against degradation by pectic enzymes. Endo- $\beta$ -1,4-galactanase could release arabinogalactans and oligomeric galactose from hairy region fractions. They could also be partially degraded when

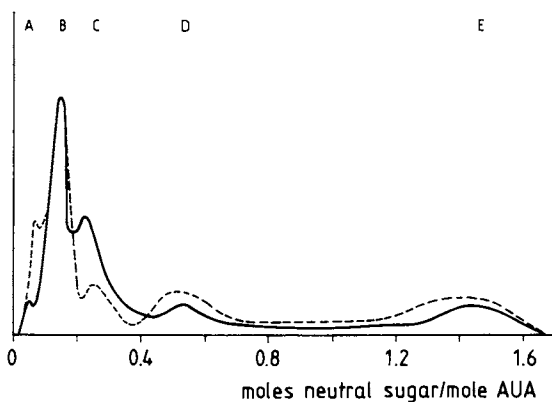
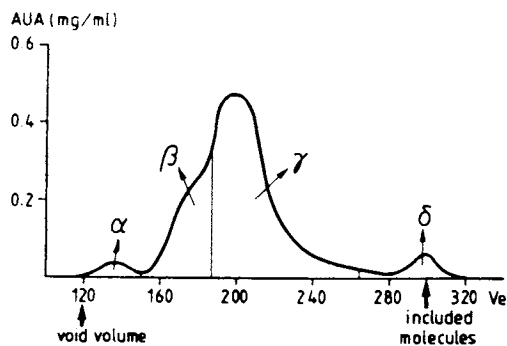


Figure 3. Neutral sugar distribution curves of pectins extracted from ripe and unripe apple AIS. The total area represents 100% of the AUA present in the four extracts. (—) Unripe apples; (---) ripe apples. (Reproduced with permission from reference 1. Copyright 1981 Carbohydrate Polymer).



	$\alpha$	$\beta$	$\gamma$	$\delta$
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

Figure 4. Gel filtration of a pectate lyase degraded pectin. AUA = anhydro-uronic acid content,  $V_e$  = elution volume, the eluent was water.



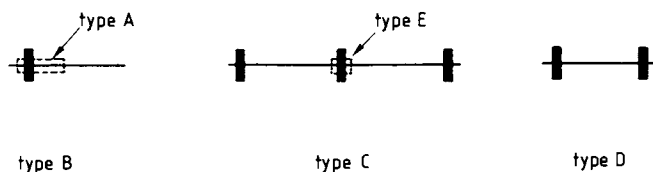


Figure 5. Model of the pectins of types A-E. Horizontal lines: rhamnogalacturonan backbone of the pectin molecule. Black areas: blocks of neutral sugar side chains. (Reproduced with permission from reference 2. Copyright 1982 Carbohydrate Polymer).

Table I. Neutral sugar composition of pectins extracted from ripe and unripe apples (AIS). The capitals 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 (1).

	ripe					
	A	B	C	D	E	on average
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	

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subjected to  $\beta$ -elimination at pH 6, 100°C for 2 hours in 0.05 M sodium phosphate buffer. Fig. 6 shows the gel filtration profiles of hairy region fractions before and after  $\beta$ -elimination and the sugar composition of degraded pectin fractions. A high proportion of xylose residues was present in the lower molecular weight fractions and this provides evidence for the presence of xylogalacturonan regions also reported by other investigators (12, 14).

#### Distribution of Methoxylated and Free Carboxyl Groups

We have already seen that the degree of esterification of the "hairy" regions is almost 100%. The homogalacturonan regions have an average degree of esterification of about 70%. The distribution of the methoxylated and free carboxyl groups was, however, unknown. The intermolecular distribution was established by estimating the degree of esterification in the many fractions obtained by extensive fractionation of many pectin fractions by gel filtration and DEAE-cellulose chromatography. These results are summarized in Fig. 7 where the amounts of fractions with a certain degree of esterification (expressed in proportion of total AUA present in all fractions) are plotted versus degree of esterification (DE). It can be seen that almost all fractions were found to have a DE of about 70-80%. Minor amounts of pectin are present in fractions with a DE of 50% and in fractions with a DE of about 95%.

Information about the intramolecular distribution was derived from studies of the oligomeric, partially esterified galacturonides obtained by extensive degradation of pectin fractions by pectate lyase and pectin lyase. The oligomers were fractionated by HPLC using an amino-bonded silica column (15). In Fig. 8 the chromatograms for pectins with 8% of the glycosidic linkages split by pectin lyase and for pectins with 7% of the glycosidic linkages split by pectate lyase are shown. The partially esterified galacturonides are separated according to the number of free carboxyl groups and not according to chain length. Peaks a, b and c represent oligogalacturonides with zero, one and two free carboxyl groups. The oligomer composition of a peak could be established by cold alkali saponification of the methylated galacturonides in the pooled fractions. Fig. 9 shows the distribution of the degree of polymerization of the methyloligogalacturonides containing one free carboxyl group (peak b) obtained from a native pectin and also the distribution found for peak b of trans-esterified pectin with the same DE as the native pectin. This trans-esterified pectin was obtained by esterification of the native pectin fraction to a DE of about 95% and subsequent saponification in the cold with a calculated amount of 0.1 M potassium hydroxide to the original DE of the native pectin. This procedure is supposed to give pectins with a random distribution pattern of the methylester groups in contrast to saponification with citrus pectinesterase which is supposed to give a blockwise distribution (16, 17). The distribution patterns obtained showed only small differences, suggesting that the native distribution and the modified distribution are similar. This could be further substantiated by theoretical considerations based on assumptions concerning the mode of action of the enzymes (number of adjacent esterified galacturonide residues necessary for bond splitting) and the intramolecular distribution of methoxyl groups (DE,

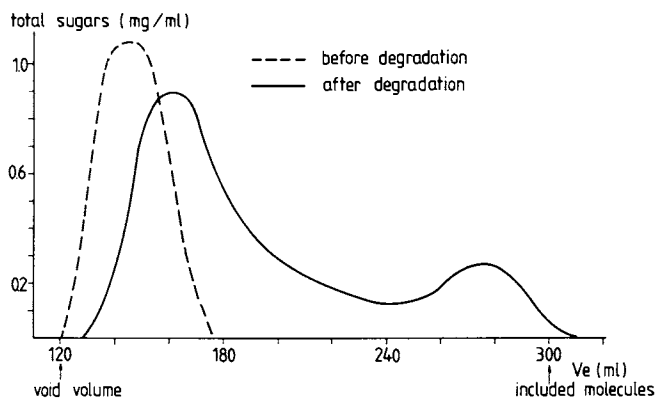


Figure 6. Gel filtration of apple pectin "hairy" regions after  $\beta$ -elimination on Sephaacryl S-300. (Reproduced with permission from reference 3. Copyright 1983 Carbohydrate Polymers).

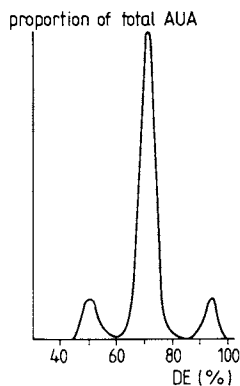


Figure 7. Occurrence of pectins with a certain degree of esterification in pectin extracts of apple AIS.

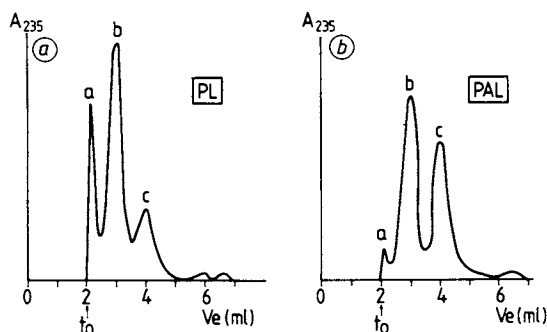


Figure 8. High-pressure liquid chromatograms of pectin fractions degraded with pectin lyase (a) and pectate lyase (b) to degradation limits, respectively, of 18% and 7%. (Reproduced with permission from reference 4. Copyright 1983 Carbohydrate Polymer).

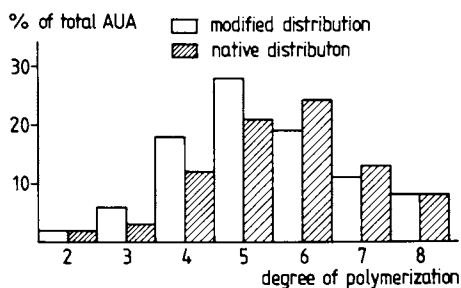


Figure 9. Distribution of the degree of polymerization of methyloligogalacturonides containing one free carboxyl group obtained from a native and from a transesterified pectin. Methyloligogalacturonides were completely deesterified (in alkali) and the degree of polymerization of oligogalacturonides was determined by HPLC. (Reproduced with permission from reference 4. Copyright 1983 Carbohydrate Polymer).

infinite chain length and random distribution). With help of statistics the frequencies of occurrence of certain sequences in a pectin molecule and the occurrence of methyloligogalacturonides with zero, one etc. free carboxyl groups can be calculated. The differences between calculated and experimental data were found to be relatively small.

The effect of different intramolecular distributions of methoxylated and free carboxyl groups on the distribution of the degree of polymerization of oligogalacturonides in enzymatic digests of pectins is illustrated in Fig. 10. These data were obtained by saponifying 95% esterified pectin to 60% esterification with citrus pectinesterase (PE, saponified) or with alkali (alkali saponified) and degrading respectively 12% and 8% of the glycosidic linkages of the two pectins with pectate lyase. The digests were saponified and analyzed for oligogalacturonide composition by HPLC. In the PE saponified pectin digest the lower oligogalacturonides were present in larger amounts. This is due to the preferential attack of pectate lyase on de-esterified regions. These results also indicate a random distribution.

### Conclusion

Chromatographic techniques in combination with pure enzymes have been used successfully as tools in the elucidation of the fine structure of apple pectins. Apple pectins were found to consist of "hairy" regions, having a backbone of rhamnogalacturonan carrying arabinogalactan side chains and xylogalacturonan and "smooth" regions, the latter being homogalacturonans with a degree of esterification of 70-80%. The intramolecular distribution of the methoxyl groups could not be distinguished from a random distribution.

Future research efforts should be directed to the problem of possible connections between these structural features of pectin and its properties as gelling agent and as dietary fibre.

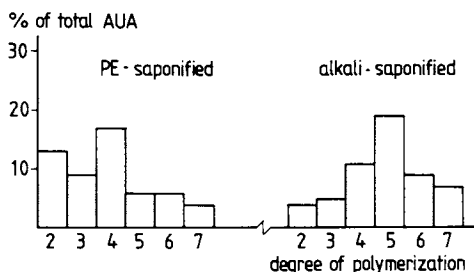


Figure 10. Distribution of the degree of polymerization of pectate lyase degraded pectins with different distributions of methyl groups. The digests were completely deesterified (in alkali) and the degree of polymerization of oligogalacturonides was determined by HPLC. (Reproduced with permission from reference 4. Copyright 1983 Carbohydrate Polymer).

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## Sugar Beet Pectins: Chemical Structure and Gelation through Oxidative Coupling

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Pectins were isolated from sugar beet pulp by sequential extraction with water, oxalate, acid, and alkali. They were found to be fairly low molecular mass products with a high degree of acetylation and a relatively high neutral sugar content. Neutral sugars were determined and their location in "hairy fragments" of the pectin molecules was studied by enzymic and chemical degradation experiments. Acetyl ester groups are bound to the anhydrogalacturonide backbone mainly. Feruloyl ester groups appear to be linked to the neutral sugar side chains. These can be used in the formation of cross-links with hydrogen peroxide and peroxidase or with ammonium persulfate. The products obtained are either pectin solutions with increased intrinsic viscosity or pectin gels. Cross-linked pectins isolated from these gels have an extremely high water-absorbing capacity.

Beet pulp is the residue left from ground sugar beet after sugar extraction. In its dried form it is available all year round at relatively low prices. Some 25% of its dry weight consists of anhydrogalacturonic acid and it is therefore a potentially rich source of pectin (1). However, several attempts in the past to commercialise sugar-beet pectins have failed, due to the poor gelling properties of the pectins, compared to those from citrus and apple. These poor gelling properties are attributable to the presence of

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acetyléster groups mainly, and further to the relatively small size of the beet pectin molecules (2).

Our recent studies on sugar-beet pectins have revealed some new features, which may increase their usefulness, and possibly lead to some new applications.

### Experimental methods

Materials. Pressed sugar-beet pulp, preserved in four volumes of ethanol was obtained from a sugar factory at Eppeville, France. Endopolygalacturonase was purified from a preparation from Aspergillus niger, as described by Thibault and Mercier (3). Endopectate lyase was obtained from Pseudomonas fluorescens (4). Endopectin lyase (type 2) was isolated from a preparation of A. niger (5). Pectinesterase was purified from an A. niger preparation according to Baron et al (6). Exo  $\alpha(1\rightarrow5)$  arabinanase and endo  $\beta(1-4)$  galactanase were also purified from A. niger (unpublished results). Horse radish peroxidase (90 U/mg solid) was obtained from Sigma Chem. Comp., St. Louis, Mo. USA.

Extraction and purification. An alcohol-insoluble residue was prepared from sugar-beet pulp. Products successively extracted from this material were: water-soluble pectin (WSP), oxalate-soluble pectin (OXP), acid-soluble pectin (HP) and alkali-soluble pectin (OHP). Extractions were done according to the procedure described by Barbier and Thibault (7). The pectins were purified by chromatography at pH 4.8 on Whatman DEAE-cellulose DE 52 under the conditions described by Barbier and Thibault (7). As the alkali-soluble pectin binds irreversibly to this column, this fraction was purified by precipitation with  $\text{CuSO}_4$  and extensive washing of the precipitate. Cupric ions were subsequently removed by dialysis against  $\text{Na}_2\text{EDTA}$  at pH 4.8.

Analytical methods. The anhydrogalacturonic acid content and neutral sugar content (expressed as anhydro-arabinose) of pectins were determined automatically by the *m*-hydroxydiphenyl method (8) and the orcinol method (9) respectively. In the latter method corrections were made for interference from anhydrogalacturonic acid. Neutral sugar residues in pectins were determined by gas-liquid chromatography of the alditolacetates (10) prepared from the sugars after hydrolysis with 2 M trifluoro-acetic acid during 1.5 h at 120°C. Methylene ester groups were determined according to Wood and Siddiqui (11). O-acetyl groups were liberated from pectins by hydrolysis with 0.1 M sodium hydroxide for 1 h at room temperature. After neutralisation, acetic acid was determined by gas-liquid chromatography with formic acid-saturated nitrogen as carrier gas (12). Feruloyléster groups were estimated spectrophotometrically at 375 nm, using freshly prepared pectin solutions in 0.1 M glycine-sodium hydroxide buffer, pH 10. The feruloyléster content was calculated using a molar extinction coefficient of 31600 (13). Polyphenols were estimated with the Folin-Ciocalteu reagent without copper treatment (14), using ferulic acid as standard. Intrinsic viscosities and viscosity-average molecular weights of pectins were determined with the method of Owens et al (15).



Chromatography. Purified pectins and their degradation products were studied by gel-permeation chromatography on Sepharose CL-2B or Sepharose CL-6B (Pharmacia), under conditions as described by Barbier and Thibault (7). High-performance size exclusion chromatography was done with a series of Biogel TSK columns types 6000, 5000, 4000 and 3000 PW (Bio-Rad Labs., Richmond, Ca., USA). The solvent was 0.1 M sodium sulfate in sodium acetate, pH 3.7, ionic strength 0.34 (16).

Degradation studies. Degradation limits by endopolygalacturonase and by  $\beta$ -elimination were determined as described by Thibault (17) and by endopectate lyase as described by Rombouts *et al* (4). For pectin lyase the reaction conditions were 0.25 (w/v) pectin, 0.01 M sodium phosphate buffer, pH 5.2 and 0.52 U/ml of pectin lyase, 30°C, 24 h. The pectin lyase reaction was monitored by measuring  $A_{235}$  nm of aliquots, diluted thirtyfold with 0.1 N hydrochloric acid and the degradation limit was calculated, using a molar extinction coefficient of 5500 (18).

Alkaline deesterification of pectins was done by dialysis of pectin solutions (4 mg/ml) against 0.05 N sodium hydroxide at 2°C for 6 h.

Conditions for enzymatic demethylation were 0.4% (w/v) pectin, 0.1 M sodium acetate buffer, pH 4.5 and 5.7 U/ml of pectinesterase, 30°C, 24 h. Inactivation of enzymes was done by heating of the reaction mixtures for 5 min in a boiling water bath. The reaction conditions for exoarabanasase and endogalactanasase were 0.75% (w/v) pectin, 0.05 M sodium phosphate buffer pH 6 and 7 U/ml of exoarabanasase and 0.66 U/ml of endogalactanasase, 30°C, 24 h. The reactions were monitored by measuring increase in reducing groups with the method of Nelson-Somogyi (19).

Crosslinking and gelation. To sugar-beet pectin solutions of varying concentrations between 0.25 and 3% (w/v) in 0.1 M sodium phosphate buffer pH 6.0 were added (per ml of pectin solution): 10 microlitres of peroxidase solution of a concentration of 1 mg of enzyme per ml and, after mixing, 0.1 ml of 0.1 M hydrogen peroxide solution. At higher pectin concentrations ( $\geq 1.0\%$  w/v) gelation occurred immediately upon mixing while at lower pectin concentrations viscosity increased. Pectin gels or solutions with increased viscosity were also obtained by treatment with 0.01 M ammonium persulfate, at 25°C for up to 15 h (measured by reduced specific viscosity).

### Results and discussion

The yields of crude pectins, as well as their anhydrogalacturonic acid content are given in Table I. This Table shows that about one third of the alcohol-insoluble residue can be solubilised as crude pectins, but the anhydrogalacturonide content of these pectins is very low. Most of the pectins are extracted with acid and subsequently with alkali. Even after these extractions not all of the pectin is solubilised from the alcohol-insoluble residue: its anhydrogalacturonic acid content is still some 5%.

Purification and composition. The purification of these pectins, carried out as described in "Experimental Methods" results in an

Table I. Pectins extracted from sugar-beet pulp

Pectin fraction		Yield (% w/w)	Anhydrogal. acid content (% w/w)
Water-soluble	(WSP)	2.2	31
Oxalate-soluble	(OXP)	0.5	50
Acid-soluble	(HP)	20	36
Alkali-soluble	(OHP)	11	41
Pectin extracted		33.7	

appreciable increase in anhydrogalacturonic acid content (Table II). Neutral sugar residues present in all four pectins are arabinose and galactose mainly and further rhamnose, fucose, xylose, mannose and glucose. Total neutral sugar content varies from 5.7 to 24.3%, in different pectins which is relatively high when compared to pectins from apple pomace and citrus wastes (20). Degrees of methylation are comparable to those of certain types of commercial pectins (from apples and citrus) which, unlike these pectins, contain few or no acetyester groups. The weight percentage values in Table II do not add up to 100% as the purified pectins still contain some protein, unbound polyphenols and sodium counterions. Viscosity-average molecular masses are low, as compared to those of pectins from apple, citrus fruits or cherries, which are in the order of 70000 to 90000 (20,7).

Gel-permeation chromatograms (Figure 1) of purified pectins on Sepharose CL-2B (WSP, HP, OHP) and CL-6B (OXP) show a fairly continuous variation of the distribution of neutral sugars over the pectin molecules of varying molecular mass, except for OHP, which apparently consists of two different populations of molecules: high molecular mass pectin rich in neutral sugars and low molecular mass pectin with a low neutral sugar content. As will be clear from degradation studies, this partition points towards breakdown of the pectin molecules during alkaline extraction.

Table II. Composition and properties of purified pectins from sugar-beet pulp.

	WSP	OXP	HP	OHP
Anhydrogalacturonic acid	54.4	77.9	65.1	54.9
Neutral sugars	16.5	5.7	18.9	24.3
Rhamnose + fructose	0.89	0.86	2.25	3.17
Arabinose	8.44	1.85	9.97	12.49
Xylose	0.14	0.16	0.17	0.23
Mannose	0.18	0.14	0.12	
Galactose	6.46	2.43	5.93	8.09
Glucose	0.39	0.21	0.44	0.31
Feruloyl ester groups	0.10	0.04	0.48	0.57
Methylester groups	7.24	8.19	7.09	0.72
Acetyl ester groups	5.71	4.04	7.53	0.54
Viscosity-average M.W.	47700	15400	42800	36400

Numbers are given as % (w/w), except M.W.

Table III. Degradation limits of sugar-beet pectins before and after alkaline deesterification.

Pretreatment and mode of degradation	Pectins			
	WSP	OXP	HP	OHP
<u>No-pretreatment</u>				
β-elimination	7.2 <sup>a)</sup>	4.9	4.6	----
pectin lyase	8.6	4.3	4.1	----
polygalacturonase	1.2	3.7	1.7	26.9
pectate lyase	1.0	4.8	1.3	23.7
<u>Alkaline deesterification</u>				
polygalacturonase	37.5	36.7	30.8	29.3
pectate lyase	33.4	34.5	31.0	26.4

a) Percentage of galacturonide bonds broken.

Degradation studies. In order to study the distribution of substituents, notably neutral sugar side chains along the rhamnogalacturonan backbone of the pectins, degradation studies were done by β-elimination (heating at 80°C and pH 6.8 for up to 6 h) and with various enzymes before and after alkaline demethylation and deacetylation. Table III summarizes the degradation limits obtained with the various methods. β-Elimination and pectin lyase degradation are known to require methylesterified anhydrogalacturonide residues. The degrees of methylesterification of WSP, OXP, HP and OHP were 76%, 60%, 62% and 7,5%, respectively. It is obvious that both break-down mechanisms were strongly promoted with pectins with higher degrees of methylesterification.

The inverse was true for degradation with endopolygalacturonase and pectate lyase. It has been shown by Rexová-Benková *et al.* (21) that acylester groups at C-2 and C-3 of anhydrogalacturonic acid residues decrease the extent of degradation by lowering the affinity of endopolygalacturonase for its substrate through blocking of binding sites. In this case a further limitation of the degree of degradation through acetylation is also observed. The degrees of acylesterification (expressed as moles per 100 moles of anhydrogalacturonide residues) are 31%, 16%, 35% and 4%, for WSP, OXP, HP and OHP, respectively. Although the degrees of methylesterification of OXP and HP are quite similar, the degradation limits of these two pectins towards endopolygalacturonase and pectate lyase are quite different, and probably reflect the difference in acylesterification. Alkali-soluble pectin (OHP) is largely de-esterified during extraction which explains the high degrees of degradation with polygalacturonase and pectate lyase.

Methylester groups and acylester groups are both completely removed by alkaline deesterification. That is why the degradation limits for polygalacturonase and pectate lyase are raised to the values shown in Table III. Higher degradation limits for polygalacturonates are reported in literature, both for endopolygalacturonase and endopectate lyase. Rexová-Benková *et al.* (21) found 38%, for pectate from citrus pectin degraded by an *Aspergillus niger* endopolygalacturonase. Rombouts *et al.* (4) found pectate from apple

pectin to be degraded to a limit of 35% by the endopectate lyase used in this study. It is likely that the differences in degradation limits of the alkali-demethoxylated and -deacetylated pectates reflect the number of neutral sugar side chains attached to the pectins. Although WSP has a much higher neutral sugar content than OXP, the alkali-deesterified products are degraded to practically the same extent. This can be explained if indeed the ratio of side chains per unit of chain length in both pectins were similar; those in WSP would then on average be four times as long as those in OXP (compare Tables II and III).

Gel-permeation chromatograms of the digests obtained in various degradation experiments reveal a general trend towards separation of the products into several peaks, in those cases where the degrees of degradation are sufficiently high. The products of sodium-hydroxide deesterified, pectate-lyase degraded pectins are shown in Fig. 2. With WSP, HP and OHP, the peaks near the void volume ( $K_{av}=0-0.5$ ) represent relatively large fragments, very rich in neutral sugar residues and relatively poor in anhydrogalacturonide residues. In contrast, the peaks in all pectin digests which elute towards the included volume ( $K_{av}=1$ ), are small products (oligogalacturonide fragments) with a very low neutral-sugar content. The oligogalacturonide fragments account for 80%, 87%, 84% and 66% of total anhydrogalacturonide present in WSP, OXP, HP and OHP digests, respectively. Intermediary sized fragments, also relatively rich in neutral sugar residues are present in all four pectin digests ( $0.7 < K_{av} < 0.9$ ).

These data confirm what is known already for certain other pectins, e.g. from apples (20) and cherry fruits (17), that the neutral sugar side chains attached to the rhamnogalacturonan backbone occur in blocks, the so-called "hairy fragments", leaving large parts of the main chain unsubstituted ("smooth fragments").

The size of the hairy fragments of alkali-deesterified pectins can be estimated by determining reducing groups with the Nelson-Somogyi method. Those of OHP with  $K_{av} \leq 0.5$ . (Fig. 2) for instance, are composed of an average of ten anhydrogalacturonide residues, and up to forty neutral sugar residues (molecular mass about 7500). As these enzyme-resistant fragments will carry a minimum of three or four neutral-sugar side chains, the average size of these would be at least ten to thirteen residues.

A study of the neutral sugar distribution in the large ( $K_{av} \leq 0.5$ ), intermediate ( $K_{av} = 0.7-0.9$ ) and small ( $K_{av} \geq 0.9$ ) hairy fragments revealed that arabinose and galactose accumulate in the large and intermediate fragments, rhamnose and xylose in the intermediate and small fragments, and fucose and mannose in the small fragments, but all sugars are present in all fragments.

Location of acetylesther groups. As shown in Table II beet pectins contain 31%, 16%, 35% and 4% acetylesther groups expressed as moles per mole of anhydrogalacturonide times 100%, for WSP, OXP, HP and OHP, respectively. There is, however, no solid evidence about the location of these acetyl groups: on the rhamnogalacturonan backbone or rather on the neutral sugar side chains (1). This problem can be studied through enzymatic degradation, and analysis of products.

Fig. 3 shows a gel-permeation chromatogram of a degraded pectin which has undergone sequential treatment with pectin methylesterase

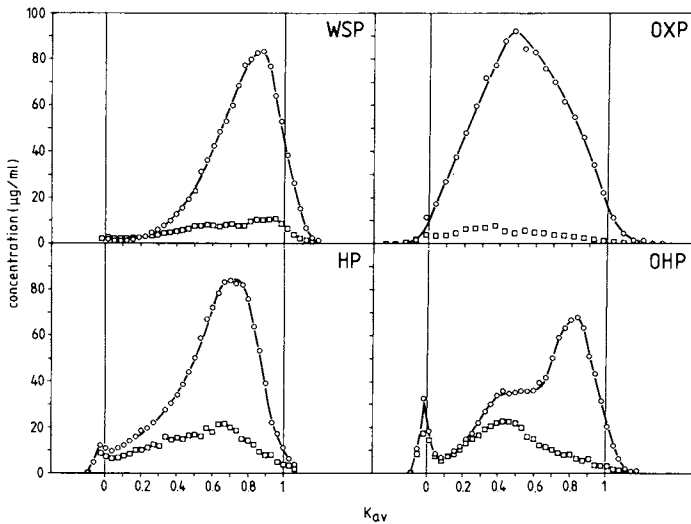


Fig. 1 Gel-permeation chromatograms of purified pectins on Sepharose CL-2B (WSP, HP, OHP) and CL-6B (OXP). Anhydrolacturonide residues ( $\circ$ ), neutral sugar residues ( $\square$ ).

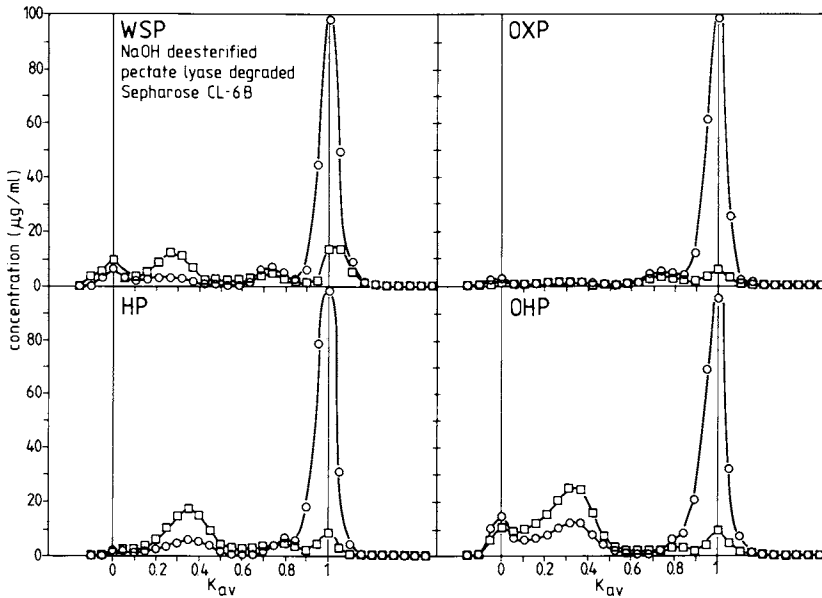


Fig. 2 Gel-permeation chromatograms of sodium-hydroxide deesterified, pectate-lyase degraded sugar-beet pectins. Anhydrolacturonide residues ( $\circ$ ), neutral sugar residues ( $\square$ ).

and polygalacturonase. It appears that the oligogalacturonic acids, which represent 92% of total anhydrogalacturonide and only 9% of neutral sugar residues, carry 75% of the acetyler groups. In addition, exoarabinanase and endogalactanase from Aspergillus niger can liberate some 50% of the neutral sugar residues, which carry only 8% of the acetyl groups. The conclusion seems justified therefore that at least 80 to 90% of the acetyl groups in beet pectin are linked to C-2 and/or C-3 positions of the galacturonide residues.

Presence and location of feruloyl ester groups. Solutions of sugar beet pectins turn yellow in alkaline medium. There is a bathochromic shift of a double absorption peak at 300 and 325 nm towards a single peak at 375 nm which causes this yellow coloration at pH 10. Also, sugar beet pectins, even when carefully purified as described in "Experimental Methods", react positively in the Folin test. Figure 4 shows an endopectate lyase digest of alkali-deesterified pectin chromatographed on Sepharose CL-6B. The elution profile shows the usual separation pattern of higher molecular mass galacturonides, rich in neutral sugar residues ("hairy fragments") and small oligogalacturonides, with few neutral sugar residues attached. Both fractions react positively in the Folin-test, but, as judged from the elution profile, the Folin-positive material present in the peak around  $K_{av}=1$  is not bound to the oligogalacturonides. However, chromatography of the "hairy fragments" on DEAE-Sepharose CL-6B confirms that Folin-positive material is bound to these pectin fragments. The absorption spectra at pH 4.8 and 10, as well as HPTLC-analysis of hydrolysis products of "hairy fragments" have shown that ferulic acid residues are ester-linked to the "hairy fragments" of sugar-beet pectins. These results are in agreement with recent work of Fry (22) who found feruloyl ester groups linked to araban and galactan side chains of pectin from spinach. This structural feature seems to be restricted to pectin from certain species in the Caryophyllales (Centrospermae) (23); indeed we have not found feruloyl ester groups in pectins from other plant sources such as potato, apple, citrus, apricots or cherries.

With the aid of the molar absorptivity value of 31600 at pH 10, as given by Fry (13), we have determined the feruloyl ester content of the four pectins. These are quite different (Table I). Acid-soluble and alkali-soluble pectins contain many more feruloyl ester groups: on average one such group per pectin molecule. It is tempting to presume a relationship between feruloyl ester content and ease of extraction of the pectins, but this requires further study.

Gelation through oxidative coupling. It has been known for some time that ferulic acid, tyrosine and isotyrosine substituents bound to biopolymers may be involved in the formation of crosslinks in the presence of hydrogen peroxide and peroxidase (24). For instance, in the case of wheat flour pentosans containing glycoproteins whose carbohydrate moiety carries feruloyl ester groups, formation of diferulic acid crosslinks results in gel formation. Following up on these observations by Geissmann and Neukom (25) we have succeeded in crosslinking and producing gels from sugar-beet pectin by using peroxidase and hydrogen peroxide according to the reaction shown in Fig. 5.

Gel formation also occurs with ammonium persulfate, but not with

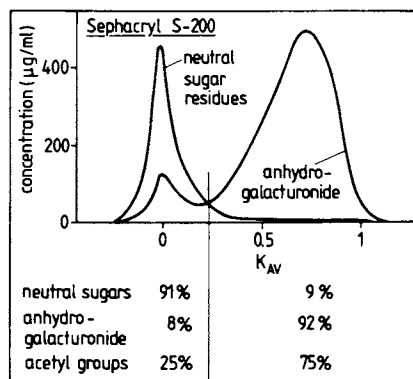


Fig. 3 Gel-permeation chromatogram of acid-soluble sugar-beet pectin degraded with endopolygalacturonase after pectinesterase demethoxylation.

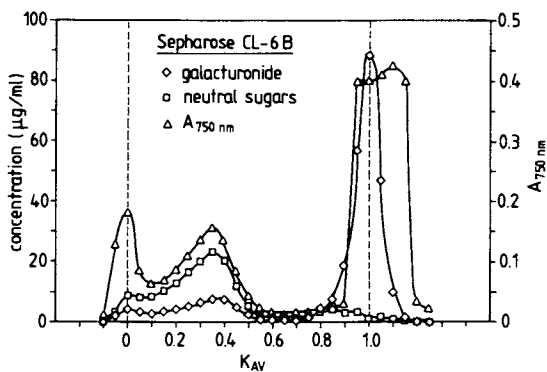


Fig. 4 Gel-permeation chromatogram of an endopectate-lyase digest of alkali-deesterified sugar-beet pectin.

Table IV. Effect of enzymic crosslinking on intrinsic viscosity and molecular mass of sugar-beet pectin.

Concentration of pectin at crosslinking (g/l)	Intrinsic viscosity (cm <sup>3</sup> /g)	Molecular mass
not crosslinked	248	46 000
6	435	70 000
9	468	73 900
15	461	73 000

other oxidation agents, such as permanganate, chlorite, periodate, or hydrogen peroxide alone.

Properties of products. Depending on the concentration of the reactants, the crosslinking reaction may be used to obtain pectins with increased molecular mass or to obtain gels with interesting properties (26). In Table IV the effect of crosslinking with peroxide and peroxidase on apparent molecular mass of the pectin is shown. In this case equimolar amounts of hydrogen peroxide and feruloyl ester groups were present in the reaction mixtures. High-performance size exclusion chromatograms of such pectins show a growing peak of material with increased molecular mass, as the crosslinking is more intense. Striking increases in reduced viscosity values can also be obtained with persulfate. As with the hydrogen peroxide/peroxidase system, gels are obtained when the pectin concentration is increased above a certain value. Pectin gels obtained by crosslinking with hydrogen peroxide/peroxidase as described in "Experimental Methods" are shown in Fig. 6.

The crosslinked pectin from such gels can be isolated by solvent-drying. The product thus obtained has a remarkable water absorbing capacity. Depending on its form (H or Na) and the ionic strength of the aqueous solution one gram of the product may absorb 50 to 160 ml of water.

### Outlook

Pectins are traditionally used as gelling and thickening agents in a rather wide range of applications (27). For these purposes pectins from apple pomace and citrus wastes have proven to be superior to those from sugar-beet pulp. In this paper we have reported the presence of feruloyl ester substituents in beet pectins, and the possibilities for enzymatic and chemical crosslinking through these substituents. This process may lead either to increased apparent molecular mass of soluble pectins, or to gel formation. In addition to the acid-sugar-pectin gel and the calcium-pectate gel, this is a third way of producing gels which is exclusive for pectins from sugar beet. The crosslinked pectins from these gels, isolated by solvent-drying have an extremely high water-absorbing capacity. We feel that this property especially should lead to some new applications e.g. as a cloud stabiliser in drinks, or as water absorbing agent in sanitary products.



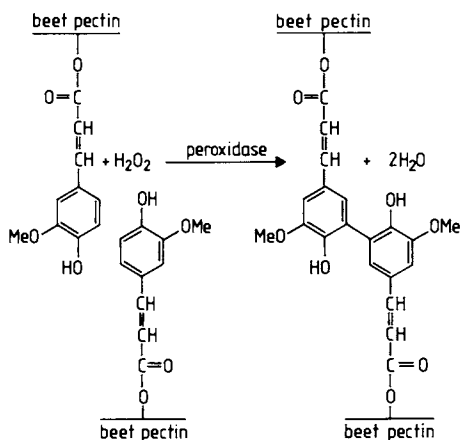


Fig. 5 Crosslinking reaction for sugar-beet pectins (modified from 25).

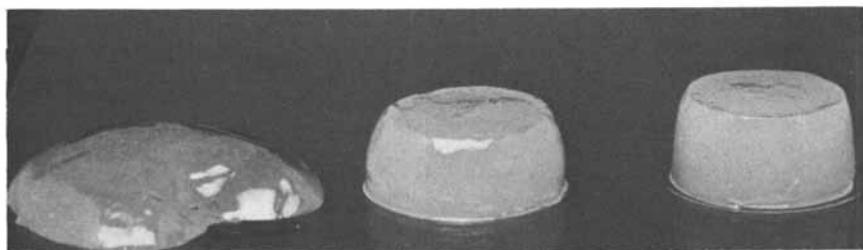


Fig. 6 Gels from acid-soluble sugar-beet pectin, obtained by crosslinking with hydrogen peroxide and peroxidase. The gels are 22 ml each and contain 0.9%, 1.8% and 2.7% pectin, from left to right. No sugar, acid or calcium salts were used.

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## Interactions of Counterions with Pectins Studied by Potentiometry and Circular Dichroism

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The aim of this study was to investigate the binding of counterions in dilute salt-free solutions of pectins with different levels and patterns of esterification. The influence of the charge parameter and of the distribution pattern of carboxyl groups on the interaction polyanion-counterion (sodium or calcium) were studied by the determination of calcium and sodium activity coefficients which were compared to theoretical values. Very low values of calcium activity coefficients (0.085) were obtained for the most charged pectin-sample. These results were completed by circular dichroism spectroscopy in order to have local informations on the conformational changes during the neutralization of pectinic acids with sodium or calcium hydroxides. The results suggest that three different conformations exist for the low-methoxyl pectins in acidic form, sodium and calcium forms.

The interactions of pectins with mono- or divalent counterions are of a great importance in many fields. It is well-known that these polyelectrolytes play an important role as natural ion-exchangers in plant physiology, phytopathology, nutrition (1-2) and their ability to form gels with calcium ions is widely used by food technologists (3).

The strong cooperative (4) calcium binding of low-methoxyl pectins may be explained by the stereochemical feature of the 1,4-linked monomeric units (5) leading to the formation of polar cavities which can be occupied by calcium ions, as well as by the chemical structure of the polymer which can be idealized by the juxtaposition of "hairy regions" where the neutral sugar side-chains are concentrated and of "smooth" regions of unsubstituted homogalacturonans (6,7).

Rees and co-workers (5,8,9) studied the calcium binding mainly by equilibrium dialysis and circular dichroism on gels. The inter-

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actions between calcium counterions and carboxyl groups were described by the "egg-box" model and a two stages process was postulated : an initial dimerization and subsequent aggregation of these preformed "egg-boxes". The intermolecular binding was confirmed by Kohn and Luknar (10) and by Ravanat and Rinaudo (11) by measuring calcium activity coefficients on calcium pectate solutions.

Our aim was to investigate the binding of counterions in dilute salt-free solutions of pectins with different levels and patterns of esterification. We used methods such as potentiometry and circular dichroism in order to obtain both general and local informations on the conformational states of the polymer depending on its ionic form.

### Material and Methods

#### Pectin samples

The starting pectin was a commercial apple pectin (Rapid-set pectin, from Unipectine, Redon, France). It was purified by precipitation with cupric ions (12). A pectin sample with a high degree of esterification was also used in this study (13).

#### Alkaline deesterification

Preparations of pectin with various degrees of esterification were obtained by partial alkaline saponification. Aqueous solutions of the initial pectin were cooled at 2°C in an ice-salt bath and the pH was adjusted to 7 with NaOH 0.1N. Then amounts of sodium hydroxide corresponding to the amount of methoxyl groups that should be saponified were added. After two days at 2°C, the pH of the solution was brought to 4.5 with HCl 1N. Pectins were then precipitated with 80% ethanol, extensively washed with 60% ethanol and dried under vacuum at a temperature less than 40°C.

#### Enzymic deesterification

A pectinesterase (E.C.3.1.1.11) from orange peel was purchased from Sigma. Pectin was dissolved in a  $4 \times 10^{-2}$  M citrate-phosphate buffer at pH 6 containing 0.1M NaCl. The amount of enzyme and the reaction time, at 30°C, were chosen in order to obtain the desired degree of esterification. The reaction was stopped by bringing the pH value down to 4.5 and the enzyme was inactivated by heating at 100°C the reaction mixture for 10 min. The samples were recovered as above.

#### Characterization of the samples

The galacturonic acid content as well as the degree of esterification (DE) were measured by conductimetry and the neutral sugar content was determined by gas chromatography, as described elsewhere (14,15). Light scattering experiments were carried out for an angle of 90° at  $25.0 \pm 0.1^\circ\text{C}$  on pectin solutions after filtration through 0.45µm Millipore filters.

### Potentiometry

The calcium and sodium activity coefficients were determined at  $25.0 \pm 0.1^\circ\text{C}$  with an Orion electrode (model 92-32) and a Radiometer electrode (model G502 Na), respectively. A saturated calomel electrode was used as the reference. Calibration curves were obtained using  $\text{CaCl}_2$  or  $\text{NaCl}$  solutions before and after each measurement. The  $\text{CaCl}_2$  and  $\text{NaCl}$  concentrations were measured by potentiometric determinations of the chlorides with silver nitrate and with a silver electrode.

### Circular dichroism

A Dichro V spectropolarimeter (Jobin-Yvon, France) was used at  $25^\circ\text{C}$ . Solutions of pectins containing 1 mequiv of carboxyl groups/l were put in a 1 mm pathlength cell. An integration time of 0.2 sec was used and 10-15 scans (0.2 nm/sec, 185-250 nm) were accumulated. The differential extinction coefficient ( $\Delta\epsilon$ ) was expressed in  $\text{cm}^{-1}$  equiv $^{-1}$ .l.

### Other methods

The acidic form of the pectin (pectinic acids) were obtained by percolation through a strong  $\text{H}^+$  exchanger column (Amberlite IRN 77). Salts (pectinates) of these pectinic acids were obtained by exact neutralization with the desired carbonate-free hydroxide.

## Results and discussion

### Characteristics of the samples

The initial pectin was precipitated by cupric ions in order to remove the neutral polysaccharides which are not linked to the pectic backbone. Ion-exchange chromatography on DEAE-Sephacrose CL-6B showed that only 0.5% of the initial material was not bound to the gel, indicating a purity of 99.5%. The purified pectins have a galacturonic acid content of 78.5%, a neutral sugar content of  $\sim 10\%$  and a degree of esterification of 72.1%.

Samples of pectins with varying degrees of esterification (0-60%) were obtained from this preparation by deesterification with alkali and with enzyme. The main characteristics of these samples are indicated in Table I. It is likely that the action of alkali leads to pectic molecules in which free and methyl esterified carboxylic acids are randomly distributed (16). The temperature was chosen in order to minimize chain-cleavage by  $\beta$ -elimination (17) and intrinsic viscosity measurements of the samples (sodium form in 0.1 M  $\text{NaCl}$ ) showed that the degradation was minimal (15).

Other pectins with degree of esterification of about 10, 30 and 40% (Table I) were obtained by enzymic deesterification of the initial preparation. An orange pectinesterase was used and the pH of the reaction was 6 in order to avoid concurrent base-catalyzed saponification. The action of plant pectinesterase is known (16-18) to result in a blockwise arrangement of free carboxyl groups in the pectic molecules. The enzymic preparation does not contain depolymerase activities as shown by the constancy of the intrinsic

viscosity values. Since the starting sample was 72% esterified, it is likely that after the enzymic deesterification, the samples contain some amounts of randomly distributed carboxyl groups. They are considered nevertheless as representative of a blockwise distribution.

The charge parameter  $\lambda$  is also indicated in the Table I. This fundamental parameter (19) governs the behavior of the polyelectrolyte polymers. Its value is given at 25°C by :

$$\lambda = \frac{e^2}{bDkT} \times (1 - DE/100) = 1.61 (1 - DE/100) \quad (1)$$

where  $e$  is the electron charge,  $kT$  the Boltzmann term,  $b$  the length of the monomeric unit and  $D$  the dielectric constant of the solvent. The  $b$  value was taken as 4.35 Å (20). The calculation of  $\lambda$  neglects the small content (< 1%, w/w) of L-Rhamnose which is present in the polygalacturonate backbone.

Table I. Characteristics of the pectins

	DE <sup>a</sup>	AG <sup>b</sup>	$\lambda^c$
Alkali-deesterified samples			
1	< 2	91.4	> 1.578
2	9.7	90.7	1.454
3	21.4	84.6	1.265
4	27.3	83.6	1.170
5	38.1	80.7	0.997
6	48.3	81.3	0.832
7	58.2	79.1	0.673
8	72.1	78.5	0.451
9	83.0	75.4	0.274
Enzyme-deesterified samples			
10	10.1	87.1	1.447
11	27.7	80.2	1.164
12	39.7	80.1	0.971

<sup>a</sup> degree of esterification

<sup>b</sup> anhydrogalacturonic acid content

<sup>c</sup> structural charge parameter

#### Counterion activity coefficients

The sodium and calcium activity coefficients were determined by specific electrodes, in dilute salt-free solutions ( $c < 10^{-3}$  equiv/l) of sodium and calcium pectinates. Results were compared to those calculated from Manning's theory (21). This model is proposed for infinitely dilute solutions of rodlike polyelectrolytes and the activity coefficients are directly imposed by the charge parameter :

$$\gamma = e^{-|z|\lambda|z|} \quad |z|\lambda \ll 1 \quad (2)$$

$$\gamma = e^{-\frac{1}{2}} (|z|\lambda)^{-1} \quad |z|\lambda > 1 \quad (3)$$

where  $z$  is the valence of the counterion.

Samples with a random distribution of carboxyl groups are characterized by experimental values which are decreasing with decreasing DE, i.e. with increasing charge parameter (15). This behavior is similar to that observed for other polyelectrolytes (22,23). Experimental sodium activity coefficients are in good agreement with the theoretical ones, except for samples with low DE which are characterized by higher values than predicted (15). Values of calcium pectate (sample 1) and for calcium salts of samples 7 and 8 (DE = 58.2 and 72.1 respectively) are close to those reported by Ravanat and Rinaudo (11) and by Rinaudo and Milas (22) for pectins with similar DE. A continuous decrease of the calcium activity coefficients was obtained whereas Kohn and Luknar (4) observed a sudden drop of the calcium activity coefficient as measured by a metal indicator method for pectinates with DE lower than 40%, although both sets of experimental values are close to each other. If the ratio experimental/theoretical calcium activity coefficient is plotted versus the DE (Figure 1), a transition is evidenced as this ratio decreases below a DE of 50%. Values of calcium activity coefficient obtained for the polymers with lowest DE are very low. These values suggest specific interactions of the pectic molecules with calcium ions. The fact that the calcium activity coefficient is about half of the theoretical value suggests that the calcium pectate behaves roughly as a polyelectrolyte with a doubled charge density, and may be ascribed to an intermolecular binding of the  $\text{Ca}^{2+}$  ions to carboxyl groups of two macromolecules leading to the formation of dimers, even in very dilute solutions (range tested  $5 \cdot 10^{-4} < c < 10^{-3}$  equiv/l).

The influence of the pattern of esterification on the binding of calcium ions is clearly shown in Figure 1. The enzyme-deesterified pectins are characterized by low values of calcium activity coefficients close to those of calcium pectate (sample 1) due to the presence in these samples of contiguous not methylated galacturonic acids (9,10), in agreement with results reported by Kohn et al. (18).

The changes in scattered light have been recorded (15) during the neutralization of some samples. The neutralization of highly esterified pectins by potassium or calcium hydroxides does not induce any important change in scattered light, as is the case during the neutralization by potassium hydroxide of the most charged samples. In contrast, the neutralization with calcium hydroxide of the samples with the highest charge density leads to an increase in scattered light. These changes may be explained by an increase in apparent molecular weight, confirming the hypothesis of an association of chains induced by calcium counterions.

#### Influence of the polymer concentration on calcium activity coefficient

In addition, the dependence of calcium activity coefficients upon the calcium pectinate concentration has been investigated. Solutions of pectinic acids of different concentrations were prepared, neutralized by calcium hydroxide and the resulting calcium salts were analyzed by potentiometry for the calcium activity coefficient.

Samples with a DE higher than 50% have constant calcium activity coefficients over the concentration range tested ( $0.8 - 3 \times 10^{-3}$  equiv/l). In contrast, pectins with lower DE are characterized by a calcium activity coefficient which is decreasing above a concentration of  $10^{-3}$  equiv/l. Furthermore, the concentration in calcium pectinates has a profound influence on the calcium activity coefficient for samples with the lowest DE or for enzyme-deesterified pectins since the calcium activity coefficient decreases to zero. With higher concentration in pectins, ( $c_p > 3 \times 10^{-3}$  equiv/l), a gelation process can be observed.

These results show that the calcium ions seem to interact largely with the polymer and can be explained by a multichain aggregate formation i.e. an association of dimers leading to an increase of the apparent charge parameter.

These results are in disagreement with those obtained by Kohn, and Luknar (4) who observed a constant calcium activity coefficient (as determined by their metallochromic method) whatever is the pectin concentration, but they prepared the solutions by dilutions of the most concentrated solution.

#### Circular dichroism measurements

In order to complete the results obtained by potentiometry, circular dichroism measurements were carried out. Pectic molecules exhibit a positive Cotton effect corresponding to a  $n \rightarrow \pi^*$  transition of the carboxyl groups (5). The optical activity of the carboxyl chromophore is determined by the environment and hence it should be affected by conformational changes due to intra- or intermolecular interactions.

The effect of level and pattern of esterification, of the degree of neutralization and of the nature of the counterion, on the circular dichroism curves were studied on dilute solution containing  $10^{-3}$  equiv/l of free carboxyl groups in order to avoid gelation in presence of calcium ions.

Values of  $\lambda_{\max}$  for the pectin samples in three ionic forms ( $H^+$ ,  $Na^+$ ,  $Ca^{2+}$ ) are shown in Figure 2. The acidic forms are characterized by a constant  $\lambda_{\max}$  value (at 210 nm), whatever is the DE and the distribution pattern of the free carboxyl groups. For the sodium form of the polymers, the  $\lambda_{\max}$  value decreases from 208 nm to 203 nm as the charge density of the polyelectrolyte increases with a transition for a DE about 40% with no influence of the distribution pattern of the free carboxyl groups. The values of  $\lambda_{\max}$  for calcium pectinates decrease from 205 nm with a broad transition and a constant value of 193 nm is obtained when the DE is less than 20%. The influence of the pattern of esterification is clearly shown by the fact that the increase in  $\lambda_{\max}$  value occurs for higher DE for enzyme-deesterified pectins. These results are not in agreement with those obtained by Kohn and Sticzay (23) who observed that the position of the absorption band was shifted only within a 2 nm range if the ionic form changes from potassium to calcium.

It can be concluded from these results that all the pectins in acidic form have only one conformational state whatever are the level and pattern of esterification. In contrast, the sodium and calcium salts of pectinic acids are characterized by a conformational transition which is stabilized if the DE is lower than 20-30%.



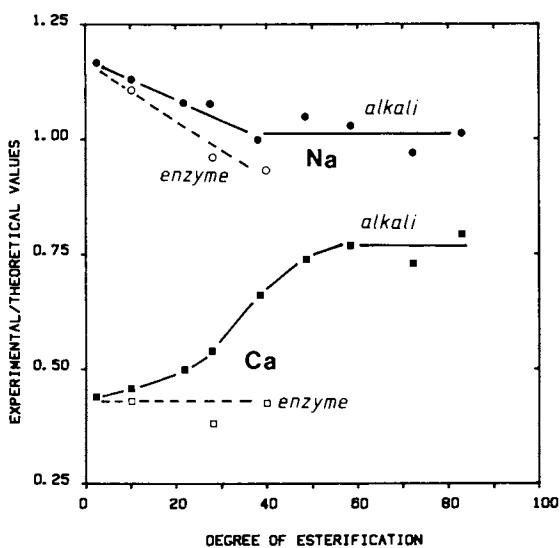


Figure 1. Variations of the ratio experimental/theoretical values of calcium ( $\blacksquare$ ,  $\square$ ) and sodium ( $\bullet$ ,  $\circ$ ) activity coefficients with the degree of esterification of alkali- (—) and enzyme-deesterified (---) pectins.

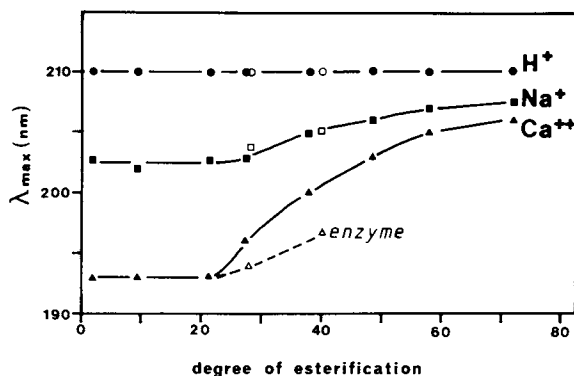


Figure 2. Variations of the  $\lambda_{max}$  values for circular dichroism spectra on acidic ( $\bullet$ ,  $\circ$ ), sodium ( $\blacksquare$ ,  $\square$ ) and calcium form ( $\blacktriangle$ ,  $\triangle$ ) of pectins deesterified by alkali ( $\bullet$ ,  $\blacksquare$ ,  $\blacktriangle$ ) and by enzyme ( $\circ$ ,  $\square$ ,  $\triangle$ ).

The evolution of the circular dichroism curves during the neutralization of the pectinic acids by sodium and calcium hydroxides were therefore followed in order to obtain more informations on the conformational changes. The neutralization of sample 5 (a pectin with a DE of 40% with a statistical distribution of the free carboxyl groups), either with sodium or calcium hydroxides leads to a regular decrease both in  $\lambda_{\max}$  and in differential extinction coefficient ( $\Delta \epsilon$ ) which progressively reach values for pure sodium and calcium forms, respectively. This is entirely consistent with a simple ionization process and all the curves pass through the isodichroic point at about 200 nm.

When a pectic acid (sample 1; DE < 2%) is neutralized by sodium hydroxide (Figure 3), a decrease both in  $\lambda_{\max}$  and in differential extinction coefficient is also observed with an isodichroic point at 200 nm. In contrast, the behavior of this sample during the neutralization with calcium hydroxide is quite different since a sudden drop in the differential extinction coefficient value is observed when the degree of neutralization is higher than 0.4 and thereafter a peak at a wavelength tending to 193 nm is increasing. Apparently one isodichroic point is also observed at about 200 nm. The great decrease in intensity at 210 nm in the solution of calcium pectate suggests a strong and specific interaction of calcium ions to the carboxyl chromophores. The Figure 4 shows the value of the differential extinction coefficient at 210 nm as a function of the degree of neutralization by sodium and calcium hydroxides.

For a degree of neutralization up to 0.4, no differences can be observed between the sodium and calcium forms, suggesting that calcium ions are bound as are bound the sodium ions and that only isolated pectic molecules exist. A transition with calcium ions occurs for higher values of the degree of neutralization and the decrease in intensity may be attributed to intermolecular linkages leading to the formation of dimers (11).

Similar curves can be obtained for other samples and a generalization was tried. The acidic form of the pectins was taken as a reference since it was shown that only one conformation exists whatever is the level and pattern of esterification. In an other hand the degree of neutralization was substituted by the effective charge parameter ( $\alpha_N \times \lambda$ ) by neglecting the autodissociation of the polyacid. A master curve can therefore be drawn where all the experimental points fall (Figure 5) and where the transition for the calcium pectinates occur for values of the charge parameter higher than 0.6. It must be pointed out that the esterification pattern has no influence in these conditions.

### Conclusion

In this work, pectins with different levels and patterns (random or blockwise) of esterification were characterized and studied in salt-free solutions by potentiometry and circular dichroism.

Concerning the measurements of ion activity coefficient, an acceptable agreement was found between sodium experimental and theoretical values in contrast with values obtained with calcium ions. Pectins with a random distribution of free carboxyl groups are characterized by a degree of binding of calcium which increases with increasing charge parameter whereas the degree of binding of calcium

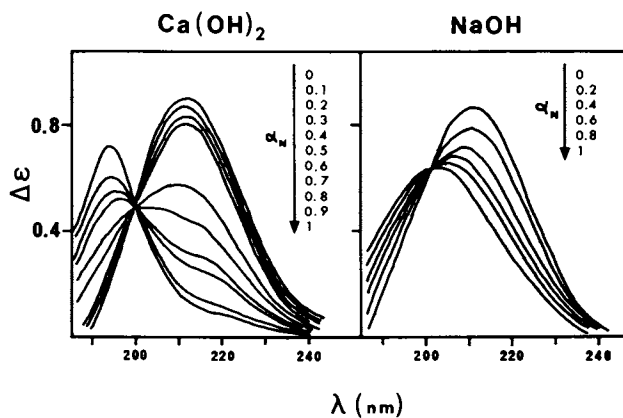


Figure 3. Circular dichroism spectra of sample 1 (degree of esterification 2%) as a function of the degree of neutralization with calcium and sodium hydroxides.

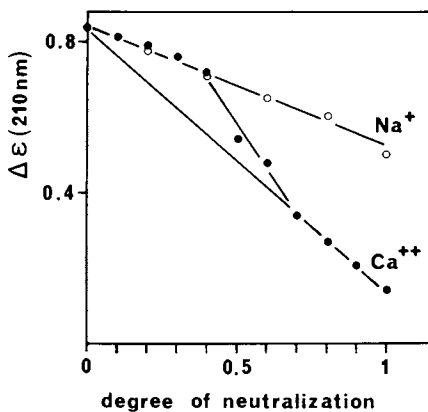


Figure 4. Dependence of the differential coefficient ( $\Delta \epsilon$ ) at 210 nm with the degree of neutralization of sample 1 with sodium (O) and calcium (●) hydroxides.

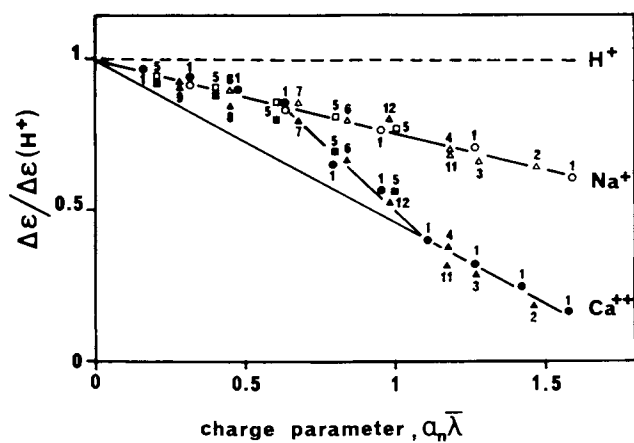


Figure 5. Dependence of the ratio differential extinction coefficient/differential extinction coefficient of the pectinic acids with the effective charge parameter ( $\alpha_N \bar{\lambda}$ ). The numbers refer to the samples (Cf. Table I).

by enzyme-deesterified pectins is roughly independent of the degree of esterification. The very low values of calcium activity coefficient when the DE is less than 40% were explained by a dimerization process, even in very dilute solutions, which can lead to aggregation and gelation when the polymer concentration is increased.

The circular dichroism measurements were performed in order to complete these results. It was concluded that only one conformation state characterizes our samples in acidic form, whatever is the DE and the esterification pattern. Neutralization with sodium and calcium hydroxides induces conformational transitions. It was demonstrated that the effective charge parameter governs the interaction : with calcium ions for values lower than 0.6, calcium counterions are not tightly bound suggesting only intramolecular interactions such as in the presence of sodium ions whereas a higher charge parameter value leads to the formation of intermolecular linkages and to a dimerization process in agreement with thermodynamic data.

From these results it is not possible to conclude definitively on the number of conformational states which exist depending on the ionic form. The results suggest that three conformations exist, one in acidic form, perhaps corresponding to a helix with a three fold symmetry, one in sodium form and another in calcium form which may be reasonably described by a helix with a two fold symmetry. In an other hand, only one isodichroic point was observed and this result may rule out the possibility of three conformations. The literature is also contradictory (24-26) in this respect and further work must be carried out.

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# Ionic Effects on the Conformation, Equilibrium, Properties, and Rheology of Pectate in Aqueous Solutions and Gels

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The effect on the conformational-aggregational properties of pectate in aqueous solution brought by the addition of specific ions ( $H^+$ ,  $Ca^{2+}$ ,  $Cu^{2+}$ ) was studied by osmometric, microcalorimetric, dilatometric, and rheological methods. Evidence is provided for the intramolecular nature of the pH induced conformational transition. The addition of divalent ions brings about at the same time a conformational change of the chain of pectate and chain-chain association.

The study of the interaction of pectate (and its partially esterified "derivatives", pectins) with specific ions in aqueous solutions is of fundamental importance to understand the properties of their solutions and gels at the molecular level. Indeed, pectate aqueous solutions in the presence of ions have been subjected to many investigations (1-5). However, only in a few cases has the "course" of the interaction been followed over a wide range of the ion to polymer molar ratio,  $R$ . The scrutiny of such a dependence for thermodynamic functions like  $\Delta H$  has allowed us to disclose an intramolecular cooperative conformational transition of pectate upon changing pH (5). Other properties investigated were consistent with the above interpretation. It was also suggested that the intramolecular conformational transition was a prerequisite for the further aggregation of chain molecules occurring in the gelling conditions at low pH. Direct evidence from molecular weight measurements will be provided here in favor of such a transition.

Among the other parameters able to induce similar macroscopic effects, the addition of divalent cations is particularly effective. Literature data are in almost complete agreement with the view that an aggregation of chains induced by ions is responsible for the gel forma-

tion (1,4). The currently accepted model envisages ions bridging parallel chains to form a linear array which is supposed to be the junction matrix for the gel network (6). This largely adopted model has eluded to direct thermodynamic confirmation because the formation of gel has been a serious limitation to the reproducibility of measurements. On the other hand, an analysis of the thermodynamic properties is necessary in order to ascertain whether the polymeric conformational "state" has been subjected to changes or not.

The results presented here on pectate solutions containing calcium or copper ions bring interesting evidence for an anomalous behavior of pectate properties in the range of ion to polymer ratio below that corresponding to the massive phase separation. The possibility that a conformational change proceeds along with the interaction with divalent ions is examined and discussed in comparison with the case of the proton-induced conformational transition.

In addition, the development of the gel phase was followed by studying the rheological behavior of a complex calcium-pectate system.

#### pH-induced Conformational Transition

On the basis of direct microcalorimetric results of enthalpy changes of dissociation and of dilution, we have proposed that by changing pH from about 3 to about 7 the pectate chain undergoes an intramolecular cooperative conformational transition in very dilute solution (5). Although nothing could be said about the actual geometry of the conformational states involved, that finding was substantiated by additional viscometric and (chiro)optical evidence (5). Pectic acid has a known tendency to give rise to chain-chain aggregation, especially so at low pH values. Therefore it was highly desirable to obtain direct evidence from molecular weight measurements for the intramolecular character of such a transition, by identifying a proper range of experimental conditions and using a suitable technique.

Membrane osmometry has been recently shown to be useful in studying the behavior of pectate in dilute solution (7). The results of osmotic pressure measurements obtained in the g/L polymer concentration range are reported in Figure 1. They have been obtained in 0.1 M ionic strength at 27°C at pH = 3.5 and pH = 6.5, i.e. for pH values corresponding to the two conformations. No problem was found as to the reproducibility of measurements on solutions previously equilibrated by dialysis. Although the points obtained at low pH are aligned on a line of negative slope (possibly indicating a poor polymer-solvent interaction), still the extrapolated value of the reduced osmotic pressure to zero polymer concentration is the same for both sets of data points. This is a clear-cut evidence that the conformational transition



that takes place in dilute solution is an intramolecular process; further aggregational phenomena will depend on increases in polymer and/or H<sup>+</sup> ion concentration, changes in temperature and ionic strength. The second virial coefficient of the reduced osmotic pressure plot of pectate changes from positive to negative on passing from neutral to acidic conditions, i.e. from the charged to the uncharged form. This finding could be in agreement with the marked decrease of the empirical Smidsrød's B parameter of stiffness upon decreasing the charge density of pectate, indicating a notable stiffening of the chain (8). Recent calculations would indicate that the value of the radius of gyration of pectate in 0.08 M phosphate buffer passes from 60 Å at pH 7.3 to 77 Å at pH 3.7, in agreement with the picture of a elongated conformation prevailing in acidic conditions despite the dramatic reduction of charge density (7).

#### Energetics (thermodynamics) of interaction with divalent ions

Pectate is known to interact very strongly with several divalent ions, although the concept of "binding" may change among different authors, to include different modes of interaction. Nevertheless, our present investigation seems to confirm such an established picture.

From equilibrium dialysis experiments (9), the amount of calcium "bound" to the polymer was taken equal to the difference between the concentration of ion in the polymeric phase and that in the polymer-free solution. In the range  $0 < R < 0.3$ , an average value of  $84 \pm 4$  % of Ca<sup>2+</sup> ions is "bound" to the pectate chains in aqueous 0.1 M NaClO<sub>4</sub>. In turn, from a previous investigation of this laboratory (10,11), the percentage of copper ions bound under the same conditions has been estimated to be larger than 99 %. This latter value includes both the fraction of ions strongly interacting with the electrostatic field generated by the macroion and the fraction (if any) of ions specifically bound on geometrically favorable sites (12).

A general result of the electrostatic interactions between counterions and polyelectrolytes (including site binding) is the large positive volume change due to the release of electrostricted water molecules from the solvated groups into the bulk of the solution (desolvation). In Figure 2 is reported the volume change of a pectate solution upon addition of divalent cations (corrected for dilution effects). The effect is largely ascribable to changes of solvation of the carboxylate groups and of the interacting counterions.

On a microscopic level the interaction process must involve the desolvation of ionic species (free ions and polymeric carboxylate) in order to approximate the groups to each other. The whole process is, therefore, characterized by a large and positive entropy change which

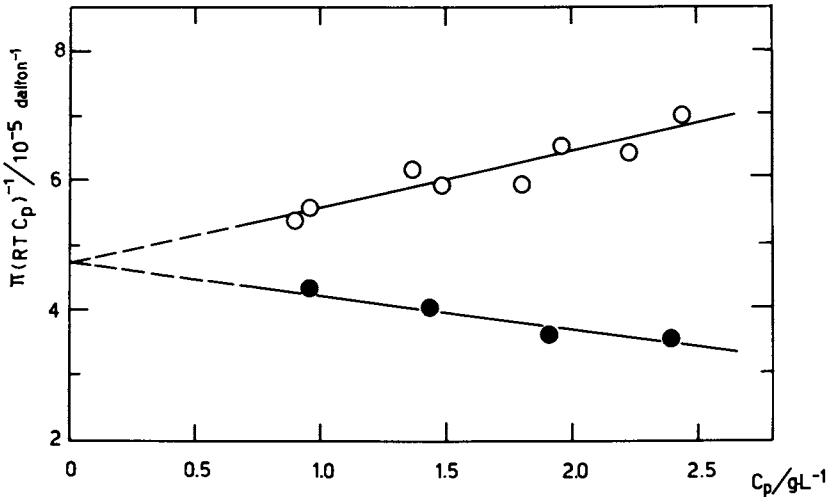


Figure 1. Dependence of the reduced osmotic pressure on the polymer concentration,  $C_p$ , of sodium pectate in 0.1 M ionic strength at 27°C: (○) pH 6.5; (●) pH 3.5.

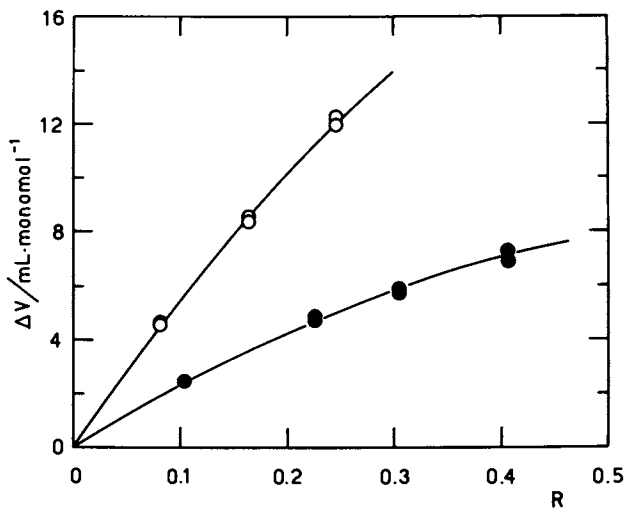


Figure 2. Dependence of the corrected volume change of mixing sodium pectate with  $\text{Cu}^{2+}$  (○) and with  $\text{Ca}^{2+}$  (●) in 0.05 M aqueous  $\text{NaClO}_4$  at 25°C. R is the ion-to-polymer repeating unit molar ratio.

provides for the favorable driving force. From the enthalpic side, such an interaction does not give an exothermic enthalpy change because the strong endothermicity of the desolvation step proper is usually not counterbalanced by other favorable contributions arising from ionic and dipolar interactions. Such a behavior is experimentally provided both by low molecular weight systems and by other polycarboxylates interacting in a highly specific mode with different divalent ions (15-18).

The enthalpy data for the mixing of a pectate solution with divalent cations ( $\text{Ca}^{2+}$  or  $\text{Cu}^{2+}$ ), in aqueous solution at  $25^\circ\text{C}$ , are reported in Figure 3. It is worthwhile to note the negative value of the enthalpy of interaction of the pectate with both ions, although the shape of the curves appears to be different. The data reported in Figure 3 represent, indeed, the excess enthalpy, i.e. they are corrected for the enthalpy changes due to the dilution of both the polymer and the divalent ion. Therefore, they represent the true temperature coefficient of the free energy of the interaction process involving the solvated polymeric chain and the ions. In the same figure the enthalpy of interaction with protons and with  $\text{Na}^+$  ions is also reported, for comparison purposes.

The general behavior of the enthalpy contribution in a process involving the interaction between charged species is a positive (endothermic) enthalpy change, as experimentally found for  $\text{Na}^+$  ions (Figure 3). This experimental evidence is substantiated by theoretical approaches based on polyelectrolyte theories (13). In some cases strong chelation may result in an enhanced endothermic enthalpy change, as discussed above about desolvation. Deviations towards exothermicity have always been reported and in all cases ascribed to the presence of ion-induced conformational transitions, which may (e.g.  $\text{Cs}^+$ -carrageenan) (14) or may not (see the curve of  $\text{H}^+$  of Figure 3 corresponding to the pH-induced transition of pectate) be accompanied by chain aggregation.

In the case of mixing pectate with copper ions, the exothermic enthalpy of interaction is absolutely unique in this class of compounds. In fact, all known polyuronates (15, 19), as well the monomeric galacturonate molecule (20), have a positive enthalpy of interaction, irrespective of the stereochemistry of the sugar residue(s) and of the ability of gel formation. Most important, a positive enthalpy has been found also for the system copper-polyguluronate (19), that is the polymer which has a behavior very similar to polygalacturonate and differs from the latter only for the stereochemistry of C(3). It is therefore mandatory to ascribe the reported exothermic behavior of pectate with  $\text{Ca}^{2+}$  and  $\text{Cu}^{2+}$  ions to the very specific change of conformation of the polygalacturonate chain.

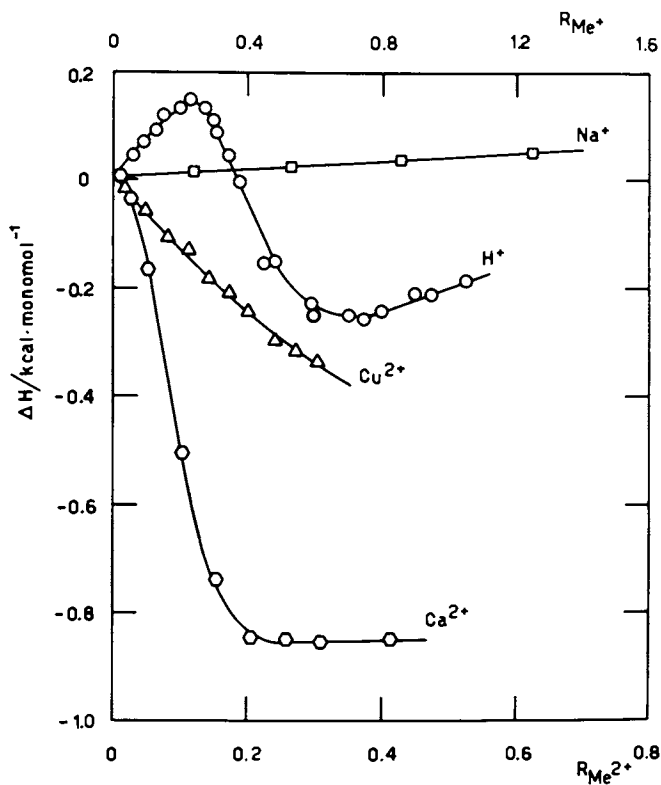


Figure 3. Dependence of the corrected enthalpy of mixing (see text) sodium pectate with different counterions in 0.05 M aqueous  $\text{NaClO}_4$  at 25°C.  $R_{\text{Me}^+}$  and  $R_{\text{Me}^{2+}}$  denote the ion-to-polymer repeating unit molar ratio for monovalent and divalent ions, respectively.

### Structural properties

Circular dichroism spectra of pectate in the presence of calcium and copper ions have been recorded as a function of the molar ratio R (sample case spectra are reported in arbitrary units in Figure 4d). The specific changes of ellipticity at different wavelengths are reported in Figure 4, a-c. They represent the development of the perturbation induced by  $\text{Ca}^{2+}$  and  $\text{Cu}^{2+}$  ions on the electronic  $n \rightarrow \pi^*$  and  $\pi \rightarrow \pi^*$  transitions of the carboxylate neighbor to dissymmetric centers (a and b) and the onset of an extrinsic chiral charge-transfer band involving the  $\text{Cu}^{2+}$  ion (c), respectively. The degree of linearity of the CD changes and the enthalpy changes for the  $\text{Cu}^{2+}$  titration are pretty similar, likely indicating a common origin for the two phenomena. In the case of  $\text{Ca}^{2+}$ , a slight difference in the dependence of the two functions on R is noticed. More information needs to be gained before a safe explanation can be inferred therefrom; for example, one might propose that the perturbation occurs at local level only, or that progressive binding of calcium occurs onto an already ordered conformation of pectate induced by very little amount of calcium ions.

Speculation on the nature of the ordered conformation induced by  $\text{Ca}^{2+}$  ions can hardly be made, not even in relation to that prevailing at low pH. Conformational calculations have been carried out in our laboratory on 1- $\rightarrow$ 4 galacturonans (21), by using standard theoretical procedures already described in the literature (22). From these calculations the allowed conformational space is located in a single region of the E( $\psi$ - $\phi$ ) diagram. Even more interesting is the fact that the pitch of the regular conformations generated by a given set of  $\psi$ - $\phi$  values changes very slightly within the allowed range of conformations (from a minimum of 4.32 to a maximum of 4.54 Å/monomer). Of course, the stability of each helical conformation is subjected to possible intramolecular hydrogen bonds and intermolecular solvation energies, in addition to the entropic destabilization due to the existence of several allowed conformations (statistical fluctuation).

### Aggregation of pectate

The aggregation of pectate in the presence of copper and calcium ions has been repeatedly reported and is commonly used as a method for the quantitative precipitation of the polymer. The actual distribution of the precipitate and the increase of the apparent molecular weight has been studied as function of R by means of sedimentation and membrane osmometry, respectively.

The curves reported in Figure 5 show the dependence of the amount of precipitate (as mass percentage) upon the addition of divalent ions. The effectiveness of

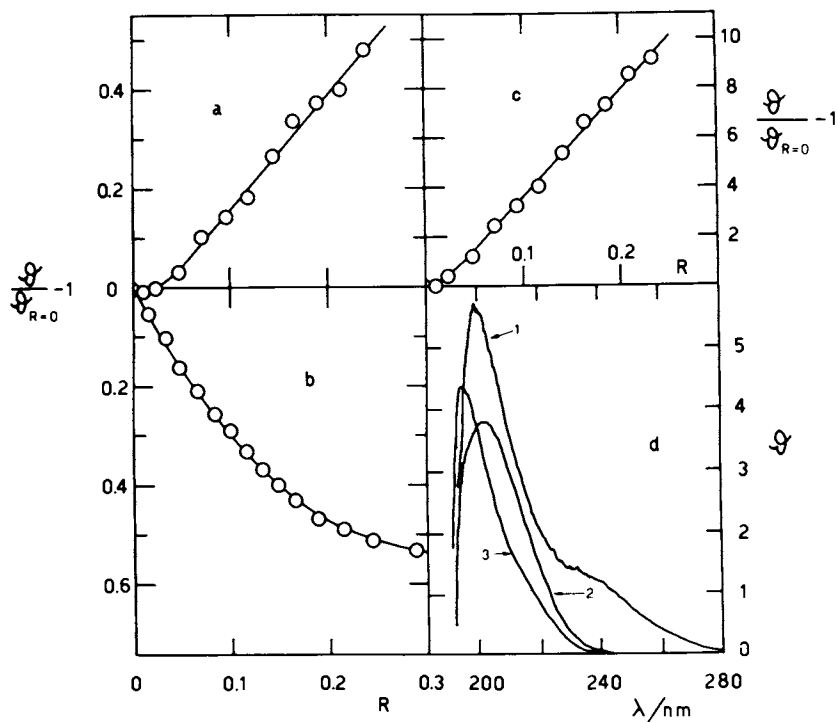


Figure 4. Dependence on  $R$  of the specific change in ellipticity of sodium pectate in 0.05 M aqueous  $\text{NaClO}_4$  at 25°C: a,  $\text{Cu}^{2+}$   $\lambda = 200$  nm; b,  $\text{Ca}^{2+}$   $\lambda = 210$  nm; c,  $\text{Cu}^{2+}$   $\lambda = 235$  nm. d: sample-case CD spectra of sodium pectate in the absence (2) and in the presence of  $\text{Cu}^{2+}$  (1),  $R = 0.24$ , and of  $\text{Ca}^{2+}$  (3),  $R = 0.29$ , respectively.

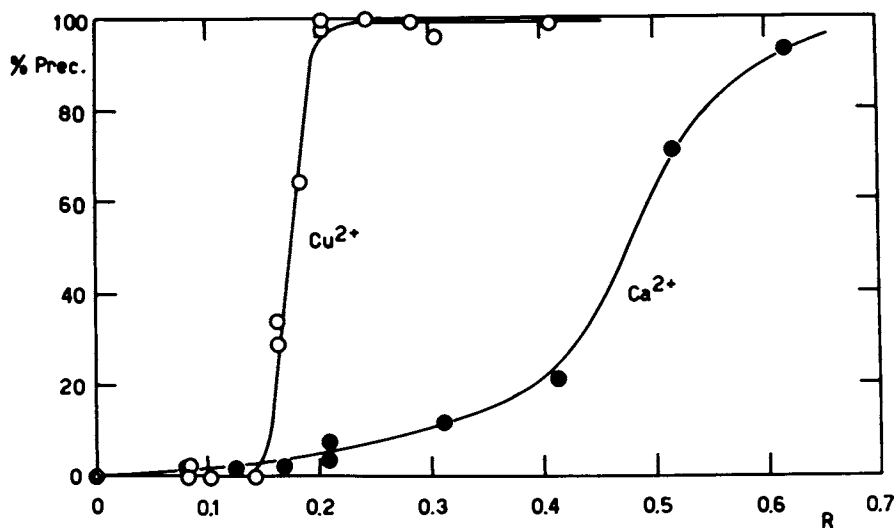


Figure 5. Weight percentage of pectate precipitated from a 0.05 M aqueous  $\text{NaClO}_4$  solution upon increasing addition of divalent ions.

copper ions is more than three times larger than that of calcium ions, in the same conditions of temperature (22°C) and ionic strength (0.05 M NaClO<sub>4</sub>). Indeed, the actual amount of precipitate is almost zero up to a value of  $R = 0.15$ , for both calcium and copper, and suddenly increases with a sigmoidal dependence, very characteristic of an all-or-none process, particularly in the case of copper.

Osmometric experiments have been carried out as function of polymer concentration ( $C_p$ ) and of  $R$ , in the presence of 0.1 M NaClO<sub>4</sub>. This ionic strength was requested in order to minimize the Donnan effect, which otherwise could seriously infirm the significance of the results. The molecular weight of the polymer (Figure 6) increases by a factor two for copper and almost four for calcium, before any visible precipitate could be detected. Apart from being the first reported data of a direct determination of the molecular weight of pectate on increasing the fraction of bound ions per repeating unit ( $r$ ), the results of Figure 6 show that, for a given amount of bound ions, the relative increase of  $\bar{M}_n$  is larger for calcium than for copper. This could stem from a higher tendency of copper ions to give rise to long, linear stretches of bound species between two pectate chains, while calcium ions might be able to form shorter but more evenly dispersed junctions, with more effective branching ability. Provided a definite correlation will be proved between the extent of chain-chain association within a junction and the strength of the resulting gel (23), then some recent rheological results showing that Cu<sup>2+</sup>/pectate gels are stronger than Ca<sup>2+</sup>/pectate gels (24) could well find a molecular counterpart in the present  $\bar{M}_n$  data.

It should be noted that the values of the  $\bar{M}_n$ , reported in the Figure 6, are calculated from the reduced osmotic pressure extrapolated to zero polymer concentration, whereas all the other results reported in this work have been obtained at finite polymer concentration and therefore may include terms arising from the concentration dependence of the investigated property.

### Rheology of mixed H<sup>+</sup>/Ca<sup>2+</sup> pectate gels

At the beginning of an investigation on the rheology of pectate gels, we were faced with the problem of finding suitable measuring conditions to determine viscoelastic parameters of such systems by using a rotating Couette type rheometer. The most effective way of preparing a gel in the measuring compartment turned out to be that developed by Toft and described in details in another Chapter of this book (25). Briefly, an amount of D-glucono- $\delta$ -lactone is added to a neutral solution containing pectate and CaEDTA. The number of moles of lactone is four times as large as that of the repeating units of pectate. The slow hydrolysis of the lactone homogeneous-



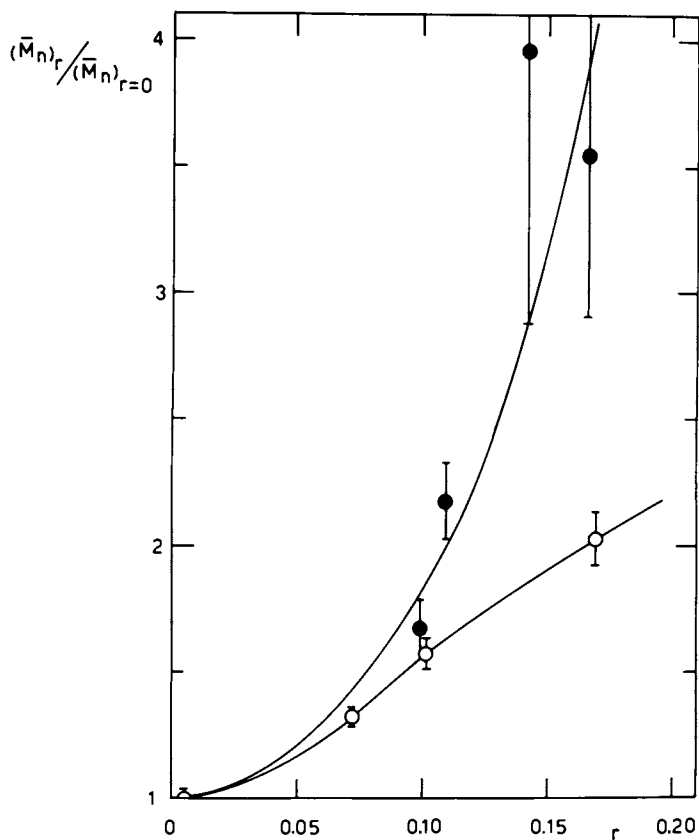


Figure 6. Dependence on the molar fraction of bound divalent ions,  $r$ , of the apparent number-average molecular weight at  $r$ ,  $\bar{M}_n$ , relative to  $\bar{M}_n$  determined in the absence of divalent ions. (●)  $\text{Ca}^{2+}$ , (○)  $\text{Cu}^{2+}$ . Solvent 0.1 M aqueous  $\text{NaClO}_4$ .  $T = 25^\circ\text{C}$ .

sly lowers the pH of the system, thereby determining a uniform release of  $\text{Ca}^{2+}$  ions and the formation of a mixed  $\text{H}^+/\text{Ca}^{2+}$  gel. It was possible to follow the time course of the reaction by using the rheometer in the oscillating mode. The measuring bob was allowed to oscillate at fixed frequency (0.05 Hz) and with constant amplitude ( $20^\circ$ ) and the  $\tau$  signal was recorded as a function of time (i.e. angle). The graphical result was a Lissajous figure, each of which was recorded in a period of time which is one or two orders of magnitude shorter than the total time course of the reaction (Figure 7, a-c). The Lissajous figures have been subjected to Fourier analysis, which showed that only the harmonics up to the third were significant. Each harmonic (corresponding to a complex viscosity  $\eta^{*,i}$ ) was in turn separated into the viscous and into the elastic components,  $\eta'^{*,i}$  and  $\eta''^{*,i}$ , respectively. The onset of gel formation is marked after about 35 min by a rapid increase of  $\eta^{*,1}$ , and of both its components, although the viscous one is dominant (Figure 7, right). The third harmonic,  $\eta^{*,3}$ , which an analysis reported elsewhere (24) shows to be of largely elastic nature, slowly increases and after about 70 min becomes larger than  $\eta''^{*,1}$ . This could indicate that the albeit small oscillating movement imposed on the gel determines the formation of (at least) two levels of structure, the second one possibly related to a reshuffling of the chains to acquire a better degree of chain-chain interaction. Work is in progress to test the effect of the many experimental variables of the rheological behavior of mixed pectate gels, and in particular to gain information on the separate enthalpic and entropic contributions to the elasticity from the analysis of temperature dependence of the determined elastic moduli.

### Experimental

Sodium pectate was obtained by purification and successive neutralization with NaOH of a sample of pectic acid purchased from Sigma Chemical Co. (Catalogue No. P-3889, sold as polygalacturonic acid). Purification and preparation of the solutions have been previously reported (5). All cations were used in the form of perchlorates. Preparation and purification of copper perchlorate have been reported (10).

Experimental methods and data elaboration of the calorimetric, volumetric, and spectroscopic experiments have been reported (5, 15). In the phase equilibrium experiments (precipitation) the determination of the polymer concentration was made by polarimetric measurement on the supernatant, after centrifugation at 3000 rpm for 30 min of the solutions which had been equilibrated for 24 h.

Osmometric measurements were carried out using a Melabs Mod. CSM-2 membrane osmometer following standard procedures. Details of the instrumentation and on the

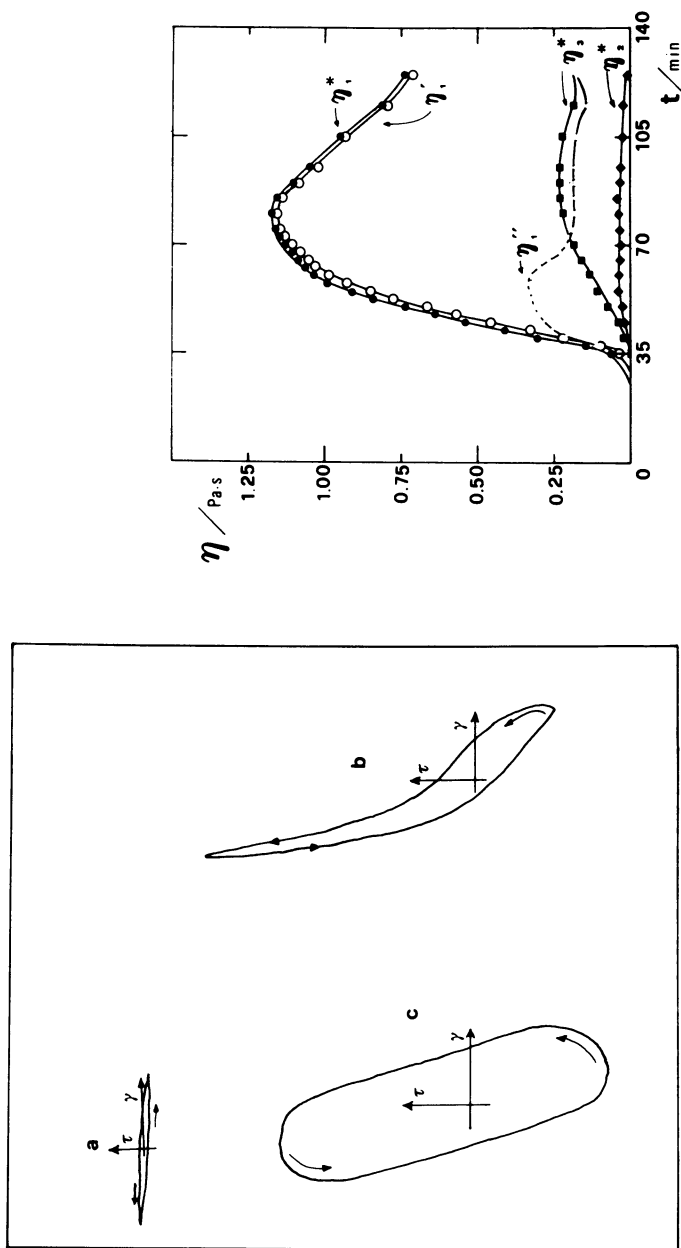


Figure 7. Rheogram of pectate solutions containing glucono-lactone and CaEDTA at 25°C. Left, a-c: Lissajous figures obtained at different times during the course of the hydrolysis reaction. Right: time course of the complex viscosity components,  $\eta''$ , 1-3, and of the viscous,  $\eta'$ , 1, and elastic,  $\eta''$ , 1, components of  $\eta^*$ , 1.

procedure of data analysis for the rheological measurements are given elsewhere (24).

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### Legend of Symbols

- $C_p$  molar polymer concentration  
 $M_n$  number average molecular weight  
 $R$  ion to polymer molar ratio  
 $r$  fraction of bound ions per polymeric repeating unit  
 $B$  Smidsrød's parameter of stiffness  
 $\psi_i, \phi_i$  conformational angles at glycosidic oxygen in  $\alpha$ -D-galacturonosyl-1,4- $\alpha$ -D-galacturonic acid dimer  
 $E(\psi_i-\phi_i)$  total conformational energy of  $\alpha$ -D-galacturonosyl-1,4- $\alpha$ -D-galacturonic acid dimer  
 $\eta^{*,i}$  complex viscosity, i-th harmonic  
 $\eta'_{,i}$  viscous component of the complex viscosity, i-th harmonic  
 $\eta''_{,i}$  elastic component of the complex viscosity, i-th harmonic

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## Pectin Internal Gel Strength: Theory, Measurement, and Methodology

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The single largest use of pectin is in the manufacture of jelly. About 80 to 90% of the 6 to 7 million kg of commercial pectin is used to make jelly and similar products. The consumer's acceptance, organoleptic properties, and quality of a jelly are largely dependent on the internal gel strength formed by pectin. Most of this pectin is high methoxyl and its gel structure is primarily stabilized by hydrogen bonding and hydrophobic interactions. The strength of the gel is affected by the pH of the product, the pectin's methoxyl content, and molecular weight.

This review discusses the above factors and the instrumentation used to measure the internal strength of high methoxyl pectin gels. These are categorized into instruments which are nondestructive of the gel and measures its elastic properties or destructive type instruments which exceed the gel's strength. Examples and advantages of each type of instrument are presented. It is hoped this review will be helpful to jelly and pectin manufacturers who are currently evaluating instruments to measure internal gel strength.

Pectin is a group designation for colloidal polygalacturonic acids, the chemistry of which has been discussed in previous chapters. Most plants contain pectin in the intercellular layer between the primary cell walls of adjoining cells. Six to seven million kg of purified pectin are produced annually, more than half of which is extracted from citrus peel (1). Of this amount, 80 to 90% is used in the manufacture of jellies, jams, and similar products (2). In this chapter, jelly will be used to denote the product formed from pectin, sugar, and acid under specific conditions. Gel will mean a similar physical state but not the commercial product (3).

In manufacturing fruit jellies, pectin is usually added to augment the pectin naturally occurring in the fruit juice in order to achieve the desired firmness or consistency of the jelly. Jelly

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is made from not less than 45 parts by weight of fruit juice with 55 parts sugar for a total of not less than 65% soluble solids. Jams and marmalades are similar to jellies but utilize the whole fruit, fruit pieces, or pieces of citrus peel (4, 5). A U.S. Grade A jelly must not score less than 90 points on a 100 point scale where consistency, color, and flavor of the gel represent 40, 20, and 40 points, respectively.

A good consistency means "the jelly has a tender to slightly firm texture and retains a compact shape without excessive syneresis (weeping)" (4). The levels of pectin use range from 0.1 to 1.0% in the final jelly or jam and are critical to its success (6). There are several good review articles which cover other uses of pectin and contain formulas and practical information (5, 7, 8-18).

Commercial pectins are generally recognized as safe (GRAS) food ingredients under FDA regulations (19) and are classified into two main categories according to their degree of methylation (DM). Low methoxyl (LM) pectins have from 25 to 50% DM and will form a gel in the presence of divalent cations, such as calcium. The second main type of pectin is the high methoxyl (HM) pectin with DM values ranging from 50 to 80% (7, 20). HM pectins will form gels in the presence of sugar and acid and are further divided into broad categories of rapid set pectins, 75 to 72% DM, which gel in 20 to 70 sec; medium set pectins, 71 to 68% DM, which gel in 100 to 150 sec; and slow set pectins, 66 to 62% DM, which gel in 180 to 250 sec (Table I) (8).

Table I. Categories of High Methoxyl Pectin (Citrus) with Approximate Degree of Methylation, Setting Times, and Temperatures

	Degree of methylation	Setting time (sec)	Setting temperature (°C)
Ultra rapid	>75	--	--
Rapid set	75 - 72	20 - 70	97 - 95
Medium set	71 - 68	100 - 150	92 - 87
Slow set	66 - 62	180 - 250	83 - 72

#### Reference (8)

This review will discuss the needs of the jelly manufacturer to measure gel strength, the theory of gel formation, factors affecting gel strength, and instrumentation used to measure gel strength.

**Jelly Manufacturer's Needs.** During the first half of this century, there was no uniform method to measure the ability of pectin to form a gel. Consequently, jelly manufacturers had to constantly adjust the amount of pectin used per batch of jelly with little assurance that the appearance and texture of the finished product would be of a high quality. After the adoption of the Institute of Food Technologist's (IFT-SAG) method in 1959, all pectins were standardized on their ability to form a gel. This method has been used by pectin and jelly manufacturers for more than 25 years. The details are discussed later in this chapter. Although the IFT-SAG method has been the pectin grading standard, it has some

deficiencies in measuring internal gel strength. So, manufacturers are looking for a supplemental method for pectin evaluation which would correlate well with spreadability, be in close agreement with commercial practice, and give reproducible results. The equipment should be durable, operator independent, and inexpensive. Ideally, the pectin should be added to the jelly test batch, as in commercial operations, so it will be in contact with the acid and sugar during the last phase of cooking. If the test were relatively rapid, it could provide real time feedback for control of the next batch of jelly. Finally, the test method should relate to the consumer's perception of texture, be in accordance with the theory of gel formation, and be based on fundamental test principles.

Theory of Gel Formation. It is important to understand the forces that stabilize a pectin gel structure and the mechanisms of pectin gelation before examining the instruments used to measure internal gel strength. Pectin was defined by Kertesz (3) as polymers of colloidal polygalacturonic acid containing a significant proportion of methyl ester groups and having capabilities of forming gels with sugar and acid. Pectinic acids are composed primarily of  $\alpha$ 1,4 linked D-galacturonic acid units. In plants, pectin is thought of as a mixture of structural, carbohydrate molecules with a generalized rather than specific composition. Pectins have a localized distribution of covalently attached neutral sugars forming hairy and smooth regions (21).

Rees (22) proposed four levels of structure for polysaccharides like pectin. Primary structure refers to the nature and mode of linkage of the component galacturonic acids. In pectin, poly-D-galacturonate forms a regular, buckled, two-fold conformation (23) except where a kink is formed by the insertion of L-rhamnose or another sugar. Secondary structure refers to the pyranose ring shapes. Nelson (20) reviewed the evidence that the 4-C-1 conformation is favored due to minimization of the steric repulsion between axial substituents. The restrictive rotational angles (anomeric carbon one to pendent oxygen) and (carbon four to pendent oxygen) determine the overall conformation of the polysaccharide chain. Tertiary structure of pectin is the specific, rigid rod-like geometry which is favored by noncovalent interactions, rigid secondary structure, efficient packing, and is inhibited by loss of conformational energy, hydration, intermolecular electrostatic repulsion, and structural irregularities. Tertiary structures may remain intact even after hydration in solution or in a gel network. Quaternary structure is the interaction of specific, rigid units of tertiary structure to form a higher level of organization (22).

Studies on the forces that stabilize the pectin gel network have contributed to our understanding of the mechanism of gelation. Owens and Maclay (24) reported that hydrogen bonding between hydroxyls of pectin, water, and sugar was promoted by a decrease in pH, due to a decrease in intermolecular, electrostatic repulsion. In addition, weak van der Waals forces between methyl ester groups contribute to the stabilization of the gel network (24). McCready and Owens (25) and Morris et al. (26) proposed that ester groups make a positive contribution to interchain association. Morris et al. (26) observed a decrease in gel strength of 72% ester pectin



gels in the presence of 8M urea and proposed that the gel network is stabilized by noncovalent forces analogous to forces stabilizing tertiary structure in proteins.

Doesburg (27) discussed the phenomenon of gelation and defined gels as two-phase systems with a discontinuous phase of solid material which restricts a finely dispersed or continuous aqueous phase. Nelson (20) proposed a gel model in which the pectin molecules were random ribbons in the sol state, progressing to less random ribbons of lower water activity in the second state, and were a gel in the third state where most of the pectin chains were involved in some type of chain stacking. In a HM pectin gel, acid, sugar, water, and pectin interact to form a gel. At lower pH values near pH 3, electrostatic repulsion is minimized due to the greater number of nonionized carboxyl groups which enhance the probability of noncovalent attraction among the methoxyl, alcohol, and carboxyl groups (24). The function of sugar has been less clear. It has been suggested that the sugar acts as a dehydrating agent (28) and promotes hydrogen bonding between pectin, water, and sugar (29). Rees (30) suggested that sugar controlled the water activity.

Further progress towards understanding the interrelationship of sugar, acid, water, and HM pectin has been made by Oakenfull and Scott (31). They proposed that hydrophobic interactions are essential to gel formation in HM pectin. However, the contribution of hydrogen bonding to the standard free energy of formation is nearly twice that of hydrophobic interactions and hydrogen bonding alone was not sufficient to overcome the entropy barrier to gelation from the loss of disorder (31). Even though sucrose increased hydrophobic interactions by 67% (32), other polyols, such as sorbitol were more efficient at stabilizing hydrophobic interactions than sucrose (32). They further reported that the formation of junction zones from 18 to 250 galacturonic acid units in length, between two pectin molecules, stabilized the pectin gel network (32, 33). The length of the junction zone depends on the hydrophobic interactions, which depend on the concentration of sugar or polyol and on the DM value of pectin (31).

Consideration of the gel formation for LM pectins has been purposely omitted because of space limitations. References helpful in understanding the formation of LM gels are 2, 23, 27, 34, 35.

#### Factors Affecting Internal Gel Strength

Several of the factors that affect gel strength will be discussed such as pH, molecular weight, type of pectin, and conditions used to test the gel. The majority of pectin is used in the manufacture of jellies and jams, so this review will focus on HM pectins. Jelly grade is defined as the number of grams of sugar with which one gram of pectin will form a 65% soluble solids gel of specified strength under suitable acid conditions (3). Olliver (36) pointed out two omissions in this definition. First, no reference is made to a standard gel strength for the "set" of the jelly. Second, from the definition, the amount of sugar is the variable; however, in practice, the sugar concentration is held constant at 65% soluble solids; therefore, it is the amount of pectin that is varied.

Pectin. The heterogeneity of pectin is difficult to control during manufacture, not well characterized, and influences gel strength in commercial jelly batches. Pectin is a naturally occurring polysaccharide which is mainly extracted from citrus peel and apple pomace. Researchers have reported on differences among the yield and jelly grade of pectins from various citrus cultivars (37, 38), between fresh and dry citrus peel (39), and in pectin extraction conditions (40, 41). Furthermore, there are differences among apple starting materials (27, 42) and differences between the resultant pectins made from citrus and apple (43).

In addition to natural variation in commercially prepared pectins, the DM of pectin influences internal gel strength. The sensitivity of pectin to divalent cations and the mechanism of gel formation is influenced by the DM value. Furthermore, jelly grade can be increased by decreasing the DM value by saponification. Doesburg and Grevers (44) showed that the jelly grade was 25% higher in a 53% DM pectin than in a 75% DM pectin made from the same starting material. Wiles and Smit (45) have a patent to prepare a low DM pectin with a high molecular weight (MW) which produces a gel with an extremely high rupture strength. Additional variability exists because DM values are heterogeneous among pectins. Walter and Sherman (46) found that at a specific DM value, the physical properties of a HM pectin are influenced by the dispersion and location of free carboxyls. Another HM pectin with a similar DM, but different distribution of unmethoxylated carboxyls, possessed different physical characteristics.

The DM interacts with gel pH to determine the maximum gel strength measurement. In the typical curve shown in Figure 1, the optimum firmness occurs at higher pH values for higher DM pectins (7, 47).

Molecular Weight. Swenson et al. (48) determined that instruments used to measure the breaking strength of pectin gels assign a higher grade to highly polymerized, higher MW pectins than do instruments measuring elasticity. Christensen (49) supported this by showing that the relationship between breaking strength and sag was not constant but depended on the MW of the pectin. Pectins whose molecular weights were near 135,000 were graded equally by these two methods. When the MW was greater than 135,000, the destructive measurements graded the pectins higher. Mitchell and Blanshard (50) wrote pectin gels have either short and very stiff or longer and more flexible chains. The elasticity modulus is influenced primarily by the short stiff chains and is independent of pectin MW above a minimum. Breaking strength is influenced primarily by the longer, more flexible chains which are largely MW dependent and remain cross linked after the short stiff chains have ruptured. Mitchell (51, 52) also found measurements of the apparent elastic modulus at short times (like the Ridgimeter reading) to be independent of MW, above a certain limiting value. Thus, a pectin with a high Ridgimeter grade would be expected to have a greater number of short stiff chains and a higher cross link density. This property may be controlled to a certain extent by pectin manufacturers and should be selected for by pectin users for products needing a high elasticity characteristic.

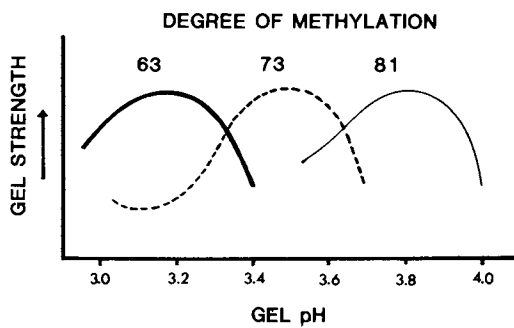


Figure 1. Relationship among gel strength, degree of methylation and gel pH (Ehrlich, 7).

Jelly pH. Another factor which influences the internal gel strength is the jelly pH. In the laboratory, Olliver (53) found pH differences between 2.82 and 3.12 could cause a variation in jelly grade of about 30%. The interactions between jelly pH and gel strength is shown in Figure 1. Acids are used to control the pH within the gelling range. The optimum pH is about 3.1 and 3.4 for slow set HM pectins and rapid set pectins, respectively (47).

Jelly Test Conditions. Other factors affecting the internal gel strength are the rate and duration of boiling a test jelly, stage of acid addition, rate of cooling, time of aging, type of sugar used (53, 54), and the use of buffer salts or synthetic fruit juices rather than water (43).

### III. Instrumentation

Several good reviews have been written on instruments used to measure pectin's gel strength, including Olliver (36), Christensen (49), Beltman and Pilnik (55), Mitchell (51, 52), and Sherman (56). Both Mitchell (51) and Sherman (56) have concise Tables which list about 20 instruments and give types of measurements which can be made. Table II gives examples of instruments used to evaluate pectin gels which will be covered in this review.

Ideally, instrumentation to measure the internal gel strength of a pectin gel should emulate the perception of gel structure by the consumer. This perception of gel structure by the consumer is an interaction of sight, taste, and texture. Visual observations are combined with the sensation of touch when a jelly is cut from a jar and spread. In the mouth, the gel is compressed, fractured, and the ruptured parts slide past each other (56). Most polysaccharide gels are composed of different textural profiles. Therefore, when a series of gels is ranked, the order can vary greatly depending on the weight the consumer places on each individual attribute (57). A sensory evaluation rating scale has been developed for several gel texture attributes (58). Models have also been developed to relate spreadability as being the inverse of the force (shear stress) exerted on the surface of a knife (59). Given that consumers use a wide range of evaluation methods to determine the texture of a gel, it is extremely difficult to relate all of the perceived textural characteristics to a single instrument.

Sherman (56) and Mitchell (51) have pointed out that despite the vast range of textural-instruments, satisfactory instruments are not yet available to measure all textural properties. The elastic modulus is defined as the ratio of stress to strain. Stress is the force per unit area producing the change in shape and strain is the change in shape or change in length per unit length of the sample (3). If the strain (displacement) is small, it is essentially linear to stress (applied force) and the gel will resume its original shape after the force is removed. The elastic limit of the gel is the largest amount of deformation that an elastic body can experience and still regain its original shape after removing the force.

Creep compliance is similar to elasticity except a constant force (stress) is applied to the gel and the deformation (strain) is followed over time. Alternatively, stress relaxation can be used

Table II. Examples of instruments used to examine pectin gels

Type of instrument	Stress applied to gel	Parameter measured	Price range
<u>I. Non-destructive</u>			
A. IFT-SAG	force of gravity	percent sag	inexpensive
B. Wageningen sag	force of gravity	amount of sag magnified 10 fold	inexpensive
C. F.I.R.A.	torquing blade immersed in gel	amount of water to make predetermined deflection	inexpensive
D. Concentric cylinders	torquing corrugated cylinders	constant stress/strain creep compliance stress relaxation	inexpensive to expensive
E. Parallel plates	sliding corrugated plates	strain creep compliance	inexpensive to expensive
F. Dynamic	oscillator	change in velocity of waves	inexpensive to expensive
<u>II. Destructive</u>			
A. Finger	pressure between thumb and forefinger	relative force to break gel	inexpensive
B. Tarr-Baker	syringe piston pressing on gel's surface	relative amount of heavy liquid to rupture gel	inexpensive
C. Luers-Lochmuller	pulling figure from a gel	weight to rupture gel	inexpensive
D. Herbstreith-Pektinometer	pulling figure from a gel	force to rupture gel	moderately expensive
E. Instron	fixtures used in either compression or tension mode	force peak height	expensive

References (51, 56)

where a constant strain is applied and the stress required to maintain a constant strain over time is measured. Additional background is found in reports by Mitchell (52, 60).

Nondestructive vs. Destructive Tests. The pectin grade depends somewhat on the evaluation method (61). Gel strength instrumentation may be nondestructive which measures the elastic properties of a gel, or destructive which measures the inelastic or breaking strength of a gel. A major pectin producer unsuccessfully tried several instruments and methods for more than 20 years to measure both elasticity and rupture strength with a single test (62).

Nondestructive tests have certain advantages including the ability to measure the elasticity of the gel on products such as jellies containing particulates. Nondestructive tests can be used to blend raw pectins to a consistent jelly grade. Also, some reports have found nondestructive instruments to be less expensive and more reliable (63). Mitchell (60) reported that large deformations outside the linear region of a stress vs. strain curve are more difficult to interpret and more difficult to measure than small deformations because rupture occurs at a defect in the gel and large deformations are not as reproducible.

Conversely, destructive tests have advantages over nondestructive tests because they are closer to a consumer's perception of spreading a jelly. The force required to rupture a gel correlated best with gel strength assessed in the mouth (57). Kuiper (64) reported that nondestructive measurements correlated well with the Ridgimeter reading, whereas destructive measurements correlated better with sensory analyses.

#### Nondestructive Tests

IFT-SAG. In the IFT-SAG method, a standardized amount of sugar is cooked with a test amount of pectin. The mixture is poured into a 7.94 cm height jelly glass which contains an excess of acid and allowed to gel for 18 to 24 hours. The jelly is demolded and the amount of sag under the force of gravity is measured with a special micrometer called a Ridgimeter.

The advantages of the IFT-SAG method have been presented (62-65). The method establishes test conditions for reproducibly measuring jelly firmness. At a pH near 2.2, minimal effect of pH on gel strength is observed (36). The effects of temperature and aging are also negligible at this pH (36, 53, 62). The IFT-SAG method uses simple and inexpensive lab equipment. It is precise, reproducible, and subject to minimal operator error. This method is the standard on which comparisons of price of pectins and predictions of the jelling capabilities have been made. Thus, raw pectin can be cut to a 150 grade or a mixture of pectins of known jelly grades can be calculated.

The IFT-SAG method did not win unanimous support at its inception because test conditions do not stimulate how pectins are used in commercial practice. The test pH of 2.2 is ten times more acid than the commercial jelly pH of 3.2 and is well below the maximum gel strength pH (7, 36, 53, 62). The test is based on a water jelly which does not account for the naturally occurring buffers and salts in fruit juice. The low gravity compression rate

of the IFT-SAG test does not adequately predict the gel performance under actual use or other high compression rates (56). The test requires 18 to 24 hours so it is not useful in real time modification during jelly manufacture. Finally, this method of evaluation underestimates the jelly grade of high molecular weight, highly polymerized pectin (49).

Wageningen Sag. Doesburg (66) developed a procedure in which sugar-acid gels were cast into a two piece cylinder. The gel was cut in half and the sag of the gel was measured by a pin attached to a pivitol pointer and a scale. This arrangement amplified the amount of sag ten times. A good correlation between the organoleptic quality of high sugar gels was found with the Wageningen sag measurements.

B.A.R.--F.I.R.A. Jelly Tester. This instrument's measurements are based on a fundamental principle where the elastic modulus is measured by the torque on a blade immersed in a jelly. The torque is applied by water flowing into a bucket and a measurement is made on the amount of water needed to produce a 30° turn of the blade--which is within the elastic limits of the gel (3, 67). Modifications have been made on this instrument using an electric motor connected to a torsion wire to turn the blade. Wires with known torsional moments are used as standards. Studies have shown a strong correlation between Ridgelmeter and F.I.R.A. grading. Other researchers have modified this jelly tester to measure a 15° turn (elasticity), followed by measurement of the breaking strength of gels and have found good correlation to sensory evaluations (55).

Concentric Cylinder Instruments. Saverborn (68) developed an instrument that requires the pectin mixture be poured between two concentric, corrugated cylinders and allowed to set. The inner cylinder is twisted by a torsion wire and the extent of torsion caused in the gel is measured. The corrugations prevent slippage of the gel. Kertesz (3) cites this as one of the finest instruments devised for jelly strength measurements.

Other instruments which use concentric cylinders include those described in (69-72) and were reviewed by Mitchell (51). These instruments can be used for fundamental measurements on gels like creep compliance, stress relaxation, and rigidity modulus.

Parallel Plate. Plashchina et al. (73) studied the creep of HM pectin gels placed between two corrugated parallel plates. Creep compliance curves were obtained for 0.5 to 2.5% pectin at temperatures from 25 to 55°C. Reversible and irreversible strain components were separated. Pectin macromolecules were characterized as being very stiff and only a slight decrease in entropy was required to form a pectin gel (73). Mitchell and Blanshard (50) used an automated parallel plate viscoelastometer to study the creep compliance on low methoxyl pectins. The value of these experiments was that a continuous response was obtained from the gel rather than a single point measurement.

Dynamic Testing. Gels can be characterized by dynamic test in which an oscillatory stress is applied to the gel and the phase

angle of the strain measured. Most gels show a phase difference less than  $90^\circ$  out of phase with the imposed stress. Gross (74) and Gross et al. (75) reviewed dynamic testing and used it to study the textures of LM pectin gels. They determined the dynamic modulus and phase angles and found that at 100 HZ, the gels that appeared to be the most firm were the weakest at 200 HZ. However, they found some good correlations between some of the dynamic tests and sensory evaluations.

#### Destructive Tests

Finger Test. The first destructive tests on a pectin gel were performed by squeezing a slice of jelly between the thumb and forefinger until the gel broke. This was made into a formalized jelly evaluation test called the Finger Test. When the analysts had adequate experience, the results were reproducible and differences of 5% or greater between the test jelly and a 'standard' jelly could be detected (3). Aside from the obvious differences among analysts, a 'standard' jelly must be prepared for each test from a 'standard' pectin which is kept refrigerated to minimize changes in the standard. The characteristics of the 'standard' jelly are not specified (49).

Tarr-Baker. The Tarr-Baker (Delaware Jelly Strength Tester) is based on the work of Tarr (76), Baker (77), and Baker and Woodmansee (78). A force is applied to the gel's surface by a syringe piston powered by compressed air. Measurements can be influenced by a 'skin' on the jelly's surface or uneven application of pressure (3). Swenson et al. (48) modified the Tarr-Baker apparatus to produce a balance-plunger type instrument. Although they found a linear stress-strain region within the elastic limits of the gel; they also found elasticity was somewhat dependent on the rate of loading. Their instrument was capable of several elastic and breaking tests on one gel. There was less than a 2% error between the true and assumed grades using breaking strength. Christensen (49) determined a ratio between a modified 'sag' grade to the breaking strength as measured by the Tarr-Baker apparatus for 22 pectins of varying MW. The breaking/sag ratio ranged from 1.38 to 0.85, but heat or enzyme treatment further reduced the ratio to 0.39. The relationship between sag grade and breaking strength was reported to be dependent on the MW of pectin (49). Doesburg (66) showed lower pH values (2.0 to 2.3) caused short setting times and reduced the internal gel strength compared to pH 3.1 as measured by the Tarr-Baker instrument. He did not observe a good correlation of organoleptic quality to Tarr-Baker measurements.

Luers-Lochmuller Pektinometer. The Pektinometer was developed in Germany (79) and measures the amount of force necessary to pull a metal figure out after being cast inside a pectin gel. A hot jelly is poured into a special container with corrugated sides containing the metal figure. The corrugated sides prevent slippage of the gel as the amount of force necessary to break the gel is measured. Steinhäuser et al. (80) used the Luers-Pektinometer in his comparison of four methods for determining gel strength and found the reproducibility was linear. Uhlenbrock (81) adopted this instrument to determine the breaking strength of sugar-acid gels.



The breaking strength varied from 500 to 600 g and the coefficient of variation was 1 to 2%.

Herbstreith-Pektinometer. Luers-Lochmuller's method has been further refined in the Herbstreith-Pektinometer which uses a specially designed clear, corrugated cup and plastic figure. One hundred grams of the pectin mixture from a laboratory or commercial batch is poured into the cup with a self centering figure. The gel is held for 2 hours at 20°C. The cup is locked in place and a hook is attached to the central figure. An electric motor pulls up on the figure rupturing the gel and the peak force required is measured on a 1 kg strain gauge. The final value is printed out in Pektinometer units.

Instron. The Instron Universal Testing Machine can be used in compression and tension experiments where the force on a load cell is measured while moving the cross head a given distance or time. In evaluating gel structure, fundamental characteristics such as brittleness, hardness, and elasticity can be quantitatively measured and related to sensory attributes such as chewiness and gumminess. Sherman (56) found sample dimension and cross head speed affect these readings. Gels were not linear in their force-compression behavior. Slow cross head speeds can lead to stress relaxation, so from low compression test rates, it was impossible to predict how a gel would behave at high compression rates as in the mouth.

Oakenfull and Scott (32) used an Instron to measure apparent shear moduli and rupture strengths. Although no satisfactory theory could be proposed to relate rupture strength and disruption of intermolecular forces, they did find that hydrophobic interactions play a role in gel formation. Mitchell (52) reviews several investigations using Instron measurements on several types of gels. Gels were tested in compression and the apparent modulus was calculated from the initial slope of the curve and the rupture strength was calculated from the peak force.

### Conclusions

This review points out why a pectin gel's internal strength measurements are important. Some of the important qualities for test methods and instrumentation to measure internal strength are given. It also relates the current theory of the formation of a HM pectin gel and the factors affecting gel strength. There are two general categories of instrumentation—nondestructive and destructive. Examples and advantages of each category are also given.

Internal gel strength measurements are currently under review. In the next few years, new methods and modifications of existing techniques and instrumentation will be developed. These tests will more fully characterize the practical aspects of a pectin's internal gel strength.

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## Characterization of Pectins

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Breaking strength measurements on commercial pectins have shown that different pectins standardized to 150<sup>0</sup> SAG will differ significantly in breaking strength depending on the peel source.

Direct correlations exist between the viscosity of a pectin solution and the breaking strength value. The use of breaking strength values as a means of grading pectin is proposed.

More than 50% of the world's pectin production is used in making jellies, jams, marmalades and confectionery products, and the ability of the pectin to form jellies and gels is therefore a most important property. Consequently it is the jelly forming ability of pectin expressed as the Jelly Grade which determines the commercial value of pectins.

The jelly grade has been determined for more than 30 years by the IFT SAG Method and commercial pectins are today standardized according to this method. Commercial pectins are usually standardized to 150 jelly grade or US SAG. When one analytical method plays such an important role, it is relevant to assure that the results from this test really give the best possible information regarding the use value of that particular pectin and due credit is given to differences arising in pectins of different origin. Also, one would expect that the results are indicative of the final characteristics of the product containing pectin.

New methods have been developed for texture analysis of jams and jellies. These indicate that the SAG test is unreliable for predicting the effect of pectin on the final product. Is the SAG method the pectin analysis for the future, or should it be complemented by other analyses to give a more complete picture of any pectin?

### Jelly Grade/SAG Method

It was realized very early that jelly grade is of outstanding importance to the pectin producer and user alike (1). Nevertheless

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it took a number of years to determine a common method that would give the best possible characterization of this very important function of pectins. In 1926 jelly grade was defined for the first time by Wilson (2). We define jelly grade as the amount of sugar that will jellify one part of pectin under certain prescribed conditions to a standard firmness.

After many years of discussion over the standard jelly for the jelly grade determination, work on a standard method was undertaken by a committee of the IFT. The committee worked for several years on pectin standardization. They selected the Exchange Ridgelimeter as the standard instrument to measure jelly SAG because it was compact, inexpensive, easy to use, and it also gave reproducible results.

Jellies for the tests are prepared in special conical glasses of defined dimensions and with sideboards. After a 24 hour setting time the sideboards are removed, a cleancut surface is made, and the jelly turned on to a glass plate which is positioned under a micrometer screw mounted on the Ridgelimeter. The micrometer screw measures the SAG or the collapse of the jelly.

Probably the pH of the test jelly was discussed most by the Committee. Most jams and jellies are produced in the pH range of 3.0-3.2. Unfortunately test results at these pH values are more erratic. Furthermore, at pH 3.0-3.2, the strength of the jellies increased more upon standing than jellies having a pH below 3.0. Moreover, soon there was strong evidence that the "acid in the glass" procedure at pH below 3.0 would give more reliable results in a shorter time than the same procedure at higher pH values. An agreement was reached on a pH of 2.3-2.4. The method, designated Method 5-54, was published in 1959 (3).

Parallel to the work in the U.S., researchers in England also were working on a method to grade pectin. Simultaneously a method was published which specified soluble solids in the test jelly of 70.5% and a pH of 3.10 (4).

In spite of the work done in England, the IFT SAG Method (sometimes called US SAG) over the years has become the most widely accepted method for the grading of commercial pectin all over the world.

#### Present Status of the SAG Method

From the beginning the IFT Committee realized that their SAG Method was a compromise between conflicting interests. Furthermore the Committee realized that no one test could measure all parameters of pectin performance in a jelly.

There are several negative aspects or shortcomings of the SAG test. The jelly used for the SAG test is not representative of a jelly as the consumer receives it, and this is particularly true with regard to the very low pH, at which the SAG test is performed.

An experiment was conducted in which the pH of SAG jellies was varied. The objective was to determine the effect pH has on SAG grade. Jellies were prepared at pH's between 2.3 and 3.1. The pH was varied by changing the amount of tartaric acid used. Pectin dosage was held constant at .433g. For comparative purposes two different pectins were used, one which is known to be a pure lime peel citrus pectin, and another citrus pectin with mixed peel

origin. As shown in Figure 1, a sharp drop in the SAG value is observed as the pH is increased. As the pH approaches 3.1, the SAG value as measured by the Ridgelimeter drops off dramatically and eventually a pH is reached where the test jellies become so soft that it is impossible to obtain a SAG value using the Ridgelimeter. Strangely, the test for grading the product fails to register or to grade as conditions approach those of actual usage. Figure 1 also shows that two pectins, which have similar SAG values at pH 2.3, will grade quite differently as the pH is increased.

As the jelly sags, the micrometer screw is used to measure the elasticity of the jelly. However, the consumer would much prefer a spreadable jelly, and is certainly not interested in an elastic jelly. Or expressed in other words, the SAG test does not give a very good expression of the jelly characteristics as desired by the consumer.

Furthermore the SAG test cannot be made on a commercial production jelly as it is removed from the production line. As shown by Figure 1, the pH is too high. At a normal jelly pH the SAG value has no meaning.

From discussions with jelly, jam, and marmalade producers in different parts of the world, it was learned that shortcomings of the SAG test with regard to final product characteristics, induced jam and jelly producers to develop other methods to determine the dosage of a particular pectin required to obtain a satisfactory end-product (5).

#### Instrumental Methods

The Ridgelimeter used for the IFT SAG Method is excellent from the point of simplicity and sturdiness. Other methods, however, have been developed with a higher degree of sophistication, but at the same time, they are also more complicated and subject to mechanical errors.

The first instrument was developed by Luers in Germany as early as 1927 and it is the so-called Pectinometer (Fig. 2) (6).

The early version of the Luers/Lochmuller Pectinometer was based on a special corrugated sample cup. A plate is placed at the bottom of the cup before the jelly is poured. After allowing the jelly to set for a standard amount of time, weights are placed on the pan to withdraw the plate from the jelly. The Pectinometer measures the weight necessary to break the jelly with the bottom plate. This instrument has gone through several stages of development, and is today represented by a sophisticated electronic version which was developed by the German company, Herbstreith, and is called the Herbstreith Pectinometer (Figure 3).

The basic principle of operation is still the same. The instrument consists of a corrugated sample cup with an open plastic insert placed a short distance over the bottom of the sample cup. The plastic insert is pulled out of the jelly by means of a motor and a pulley, on which is mounted a load cell. Loads are registered on a digital display. At the end of a test cycle the peak value in Breaking Strength Units will be printed out automatically.

Increasingly, this instrument is gaining popularity as the basis of a fast and reliable method for measuring Breaking Strength

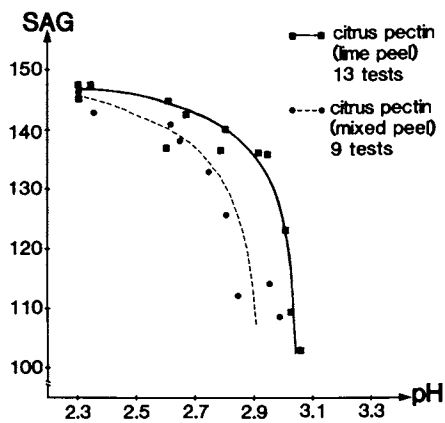


Figure 1 - Effect of pH on Measured SAG of Pectin Jellies

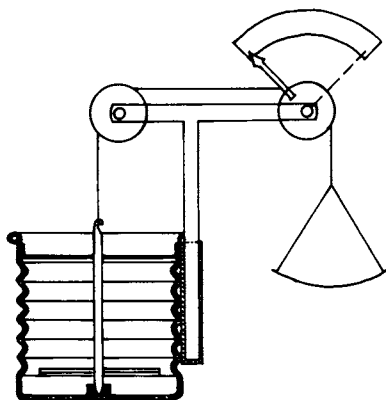
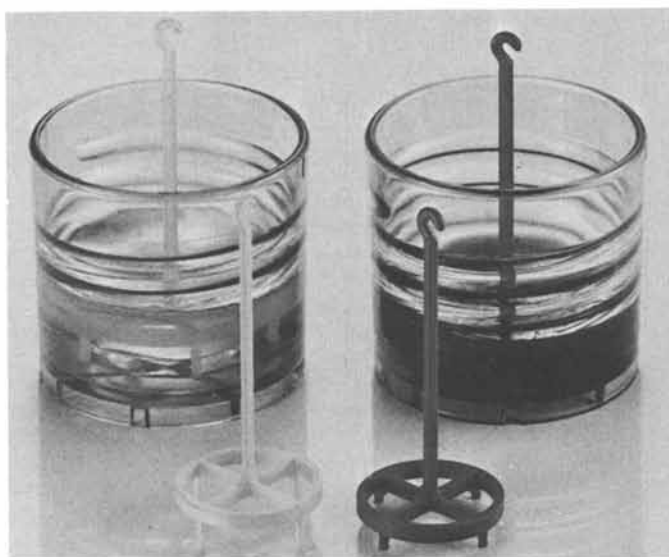
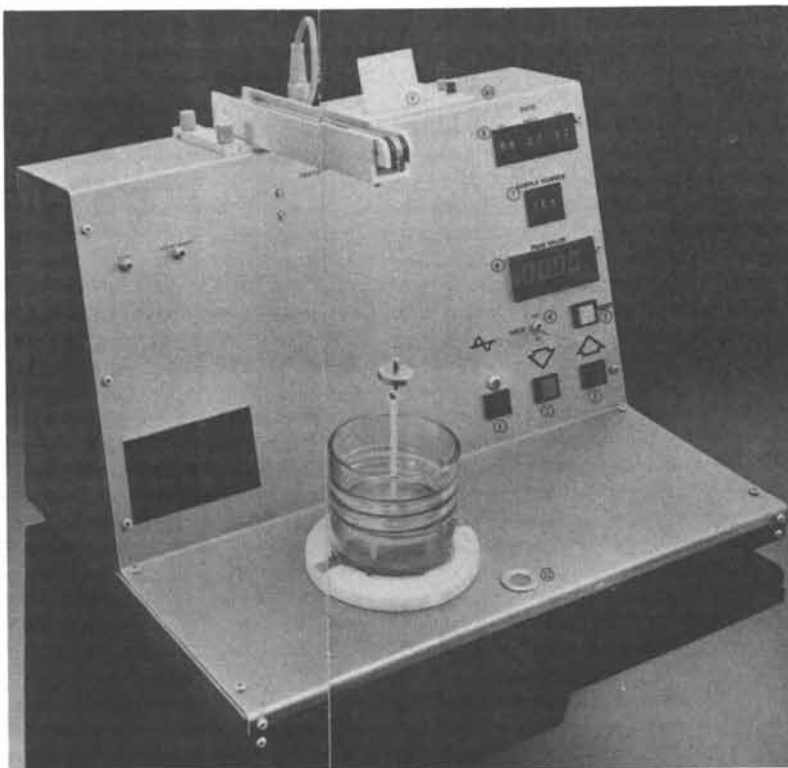


Figure 2 - The Luers and Lochmuller Pectinometer





**Figure 3. The Herbstreith Pectinometer.**

of pectins. The pH of the test jelly is 3.05 and is obtained by using a buffered pectin solution. Measurements are made after only two hours of setting, which makes it one of the quickest methods available. This method is also recommended for use on samples straight off the production line. As far as is known, this is the only test method which measures Breaking Strength from inside the jelly and thereby eliminates any problems with skin formation on the surface of the test jelly.

In recent years various test instruments have been developed to measure texture of a number of food products. Some of these instruments also have been used to test jellies. One of these is the Voland Stevens Texture Analyzer (Fig. 4).

The instrument is programmed to lower a plunger into a gel at a specific speed and to a specific depth. A load cell measures resistance which can be obtained from a digital display or a connected recorder.

When the instrument is used on a jelly with a plunger velocity of 0.5 mm/sec. and a penetration of 5 mm, a typical curve shown in Figure 5 is obtained. The penetration is chosen to exceed the elastic limit of the jelly. Above the elastic limit a sharp increase in the force or the load is seen until the jelly breaks, and then a sharp drop-off is observed followed by some compression effects.

#### Comparison of Various Methods for Pectin Grading

Limitations of the IFT SAG method with respect to pH have been demonstrated. Similar limitations have been reported by other laboratories (7). Since the Herbstreith Pectinometer and the Voland Stevens Texture Analyzer have been developed recently, it could be of interest to see how the jelly characteristics measured by these instruments correspond to the official results as expressed by IFT SAG.

Standard apple jellies were prepared having a soluble solids of 65% and a pH of 3.15. Five commercial citrus pectins standardized to 150° SAG were used in the study. Pectin dosage was varied between 0.1% and 0.5%. The resulting jellies were tested for Breaking Strength or internal strength on the Voland Stevens Tester. The results are shown in Figure 6.

Internal strength was plotted against pectin dosage. Four of the pectins are relatively close together whereas one is at a constantly higher level. At a pectin dosage of 0.35%, which would be considered fairly normal in the industry, an internal strength of approximately 55 g. is obtained which could be considered as an average value as this is grossly the value represented by four of the five pectins in the test. For pectin No. 1 an internal strength equal to the other four pectins is obtained with a dosage of only 0.27-0.28%. By the SAG test, all five pectins were judged to be of the same quality. Four of the pectins in the test were commercial citrus pectins of mixed peel origin, whereas sample No. 1 with the constantly higher internal strength readings is a pectin known to have been made from pure lime peels.

These surprising results on five pectins which were supposed to be equal led to further testing where other pectin samples were tested using the Pectinometer in addition to the Voland Stevens

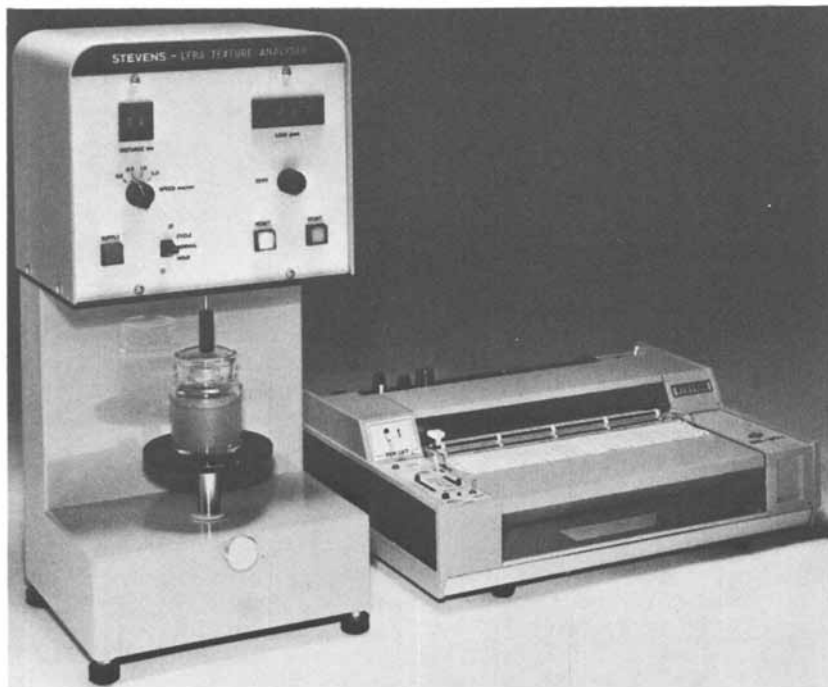


Figure 4 - The Voland Stevens Texture Analyzer

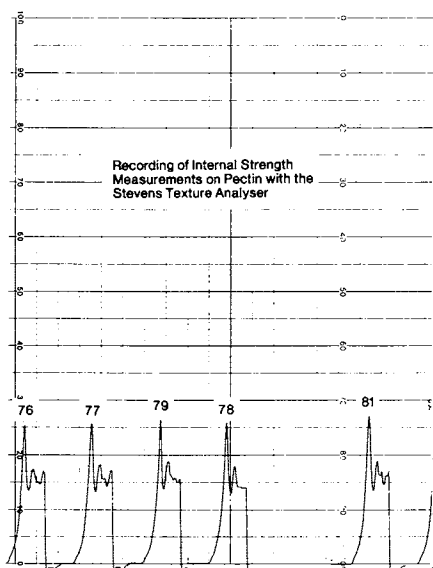


Figure 5 - Internal Strength Curves Obtained with Voland Stevens Texture Analyzer

Tester. Several tests were conducted at the Food R. A. in England (8) using four different commercial pectin samples, of which three are citrus pectins of mixed peel origin and one sample is a pure lime pectin.

The first test was made on a Stevens Texture Analyser using a plunger slightly different from the one used in our laboratories, but also made on an apple jelly with pectin dosages ranging from 0.1% to 0.5%. The break load level is slightly different from those obtained in our laboratories, but the trend is the same. Once more, 150° SAG pectins, supposedly of the same strength, when measured by a Texture Analyser under usage conditions appear to be significantly different (Fig. 7). The degree of methoxylation (DM) for all four pectins was  $65 \pm 2$ .

The same test was repeated using the Herbstreith Pectinometer. The same pectins, jelly composition, and pectin dosages were used. The pectinometer gives Breaking Strengths at a totally different level from those obtained using the Volland Stevens Tester, but again a marked difference between the four pectins in question is observed (Fig 8).

Even in the very early days of the commercial pectin industry, molecular weight of the pectin molecule was believed to play an important part in the gel formation, and in particular in the gel strength. As early as 1953 one of the major pectin producers (9) stated that the relationship between SAG Grade Value and Breaking Strength Value of pectin is not constant, but depends on the molecular weight of the pectin. It was further stated that it would be impossible to alter the existing methods of grade determination so that the SAG method would give results which would coincide with Breaking Strength values.

An investigation was conducted to determine to what extent the differences observed between the four 150° SAG pectins used in the breaking strength study could be attributed to differences in molecular weight.

Gel Permeation Chromatography studies were conducted by the Food R. A. on the four pectins used in the Breaking Strength study. Using the results of duplicate runs for the four samples, the distribution coefficients (K) were calculated for each run giving the following results:

#### RESULTS FROM GPC

Sample	K value
CP MP SS200	0.31, 0.29
CP 7384	0.36, 0.38
CP 7978	0.40, 0.42
CP 5725	0.45, 0.45

While it was not possible to determine actual molecular weights from this GPC method at the time of testing, it is possible to make the following assumptions:

The K values are inversely related to average molecular weight, and the results suggest that sample CP MP SS 200 has a

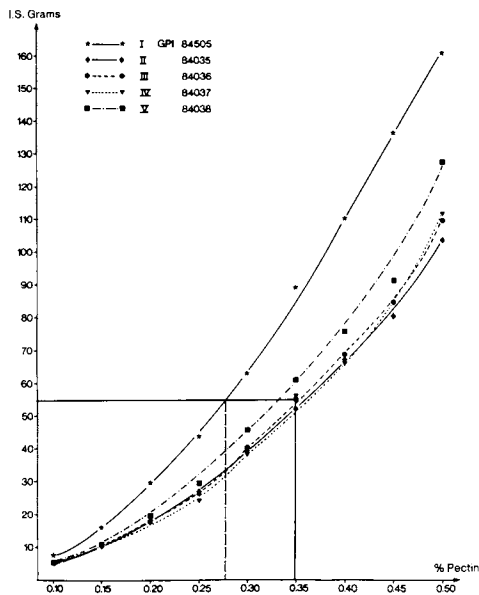


Figure 6 - Internal Strength vs. Pectin Dosage Using the Voland Stevens Texture Analyzer

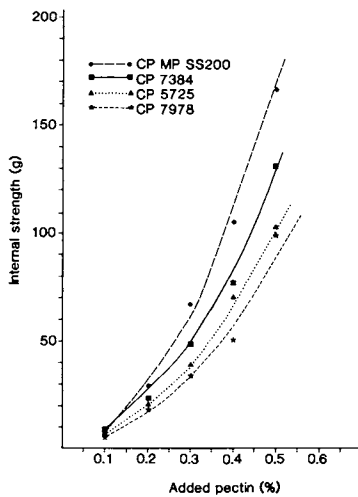


Figure 7 - Internal Strength vs. Pectin Dosage Using the Voland Stevens Texture Analyzer

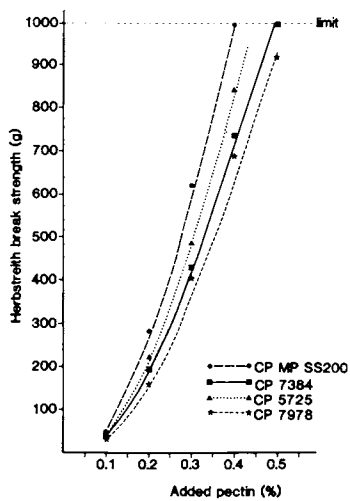


Figure 8 - Internal Strength vs. Pectin Dosage Using the Herbstreith Pectinometer

significantly higher average molecular weight than the other samples.

The four pectin samples used for the GPC were further investigated by the North East Wales Institute (10), using a different technique, which is size exclusion chromatography, to give an index of the relative molecular weights and the molecular weight distributions of these samples.

The elution profiles for the four samples are shown in Figure 9. In each case the broad peak represents the uronic acid, which corresponds to the pectin being eluted. The higher molecular weight samples will have peaks near  $V_0$ . On the side of the curves we have the resulting weight average molecular weight,  $MW$ , and number average molecular weight,  $M_n$ . The range for the weight average molecular weight is from 65,000 up to 150,000, which is a range confirming the results from the GPC method. In the middle row we have the indexed values of  $MW$ .

The results from the GPC and the size exclusion chromatography studies together with a number of intrinsic viscosities that were measured in our laboratories have clearly demonstrated that on a constant and consistent basis citrus pectins derived only from lime peels have a molecular weight superior to citrus pectins of normal mixed peel origin. A citrus pectin of mixed peel origin can consist of any proportion of peels from orange, grape fruit, lemon, and lime.

The differences that were observed in breaking strength or internal strength between the four pectin samples, all of which had a 150 SAG and a degree of methoxylation (DM) of 65, can be attributed to differences in molecular weight. Most investigations so far have worked on slow set pectins, which on the U. S. market is by far the most widely used pectin type.

However, if we look at a wide range of pectin types starting with an extra slow set pectin with a low degree of methoxylation and progress through the various intermediate types up to a rapid set pectin with a high degree of methoxylation, a sharp increase in breaking strength is observed (Fig. 10).

Viscosity values of the same pectins also were measured where the values represent scale units of a Haake Viscosimeter on a two SAG pectin solution. Also here we see a linear relationship between DM value and viscosity. Viscosity measurements are made on solutions which are considerably easier to make than test jellies, and when working under well defined conditions we have found that viscosimetry is a great advantage when studying pectins. Working with viscosimetry we have the added advantage of having a method with direct influence from differences in molecular weights.

A good correlation between breaking strength and two SAG viscosity was found. A regression coefficient of 0.90 was obtained. With better standardization of the cooking procedure, we expect test jellies to give an even higher correlation between breaking strength and viscosity.

We conclude that pectin molecular weight is proportional to degree of methoxylation in that a rapid set pectin with a high degree of methoxylation will require the most gentle type of extraction whereas lower levels of methoxylation are exposed to more adverse extraction conditions. In demethoxylating the pectin molecule, the pectin molecule is also degraded to a certain degree

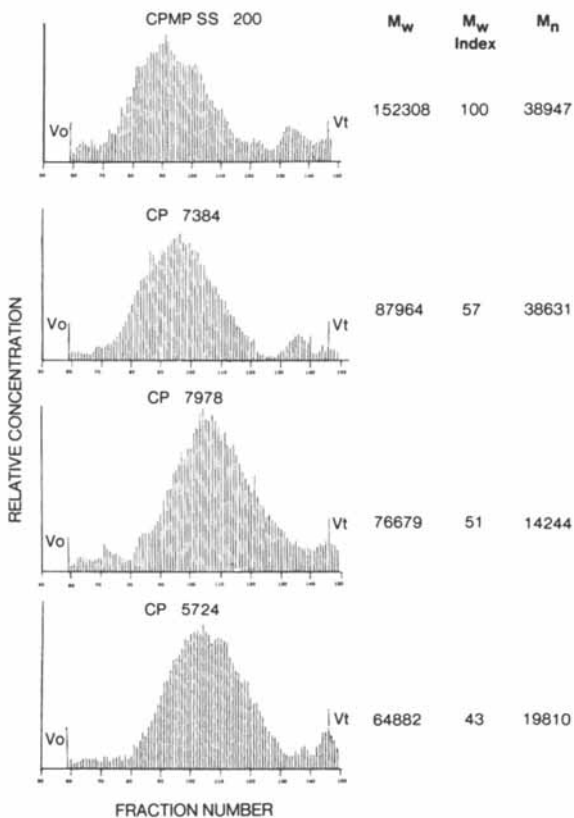


Figure 9 - Elution Profiles from Size Exclusion Chromatography Studies of Pectins

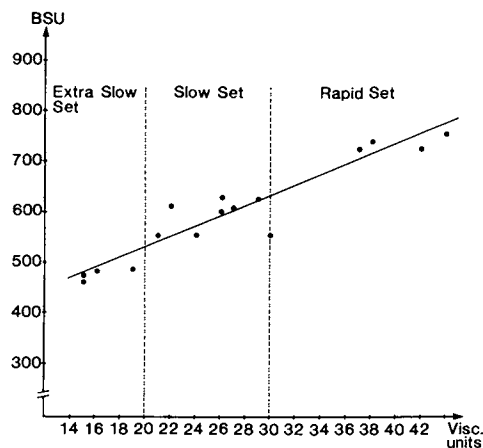


Figure 10 - Breaking Strength of Pectin Jellies and Viscosity of Pectin Solutions Prepared with Pectins of Varying Degrees of Methoxylation



resulting in a decreasing molecular weight. We theorize that the correlation between breaking strength and viscosity at a specific degree of methoxylation is the result of the molecular weight of the pectin.

At the time of the introduction of the SAG method around 1950, apple pectins played an important part of the over-all pectin consumption in the United States, and at the time it was asked whether the SAG method would be fair to apple pectins, which often gave a stronger jelly than a corresponding citrus pectin. Since then the pectin market has changed, and today the predominant type on the U.S. market is citrus pectin, which also has been the basis for this present study, although a differentiation has been made between citrus pectin of mixed peel origin and citrus pectin based on lime peels only. However, high quality apple pectins are still being produced and are used around the world.

Available data indicates that by careful processing it is possible to produce apple pectins with high breaking strength similar to that of lime pectins, and it is no coincidence that the pectinometer in its present form has been developed by an apple pectin producer.

### Conclusion

The SAG method for jelly grade determination has served everyone concerned with pectin for more than 30 years, but we believe that we may have come to the time when aspects other than jelly grade are taken into consideration when evaluating pectins. Everything is becoming more and more consumer oriented, and it would therefore be a logical consequence that the final characteristics of the jam as the consumer receives it are taken into consideration in pectin grading. One of the most important characteristics of a consumer jelly is spreadability which essentially is a rupture of the jelly, and this is one very important characteristic that the SAG test will not describe, whereas the spreadability can be expressed to a certain extent through the use of internal strength or breaking strength.

We have demonstrated that pectins of different origin will differ in breaking strength and thereby give jellies of differing firmness.

However today, the determination of breaking strength has reached a high degree of accuracy and can be quantitative. Thus, pectins can be standardized to a constant internal strength or breaking strength, as is done today with the SAG method. It would be technically possible to produce commercial pectins with standardized internal strength of the same level from one batch to the next. This standardization to internal strength can either be made on a basis where the present SAG value is forgotten, or a commercial pectin could be standardized to 150<sup>0</sup> SAG as well as to a well defined internal strength value.

The most advanced jelly and jam companies have for several years been working with methods whereby they determine the internal strength of each individual pectin lot in order to adjust the dosage accordingly. This to us demonstrates the need for the pectin industry to standardize to constant internal strength so that the jelly and jam maker can be assured of a constant and equal pectin dosage from one pectin lot to the next.

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## Synergistic Gelation of Alginates and Pectins

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Mixtures of alginate and high methoxy pectins formed thermoreversible gels below pH 3.8 without any addition of sugar, provided that D-glucono-delta-lactone (GDL) was used as a slow acidifier in the cold. The gelling occurred at conditions where neither alginate nor the high methoxy pectin gelled alone. The strength and melting points of these synergistic gels increase with decreasing pH and increasing content of L-guluronic acid residues in the alginates. The gelling effect was correlated to the sequential distribution of the two monomers, D-mannuronic (M) and L-guluronic acid (G) residues, in the alginate chain. The results indicated that "blocks" of at least four contiguous G-units were necessary for gelling to occur. The results were discussed in view of existing molecular theories for synergistic gelation.

In a search for thermoreversible gelling systems that could be used in low-sugar, low-calorie jams and jellies, Toft discovered that mixtures of high methoxy pectins and alginates with a high content of L-guluronic acid residues formed gels under conditions where neither alginate nor pectins gelled alone (1). These warm-setting gels were formed below pH 3.8, and could be made with a sugar content as low as 20%. Later Toft reported (2) that thermoreversible gels could be formed without any requirement for sugar, by using D-glucono-delta-lactone (GDL) in the cold as a slow acidifier. Since the number of gel forming polysaccharides allowed for use in the food and pharmaceutical industries is limited, the discovery of new synergistic gelling systems may have potential commercial value, as have the well-known

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synergistic interactions of certain galactomannans with xanthan, agar and kappa-carrageenan (3). Since our molecular understanding of synergistic gel-formation is very limited (4), the pectin-alginate system offers an opportunity to get a general insight into the mechanism of synergistic gelation. This was the object of Thom et al., who used circular dichroism spectroscopy to study the warm setting alginate-pectin gels (5). They proposed a model for the junction zones in the mixed gels in which "blocks" of esterified  $\alpha$ -D-galacturonic acid residues in the pectin chain interacted with "blocks" of  $\alpha$ -L-guluronic acid residues in the alginate chain. This interaction could occur when the uronic acids were mainly in their protonated form, and was sterically favoured because both types of "blocks" contain glycosidic linkages between axial substituents (0-1 and 0-4) thereby giving the same set of repeating distances along similarly ordered chain segments. In the present work we use alginate of different monomer sequence to test this idea. Alginates may now be characterized by both diad and triad-frequencies, due to the development of powerful NMR-methods (6-7) which offer the opportunity to study the sequence requirements in the alginate chain for the interaction with pectin to occur. For lack of similar methods for studying the distribution of methoxy groups along the pectin chain, only one pectin sample with a high degree of methoxylation (70%) is used. Although one paper has recently been published dealing with cold setting alginate-pectin gels (8), it is necessary here to include some results on the effect of pH and calcium ions, and on varying alginate-pectin ratios, before alginates of different sequences are tested.

#### MATERIALS AND METHODS

Only one sample of pectin was used throughout this study, a commercial sample provided by Grindsted Products A/S and labelled Mexpectin RS 400. The degree of methylation of the carboxylgroups as measured by the supplier, was 70%. No information about the sequence of the ester groups or the content of neutral sugar was obtained, but according to the supplier the pectin had been produced from limes (*Citrus aurantifolia*).

Seven different alginate samples were used. Some were supplied by Protan A/S, Drammen, Norway, and others were prepared in the laboratory by standard procedures (9). They all had a weight average degree of polymerization above 500, the lower limit at which chain length affects gel strength in calcium alginate gels (10). They were analyzed for their fractional content of L-guluronic acid, ( $F_G$ ), and D-mannuronic acid, ( $F_M = 1 - F_G$ ), and their fractions of the four diads,  $F_{MM}$ ,  $F_{GG}$ ,  $F_{MG}$  and  $F_{GM}$  by

the NMR-method of Grasdalen *et al.* (11).  $F_{MM}$ ,  $F_{GG}$  etc. are the weight fractions of the sequence  $MM$ ,  $GG$  etc. such that  $F_{MM} + F_{GG} + F_{MG} + F_{GM} = 1$ . The G-centered triads were measured according to the method of Grasdalen (7). There are eight possible triads in a linear binary heteropolysaccharide with weight fractions  $F_{MMM}$ ,  $F_{MMG}$  etc. such that  $F_{MMM} + F_{MMG} + F_{MGM} + F_{GMM} + F_{MGG} + F_{GGM} + F_{GMG} + F_{GGG} = 1$ . For long chains  $F_{MG} = F_{MGG}$  and  $F_{GGM} = F_{MGG}$ . Data for the seven alginate samples are summarized in Table 1.

### Preparation of gels

Pectin-alginate solutions were prepared by dissolving 1.65 g of the polymer mixture slightly moistened with 5 ml ethanol in 125 ml distilled water. Appropriate amounts of GDL were dispersed in 2.5 ml distilled water, and, after complete dissolution immediately stirred into 25 ml of alginate-pectin solution. A portion of these solutions was poured into cylindrical perspex tubes, covered with dialysis membranes at both ends (12) and allowed to gel. Cylindrical pellets of gels with a height of 15 mm and a diameter of 14 mm were then obtained. The remaining portions of the solutions were allowed to gel in the beakers, and used for measurements of pH.

### Determination of mechanical properties of gels

The gels were tested in a Stevens-LFRA Texture Analyzer. The modulus of rigidity,  $E, Pa(N/m^2)$ , was calculated from the load causing compression of one or two millimeters, in which range the stress-strain curve was linear. The breaking load (in grams) was also determined. The standard error in both types of measurements was  $\pm 10\%$ . Melting points were determined by heating the gels in a thermostat at a rate of  $0.5^\circ C$  per min. Several small glass spheres (diameter  $\sim 3$  mm) were placed on the surface of the gels, and the melting point was taken as the temperature at which the spheres started to sink. The accuracy was  $\pm 5^\circ C$ .

### RESULTS AND DISCUSSION

For warm-setting pectin-alginate gels Toft reported (1) that calcium levels above 5 mM, weakened the gels. In those experiments, the calcium was present in the water before the pectins and alginates were dissolved. In similar experiments with gels prepared by the GDL-method, we also found that the gels weakened when the calcium concentration was 4 mM or higher. It was observed, however, that during the preparation of the pectin-alginate solution prior to mixing with GDL, the solutions contained small islands of gels (microgel). It

TABLE 1. Characteristics of alginate samples.

Type of alginate	$F_G$	$F_{MM}^{MG}$	$F_{GM}$	$F_{GG}$	$F_{GGG}^{MGG}$	$F_{GGM}$	$F_{MGM}$
PROTANAL LF 250							
<u>Laminaria hyperborea</u> , stipe	0.70	0.19	0.11	0.59	0.55	0.04	0.07
PROTANAL LF 120 DL,							
<u>Laminaria hyperborea</u> , whole plant + leaf	0.57	0.28	0.14	0.43	0.39	0.04	0.10
Lab.prep. from							
<u>Laminaria hyperborea</u> leaf	0.50	0.32	0.18	0.32	0.26	0.06	0.12
Lab.prep. from							
<u>Laminaria digitata</u> , leaf	0.43	0.41	0.16	0.27	0.22	0.05	0.11
PROTANAL LF 200 MA							
<u>Ascophyllum nodosum</u> + <u>Laminaria hyperb</u> , leaf	0.41	0.41	0.18	0.23	0.18	0.05	0.13
Lab.prep. from							
<u>Ascophyllum nodosum</u> lower part	0.43	0.34	0.23	0.20	0.12	0.08	0.15
Lab.prep. from							
<u>Ascophyllum nodosum</u> fruiting bodies	0.02			0	0		

was therefore possible that the limited solubility of alginate in the calcium-containing water caused the diminished reaction with pectin, and that calcium was not antagonistic per se to the formation of mixed pectin-alginate gels. To test this idea, some gels were made with calcium-free water in the standard way, and thereafter soaked in calcium chloride solutions of different strength. The introduction of calcium ions in this way increased the gel strength compared to the calcium-free gels. In fact, some gels more than tripled in strength as measured by the breaking load, and they even regained the original form after compression above the breaking load. This was obviously an interesting new way of preparing mixed acid-calcium pectin-alginate gels, but no further studies were performed. In the rest of this work only calcium-free systems are considered.

Alginate samples 1 and 5 (Table 1), containing about 70 and 40%  $\underline{\text{L}}$ -guluronic acid, respectively, were used to test the effect of varying the concentration of GDL in this system. The weight ratio between alginate and pectin was kept 1:1 in the series. The results (Fig.1) clearly demonstrate that increasing amounts of GDL increase the gel strength as measured by the modulus of rigidity. In Fig.2 the same results are plotted against the pH in the gels. The curves are drawn to zero around pH 3.8, the upper limit of pH at which self-supporting gels were formed. Since the  $\text{pK}_a$  values of the three uronic acid residues in alginate and pectin are between 3.4 and 3.7, the results indicate that the polyuronides have to be partly protonated for gelation to occur. The results of the breaking load measurements on the same gels are given in Fig.3. The data are qualitatively very similar to the results in Fig.1 and 2, and show that the breaking load and modulus vary in a highly correlated manner.

In a similar series of experiments the melting points of the gels were measured. The results (Fig.4) show that the melting point also increases in a systematic manner with increasing concentration of GDL in the system. The results Figs.1-4 all demonstrate that the stability of the gels, whether characterized by the modulus, the breaking load or the melting temperature, is higher for the alginate with the higher fraction of  $\underline{\text{L}}$ -guluronic acid residues. This points to a key role of  $\underline{\text{L}}$ -guluronic acid residues in developing the network in harmony with earlier suggestions (5). Before pursuing this point further, some data on varying the pectin alginate ratio will be given.

The weight ratio between alginate and pectin was varied in steps between 0.1 and 0.9. Since the buffer capacity of the non-esterified alginate is higher than that of the pectin sample, a series of GDL-concentrations in the range from 0.7 to 1.8 g/100 ml was used for each polymer mixture, giving different pH-values in gels. This

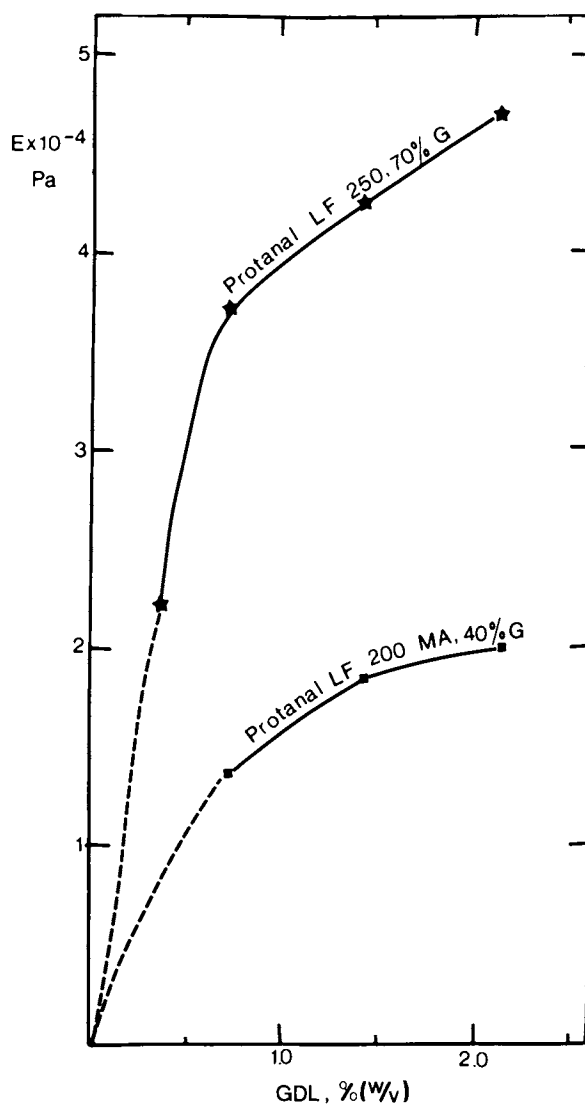


Figure 1. Modulus of rigidity,  $E$ , for different concentration of  $D$ -glucono- $\delta$ -lactone, GDL. Total polymer concentration is 1.2% (w/v) in a 1:1 mixture of Mexpectin RS 400 and the two alginates shown on the curves.



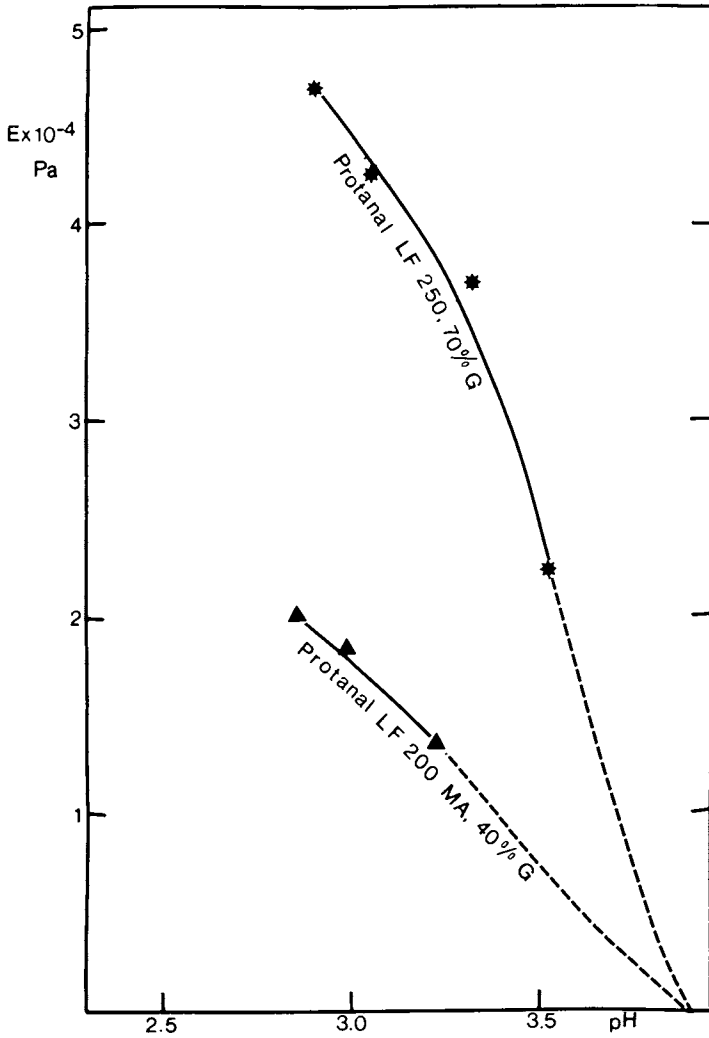


Figure 2. The data from Fig.1 plotted against pH of the the gels.

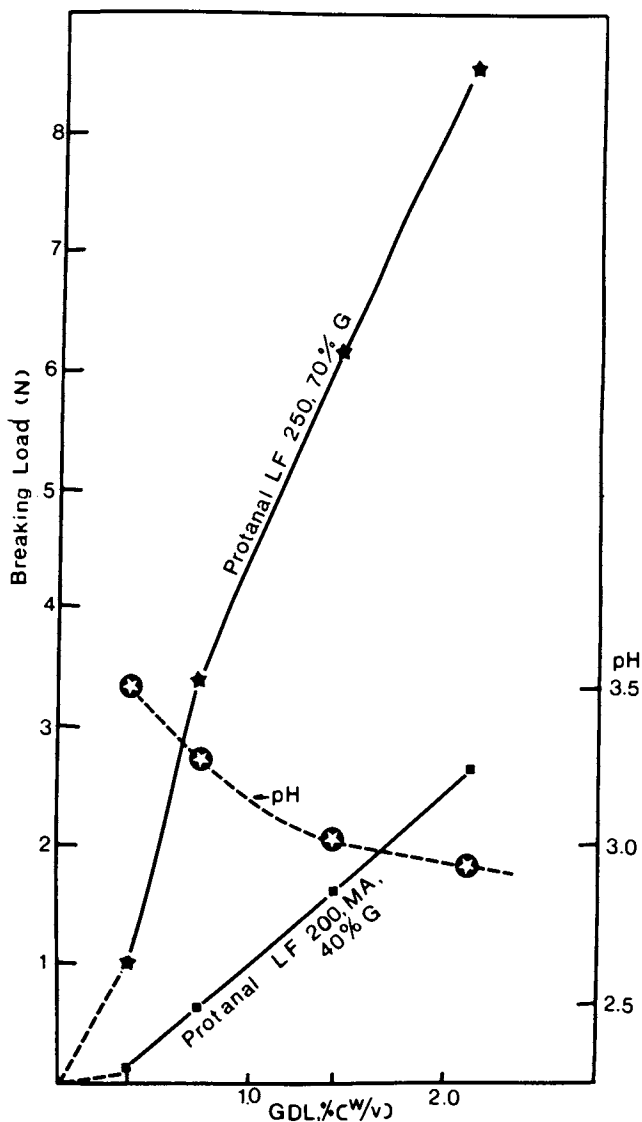


Figure 3. Breaking loads for different concentrations of GDL and the resultant pH's of the gels. Total polymer concentration is 1.2% (w/v) in 1:1 mixtures of pectin and the two alginates shown on the curves.

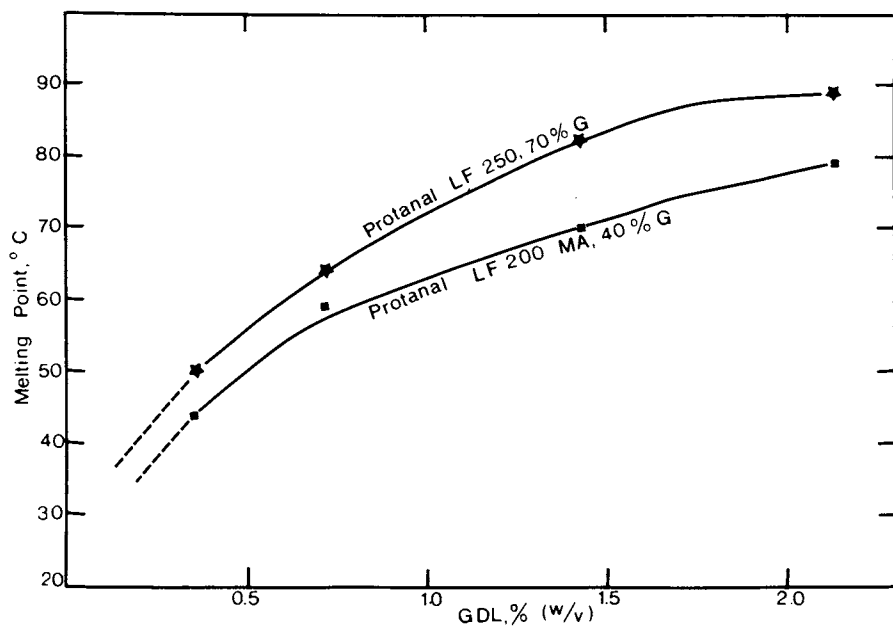


Figure 4. Melting point for different concentrations of GDL. Total polymer concentration is 1.2% (w/v) in 1:1 mixtures of pectin and the two alginates shown on the curves.

allowed interpolation or extrapolation of the gel strength to a single pH value of 3.1 for comparison. Only the alginate sample No.1 was used in this study. The results for the moduli and the breaking loads are given in Figs.5 and 6, respectively. Both sets of data indicate maximum gel strength at ratios of alginate to pectin of 1 to 1. Since the alginate sample contains 70% L-guluronic acid and the pectin 70% methyl ester groups, these results are in harmony with the gelling mechanism discussed earlier, although not representing any independent evidence.

Before presenting the results for different alginates, some basic features of the monomer sequence in alginates have to be discussed. In a series of papers, Haug, Larsen, Painter and Smidsrød (for an excellent review see T. Painter (13)) showed that alginates were not physical mixtures of polymannuronic acid and polyguluronic acid, that they did not have any regular repeating unit, and that their monomer sequence could not be described by a random (Bernoullian) distribution of residues along the chain. Fractionation of partly hydrolyzed alginates suggested a block-wise distribution of monomer residues with blocks of homopolymeric sequence of both types ("MM-blocks" and "GG-blocks") and blocks enriched in alternating sequences. Attempts were also made to describe the sequence with Markov chain statistics of different order, i.e., with the probability of finding a certain monomer next to another depending on the nature of the preceding units. With such a complicated sequence, a population of alginate chains is not fully characterized by measurement of the average composition ( $F_G$  and  $F_M$ ) only.

To illustrate this point further, we may consider three different types of alginates with well-defined structures, all having  $F_M = 0.5$ , and calculate the diad and triad frequencies  $F_{MM}$  and  $F_{MMM}$  for each. The first example is an alginate with a strictly alternating structure. In this case,  $F_{MM} = F_{MMM} = 0$ . At the other extreme is an alginate with infinitely long blocks of M-units and G-units, which is statistically not different from a physical mixture of the two homopolymers. In this case,  $F_{MG} = 0$ , and  $F_{MM} = F_{MMM} = 0.5$ . The third example is a Bernoullian distribution. Here the monomer is completely randomly distributed, which means that the probability of finding (and hence the fractional content of) a given diad or triad is just the product of the probability of finding their constituent-monomers. Thus, in our example,  $F_{MM} = F_M \cdot F_M = 0.25$  and  $F_{MMM} = F_M \cdot F_M \cdot F_M = 0.125$ . We see from these examples that alginates with widely different monomer sequence (and physical properties) may have the same average chemical composition. Since alginates in general do not follow any of the statistical rules of the three examples above, they have to be

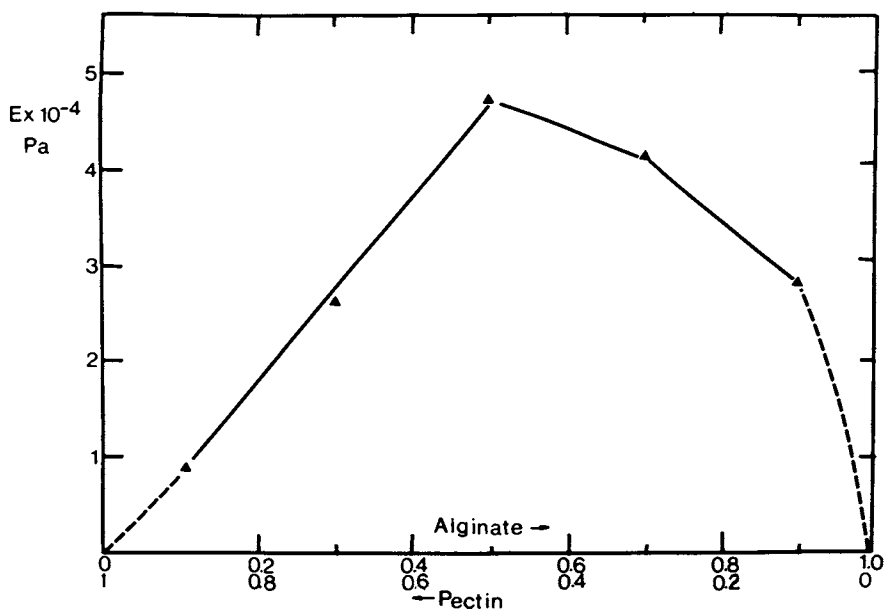


Figure 5. Modulus of rigidity,  $E$ , for different mixing ratios of pectin and alginate (Sample No.1). Total polymer concentration is 1.2% (w/v) and the pH is 3.1.

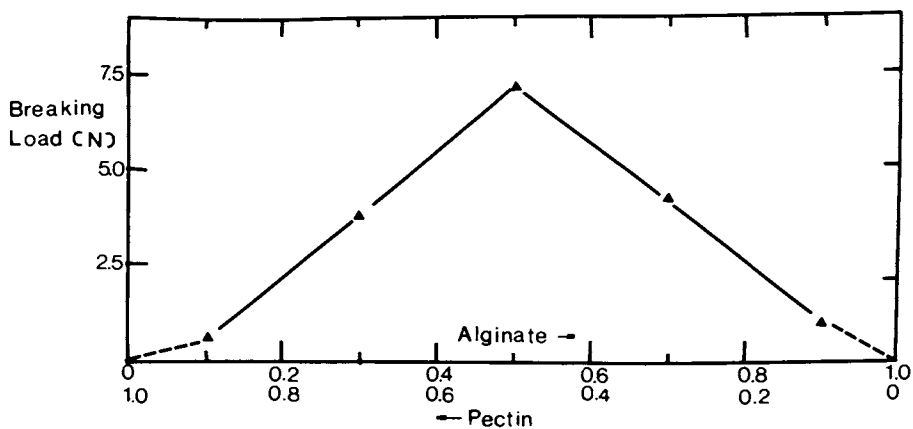


Figure 6. Breaking load for different mixing ratios of pectin and alginate. The gels are the same as in Fig.5.

characterized by as many measured diad, triad and higher order frequencies as possible.

In a series of papers Grasdalen *et al.* (6,7, 11, 14) showed that the four diad frequencies ( $F_{MM}$ ,  $F_{MG}$ ,  $F_{GM}$  and  $F_{GG}$ ) and the eight triad frequencies ( $F_{MMG}$ ,  $F_{MMG}$ ,  $F_{MGM}$ ,  $F_{GMM}$ ,  $F_{MGG}$ ,  $F_{GGM}$ ,  $F_{GMG}$  and  $F_{GGG}$ ) could be measured with different NMR-techniques. Such measurements have, on the one hand, made it possible to test what type of statistics best explain the sequence in different alginate samples, and, on the other, to calculate the average length of the different blocks in alginates.

In the present work, where L-guluronic acid residues obviously play a dominant role in the interaction with pectin, the alginates have been characterized with only the four G-centered triads in addition to diad frequencies. These values allow the calculation of the average block length of G-units,  $\bar{N}_G = F_G / F_{MG}$ . By excluding all the G-units lying in between two M-units, the average length of G-blocks longer than one unit,  $\bar{N}_{G>1}$ , may also be calculated (6):

$$\bar{N}_{G>1} = \frac{F_{GGG} + F_{GGM} + F_{MGG}}{F_{GGM}} = \frac{F_G - F_{MGM}}{F_{GGM}}$$

In Table 2 are given the results of gel strength measurements of 1:1 mixtures of alginate and pectin at pH 2.9. Just some relevant structural characteristics of the alginates, namely  $F_G$ ,  $F_{GG}$ ,  $F_{GGG}$  and  $\bar{N}_{G>1}$  are given in the Table.

The general tendency shown by the data in Table 2 is that increasing the fraction of L-guluronic acid residues results in increasing the gel strength. However, there are clearly exceptions to this general rule. The samples 4, 5 and 6 have almost identical  $F_G$ -values, but only two of them (4 and 5) give a gel of measurable gel strength. This is most probably, caused by the lower values of  $F_{GG}$ , and, in particular,  $F_{GGG}$  of sample No.6. This sample must obviously contain few and short "GG-blocks" compared to the other two samples of similar average composition.

The data for the modulus measurements are plotted against  $F_G$ ,  $F_{GG}$  and  $F_{GGG}$  in Fig.7. These plots clearly indicate that the correlation with  $F_{GGG}$  is the best, pointing to the importance of the "GG-blocks" in the interaction with pectin.

In Fig.8 the same gel strength data are plotted against the average lengths of the "GG-blocks",  $\bar{N}_{G>1}$ . The figure indicates that the average number of G-units in the blocks must exceed 4 for the interaction with pectin to occur. One must, of course, remember that  $\bar{N}_{G>1}$  is an average value, and that the distribution of block lengths is probably broad in all alginate samples. In the physical situation it may well be that mainly the

TABLE 2. Gel strength measurements of 1:1 combinations of different alginates with Mexpectin RS400 at pH 2.9. Total polymer concentration is 1.2% (v/w).

$F_G$	$F_{GG}$	$F_{GGG}$	$N_{G>1}$	Breaking load (g)	$\text{Ex}10^{-4}, \text{Pa}$
0.70	0.59	0.55	15.8	1000	4.85
0.57	0.43	0.39	11.8	830	3.9
0.50	0.32	0.26	6.3	460	3.0
0.53	0.27	0.22	6.4	230	2.5
0.41	0.23	0.18	5.6	250	1.9
0.43	0.20	0.12	3.5	0	0
0.02	0	0	2	0	0

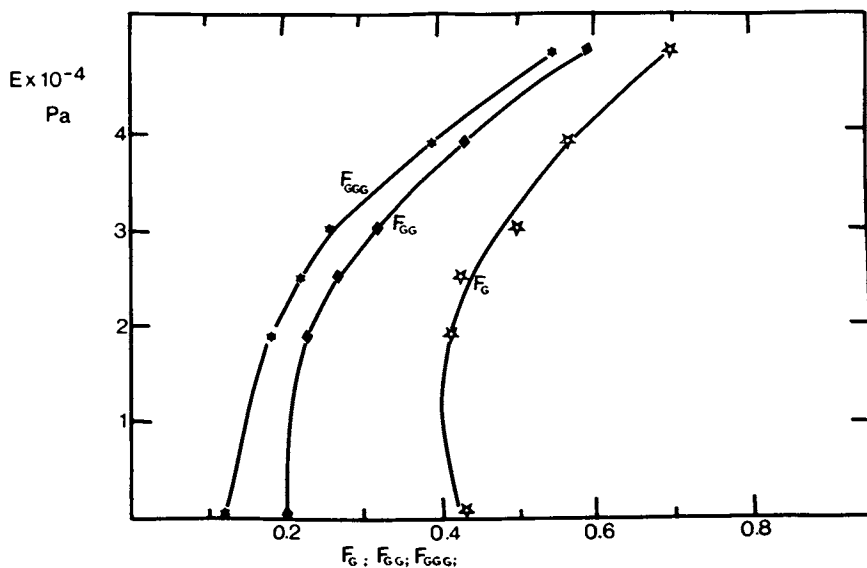


Figure 7. Modulus of rigidity against  $F_G$ ,  $F_{GG}$  and  $F_{GGG}$  for different alginate samples. Total polymer concentration is 1.2% (w/v) in 1:1 mixtures of pectin and alginates.

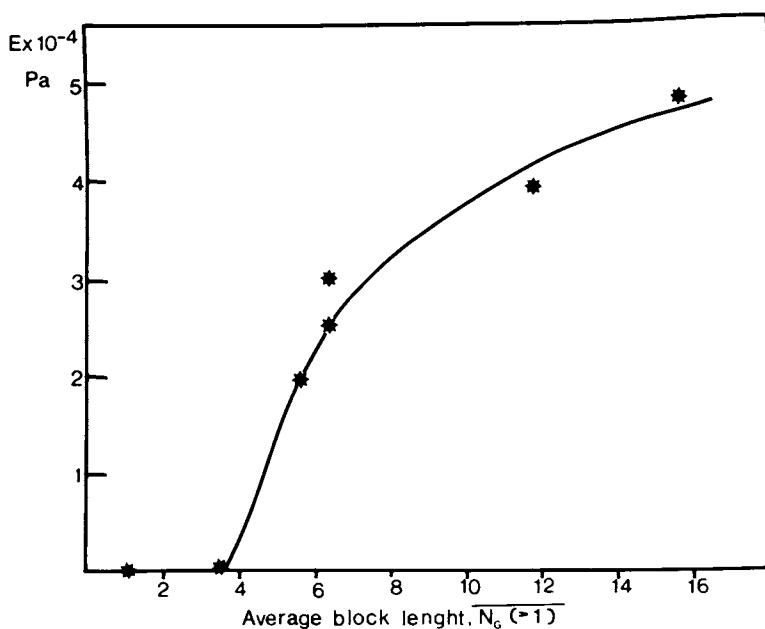


Figure 8. Data from Fig.7 plotted against average block length of G-units excluding the singlet G-units,  $N_{G>1}$ .



tail of the distribution with higher block lengths than 4 takes part in the interaction with pectin. However, the importance of the "GG-blocks" for the interaction to occur is evident.

The data in this paper taken together give full support to the idea of Thom et al.<sup>5</sup> referred to earlier, that fully or partly protonated "GG-blocks" may interact with esterified pectin chains giving junctions in the gel network. To exclude the possibility that non-esterified pectin chains take part in the interaction, some studies with pectin samples better characterized with respect to the distribution of methoxy groups should be carried out. The details of the interaction are not known, but since the chains of  $\alpha$ -D-galacturonic acid and of  $\alpha$ -L-guluronic acid are mirror images, apart from the configuration around C-3, there should be a number of regularly spaced atomic groups along the two chains to give possibilities for a strong, cooperative, inter-chain contact.

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## Control of Pectin Synthesis and Deposition during Plant Cell Wall Growth

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The assembly of the cell wall outside the protoplasts is controlled at 3 levels: a) at the synthase systems which use the nucleoside diphosphate sugar donors b) at the operation of transport mechanisms for movement of material into the endomembrane system and c) the regulation of the directed movement and fusion of vesicles derived from the endomembranes. Pectin is deposited in the wall during primary growth only. The nucleoside diphosphate sugars for pectin synthesis arise by epimerases from the corresponding nucleotides of the glucose series. The epimerases are active during secondary growth so that little control is exerted at these steps. Assembly of pectin occurs in the Golgi apparatus by synthases which probably do not use lipid intermediates. These synthases are controlled and are shut down during secondary growth. The sugar nucleotides are specifically transported into the Golgi cisternae and regulation can occur at these sites. Vesicles, containing pectin, are moved to the plasma-membrane probably directed by microtubules. Fusion of these vesicles with the cell membrane is partly controlled by the level of  $\text{Ca}^{2+}$  at the points of fusion.

Pectin is a mixture of complex polymers (1-3). These are formed during primary wall formation while the cell wall is expanding in surface area. At these early stages of growth the pectin polymers contribute in a major way to the texture of the wall especially to its ability to expand and stretch. The wall is much more a fluid structure at this time and water is an extremely important constituent. The primary wall when the matrix is non-lignified can be considered as a fluid plastic structure so that any load applied to the wall is transmitted to the microfibrils by the viscous drag of the plastic deformation of the matrix. This can alter very much with the composition and physical state of the matrix materials, especially with that of the pectin complex (3).

The pectin polymers are constituents of the cell plate during cell division and they are the first set of polysaccharides to be

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secreted by protoplasts during the formation of a new wall (4,5). The composition of the pectin polymers can be altered by the application of plant growth factors (6). The texture of fruit and the ripening process depends upon the alteration of the pectin components of the cell walls of fruit tissue. It is apparent that the pectin polymers are a variable and important feature of the young, growing wall.

Numerous slimes and mucilages secreted by plant cells have chemical compositions which are related to the pectins (polymers containing arabinose, galactose and galacturonic acid) and these materials are formed to lubricate such organs as roots and to retain water within a gel structure to keep the organs moist at the various stages of growth of the plant (7,8).

The formation of pectin and its assembly for final export across the plasmamembrane will be considered here as a production line to indicate the various limiting steps which are controlled to monitor its production. The various channels for the movement and synthesis will be described separately so that the control points can be assessed. The channels which operate for production and movement of the polymers within the cytoplasm and endomembrane system of the cells are:- Channel 1. Production and movement of nucleotide sugar donors. Channel 2. Synthesis and compartmentalization of the pectin polymers within the endomembrane system. Channel 3. Movement of vesicles and fusion with the plasmamembrane for assembly and deposition within the wall.

During fruit ripening the pectin is degraded and this is also a controlled process brought about by the induction of degradation enzymes such as polygalacturonase (9). Degradation or turnover may go on to a limited extent in the normal growing and thickening wall during differentiation. However, there is evidence that the pectin material once deposited in the wall remains there (10) so that the main controls of the amount present during differentiation are those which operate for its synthesis and deposition.

#### Channel 1. Production and Movement of Nucleotide Sugar Donors

The nucleotide sugar donors are formed outside the endomembrane system in the cytosol. Those for pectin arise by epimerase reactions from the corresponding nucleotide sugar compounds of the glucose series, Figure 1. The epimerases are active throughout growth. Thus the potential for the production of UDPGal, UDPGalAc and UDPPara, which are the donors of the pectic material, is present even during secondary thickening when no pectin is deposited in the wall (11,12). The epimerase reactions are therefore not sites at which control of the polysaccharide synthesis is exerted.

The nucleotide sugar compounds are hydrophilic and it is necessary to transfer them to the sites of the synthases within the endomembrane system. Their transport across the membrane is an obvious control point. Not only is the rate of polysaccharide synthesis controlled by this mechanism but in addition the qualitative nature of the polymer found in the membrane compartment may be determined. Thus although the synthases may be present in a particular part of the membrane system e.g. endoplasmic reticulum, the assembly of the polysaccharide does not occur at this level if the nucleotide sugar cannot enter the lumen of the compartment (13). The main assembly of the polysaccharides occurs at the Golgi apparatus, Figure 1.

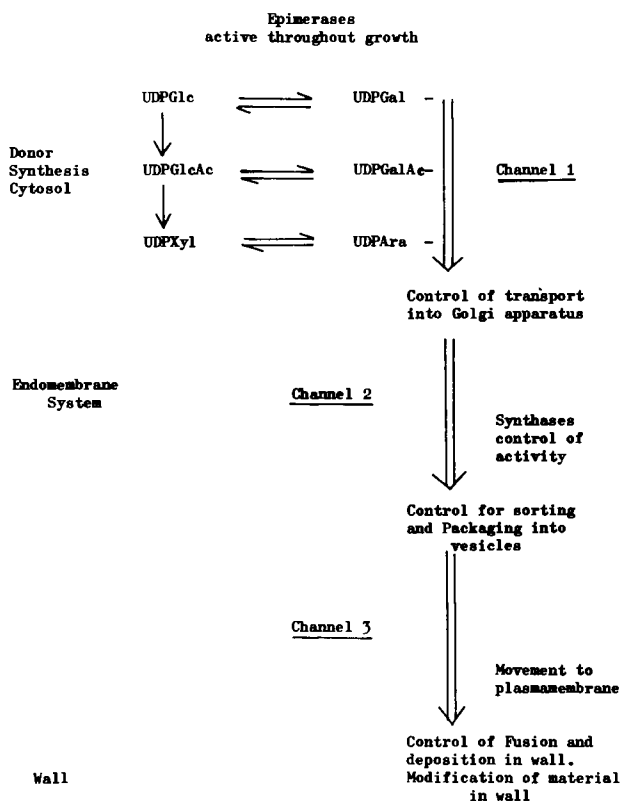


Figure 1. Epimerase reactions for the formation of nucleotide sugar donors.

The assembly of the pectic polysaccharides, like most of the cell wall polysaccharides, with the possible exception of cellulose, appears to take place by transfer of the glycosyl group from the nucleotide-sugar compound to the growing polymer without the necessity for a dolichol phosphate intermediate acceptor. This has consequences for the mechanism of the method of transport of the sugars across the membrane. For the assembly of the sugar portion of N-linked glycoproteins which occurs in the endoplasmic reticulum, isoprenoid phosphates act as intermediate acceptors and several mechanisms of transport involving these lipid sugar compounds have been suggested (14). It is also clear that when the glycosylations occur within the Golgi apparatus these methods of transport are not involved and direct entry of the nucleotide-sugar compounds by specific transporters may take place (15).

#### Channel 2. Synthesis and Compartmentalization of the Pectin Polymers within the Endomembrane System

The synthases or transglycolyses which will transfer the galactose, galacturonic acid and arabinose to the pectic polymers are the principal enzymic points at which control is maintained for the synthesis of the pectin (13, 16-20). The activity of these enzymes decreases during secondary thickening of the wall when pectin deposition ceases. By the careful use of inhibitors of transcription and translation and by the use of a monoclonal antibody it was shown that the changes in activity of enzymes were correlated with changes in the amount of enzyme protein present at the membrane (19,21). Thus this control is operated at the level of the genome and the changes in the cell wall are part of a developmental process.

One of the main control points of polysaccharide synthesis, therefore, is the amount and consequently the activity of the transglycosylases or synthases which operates for the particular nucleotide sugar at the lumen of the membrane. However there is a further complication which arises with complex polymers such as those in pectin. These are heteropolymers e.g., the rhamnogalacturonan, the arabinogalactan and the rhamnogalacturonan-arabinogalactan. There are also some, like the arabinogalactan which are highly branched polysaccharides (3,22) and, in addition, minor sugars such as fucose and xylose occur in these complex polysaccharides. If the sugars are added directly to the growing polymer one at a time, then the synthesis of the main chains may depend to some extent on the incorporation at various stages of a different sugar or branch point so that the activity of the transglycosylase forming the chain is maintained. Alternatively smaller subunits of the main polymer may be preformed on intermediate carriers, such a lipid or protein, and then incorporated as blocks into the polymer (23). This method of synthesis would resemble that for the formation of some glycoproteins in animals and bacteria. It would result in a certain amount of regularity in the chemical composition of the polysaccharide that is formed and it would introduce secondary stages at which control of the assembly could be exerted.

In addition to the control of the amount of synthase which defines the total activity of the enzyme, the activity of some synthases are modulated by the energy status of the cell since they are inhibited by levels of nucleoside mono and diphosphates (17).

As the polysaccharide is being formed it must be transferred to particular parts of the membrane system and finally packaged for export to definite regions of the periphery of the cell, Figure 1. Very little is known about the control of this important part of the assembly system. Some ideas for the mechanisms of the sorting and transport processes may be obtained from the limited evidence known for the synthesis and export of some glycoproteins from the Golgi apparatus and their carriage between the endoplasmic reticulum and Golgi apparatus. It has been shown for instance that the cisternae of the Golgi apparatus can be divided into cis, medial and trans regions and that different transglycosylases are located at different regions (24,25). It has also been shown for a few polymers that during their packaging and export from one membrane compartment to another, they are separated from others by sequestering them to particular regions or cisternae. This is done by the provision of specific recognition markers on the polymer such as removal of the terminal glucose units to expose mannose radicals (26) (for transport from endoplasmic reticulum to Golgi apparatus) or formation of mannose-6-phosphate at the non-reducing end of the oligosaccharide (for transport from Golgi apparatus to lysosome) (14,25-29). The enzymic reactions which enable the marker to be developed upon the oligosaccharide of the glycoprotein do however recognise the protein portion of the polymer in addition to the oligosaccharide. In this way, for example, distinctions can be made between glycoproteins that carry mannose since only some of these will be transported to the lysosome.

### Channel 3. Movement of Vesicles and Fusion with the Plasmamembrane for Assembly and Deposition within the Wall

Vesicles containing the pectin polysaccharide arise from the Golgi apparatus. The membranes of the cisternae of the Golgi apparatus, where dispersal occurs, become modified so that the membranes of the secretory vesicles resemble, both in their chemical composition and ultrastructural appearance, the plasmamembrane. The vesicles are transported to specific areas of the cell surface when the cell is expanding in surface area. In some cells it can be seen that vesicle fusion depends upon a characteristic distribution of protein particles within the membrane (30,32). There is thus an elaboration of the membranes both chemically and by ultrastructural changes so that fusion is possible at the required site. The vesicles produced from the membrane system may be directed, in part, to particular cell surfaces by microtubules. Although this is not so apparent at the early stages of growth, when pectin is being deposited in the wall, as it is during secondary wall formation. During the development of xylem elements, for instance, when a spiral or reticulate secondary wall is formed, the microtubules are distributed just under the plasmamembrane at the sites of secondary thickening (33,34). However at this stage no pectin is being accumulated in the wall and the distribution of the microtubules reflects the additions of hemi-cellulose and cellulose within the thickening. During the formation of the primary wall the microtubules are distributed just under the plasmamembrane but they are not so obviously localized. They occur in groups of three or four randomly distributed in a circular or

helical arrangement around the cell. It may be that the pectin is deposited fairly evenly over the wall at this time.

Pectin-like material is secreted from suspension tissue culture cells of sycamore and the rate of secretion of these polysaccharides can be measured using radioactive arabinose which is incorporated into arabinogalactans. The addition of  $\text{Ca}^{2+}$  increased the steady state rate of secretion within seconds. The stimulation was brought about by an action on the normal mechanism of cell-wall polysaccharide export from the cytoplasm. It seemed therefore that the fusion of the vesicles with the plasmamembrane was a rate-limiting step and is probably a control point. The action of the  $\text{Ca}^{2+}$  was to increase the rate of fusion at the cell membrane so that there was an immediate increase in the amount of polysaccharide secreted (35). The control could be established if at any one time there was at the membrane more vesicles than those that were fusing. However in order to sustain the increase in secretion it would be necessary to transmit the signal back to the synthetic system and packaging process at the Golgi apparatus so that a new steady state system was obtained. This would produce the requisite number of vesicles containing the polysaccharides necessary to maintain the new rate of fusion and secretion. The turnover of the Golgi apparatus can be very fast in plant cells and times of 5-40 minutes have been calculated (36). In vitro experiments which involved isolation of membrane fractions from maize-root cells also showed that  $\text{Ca}^{2+}$  was necessary for membrane fusion (37). Analysis of the membranes indicated that the  $\text{Ca}^{2+}$  dependence involved membrane proteins and one of these was a  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -dependent ATPase (38).

Once deposited in the wall the pectin may undergo further chemical modification by transglycosylases which join particular polymers such as the arabinogalactans to polygalacturonans (39,40). Even ester linkages with diferulic acid may occur to give bridges between different polysaccharides of the wall (41). It is well known that  $\text{Ca}^{2+}$  form ionic bonds between the acidic groups of the pectin polymers. However, regardless of the presence of these intermolecular covalent and ionic bonds, in the primary wall when it is unignified, the main cohesion between the constituents must be the hydrogen bonding that allows a flexibility to the structure of the wall which depends on its most variable feature the water content especially in its relationship to pectin (3). When lignification occurs during secondary thickening the polysaccharides become enclosed in a cross-linked polymer cage and bridges between the polysaccharides and the phenylpropanoid radicals are formed. The wall thus develops great tensile strength because of the microfibrils and it becomes a rigid structure because of the lignified matrix in which the hydrophobic lignin has replaced the water. The pectin at this time represents only a minor constituent of the wall and contributes very little to its texture or properties.

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## Comparative Analysis of Pectins from Pericarp and Locular Gel in Developing Tomato Fruit

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Pectins from tomato pericarp and locular gel were examined to ascertain the relative importance of these polysaccharides in the texture changes which accompany development of the respective tissue types. Ripening of pericarp was marked by increased quantities and depolymerization of soluble pectins. Soluble pectins from ripe fruit exhibited a lower degree of esterification, a lower average molecular size and a decreased neutral sugar content compared to pectins from unripe fruit. Pectins from gel showed little evidence of depolymerization during the active period of gel autolysis, consistent with the observation that only trace levels of endo-D-galacturonase were found in this tissue. Degree of esterification of gel pectins was high and showed no change during development. Gel pectins were rich in arabinose, galactose, and xylose, all of which decreased during early gel formation.

The ripening of many fruit types includes as a major event extensive modifications in texture, often attributed to the action of specific cell-wall hydrolases, notably the D-galacturonases (1,2). The temporal relationship between softening, pectin solubilization and depolymerization, and the appearance of endo-D-galacturonase (EDG, E.C. 3.2.1.15) activity together strongly underscore the dominant role of these enzymes (3-11).

It is becoming increasingly evident that processes other than those involving EDG are operative in the cell-wall metabolism of ripening fruit. The cell-wall neutral sugars galactose and/or arabinose decrease during ripening of many fruit types (8,12-14) including rin tomato fruit (15,16), which has no EDG (17,18). In a species survey, Gross and Sams (19) reported losses of cell-wall galactose and arabinose during ripening in fourteen of seventeen fruit types examined. In both tomato (11) and strawberry fruit (20), ripening was accompanied by decreases in the molecular size

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of hemicelluloses and the absence of EDG in strawberry (20-22) indicates that the altered alkali-soluble polymers did not arise as an indirect result of pectin degradation. Of equal interest in strawberry fruit was the observation that levels of soluble pectins increased in the apparent absence of EDG. Soluble pectins showed no evidence of the progressive depolymerization exhibited by soluble pectins from tomato (11), watermelon (23) and peach (4), all of which contain EDG. In addition to strawberry, plum (24), hot pepper (25) and muskmelon (3,26) represent other fruits which show increased levels of soluble pectins during ripening yet apparently contain no EDG. It is apparent from these studies that increased pectin solubilization during ripening is not in itself sufficient evidence for inferring that EDG is responsible.

Tomato fruit appeared to represent a unique system for investigating possible variability in the functions of pectins in texture modifications. Firstly, the tomato fruit is composed of distinct tissue types, including locular gel and pericarp which exhibit markedly different patterns of tissue autolysis. Locular gel develops prior to ripening of pericarp and eventually, toward the terminal stage of ripening, exhibits an almost liquid nature. This developmental scenario is in some respects similar to the ethylene-induced maceration of watermelon fruit (27), a process involving greatly increased levels of EDG and extensive solubilization and depolymerization of pectins (23). In contrast, tomato locular gel has been reported to contain little (5) or no EDG activity (28). The objective of this study was to examine some of the structural features of pectins from tomato pericarp and gel, and to relate this information to the textural characteristics of these tissues.

### Materials and Methods

Plant Material. Tomato (*Lycopersicon esculentum*, Mill. var Sunny) fruit were harvested from plants grown at the IFAS Horticultural Farm near Gainesville, FL. Fruit were selected at five stages of development: immature green (undeveloped fruit, gel tissue firm), mature-green (gel formation initiated), turning, pink and ripe (red). Fruit were surface sterilized with 0.05% Na-hypochlorite and rinsed. Fruit were quartered, the pericarp and locular gel separated, and both stored at -20°C.

Preparation of Ethanol-Insoluble Solids. Pericarp or gel (100 g) while frozen was placed in 400 ml cold (4°C) ethanol and partially thawed. The tissue was homogenized in a Sorvall Omnimixer for 1 min and then refluxed at 84°C for 30 min to inactivate pectin hydrolase activity. This technique is effective at inactivating wall-bound pectin-hydrolyzing activity (29) and results in no apparent changes in the characteristics of pectins (30). After refluxing, the suspension was rinsed through Miracloth and washed, in succession, with 80% ethanol (11), 80% acetone (11), and 100% acetone (11). The resulting powders were air-dried at 40°C and stored covered at room temperature.

Measurement of Total and Soluble Pectins. Total pectins in ethanol-insoluble powders were measured using the procedure described by Ahmed and Labavitch (31). Pectins were assayed colorimetrically using the method of Blumenkrantz and Asboe-Hansen (32). For measurement of soluble pectins, 20 mg ethanol-insoluble powder were placed in 7 ml of either distilled water or Na-acetate buffer (40 mM, pH 5.0) containing 20 mM EDTA and incubated with shaking at room temperature for 6 hr. Aliquots (0.5 ml) were filtered through glass fiber filters and analyzed for soluble pectins.

Gel Filtration and Ion-Exchange Chromatography. Pectins for gel filtration and ion-exchange chromatography were solubilized from powders using the Na-acetate-EDTA buffer at room temperature. This extraction procedure was found to maximize the yield of pectins (relative to water extractions) and produced no apparent modifications in pectin molecular size and neutral sugar content, two variables of major concern in these studies. Details of the gel filtration and ion-exchange chromatography of pectins have been described (20).

Neutral Sugar Analysis. Analyses of neutral sugars associated with tomato pectins were performed using polymers recovered from ion-exchange profiles. Gradient fractions were combined, concentrated to a volume of 10 ml and dialyzed (Spectrapor 2,000) against distilled H<sub>2</sub>O for 36 hr at 4°C. Samples were air-dried under a stream of filtered air, hydrolyzed and acetylated (33), and examined using gas chromatography on a column of GP 3% SP-2340 (Supelco) at 225°C. Neutral sugar analyses were performed on pectins prepared from three separate tomato fruit isolates.

Percent Esterification. Ethanol-insoluble powder (200 mg) was suspended in 10 ml 0.5 N NaOH containing ethanol as internal standard. After 1 hr, the suspensions were filtered, distilled under vacuum until 5 ml were collected, and the distillate analyzed for methanol using gas chromatography on a column of Super Q (Alltech) operated at 160°C.

Hemicellulose Extraction and Analysis. Hemicelluloses were prepared from  $\alpha$ -amylase-treated ethanol-insoluble powders in the manner previously described (11). Approximately 4 mg hemicellulose in 2 ml Na-citrate-phosphate buffer were fractionated on a column (1.5 cm x 60 cm) of Ultrogel AcA 34 eluted with Na-citrate-phosphate buffer (20mM, pH 5.5) containing 50 mM NaCl (11). Fractions of 0.5 ml were analyzed for total sugar using the phenol-sulfuric acid procedure (34), and for xyloglucan using Kooiman's iodine staining technique (35) as described by Nishitani and Masuda (36).

Enzyme Assays. Endo-D-galacturonanase activity in tomato pericarp and gel was extracted and assayed as previously described for pericarp tissue (11). Protein was measured using the method of Bradford (37) employing bovine serum albumin as standard.

## Results

Initial experiments employing aqueous (water or buffer) extraction procedures for preparing wall isolates from tomato pericarp and gel disclosed that significant quantities of pectins were lost during washing of cell wall. This problem was most serious in pericarp at advanced stages of ripening and with gel at all developmental stages examined. For this reason, ethanol-insoluble powders were used as starting material for all pectin analyses. As another advantage, inactivation of endogenous enzymes which remain bound and autolytically active in cell-wall isolates (29) is more readily accomplished in ethanol homogenates. Heat treatment of aqueous extracts promotes  $\beta$ -eliminative degradation of pectins (38), whereas we have noted no apparent effect of boiling 80% ethanol on the solubility, molecular size or neutral sugar content of pectins solubilized from powders so prepared (30).

Analysis of total pectins in ethanol powders disclosed a trend of increasing pectin (on a per mg powder basis) in both gel and pericarp (Table I). Pericarp levels greatly exceeded those in

Table I. Total and Soluble (Acetate-EDTA Buffer) Pectins in Ethanol Powders from Tomato Pericarp and Gel. Average of 3 replications. Numbers in parentheses represent total pectins expressed on tissue fresh weight basis.

Stage of development	Pericarp		Gel	
	Total	Soluble	Total	Soluble
	$\mu\text{g} \cdot \text{mg powder}^{-1}$ ( $\mu\text{g} \cdot \text{mg fresh wgt}^{-1}$ )			
Immature green	258.7 (6.9)	61.4	143.8 (2.7)	94.4
Mature green	298.1 (6.9)	91.9	168.2 (1.8)	145.6
Turning	310.9 (5.7)	105.7	196.4 (1.7)	117.0
Pink	328.4 (5.6)	192.2	188.5 (1.8)	122.8
Ripe	334.6 (6.1)	201.3	211.1 (2.2)	164.9

gel at all stages of development. It should be noted that all subsequent analyses are performed using only those pectins solubilized from powders by acetate-EDTA buffer. In pericarp, the quantity of pectins solubilized as a percentage of total pectins ranged from 24% at the immature-stage to 60% at the ripe stage. In gel, greater than 60% of the total pectins were readily soluble in buffer and were nearly equally soluble in water alone (not shown). This latter observation underscores the importance of avoiding aqueous procedures when preparing isolates of structural polysaccharides from gel. Prior to chromatography, extracts containing soluble pectins were centrifuged and filtered through glass fiber filters. Including these steps did not affect pectin levels and greatly improved column performance and longevity in terms of both flow rate and elution characteristics. It is understood that the behavior of polysaccharides, particularly the anionic pectins, on size exclusion gels should be interpreted with

caution. The known tendency of the pectins to form intermolecular aggregates, via hydrogen, ionic and/or hydrophobic interactions (39-43), is one potential problem. Furthermore, the extreme structural diversity of the pectins (44) and consequently the variable conformational features of these polymers (45) also impairs the assignment of accurate molecular size information. For these reasons, the gel filtration data presented are considered only in terms of how elution behavior differs between tissue types and during fruit development.

Gel filtration profiles of pectins from gel and pericarp from fruit at selected stages of development are shown in Figure 1. In undeveloped fruit, soluble pectins from both gel and pericarp were excluded from Ultrogel Aca 34. Little change was observed in gel pectins throughout development whereas pectins from pericarp exhibited a clear trend of depolymerization. The differential fractionation patterns were generally consistent with the relative levels of EDG activity found in each tissue (Table II).

Table II. Endo-D-Galacturonanase Activity in Pericarp and Gel

Stage of development	Endo-D-Galacturonanase Activity ( $\mu\text{g}$ reducing equiv. $\cdot$ mg protein <sup>-1</sup> $\cdot$ hr <sup>-1</sup> )	
	Pericarp	Gel
Mature green	0.18	0.02
Turning	2.36	0.12
Pink	6.70	0.24
Ripe	8.23	0.32

Gel tissue contained only trace levels of EDG activity. The fact that extracts from three separate lots of fruit exhibited similar activity is an indication that the presence of the enzyme in gel was not a consequence of handling-induced contamination from pericarp tissue. Evidence for *in situ* action of the enzyme was apparent when gel pectins were examined on Ultrogel Aca 22, a filtration matrix with a broader fractionation range than that of Ultrogel Aca 34 (Figure 2). On Aca 22, pectins from gel showed evidence of depolymerization, albeit less extensive and first apparent much later in fruit development (ripe stage) than that affecting pericarp pectins.

Ion-Exchange Chromatography. Soluble pectins from gel and pericarp showed markedly different elution behavior on DEAE-Sephadex, although within each tissue type little change in elution pattern was evident during development (Figure 3). Pectins from gel eluted in a range of the gradient corresponding to a concentration of 200 mM NaCl whereas those from pericarp eluted at higher concentrations, averaging about 500 mM. These observations were consistent with the range of values of from 70-80% obtained for percent esterification of gel pectins. Pericarp pectins showed a lower percent esterification, the degree of which decreased progressively during development from a high of 40% at the mature-green stage to 25% at the ripe stage.

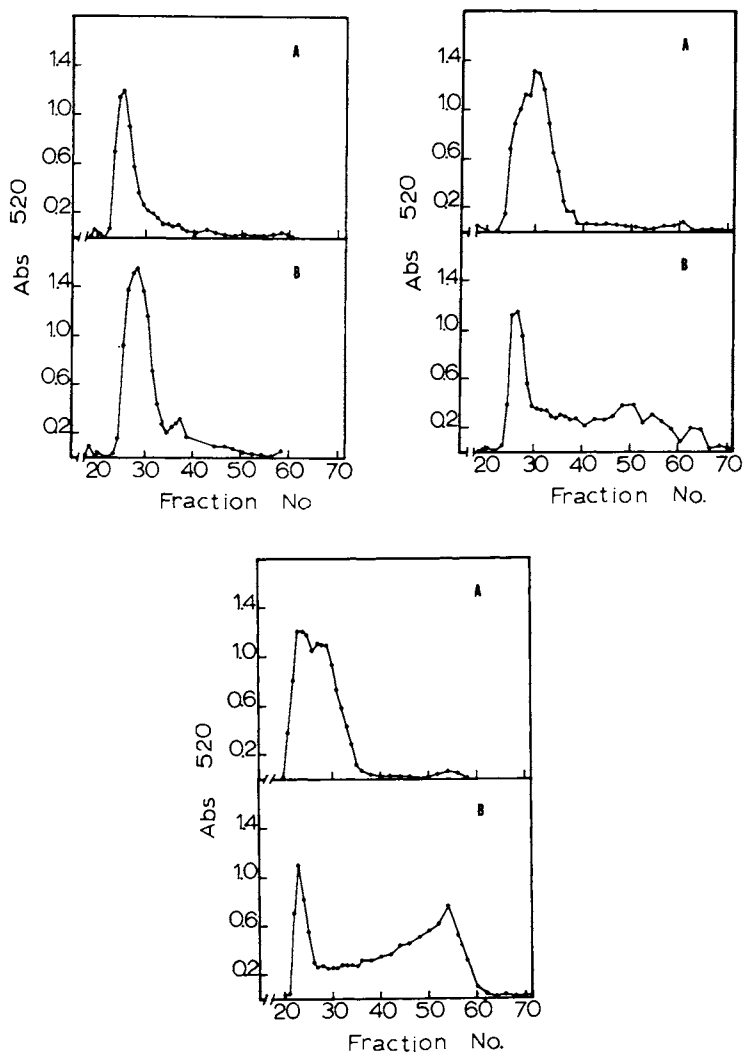


Figure 1. Ultragel Aca 34 profiles of pectins from tomato gel (A) and pericarp (B). Tissues were prepared from mature green (top left), pink (top right), and red (bottom) fruit. Fractions were analyzed for acid sugars ( $A_{520}$ ).

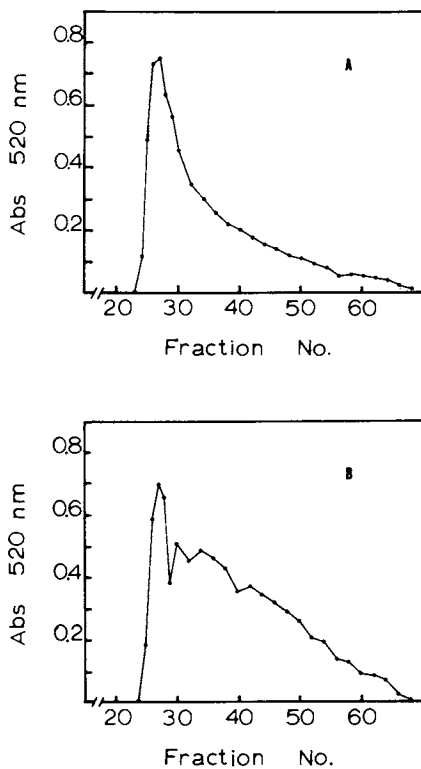


Figure 2. Ultrageel AcA 22 profiles of gel pectins from tomato fruit at mature-green (A) and ripe (B) stage of development. Acid sugars ( $A_{520}$ ).

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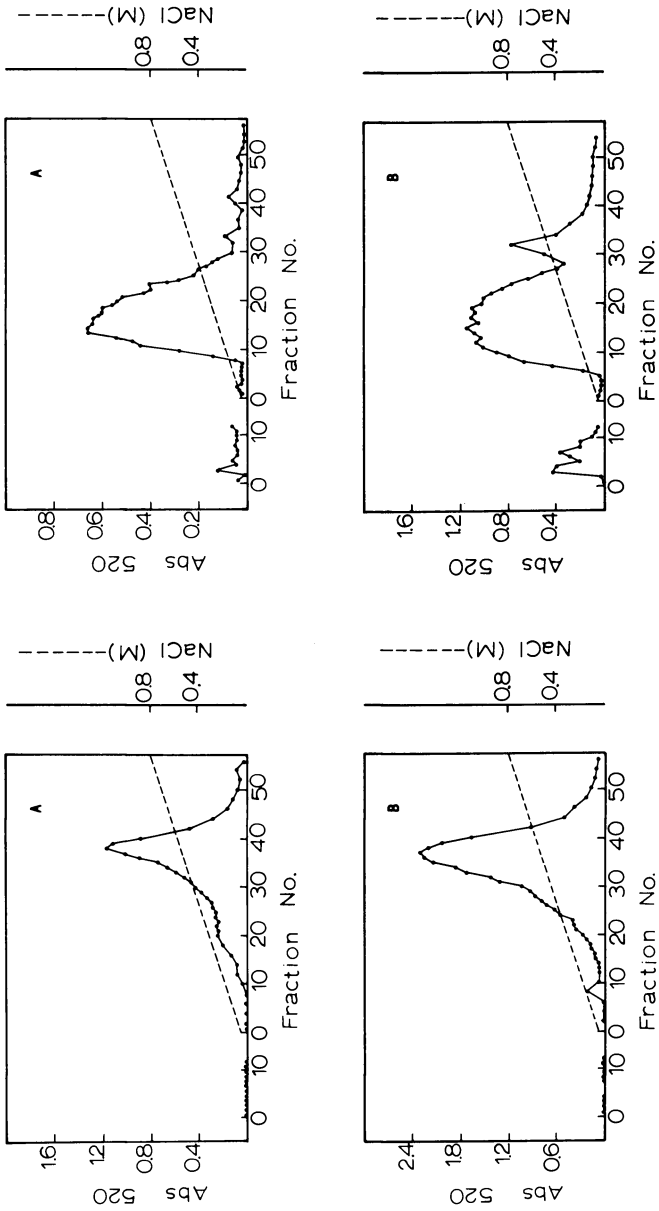


Figure 3. DEAE Sephadex profiles of pericarp (left) and gel (right) pectins from tomato fruit at immature green (A) and ripe (B) stage of development. Acid sugars (A<sub>520</sub>).

Neutral Sugar Analysis. Figure 4 illustrates the trend of neutral sugar changes in soluble pectins from pericarp and gel. Total neutral sugars were considerably higher in gel, accounting for nearly 40% (mole ratio basis) of the pectin. In pericarp pectins, neutral sugars never exceeded 12-13%. The quantitative trend in neutral sugar levels in gel and pericarp pectins were temporally distinct, with gel levels decreasing nearly 62% during the transition from immature-green to mature-green, the most active period of gel formation. This decrease was due to losses in galactose (64%), arabinose (53%) and xylose (62%) (Figure 4 C). Of particular interest regarding the gel pectins was the presence of high absolute quantities of xylose, which at 10% exceeded the levels of arabinose (8%). Little change in neutral sugar content occurred beyond the mature-green stage. Total neutral sugars of pericarp pectins changed little or increased slightly through the mature-green stage and decreased 25% during ripening. Figure 4 B illustrates that the neutral sugar decrease in pericarp soluble pectin was a reflection of decreases in galactose (54%) and to a lesser extent arabinose (13%).

Hemicellulose Analyses. Hemicelluloses (4N alkali-soluble) from both gel and pericarp exhibited a trend of decreasing high-molecular-weight polymers and increasing quantities of low-molecular-size polymers (Figure 5). The profiles shown represent hemicelluloses from mature-green and ripe fruit; however examination of fruit at intermediate stages of ripening disclosed that the change was gradual. Polymers from tissues from immature fruit resembled those derived from mature-green fruit. The molecular weight shift was accompanied by a change in the elution behavior of xyloglucan, as indicated by the Kooiman's iodine-staining test.

### Discussion

It has for some time been evident that fruit softening can not in all cases be attributed solely to the action of endo-D-galacturonanase (EDG). In some fruits, including muskmelon (3,26), plum (24), and strawberry (20-22), softening occurs in the apparent absence of this enzyme. Apple fruit contain exo-D-galacturonanase (46); however it seems unlikely that this enzyme is responsible for the solubilization of polymeric pectin during ripening. Even in fruits known to contain EDG, wall changes apparently unrelated to EDG action have been observed during ripening and softening. In some cases (discussed below) the participation of wall polymers other than pectins has been implicated. This study addressed potential variability in the contribution of pectins in particular to texture changes in tomato pericarp and locular tissue (gel). The results demonstrate that the changes affecting pectins in these tissues are quantitatively, qualitatively, and temporally distinct. In studies with tomato pericarp and other fruit types, pectin changes, particularly increased solubility and/or depolymerization, have been shown to be temporally associated with increased EDG activity and with ripening in general (3-11). This is not the case with tomato gel. This tissue undergoes dramatic anatomical changes

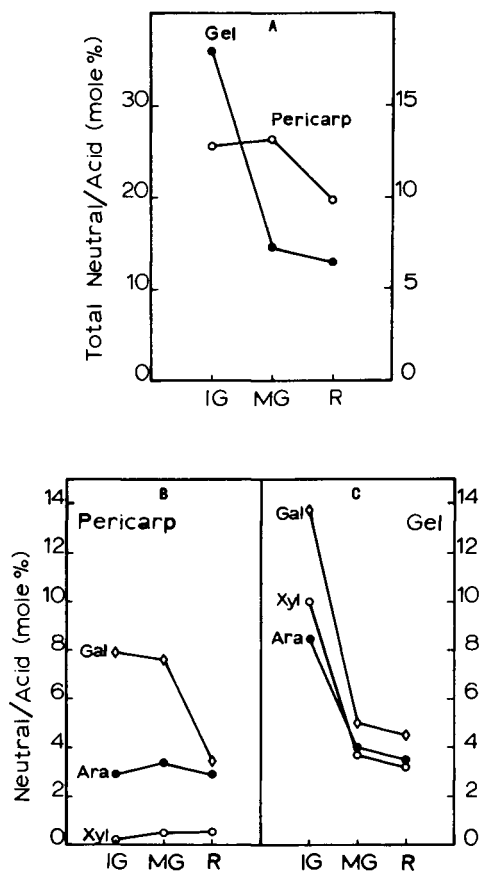


Figure 4. A, Total neutral sugar levels of soluble pectins derived from tomato fruit at the immature green (IG), mature-green (MG), and ripe (R) stage of development. Left ordinate, gel; Right ordinate, pericarp. B and C. Individual neutral sugars from pericarp and gel pectins. Gal (galactose), Xyl (xylose), Ara (arabinose).

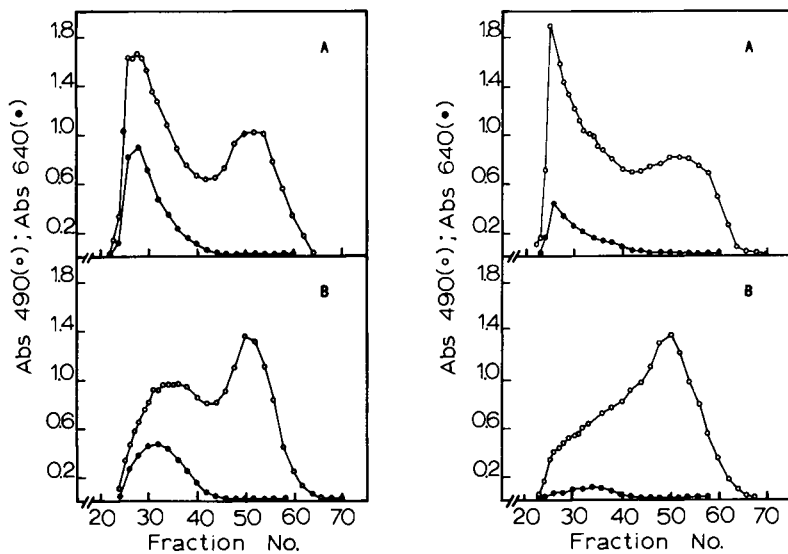


Figure 5. Ultrahydrogel Aca 34 profiles of hemicelluloses from pericarp (left) and gel from fruit at mature-green (A) and ripe (B) stage of development. Total sugars ( $A_{490}$ ), xyloglucan ( $Abs_{640}$ ).

during initial gel formation, due in part to enlargement and extensive thinning of the walls of cells formed during the period shortly following pollination (47,48). This differentiative event occurs prior to visible signs of gel ripening, such as the appearance of lycopene, which occurs following initial gel development but prior to ripening of pericarp. Levels of soluble pectin in gel were high throughout development, although a significant increase in soluble pectins occurred during the transition of fruit to mature green, the initial period of gel development. Beyond the mature-green stage, levels of soluble pectins remained high but showed no consistent pattern and were not well correlated with activity of gel EDG. As reported for tomato (pericarp) and other fruit types, increases in soluble pectins in pericarp closely paralleled EDG activity. The seemingly high level of soluble pectins in immature (24%) and mature pericarp (30%) is explained by the fact that chelator (EDTA) was included in pectin extraction media.

Gel Filtration of Tomato Pectins. Gel filtration of pectins from 'Sunny' pericarp revealed a trend of depolymerization similar to but less extensive than that reported for 'Rutgers' tomato fruit (11). The difference is likely due to the inherent firmer texture and lower EDG activity of the cultivar 'Sunny' (30,49). Other investigators have reported lower levels of soluble pectins and/or EDG activity in firmer tissues or cultivars of a given fruit type (28,50,51). Although the cultivars 'Sunny' and 'Rutgers' differ in levels of soluble pectins, examination of these polymers on Bio-Gel P-10 revealed an overall similarity in molecular size (data not shown). Possibly, absolute levels of EDG influence the quantity of rather than the extent to which pectins are depolymerized. In gel, pectin depolymerization was less extensive and not apparent until late in development, after the transition of fruit from immature to mature green. Although gel is well developed in mature-green fruit, the consistency of this tissue continues to change in the period during which pericarp tissue ripens, thus possibly the trace levels of EDG activity are operative during the terminal stages of gel development. The potential influence of degree of esterification on the intermolecular associations of pectins is well appreciated (40,41,52). However, a role for this variable in gel formation would appear unlikely since esterification of total pectins showed no significant change throughout development. The high degree of esterification of gel pectins clearly identifies them as "high methoxyl pectins" (42), and the overall consistency of gel is undoubtedly largely due to the influence of these polymers. The high esterification of these polymers, in addition to the low levels of EDG might explain the more restricted depolymerization of gel pectins (53,54). Percent esterification decreased in pectins from pericarp providing evidence that pectinmethylesterase was operative (54).

The rather limited and delayed depolymerization of gel pectin and the constancy of esterification indicates that the initial period of gel formation involves a mechanism other than the classic PME→D-galacturonanase scenario.

Neutral Sugar Changes. Neutral sugars are known to exist as structural components of pectins (44,55,56). Arabinose and galactose comprise the predominant neutral sugars, present as side chains of variable length, along with minor quantities of other, sometimes rare sugars or derivatives. The precise structural and positional characteristics of these neutral sugars, along with the quantity and distribution of the intrachain sugar rhamnose, will clearly influence the conformational features of the polymers (45). There is also evidence that branches may be nonrandomly distributed resulting in the structural distinction between 'hairy' and 'smooth' regions (57,58). A common feature of the pectins from gel and pericarp was the decrease in covalently associated neutral sugars. These decreases were temporally distinct, occurring first in gel and involving nearly a 60% decrease in total neutral sugar which initially constituted 40% of the pectin (mole basis). This decrease occurred during the initial period of gel formation with little further change during subsequent fruit development. The trends observed in arabinose and galactose levels have been reported for other fruit, although the mechanism of their removal and the nature of the parent polymers are in many instances unknown. As Ahmed and Labavitch (8) have stated, compositional analyses of total cell wall offers little information regarding the nature of the parent polymer(s) involved.

Ahmed and Labavitch (8) demonstrated for ripening 'Bartlett' pear fruit that the decrease in cell wall arabinose was largely explained by the endo-D-galacturonanase-mediated release of arabinose present as a structural component of pectins. Knee (12) attributed decreases in pectin-associated arabinose and galactose in ripening apple to the action of  $\beta$ -galactosidase and  $\alpha$ -arabinosidase. Similar enzymes were implicated in the proportional loss of cell wall arabinose and galactose from an "acid-soluble hemicellulose" in ripening Japanese pear (14). Compositional data indicated that this fraction consisted largely of pectin. Boothy (24) reported no change in the proportional composition of neutral sugars associated with pectins in ripening plum. Examining a soluble pectin fraction isolated from tomato pericarp cell wall, Gross (16) found that galactose levels decreased while arabinose increased during the transition from turning to ripe. However, his neutral sugar data were expressed in terms of proportional composition, thus the increase in arabinose possibly reflected, in part, the large decrease in galactose. Even so, Gross' data and those presented here are not directly comparable. His studies were performed using aqueously (buffer) prepared cell walls, which in tomato show reduced levels of pectin during ripening (15), presumably due to the generation of freely soluble pectins by EDG. Ethanol-insoluble powders, while not constituting a homogeneous cell wall preparation (59), retain the majority of enzymically generated pectins and allow assessments of neutral sugar changes occurring independently of major losses in pectins. In the present study, absolute levels of arabinose associated with pericarp soluble pectins actually decreased (13%). The marked decrease in arabinose (50%) in gel pectins provides further evidence for a mechanism of arabinose removal independent of EDG action and of proportional losses in other neutral sugars.

In tomato pericarp, the loss of pectin-associated galactose appears to occur via at least two mechanisms. One involves the EDG-mediated release of a galactan-rich pectin, as demonstrated by Pressey (60). Pressey has also shown that a  $\beta$ -galactosidase from ripe fruit will depolymerize this galactan *in vitro* (61), representing an EDG-independent means of galactose loss. Gross (16) has recently shown that galactose loss in ripening tomato fruit may involve another, presumably non-pectin galactose polymer present as a component of  $\alpha$ -cellulose. In addition to the presence of both arabinose and galactose in gel pectins, xylose levels were unusually high (nearly 10%) and showed a loss concomitant with the loss of arabinose and galactose. Cell wall xylose levels are generally reported not to change during fruit ripening, although Gross (19) reported for apricot that xylose was the major cell-wall neutral sugar lost during ripening.

Hemicellulose Changes. The changes observed in pericarp alkali-soluble polymers are similar to those reported previously for 'Rutgers' tomato fruit (11). Since 'Rutgers' and 'Sunny' represent cultivars differing significantly in firmness and texture changes during ripening, the overall similarity in hemicellulose changes indicates that firmness differences are not, in this instance, explained by variation in the behavior of hemicelluloses. Levels of EDG and/or calcium appear to be of more importance as determinants of firmness and texture changes (50,51). It is evident from the present study that hemicelluloses from gel exhibit a similar shift toward lower-molecular-size polymers. These changes were not evident until after the initial period of gel formation, indicating that if these polymers participate in gel formation, then their role is restricted to the latter stages of development. Hemicellulose changes in tomato pericarp occur gradually during ripening, simultaneous with the appearance of EDG activity and pectin degradation products (11). However, similar changes observed in ripening strawberry fruit (20), which contain no EDG, and in tomato gel, which has low levels of the enzyme provide additional evidence that the modifications in alkali-soluble polymers are the consequence of reactions or processes unrelated to pectin depolymerization.

These studies demonstrate the diverse nature of the role of pectins in the texture changes of ripening tomato fruit. The inverse relationship between EDG activity and the extent of softening in gel and pericarp emphasize that EDG does not play a major role in the early, dramatic period of gel formation. The marked losses in pectin neutral sugars, including xylose, occurred simultaneously with initial gel formation although the mechanism and function of these losses in this tissue remain unknown.

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## Polygalacturonases in Higher Plants

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Polygalacturonase (PG) is widely distributed in higher plants. The richest plant source of PG is ripe tomato fruit. Extracts of tomatoes contain two forms of the enzyme (PG I and PG II) which differ markedly in molecular size and stability to heat. A protein (PG converter) has been isolated from tomatoes that reacts with PG II to form PG I. PG II can be recovered by treating PG I with mild alkali (pH 11). Both tomato enzymes are endo-PG's. Some fruits that soften markedly during ripening such as pears and freestone peaches contain not only endo-PG but also exo-PG. Other fruits such as apples and clingstone peaches contain only exo-PG, consistent with slow softening characteristics. Low levels of exo-PG are found in many vegetative and storage tissues.

Pectin occurs in most plant materials but is particularly high in young and fruit tissues. It is an important constituent of the cell wall where it may be involved in interlinking of other structural polysaccharides and proteins (1). Pectin is also the major component of the middle lamella which has led to the classical view that it is an intercellular adhesive.

Pectin appears to vary greatly in composition from source to source and even from the same plant at different stages of growth. A serious problem in characterization of pectin is that the native polysaccharide is essentially insoluble and solubilization can be accomplished only by degradation. Other polysaccharides are solubilized along with pectin and separation is difficult. Thus the pectin that is isolated is partly degraded, quite heterogeneous in terms of molecular size, and may contain contaminating neutral polysaccharides. Nevertheless, it is known that pectin is basically a linear  $\alpha$ -1,4-galacturonan in which at least some of the carboxyl groups are esterified with methanol. Variable amounts of neutral sugars are usually associated with the galacturonan (2).

The galacturonan chain is interrupted by  $\alpha$ -1,2-linked

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rhamnose residues, but the amount of rhamnose in pectin is highly variable from source to source. For example, the pectin from suspension cultured sycamore cells contains nearly ten times more rhamnose than the pectin from apple fruit (2, 3). Two other neutral sugars, arabinose and galactose, are also covalently bonded to the galacturonan. These sugars occur as side chains of  $\alpha$ -1,5-arabinan and  $\beta$ -1,4-galactan (1, 3, 4, 5). It has been proposed that branching of the galacturonan chain is more prevalent in the pectin from cell walls than that from the middle lamella (6, 7).

Two classes of enzymes degrade the  $\alpha$ -1,4-galacturonan in pectin. Lyases or transeliminases cleave the  $\alpha$ -1,4-galacturonosidic bond by a trans elimination of the proton on carbon 5 of galacturonic acid with the oxygen of the glycosidic bond (8). This cleavage produces an unsaturated bond between carbon atoms 4 and 5 of the galacturonic acid residue at the nonreducing end of the fragment released. Most lyases are specific for esterified galacturonans, but some lyases cleave de-esterified galacturonans (9). The end-product of lyase action is an unsaturated monomer that rearranges to the 2-keto-uronic acid (10). Lyases appear to be common in microorganisms, but Albersheim and Killias (11) reported a transeliminase in an extract of pea seedlings.

The galacturonan chains in pectin are also cleaved by poly- $\alpha$ -1,4-galacturonide glycanohydrolase, EC 3.2.1.15, which is usually referred to as polygalacturonase (PG). This enzyme is produced by microorganisms along with lyases, but polygalacturonases also occur in higher plants. In contrast to the lyases, polygalacturonases are specific for de-esterified galacturonans, i.e. polygalacturonic acid. The rate and extent of hydrolysis by polygalacturonase are functions of the degree of de-esterification of pectin (12). It has been suggested that at least two adjacent free carboxyl groups are necessary for polygalacturonase action to occur (12). This requirement for de-esterification suggests that pectin hydrolysis in plants may be controlled by the enzyme pectinesterase. However, evidence has not been provided that pectinesterase is a controlling factor in fruit softening. For example, tomatoes contain a high level of pectinesterase but the activity does not appear to be related to tomato softening (13).

Polygalacturonase Assay. Polygalacturonase can be measured by the change in viscosity of pectate in an Ostwald or similar viscometer. This is a very sensitive assay for random-cleaving enzymes. However, methods that measure the increase in reducing end groups during hydrolysis of polygalacturonates are more convenient and are applicable for both endo- and exo-enzymes. Several colorimetric methods have been used (14, 15, 16) but I prefer the arsenomolybdate method (17). Commercially available pectate of citrus origin is a suitable substrate for the assay. I have prepared three fractions of polygalacturonates of lower molecular weights by controlled enzymatic hydrolysis of pectate (18). More extensive hydrolysis of pectate produced oligogalacturonides with a degree of polymerization from two to seven which were separated on DEAE-Sephadex A-50 (19). Substrates such as these with a wide

range of molecular weights are useful for characterizing polygalacturonases.

Polygalacturonase in Tomatoes. PG in higher plants was first found in ripe tomato fruit (20) which remains the richest plant source of this enzyme. PG in tomatoes has received considerable attention, especially in recent years, in relation to tomato ripening and softening. Although it has been assumed that green tomatoes do not contain PG activity, I recently detected a very low level of exo-PG in green fruit (21). Nevertheless, it is well known that endo-PG appears at the onset of tomato ripening and increases sharply during ripening (22). The level of endo-PG in ripe tomatoes is about 600 times higher than that of exo-PG in green fruit. Numerous studies have established that the fruit from firm tomato cultivars has less PG activity than that from soft cultivars (23, 24, 25). The non-ripening tomato mutants nor and rin do not develop PG activity (26), and the landraces of tomato Longkeeper and Alcobaca in which the fruit have prolonged keeping qualities develop very low levels of PG (27, 28). Thus it is generally accepted that PG has an important role in the ripening and softening of tomatoes.

In early work on tomato PG, McColloch and Kertesz (29) found that most of the activity in crude extracts of ripe tomatoes was destroyed by heating at relatively low temperatures, while the remainder survived heating to 90° C. This suggested the presence of two enzymes in tomatoes. In 1973, we separated the two enzymes by chromatography on DEAE-Sephadex A-50 (30). The enzymes have been designated PG I and PG II in the order of their elution off the column. Separation of the PG isoenzymes can be achieved by gel filtration (Figure 1) and by HPLC on a strong cation exchanger (31).

Tomato PG I and PG II are similar in many respects. Both enzymes are endo-PG's although PG II is more effective in reducing the viscosity of pectate (30). Their pH optima are near 4.5, and both enzymes exhibit broad peaks of activity extending from pH 1.5 to 5.5 when hydrolyzing short-chained substrates (30). They are basic glycoproteins with pI's of 8.6 and 9.4 for PG I and PG II, respectively (32). Antibodies raised against PG II react also with PG I (32). The same polypeptide is obtained when PG I and PG II are denatured in SDS solutions (32, 36). However, the enzymes differ markedly in molecular size and stability to heat. The molecular weights, as determined by gel filtrations are 100,000 and 44,000 for PG I and PG II, respectively (30, 32). PG II is inactivated after 5 min at 65° C but PG I is inactivated only after 5 min at 90° C (30).

The evidence that PG I reacts with the antibody raised against PG II and that both enzymes yield the same polypeptide in SDS solutions indicates a common feature in the enzymes. Tucker and Grierson (33) proposed that PG I is a dimer of PG II which is consistent with the approximately 2-fold difference in molecular weights of the enzymes. In a study undertaken to verify the monomer-dimer relationship, I did not observe formation of PG I from purified PG II at various conditions of enzyme concentration, pH, and ionic strength. However, I discovered that PG I is converted to PG II at alkaline conditions (34). This was

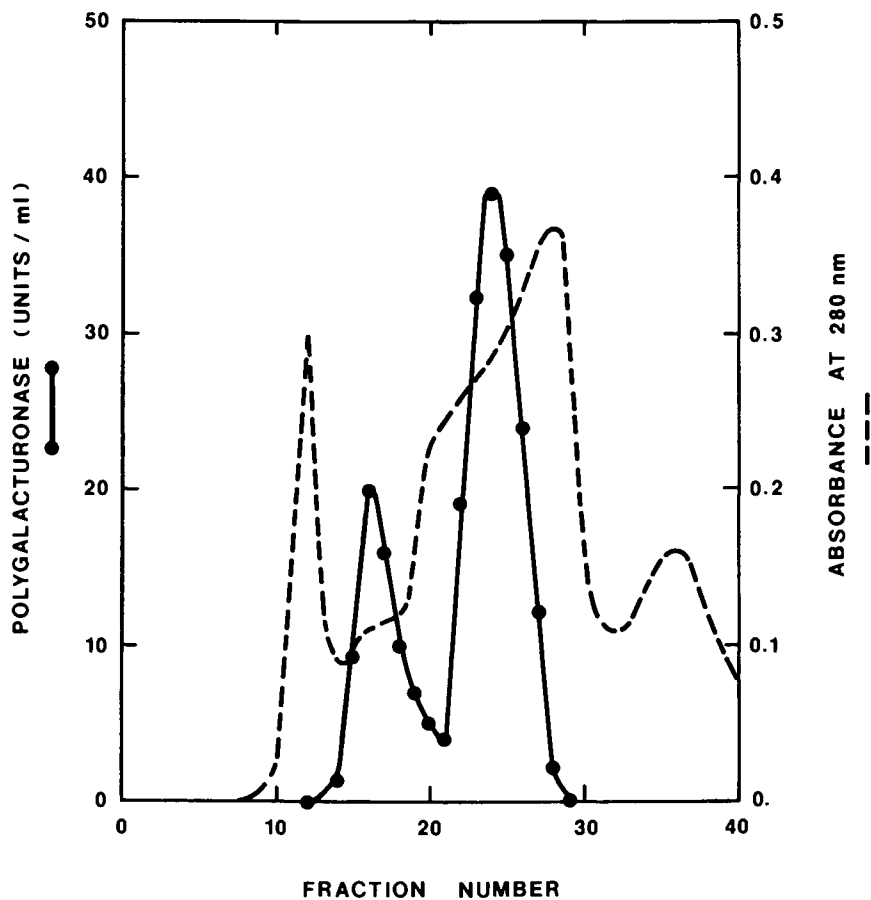


Figure 1. Separation of tomato PG I and PG II by chromatography on Sephadex G-100. Reproduced with permission from Reference 31.

demonstrated by diluting a solution of purified PG I with an equal volume of 0.2 M glycine, pH 11, and incubating at 37° C for 1 hr. The pH of the solution was then lowered to 5 with acetic acid. There was no loss of polygalacturonase activity after the treatment of pH 11, but 81% of the activity was sensitive to heating for 5 min. at 65° C. The solution was then analyzed for PG I and PG II by column chromatography on Sephadex G-100. A small peak corresponding to PG I was obtained but most of the activity was in the PG II peak.

The conversion of PG I to PG II does not occur at neutral and acid conditions. At pH 5, heating solutions of PG I at 100° C for 7 min. resulted in complete loss of PG activity. However, I found that the heated solution of PG I contained a component that produced PG I on addition to purified PG II. Because this component converts one isoenzyme to the other, it was named PG converter. It can be assayed by measuring the quantity of PG I formed from PG II and solutions of the converter. PG I can be determined as the residual activity in solutions heated at 65° C or by various chromatographic methods. A unit of PG converter has been defined as the amount that converts 1 unit of PG II to PG I.

PG converter has been highly purified according to the following procedure (34). Ripe tomatoes were extracted first with 0.5 M NaCl at pH 5.5 to remove most of the PG II and other soluble components. The insoluble fraction was then extracted with 1.2 M NaCl at pH 6. The extract was concentrated by ultrafiltration, adjusted to pH 5, and heated 7 min. at 100° C to inactivate the polygalacturonases. The PG converter in the heated solution was then purified by chromatography on Sephacryl S-200 and Polyanion SI and by chromatofocusing on a Mono P column. A 28-fold increase in the specific activity of the converter was achieved with an 81% recovery of total activity (Table I).

Table I. Summary of the purification of tomato PG converter.

Step	Volume	Protein	Activity	Specific Activity
	ml	mg	units	units/mg
1.2 M NaCl extract	25	172	-	-
7 min at 100 °C	23	156	3670	24
Sephacryl S-200	10	21	3420	163
Polyanion SI	6	9.6	3180	346
Chromatofocusing	5	4.4	2960	673

Source: Reproduced with permission from reference 34. Copyright 1984 FEBS.

Chromatofocusing of the purified PG converter yielded a single peak of protein corresponding to converter activity. Elution of the converter off the column at pH 5.1 indicates a weakly acidic molecule. The converter gave positive reactions with biuret and Folin-Ciocalteu reagents but not with Coomassie blue G-250 reagent. The absence of a reaction with Coomassie blue probably explains why

the converter was not found along with the PG II polypeptide in SDS solutions of PG I (32). The converter was stable to heating at 100° C when the pH was between 2 and 9. It was also quite stable in 0.1 M HCl, but it was very sensitive to alkaline conditions with a complete loss of activity in 0.1 M NaOH at 24° C and in pH 11 solutions at 100° C. The converter was susceptible to proteases, with complete inactivation by pronase and papain. A low amount of carbohydrate was present in the purified converter. The properties of the PG converter are thus consistent with that of a glycoprotein.

The converter does not affect the activity of PG II when they react. The only changes in the enzyme on binding to the converter appear to be a larger molecular size and a greater resistance to inactivation by heat. The interaction occurs rapidly and irreversibly at physiological pH (34). As will be shown below, the converter is found in green tomato fruit well before PG activity can be detected. What is the physiological function of this glycoprotein that appears to specifically bind PG II? A possible role is to immobilize the enzyme in the cell wall and thus regulate its action. Morgens et al. (35) recently obtained evidence that PG may indeed be sequestered in tomato fruit.

PG is usually extracted from tomatoes with 1 M NaCl at pH 6 after washing the cell wall fraction with water at the endogenous pH (30, 36). Because of the importance of detecting PG when it first appears in relation to other aspects of the ripening process, I recently conducted a detailed study on the solubility of the enzyme (37). The PG was practically insoluble in water at pH 3 (Figure 2). This allows washing of the cell wall fraction at pH 3 with water to remove the soluble components including the reducing sugars which interfere in the PG assay. Samples of washed cell walls were then extracted with water at pH's lower and higher than 3. The highest amount of PG activity was solubilized at pH 6 and higher (Figure 2), and the amount extracted was about 80% of that extracted by 1.2 M NaCl at pH 6. Analysis of the pH 6.5 water extract showed that it contained 46% PG I and 54% PG II. The solubility of PG increased also as the pH of the cell walls in water was lowered below 3 (Figure 2). The amount extracted was highest at pH 1.8 although it was considerably less than that extracted at pH 6. The PG activity extracted at pH 1.8 was due exclusively to PG II.

The effects of salts on the solubility of tomato PG was found to be highly dependent on the pH of the extraction solution. At pH 1.8, the solubility of PG increased with increasing NaCl concentration to a maximum at about 0.4 M NaCl (Figure 3). Only PG II was extracted at pH 1.8 by NaCl solutions as high as 0.1 M. As the salt concentration was increased further, PG I appeared and increased at the expense of PG II. The results can be explained by the involvement of PG converter in the formation of PG I. Apparently the converter is insoluble in NaCl at low concentrations at pH 1.8 and thus is not available to react with soluble PG II. However, the converter is solubilized from the cell wall fraction at higher concentrations of NaCl and as it is solubilized, the converter reacts with PG II to yield PG I.

At pH 6, the addition of NaCl to the extraction solution decreased the solubility of total PG, PG II, and especially that of

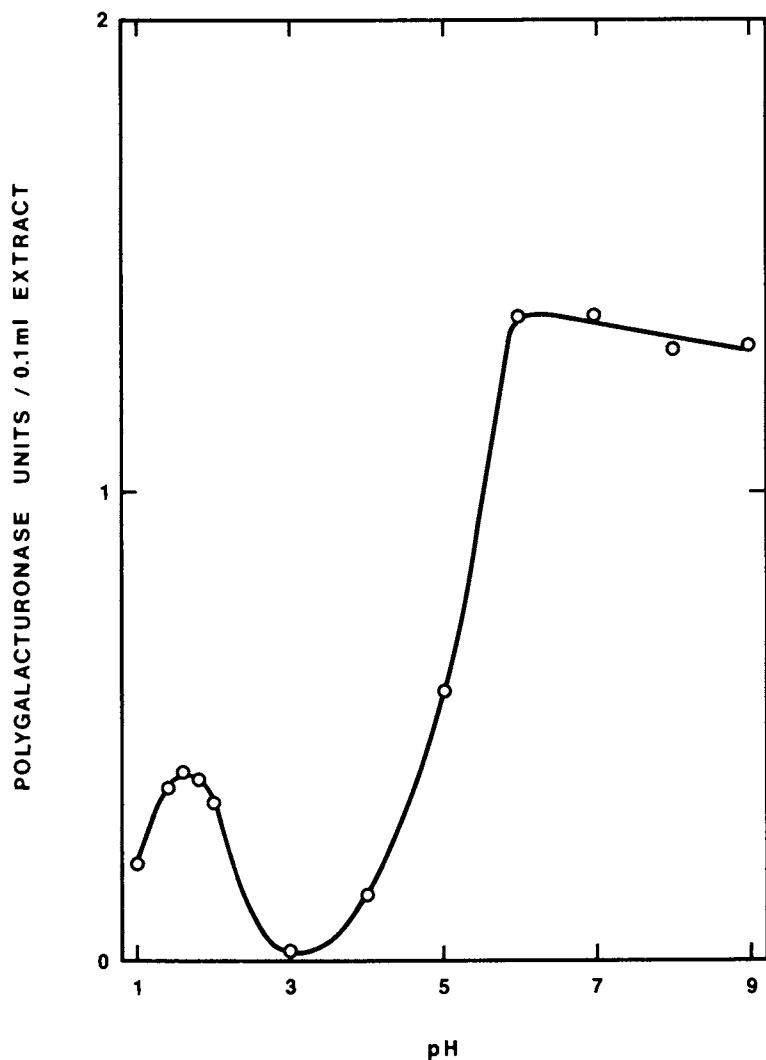


Figure 2. Effect of pH on the extraction of polygalacturonase from the cell wall fraction of tomato. Reproduced with the permission of Reference 37.



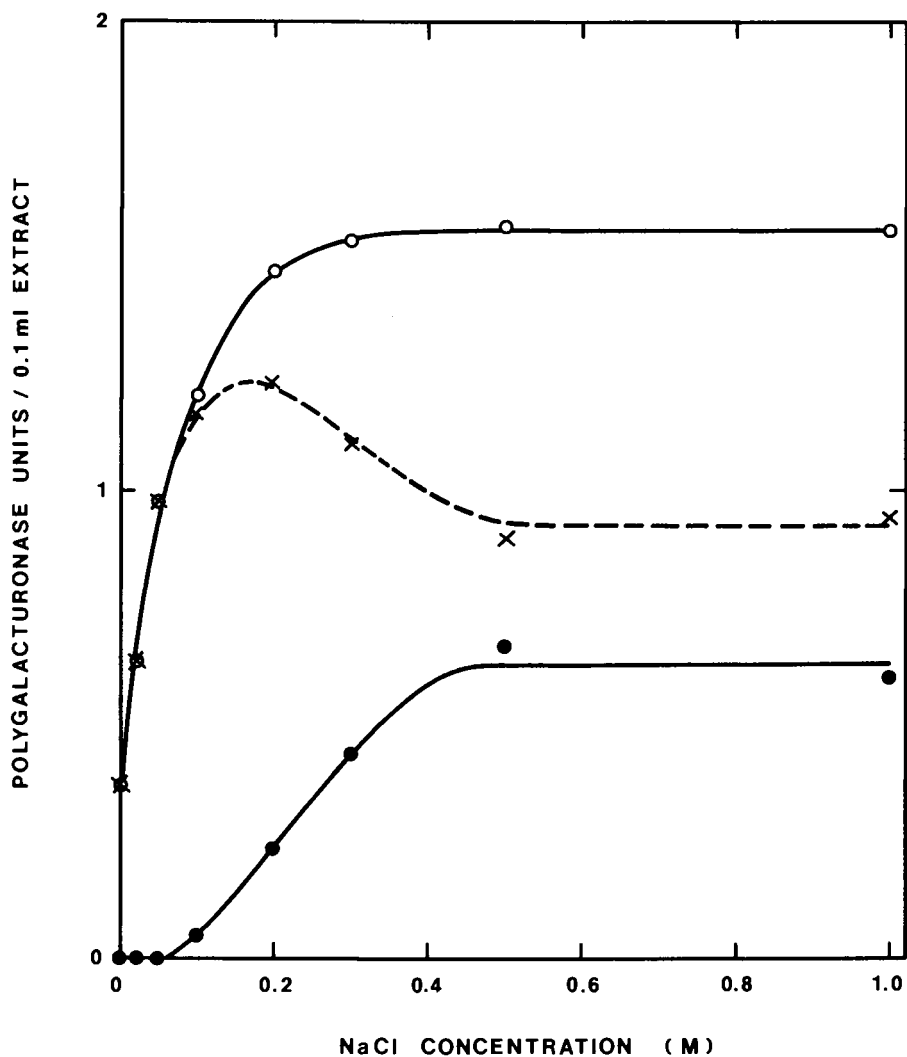


Figure 3. Effect of NaCl concentration on the extraction of tomato polygalacturonases at pH 1.8. o, total PG; ●, PG I; x, PG II. Reproduced with the permission of Reference 37.

PG I, to minima at about 0.15 M NaCl (Figure 4). A possible explanation for the lower recovery of PG activity involves the role of pectinesterase (PE) which accompanied PG in these extracts. The high level of PE in the salt extracts plus the enhancement of PE activity by the cations leads to rapid de-esterification of pectin in the cell wall fraction. The negatively charged pectates then adsorb the cationic PG's and thus remove them from solution. Extraction of cell wall fraction at pH 6 with NaCl solutions higher than 0.15 M increased the yields of total PG activity and PG I to maxima at about 1.2 M NaCl (Figure 4). However, the highest amount of PG II was obtained with 0.5 M NaCl. This again indicates differences in solubilities of PG II and PG converter in relation to NaCl concentration. The amount of total PG activity extracted by 1.2 M NaCl was relatively independent of pH over the range of 2 to 9. The yield of PG decreased only when the pH was lowered to 1, which may reflect instability of the enzyme at highly acid conditions.

Another consideration in the assay and isolation of tomato PG is the method for concentrating the activity in crude extracts. The method that is commonly used is precipitation with ammonium sulfate followed by dialysis of the re-dissolved protein against dilute NaCl or even water (30, 36, 38). A study was conducted to determine the recovery of PG activity by this method compared with ultrafiltration. An extract of a washed cell wall fraction was prepared in 1.0 M NaCl at pH 6. One-third of the extract was ultrafiltered to 20 ml and dialyzed against 1.0 M NaCl. The remaining two-thirds of the extract was treated with ammonium sulfate at 75% of saturation. The precipitate was dissolved in 40 ml of 1.0 M NaCl, and aliquots of the solution were dialyzed against 0.15 M and 1.0 M NaCl. Relative to the PG activity in the ultrafiltrate, only 43% and 78% of the activity was recovered in the ammonium sulfate fractions dialyzed against 0.15 M and 1.0 M NaCl, respectively. The much lower activity in the fraction dialyzed against 0.15 M NaCl was due primarily to the loss of PG I. These results explain the low levels of PG I reported by Tucker et al. (36). Their procedure for preparing extracts included precipitation with ammonium sulfate followed by dialysis against 0.15 M NaCl. They found that only about 10% of the activity was due to PG I in extracts of the fruit of two cultivars of tomatoes.

I have used the methodology described above to study the changes in PG I, PG II, and PG converter in ripening tomatoes (39). Fruit were selected at six stages of ripeness. The fruit softened markedly during ripening and the level of water-soluble pectin increased sharply. PG activity was first detected in extracts of fruit at the turning stage, in agreement with earlier reports (23, 32, 26). PG I was the only enzyme present in these extracts. PG II was first found in extracts of fruit at the pink stage. On further ripening, both enzymes increased markedly but PG I remained the major isoenzyme even at the over-ripe stage. This is in contrast with the results of Tucker et al. (36) mentioned above and also with those of Moshrefi and Luh (38) that PG I was absent in extracts of ripe tomatoes. The low recovery of PG I in both of these studies may be attributed to precipitation of PG I during dialysis of the extracts against 0.15 M NaCl (37).

Whereas the polygalacturonases appeared after ripening of

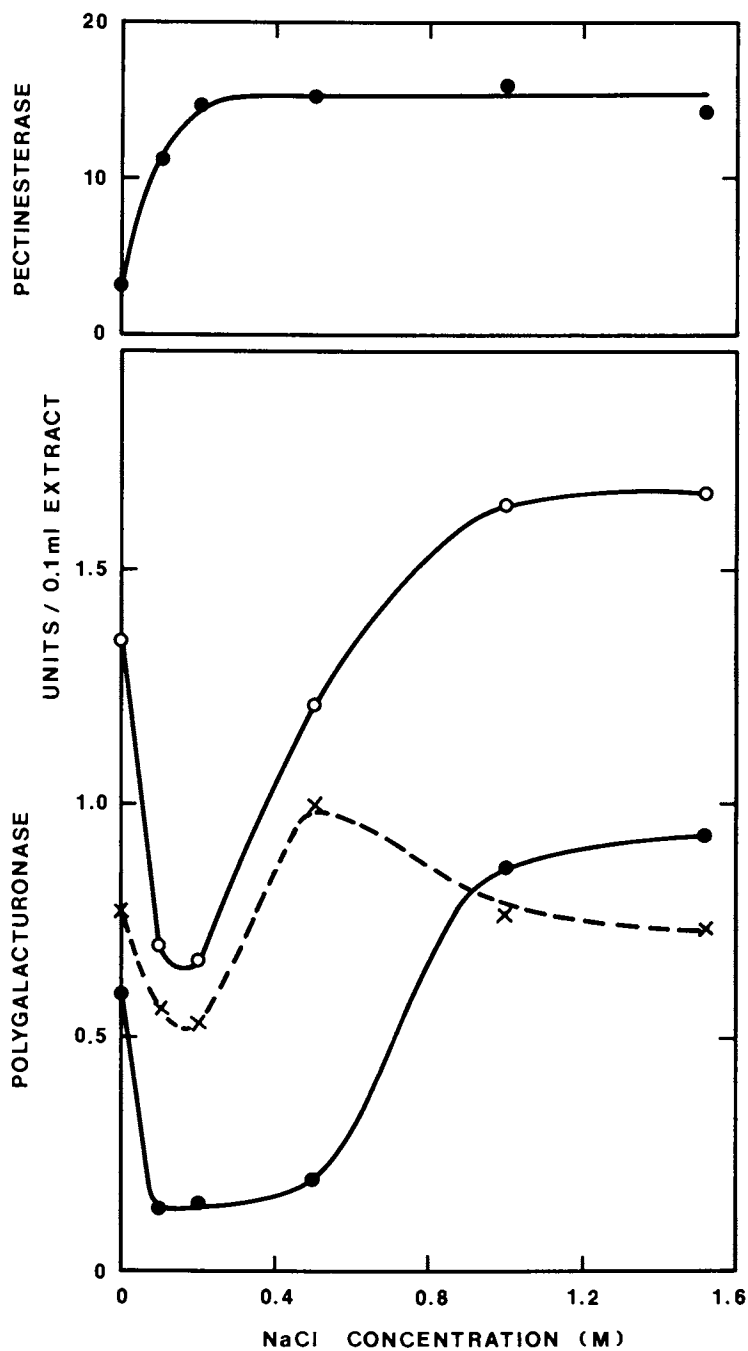


Figure 4. Effect of NaCl concentration on the extraction of tomato polygalacturonase at pH 6. o, total PG; ●, PG I; x, PG II. Reproduced with the permission of Reference 37.

tomatoes began, PG converter was present in the fruit at the green stage. The level of PG converter remained low through the breaker stage and then increased with ripening. The presence of the converter in unripe tomatoes raises the possibility that PG I may be formed during extraction of ripening fruit. As PG II appears in the fruit, during the preparation of extracts the enzyme reacts with the excess of converter to form PG I. Thus free PG II is not found in the extracts until the enzyme exceeds the level of the converter.

Polygalacturonases in Peaches. Freestone peaches soften markedly during ripening, resulting in the characteristic juicy texture of peaches. Accompanying peach softening is a conversion of insoluble pectin to water-soluble forms (Figure 5). The solubilization of pectin suggests that degradation of pectin occurs, but PG activity was not detected in peaches in early studies (40). The reason it was not detected is that the level of activity in ripe peaches is much lower than that in tomatoes (41). The activity is so low that it is necessary to use very long assay incubation period (41) or to concentrate the enzyme by ultrafiltration (42) to measure it. But as in tomatoes, PG activity appears in peaches near the onset of ripening and then increases sharply during ripening (Figure 5).

The PG activity in ripe freestone peaches is due to two enzymes (42). In contrast to tomatoes which contain two endo-PG's, peaches contain an endo-PG but also an exo-PG (42). The peach enzymes can be separated by chromatography on a Sephadex G-100 column. The endo-PG has a pH optimum near 4 and is very effective in solubilizing pectin from peach cell walls. The discovery of exo-PG in peaches represented the first time that this enzyme was found in fruit tissue. The exo-PG has a pH optimum near 5.5 and requires  $\text{Ca}^{2+}$  for activity with an optimum concentration of 0.4 mM. It removes monomer units from the nonreducing ends of the substrate molecules. This enzyme does not solubilize the pectin from isolated peach cell walls.

Ripening clingstone (non-melting flesh) peaches soften considerably less than do freestone peaches. They also retain more insoluble pectin, suggesting that less pectin degradation occurs. An examination of a number of cultivars of peaches (43) showed that all ripe freestone peaches contain approximately equal levels of endo-PG and exo-PG whereas clingstone peaches contain a high level of exo-PG but zero or very low endo-PG (Table II). The absence of the endoenzyme in clingstone peaches is consistent with the low solubilization of pectin and retention of fruit firmness in these peaches. Thus the markedly different textural characteristics of the two types of peaches are accounted for by the difference in polygalacturonase composition.

Polygalacturonases in Other Fruits. It has long been known that ripe pears contain a low level of PG (44). Yamaki and Matsuda (45) found that PG activity was present in pears during the cell division stage and that it decreased during the enlargement stage before it increased markedly during ripening. We have extracted the PG activity from ripe D'Anjou pears and separated it into two

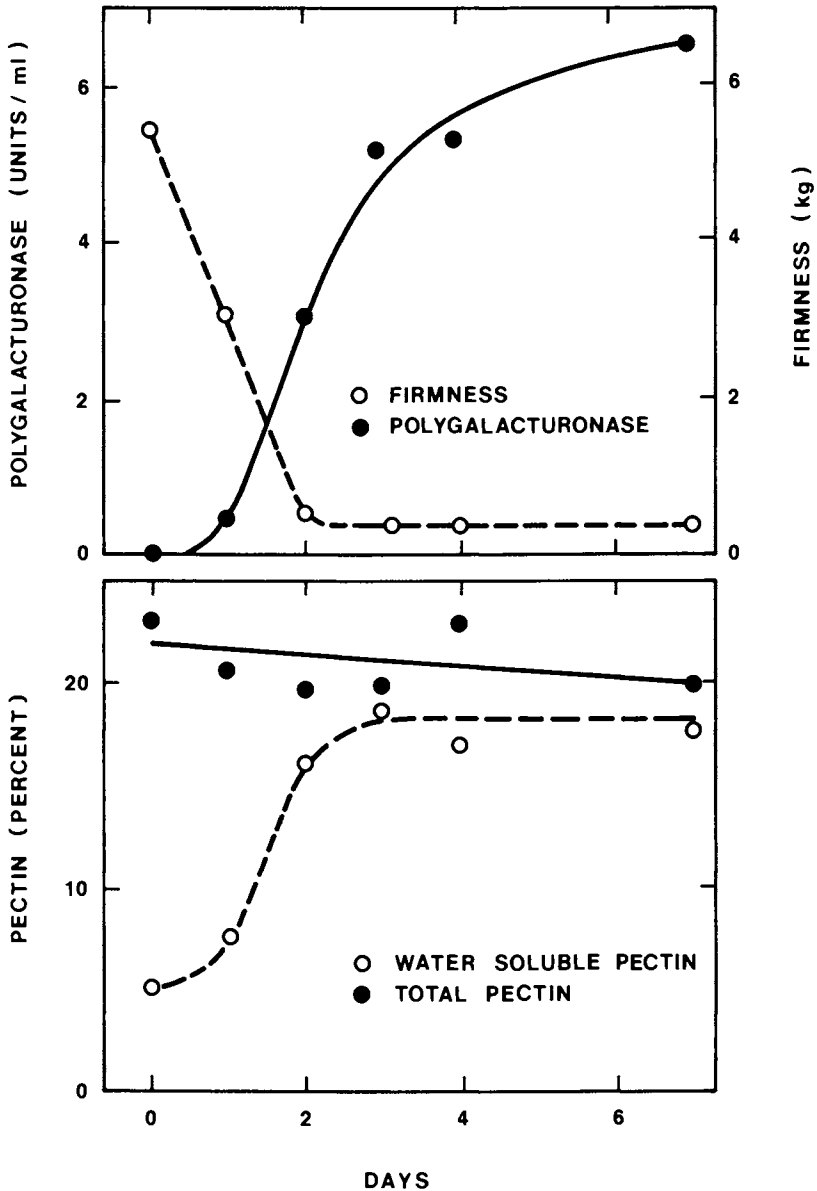


Figure 5. Changes in fruit firmness, polygalacturonase, water-soluble and total pectin in peaches during postharvest ripening (41). Reproduced with the permission of Reference 41. Copyright 1971, Institute of Food Technologists.

Table II. Polygalacturonase activity in ultrafiltered extracts of peaches.

Variety	Stone freeness	Polygalacturonase	
		Endo	Exo
		units/ml	
Baby Gold 6	cling	0.2	3.0
Mountain Gold	cling	0.2	2.6
Suncling	cling	0.2	4.2
Fay Elberta	free	4.6	4.8
Sullivan Elberta	free	4.0	3.8

peaks by chromatography on Sephadex G-100 (Figure 6). The enzyme in the first peak was very effective in reducing the viscosity of pectate and thus is an endo-PG (46). The second enzyme had a pH optimum of 5.5, required  $\text{Ca}^{2+}$  for activity, and its mode of action was consistent with that of an exo-PG (46). The exo-PG was the major PG in these pears.

Another fruit that contains both endo-PG and exo-PG is papaya (47). PG activity has also been found in ripe avocados (48), dates (49), and mangoes (50), but these enzymes were not characterized for their modes of action. Bartley (51) extracted a low level of PG activity from ripe apples which he identified as an exo-PG. The apple enzyme degraded cortical cell walls releasing low molecular weight uronic acid residues and polyuronide. Immature cucumbers contain PG activity that increases during growth of the fruit (52). The enzyme is an exo-PG but differs from other exo-PGs in that it is readily soluble in water and it exhibits the highest rate of reaction with pectate, the largest substrate. McFeeters et al. (53) have isolated and characterized an endo-PG from ripe cucumber fruit.

Polygalacturonases in Other Plant Tissues. PG activity is usually associated with ripe fruit tissues and, as we have seen, the activity can be due to an endo-PG, exo-PG, or to both enzymes. However, I have already mentioned the occurrence of PG activity in unripe tomatoes (21), pears (45), and cucumbers (52). The enzymes in green tomatoes and cucumbers have been characterized as exo-PG's.

Exo-PG has also been reported to be present in carrot roots (54) and citrus leaf explants (55). It occurred to me that this enzyme may be widely distributed in higher plants and an examination of various tissues showed this to be true (56).

PG has been found in seedlings of various plants including beans, corn, oats, and peas (Table III). In oat coleoptiles, the activity was highest near the growing tip and it decreased markedly as the distance from the tip increased. Similarly in pea seedlings, PG activity was highest in the plumules and hook, but the activity was quite high in the 1 cm section below the hook and considerably lower in lower sections of the epicotyl (57). However, PG was also found in stem and leaf tissues from these plants.

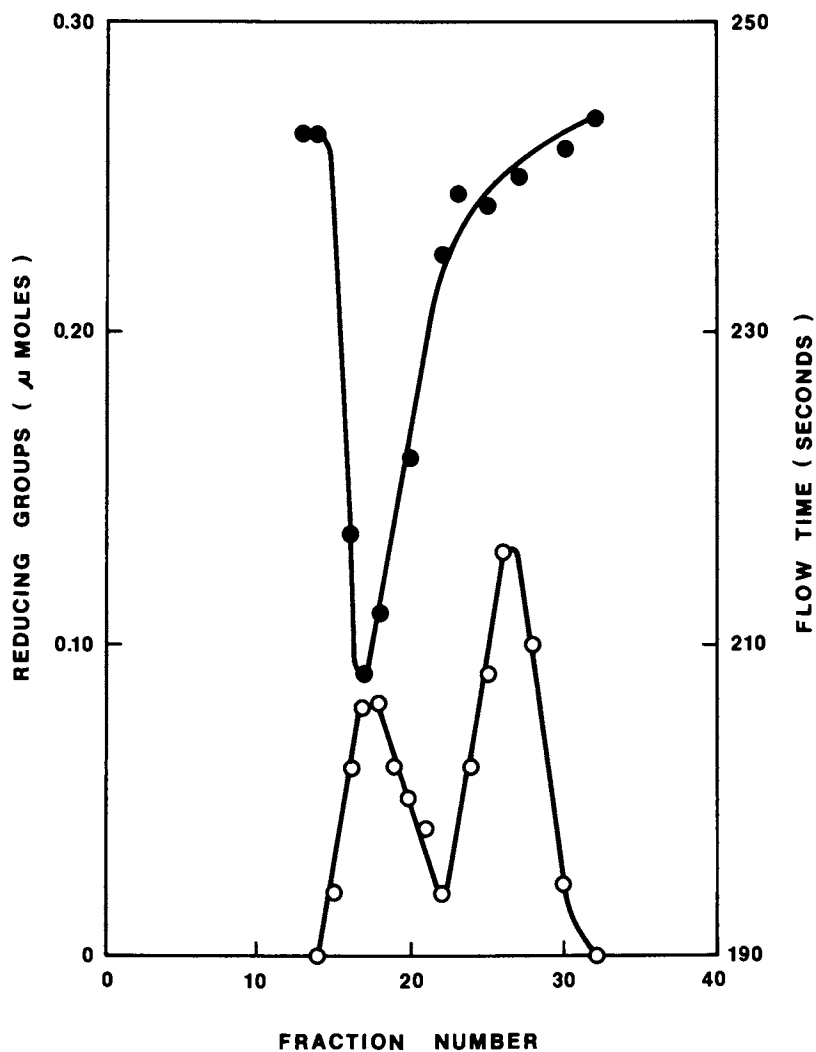


Figure 6. Separation of pear polygalacturonases by chromatography on Sephadex G-100.

The PG in oat seedlings has been partially purified and characterized (56). The molecular weight of oat PG was 63,000, as determined by gel filtration. The enzyme was optimally active between pH 5.0 and 5.5 in the absence and presence of added  $\text{Ca}^{2+}$  (Figure 7). The purified enzyme had low activity which was further reduced by EDTA and citrate.  $\text{Ca}^{2+}$  stimulated the enzyme, with an optimum concentration of 0.4 mM that produced a 7-fold increase in reaction rate over the control. Higher concentrations of  $\text{Ca}^{2+}$  were inhibitory, and the inhibition was greatest for the largest substrate, suggesting that the effect is due to substrate insolubilization rather than to actual inhibition of the enzyme.  $V_{\text{max}}$  and  $K_{\text{m}}$  were determined for oat PG acting on oligogalacturonides, polygalacturonates and pectate (Table IV). The highest reaction rates were obtained with moderately long substrates, but the enzyme had the highest affinity for pectate, the largest substrate. It was established that oat PG is an exo-enzyme that removes monomer units from the nonreducing ends of the substrate molecules (56).

Table III. Polygalacturonase activity in a variety of plant tissues.

Tissue	Polygalacturonase units/g	pH optimum	Activation by 0.6 mM $\text{Ca}^{2+}$ %
Red Kidney bean hypocotyls	0.52	5-5.5	65
Blue Lake bean pods	0.34	5	160
Seneca Chief corn seedling	0.54	5-5.5	43
Seneca Chief corn plants	0.62	5-5.5	86
Alaska pea seedling	0.24	4.5-5	100
Swiss Chard stalks	0.21	5-5.5	44
Pokeweed stems	0.17	5-5.5	--
Better Boy tomato stems	0.18	5.5	84
Butterbar squash stalks	0.10	4.5	--
Asparagus shoots	0.72	5.5	66
Turnip roots	0.42	4.5-5	64
Red beet roots	0.30	5	--

The PG in pea seedlings has also been determined to be an exo-hydrolase (57). Based on small effects on the viscosity of pectate and on activation by  $\text{Ca}^{2+}$ , the PG's in the other sources listed in Table IV appear to be exo-PG's. Endo-PG activity appears to be extremely low or absent in vegetative and storage tissue. Other workers have found exo-PG in potato tubers (58) and in suspension cultures of a liverwort (59).



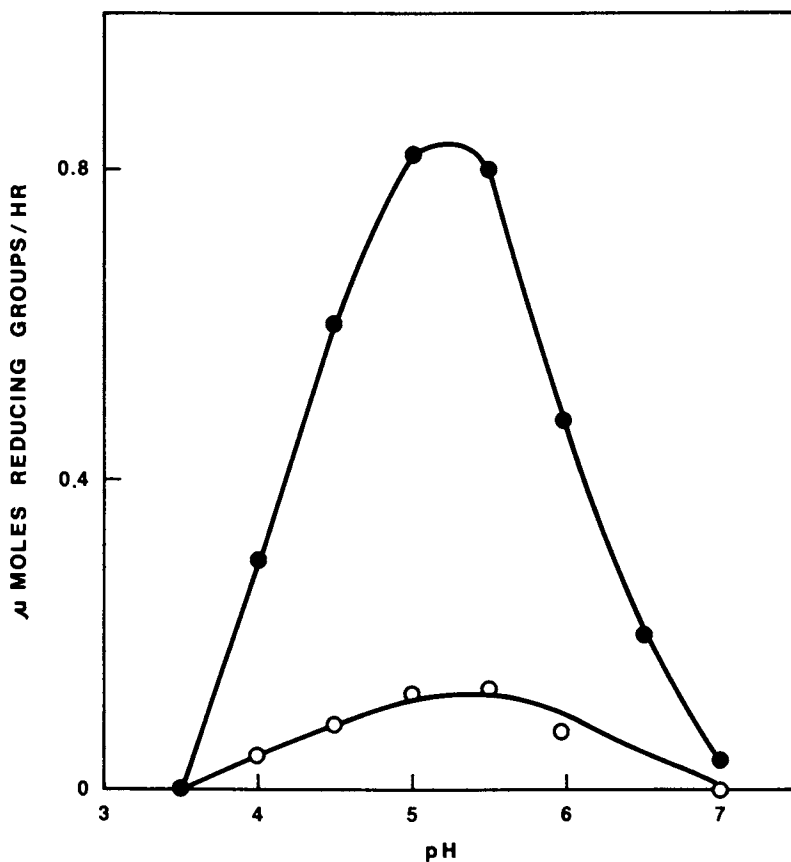


Figure 7. Effect of pH on the activity of oat seedling polygalacturonase.  $\circ$ — $\circ$ , in the absence of added  $\text{Ca}^{++}$ ;  $\bullet$ — $\bullet$ , in the presence of 0.4 mM  $\text{Ca}^{++}$ . Reproduced with the permission of Reference 56. Copyright 1977, American Society of Plant Physiologists.

Table IV. Kinetic parameters of the hydrolysis of galacturonans by oat polygalacturonase.

Substrate	$K_m$	Maximum Velocity
	$\mu M$	Relative
Digalacturonate	290	0.2
Trigalacturonate	210	0.6
Tetragalacturonate	180	1.7
Pentagalacturonate	160	1.9
Reduced Pentagalacturonate	130	2.0
Hexagalacturonate	140	2.3
PGA III	74	3.0
Reduced PGA III	68	2.9
PGA II	46	3.8
Reduced PGA II	48	4.1
PGA I	12	3.1
Pectate	6	1.0

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## Paramagnetic Ion Spin-Spin Coupling as Direct Evidence for Cooperative Ion Binding to Higher Plant Cell Walls

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Electron spin resonance {ESR} spin-spin coupling experiments were performed to estimate  $\text{Mn}^{2+}$  and  $\text{Cu}^{2+}$  near neighbor distances, thereby determining if carboxylate-divalent cation complexes potentiate ion association at adjacent sites on cell wall polyuronides. Distances were estimated to be 12 and  $14\text{\AA}$  for  $\text{Cu}^{2+}$  and  $\text{Mn}^{2+}$ , respectively. At the maximal bound ion concentration, the lattice constant  $\{\kappa\}$  was ca. 2.5 indicating that approximately 6 paramagnetic ion near neighbor spin-spin interactions occur per dipole in the nearly-filled lattice. Competitive ion exchange with  $\text{Ca}^{2+}$  was found to reduce the  $\text{Mn}^{2+}$  spin-spin line broadening at similar total bound  $[\text{Mn}^{2+}]$ ; this could only be observed if  $\text{Ca}^{2+}$  competes with  $\text{Mn}^{2+}$  at adjacent sites. These data offer strong support for the sequential-cooperative ion binding mechanism.

Polyuronides are a major component of the primary cell wall and middle lamella of higher plant cortical tissues (1, 2). These polymers are efficient cation exchangers (3, 4) and, thus, can affect ion activity, transmembrane potential and electrolyte flux (5). In addition, the divalent salts of pectic substances

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appear to play some structural role in adjacent cell wall-to-wall adhesion (6-9) as well as exert steric control over the activity of certain hydrolytic enzymes (7, 8).

Solution studies on the binding behavior of polygalacturonate and polyguluronate (10-16) indicate that a molecular size dependent cooperative ion binding takes place (12, 13). Some authors (11, 15) have proposed that these acidic polymers bind divalent cations in electronegative cavities between chains like "eggs in an egg box". Little information has been available with regard to the ion binding mechanism or the polyuronide-cation aggregate structure in solid matrices (3-5, 17).

In this paper we will present direct physical evidence (18, 19) which supports the concept that carboxylate group-divalent cation complexes potentiate ion association at adjacent binding sites (the sequential binding mechanism, 18). We will also present evidence that the cation-polyuronide aggregate structure is similar to the model proposed for polyuronides in solution (11), that polymer blocks exist in three dimensional lattices low in methyl ester content and that subtle conformational changes can be observed with this ESR technique (19). This method could prove useful in the elucidation of the structural mechanisms of certain developmental changes in plant tissues (e.g., ripening, abscission, extensive growth, etc.; 6-9, 19).

#### Materials and Methods

Intact cortical tissues of Malus pumila {cv. Golden Delicious} fruit were used throughout these experiments. Tissue fixation and determination of free {e.g., nonmethylated} uronic acid concentrations were described previously (18).

For the ESR experiments, intact tissues {1x1x4mm} were equilibrated at pH 5 in water for 15 min. and decanted; this procedure was repeated thrice. About 10-40 mg dry weight of cell wall complex was used for each treatment combination. Samples were equilibrated {22±2°} in 10 ml of various concentrations of  $Mn^{2+}$  (18, 19),  $Mn^{2+}$  with  $Ca^{2+}$  (18) or  $Cu^{2+}$

(19). After ca. 24 hrs., the solutions were decanted and tissue specimens washed thrice in {pH 7} water, allowing 15 min. equilibration in each wash. After an additional 1 hr. soak, this washing procedure was repeated. Samples were dehydrated with ethanol and critical point dried as described previously (18, 20). Some samples were rehydrated in a saturated water vapor chamber (19) at 160 torr and 22°C. All samples were immediately loaded and sealed in 3x120 mm quartz ESR tubes. After each experiment, the samples were removed, washed in methanol, vacuum dried at 35°C, weighed, and dry-ashed for atomic absorption spectrophotometric analysis of  $Mn^{2+}$ ,  $Ca^{2+}$  and/or  $Cu^{2+}$  using standard procedures (18, 19).

$Mn^{2+}$  and  $Cu^{2+}$  ESR spectral parameters,  $Mn^{2+}$  linewidths  $\{\Delta H_{[dI(H)/dH]_{max}}\}$  and dimer-only nearest neighbor distance parameter  $\{d\}$  calculations were as previously described (18, 19). All experiments in this report were run at near liquid  $N_2$  temperatures  $\{-176$  to  $-180^\circ C\}$  to avoid certain relaxation time phenomena which can be problematic (19).  $Cu^{2+}$  linewidths were calculated directly from the first derivative  $g_{\perp}$  component (19) as the difference in field strength  $\{G\}$  between the maximum and minimum amplitude. This empirical measure of linewidth was found to be directly proportional to the true linewidth as determined by an anisotropic simulation routine (21); this simulation showed that the true linewidth  $\{\Delta H_{[dI(H)/dH]_{max}}$ , Figure 3} was equal to the empirical measure, described above, divided by 1.17 (19).

### Results and Discussion

The results in Figure 1 demonstrate that cell wall bound  $Mn^{2+}$  is associated with an acid titratable site; similar results have been obtained with  $Ca^{2+}$ . Polyuronides are the most likely ion binding species since they are the predominant anionic component (2, 20) in these tissues.

From the standpoint of the ESR experiment, ionically bound paramagnetic ions can be treated as fixed points which interact

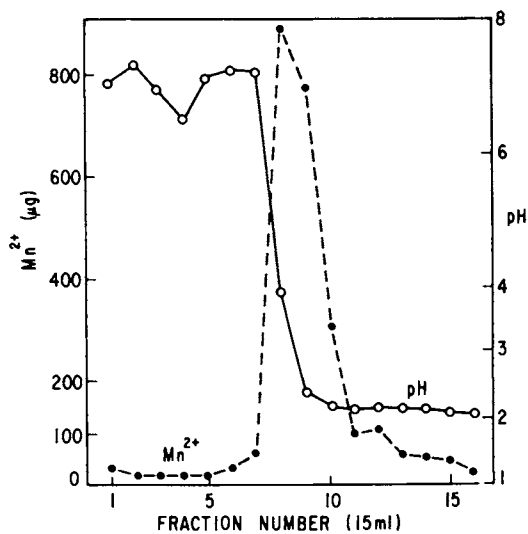


Figure 1. Titration of  $Mn^{2+}$  off intact cell wall matrices under mildly acidic conditions. Reproduced with permission from Ref. 18. Copyright 1984, Biochimica et Biophysica Acta.

only through their couplings with each other (22). Thus, any *i*th spin in the midst of other paramagnetic transition ions bound within a solid matrix, such as these cell wall matrices, has a total spin interaction profile equal to the sum of the spin-spin and exchange interactions (22). In this summation, the series converges to a value which depends only on those dipoles in the vicinity of each *i*th spin and, thus, is the same for all the dipoles of the same type in the lattice. Of these terms, spin-spin interactions cause line broadening (23) and are proportional to the inverse cube of the distance between interacting spins ( $1/r_{ij}^3$ ; 22-26) as described by the square root of the second moment ( $\langle \Delta\nu^2 \rangle^{1/2}$ ; 23, 26, 27) of a Gaussian line:

$$\langle \Delta\nu^2 \rangle^{1/2} = \{3/5 \cdot g^4 \beta^4 h^{-2} \cdot S[S+1] \cdot \sum_i 1/r_{ij}^6\}^{1/2}; \quad (1; \text{units in Hz})$$

whereupon *g*,  $\beta$  and *h* have their usual values and *S* represents the total electron spin of the ion {*S*=5/2 for  $\text{Mn}^{2+}$  and 1/2 for  $\text{Cu}^{2+}$ }. In nonmagnetic insulators (28), linewidths and -shapes agree with basic Van Vleck theory (23). However, as paramagnetic ions approach one another ( $r < 5\text{-}10 \text{ \AA}$ ; 25) the linewidths are affected by exchange coupling (29). While  $\langle \Delta\nu^2 \rangle$ 's are independent of the exchange term ( $2J_{ij} \cdot \bar{S}_i \cdot \bar{S}_j = 2J_{ij} S_i S_j \cos\theta$ , where  $J_{ij}$  is the "exchange integral" and  $\theta$  is the angle formed between the *i*th-*j*th spins and the magnetic field; 22, 30) the lineshapes no longer remain Gaussian, become mixed (27) and line narrowing results (23, 29, 31, 32). Figures 2 and 3 illustrate that, relative to the spin-spin interactions, we have little exchange since the linewidths of the bound dipoles only broaden as the concentration increases.

We (18) have simplified the  $\langle \Delta\nu^2 \rangle$  relationship by making use of the fact that, for a Gaussian line, the linewidth is  $2\langle \Delta\nu^2 \rangle^{1/2}$ . In this relationship

$$\Delta\Delta H_{[dI(H)/dH]_{\max}} = 28690 \cdot \text{GA}^3 \cdot \{S[S+1]\}^{1/2} \cdot \kappa/d^3; \quad (2; \text{units in G})$$



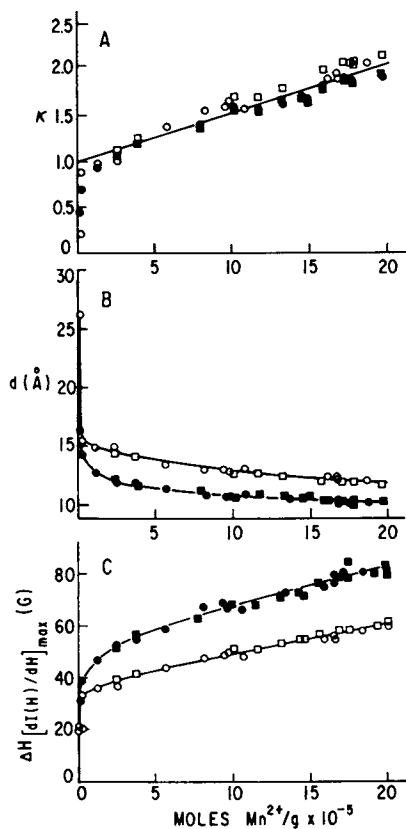


Figure 2. Cell wall bound  $Mn^{2+}$  concentration dependency of linewidth  $\{\Delta H [dI(H)/dH]_{max}\}$ , dilute limit nearest neighbor distance parameters  $\{d \text{ assuming } \kappa=1\}$  and lattice constants  $\{\kappa\}$ . Open symbols represent hydrated samples, closed symbols represent dry samples. Squares and circles represent the results of two independent experiments. The  $d$  values used to calculate  $\kappa$  were 13.72 and 16.3Å for dry and hydrated tissues, respectively. Reproduced with permission from Ref. 19. Copyright 1985, Biochimica et Biophysica Acta.

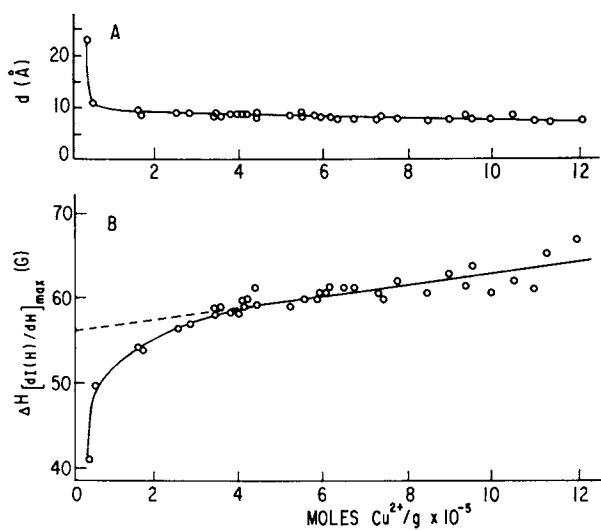


Figure 3.  $\text{Cu}^{2+}$  linewidth  $\{\Delta H_{[dI(H)/dH]_{\max}}\}$  and dilute limit nearest neighbor distance parameters' dependency  $\{d$  assuming  $\kappa = 1\}$  on cell wall bound  $\text{Cu}^{2+}$  at  $-180^\circ \text{C}$ . All samples were dehydrated. The dilute limit  $\Delta H_{[dI(H)/dH]_{\max}}$  intercept  $\{\text{dotted line}\}$  is ca. 56 G. Reproduced with permission from Ref. 19. Copyright 1985, Biochimica et Biophysica Acta.

$\Delta\Delta H_{[dI(H)/dH]_{\max}}$  is the incremental change in linewidth relative to a dilute glassy matrix containing  $Mn^{2+}$  or  $Cu^{2+}$ ;  $d$  is the nearest neighbor distance parameter. The lattice constant  $\{\kappa\}$  depends upon the arrangement of the ions in the lattice and is unity for a 2-spin system, 1.42 for a linear array of spins and about 2.85 for a filled cubic lattice (18). We can closely approximate the dimer-only  $d$  values  $\{\text{when } \kappa=1\}$  from the extrapolated zero concentration- $\Delta H_{[dI(H)/dH]_{\max}}$  intercept for  $Mn^{2+}$  {Figure 2} and  $Cu^{2+}$  {Figure 3}; this calculation provides the most reliable results when utilizing Van Vleck theory (23). Using this approach, the dimer-only  $d$  values were calculated {equation 2} to be ca. 12 and 14Å {dehydrated samples} for  $Cu^{2+}$  and  $Mn^{2+}$ , respectively. This small difference in  $d$  could be due to the fact that  $Cu^{2+}$ , unlike other transition ions such as  $Mn^{2+}$ , loses a portion of its shell of hydration upon binding (33, 34) which causes these ions to have an almost covalent character probably resulting from a more compact lattice structure (19). These distances (12x the intrachain carboxylate spacing; 15, 35) are observed at very low bound ion concentrations and argue for a special form of cooperative ion binding which we have referred to as sequential (18). This mechanism is also suggested by the relatively small change in  $d$ , after the initial rapid increase, as the lattice fills with  $Mn^{2+}$  or  $Cu^{2+}$ . The relatively small increase in  $d$  as the binding sites gradually fill is likely due to a change in the number of near neighbors ( $\sim\kappa^2$ ; 19, 26). Figure 2A shows calculated  $\kappa$  values assuming a constant  $d$ . If the sequential ion binding model is true,  $\kappa$  should approach zero only at low concentrations {e.g., ca.  $10^{-5}$  moles/g} where a significant portion of the  $i$ th dipoles experience no interactions with neighboring spins. Figure 2A clearly demonstrates this feature. From the  $\kappa$ - $[Mn^{2+}$  or  $Cu^{2+}]_{\text{bound}}$  relationship we calculate that the  $\kappa_{\max}$  value is ca. 2.5. We can utilize  $\kappa_{\max}^2$  to closely approximate the number of nearest neighbors even for samples having an extended array because of the decreased weighting of distant interactions. The

values for  $\kappa_{\max}^2$  indicate that the average number of near spin-spin interactions per  $i$ th spin is ca. 6 and is evidence for an egg box-like divalent cation-polyuronide aggregate structure (10, 11, 16, 36, 37) of the nearly-filled lattice. The large  $\kappa_{\max}^2$  also argues for the existence of pockets or regions of nonmethylated polymer existing contiguously in the cell wall/middle lamella matrix (19) since  $\kappa_{\max}^2$  is larger than expected for a highly methylated pectic polysaccharide matrix (20). Upon equilibrium hydration {Figure 2B and C, open symbols} the dimer-only distance parameter increases about  $2\text{\AA}$  {10G decrease in  $\Delta H[dI(H)/dH]_{\max}$  as  $[\text{Mn}^{2+}]_{\text{bound}}$  approaches 0}. This change is not likely to be due to the hydration of the transition ions alone because bound divalent cations, such as  $\text{Mn}^{2+}$ , have a rather significant aquoshell even in the dehydrated state (19). This observation, in conjunction with other data (19), indicate that the higher order structure of cell wall polyuronides changes as a function of temperature, degree of hydration and polymer size.

Lastly, if the sequential ion binding hypothesis is true, competitive exchange with a nonparamagnetic ion should reverse the broadening effect. We find that the  $\text{Mn}^{2+}$  spin-spin line broadening effect is largely lost when cell wall material is exchanged with both  $\text{Mn}^{2+}$  and  $\text{Ca}^{2+}$  {Figures 4 and 5} while keeping the total bound  $\text{Mn}^{2+}$  constant. For example, both first derivative spectra in Figure 4 are obtained from wall material having equivalent levels of bound  $\text{Mn}^{2+}$  {ca.  $4 \times 10^{-5}$  moles/g}; however, spectrum B is qualitatively similar to those obtained from dilute  $\text{Mn}^{2+}$  glycerol/water solutions at  $-176^\circ\text{C}$ . The small hyperfine lines seen in the least broadened spectrum {B} are due to the effect of the crystal field (38) on the Zeeman levels. Spectra with qualitatively identical crystal field splittings were obtained from dilute  $\text{Mn}^{2+}$  glasses at  $-176^\circ\text{C}$  and are evidence that the crystal field contribution to lineshape is effectively the same in both cases. We have also found that the  $\text{Mn}^{2+}$  line broadening, which results from  $\text{Mn}^{2+}$  near neighbor

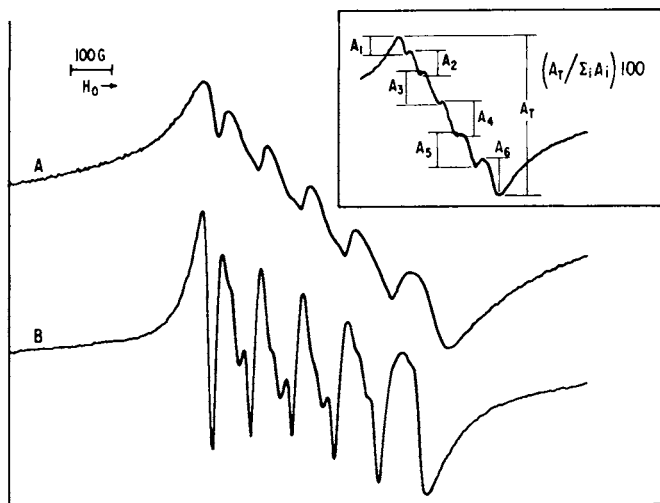


Figure 4. First derivative ESR spectra of cell wall absorbed  $\text{Mn}^{2+}$  as a function of bound  $\text{Ca}^{2+}$ . The sample represented in spectrum A has ca.  $4 \times 10^{-5}$  moles  $\text{Mn}^{2+}/\text{g}$  dry weight  $\{X_{\text{Mn}^{2+}} = 1\}$ ; the sample represented in B has an equivalent level of paramagnetic ion but  $X_{\text{Ca}^{2+}} = 0.7$ . Inset spectrum: Illustration of the empirical line broadening factor,  $\text{LBF} = \{A_T / \sum_i A_i\} 100$ . Reproduced with permission from Ref. 18. Copyright 1984, Biochimica et Biophysica Acta.

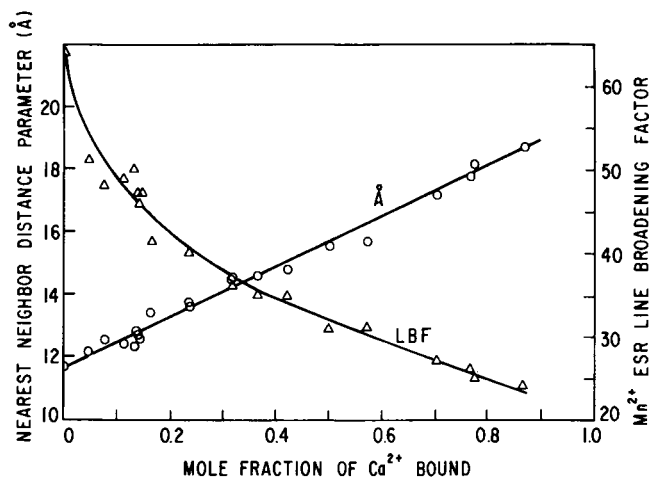


Figure 5. Changes in linewidth as measured by the line broadening factor {LBF, triangles} and calculated paramagnetic ion nearest neighbor distance parameters { $d$ , circles} as a function of  $X_{\text{Ca}^{2+}}$ . The fraction of sites filled by  $\text{Mn}^{2+}$  are approximately the same ( $22.4 \pm 1.5\%$ ) for each sample though the LBF values vary between 64 and 30; thus, the change in line broadening can only be associated with a competitive binding effect. Reproduced with permission from Ref. 18. Copyright 1984, Biochimica et Biophysica Acta.

spin-spin interactions, is reduced greatly as the mole fraction of  $[Ca^{2+}]_{bound}$   $\{X_{Ca^{2+}}$ , Figure 5 $\}$  increases at the same total bound  $[Mn^{2+}]$ . The linear relationship observed between  $d$  and  $X_{Ca^{2+}}$  is expected if a lattice consists of a uniform sequential array of bound ions (39). These data also indicate that the binding of  $Ca^{2+}$  to cell wall polyuronides is similar to  $Mn^{2+}$ .

### Conclusions

From these studies (18, 19) we conclude that certain divalent cations bind to the cell wall polyuronides in a spatially sequential fashion. This form of association can be thought of as a special case of cooperative whereby the binding of some initial cation lowers the potential energy for binding only at nearby sites {e.g., 1-2x the intrachain carboxylate distance}. This type of ion binding is implied by the proposed egg box structure (11) and is supported by these data (18, 19). Our results also indicate that a large proportion of pectic substances exist contiguously in three dimensional lattices low in methyl ester content and that the relative positions of the point dipoles increases ca.  $2-3\overset{\circ}{\text{A}}$  upon hydration, probably under the influence of alterations in the conformation of the polyanionic ligand. Other data (19) indicate that hydration-induced changes {Figure 2} in the apparent relative position of bound  $Mn^{2+}$  is at least partially controlled by the polymer's structure.

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## Softening of Cooked Snap Beans and Other Vegetables in Relation to Pectins and Salts

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The use of monovalent salt solutions as cooking media caused a greater softening than when vegetables were cooked in distilled water. Removal of endogenous salts by leaching before cooking reduced softening. The additional softening with salts was the result of two separate actions. One action, associated with the solubilization of part of the tissue  $\text{Ca}^{++}$ , took place when the salt was used either before or after cooking. The other action, requiring the presence of salt during cooking, was associated with the appearance of higher liquor pectin concentrations. A blanch temperature of  $71^{\circ}\text{C}$  resulted in snap beans that softened more slowly when cooked in distilled water and had a slower rate of salt-induced softening than was the case with snap beans blanched at  $90^{\circ}\text{C}$ .

Two concepts have dominated the interpretation and design of research concerned with the softening of vegetables during heat processing. One idea has been the association of pectin depolymerization and solubilization with the loss of firmness (1). The other idea deals with the ability of  $\text{Ca}^{++}$  to increase firmness and tissue adhesiveness in cooked tissues (2, 3). In well cooked non-fibrous vegetables the firmness is determined largely by the strength of intercellular adhesion localized at the middle lamella. Variations in firmness that are produced in such tissues can be considered the result of reactions and interactions that take place with the pectins and other components of this restricted region.

Softening of fruits and vegetables during heating appears to occur through two different pectin degrading reactions depending on the pH of the tissue. Doesburg (1) has proposed a useful generalization that softening below pH 4 is consistent with an acid catalyzed cleavage of the cell wall polysaccharides, while at pH levels over 5, the normal conditions for vegetables, softening is consistent with a pectin depolymerization reaction that has the characteristics of a  $\beta$ -elimination reaction catalyzed by hydroxyl ions (4) and inhibited by demethoxylation of pectins (5).

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A large body of research indicates that  $\text{Ca}^{++}$  functions largely through its interaction with pectin (6, 7, 8). Removal of a part of the pectin limits the ability of  $\text{Ca}^{++}$  to restore firmness to original levels in tissues like potatoes (9), while extraction of  $\text{Ca}^{++}$  with chelating agents results in the solubilization of a large part of the cell wall pectin.

#### Effects of salts present before cooking

Many studies have demonstrated that the addition of monovalent salts to vegetable tissues before cooking results in a softer cooked product. Some of the earlier work was done with potatoes. Investigations by Davis and LeTourneau (10), Table I, using higher cooked potato weight as an index of higher cohesiveness, showed a decreased cohesiveness when NaCl and KCl were used. The cooked potato weight, measured after draining on a sieve, was lower when monovalent salts were present in the cooking solution.  $\text{CaCl}_2$  increased cohesiveness. Hughes et al. (11) obtained less resistance to compression for potatoes cooked with monovalent salt. Peas also respond to NaCl (12, 13), giving a softer texture when higher concentrations of salt were added before the sterilization process, Table II. Sterling (14) found that carrots were softer when cooked with monovalent salts or when higher pH levels were used. Similar results were found by Mattson (15) using dried peas as the test material.

Table I. Effect of salts on the cohesiveness of cooked potatoes

Salt in cooking solution		Cohesiveness, cooked potato weight units
NaCl	0.008 M	85
KCl	0.008 M	87
KCl	0.512 M	10
$\text{CaCl}_2$	0.008 M	120
Distilled water		101

Cooked for 24 min at 100 °C. From Ref (10).

Table II. Effect of NaCl on canned pea firmness

Pea variety	Shear press values, lb			
	% NaCl in brine			
	0	1	2	3
Alaska	245	222	201	197
Alaska	236	220	203	175
Perfection	229	244	221	202

Cooked for 30 min at 240 °F. From Ref (13).

The major cation present in fruits and vegetables is  $K^+$ . Its concentration can be affected by environmental factors, principally the amount of available K in the soils or root media (16). Different applications of K resulted in more than a two-fold difference in snap bean pod K (17), Table III. Higher pod K levels were associated with softer beans when measured after a standard canning procedure. Firmness values in this table and in Tables IV-V1 and in Figures 1 and 2 were obtained as kg force resistance to compression in a back extrusion cell (17).

Table III. Effects of K fertilization on the firmness of canned snap beans grown in sand culture

Fertilization with K meq/l	Blanch treatment	Canned bean firmness kg force	K, mg/g	
			Fresh pods	Can liquor
0.3	82 °C/2 min	59		0.45
	None	41	0.98	0.56
6.0	82 °C/2 min	42		1.21
	None	33	2.60	1.17

Canning heat treatment was at 115 °C for 20 min. Source:  
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Table IV. Effect of leaching and NaCl on snap bean firmness

Beans	Treatment Can media	Firmness kg force	Liquor Ca, µg/ml
Leached	0.145 M NaCl	38	63
Not leached	Distilled water	43	79
Not leached	0.145 M NaCl	34	90

Leachings were after blanching and overnight in distilled water at 2 °C. Snap beans cooked 20 min at 115 °C. Source:  
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Since higher tissue salts caused softening, it was of interest to see if the removal of salts prior to the canning process would result in firmer beans (18). Salts were removed by leaching blanched beans overnight in distilled water. After canning, the firmness of the leached beans was higher than for those not leached, Table IV. The use of NaCl in the canning media led to softer cooked beans than when the canning media was distilled water.

#### Effects of salts present before or after cooking

Since the firmness of heat treated vegetables can be influenced by both the extent of pectin depolymerization and pectin interaction

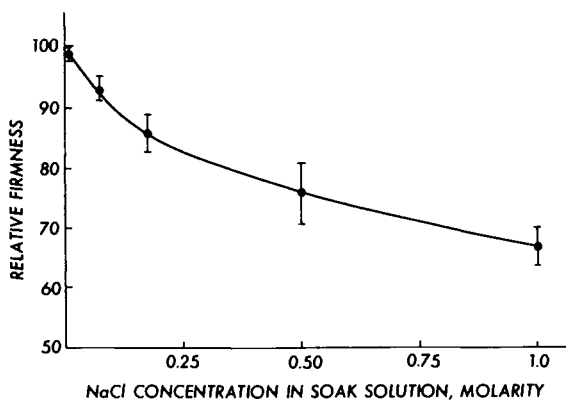


Figure 1. The effect of soaking canned snap beans in NaCl solutions on the relative firmness of the soaked beans. Reproduced with permission from Ref 19. Copyright 1984, 'Institute of Food Technologists'.

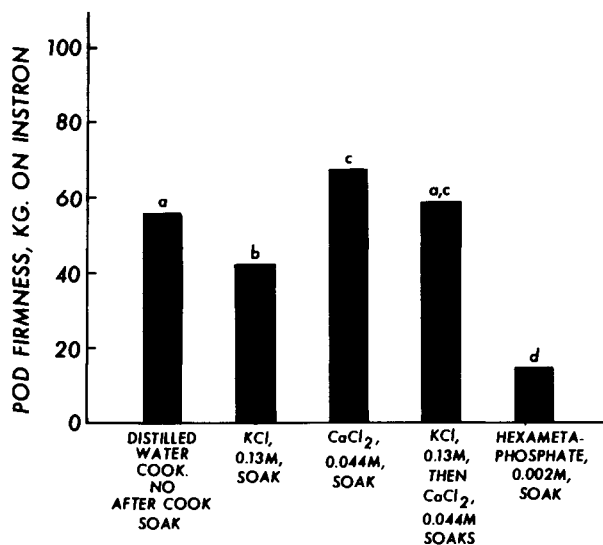


Figure 2. The effect of soaking canned snap beans in KCl, CaCl<sub>2</sub>, or Na hexametaphosphate solutions on their firmness. Reproduced with permission from Ref 19. Copyright 1984, 'Institute of Food Technologists'.

with  $\text{Ca}^{++}$ , both these mechanisms could have been involved to produce the firmness effects seen with monovalent salts present during the cooking. This possibility was tested in experiments where salts were added either before (salt cook) or after (salt soak) canning, Table V (18). Snap beans canned without NaCl were firmer, and their can liquor had less soluble pectin and  $\text{Ca}^{++}$ , than was seen for beans where NaCl was present during canning. When beans, previously canned without added salts, were soaked for an extended period in their own can liquor augmented with NaCl the resulting soaked beans had an intermediate firmness and liquor  $\text{Ca}^{++}$  concentration between those of samples cooked with and without NaCl. No change was seen in the liquor pectin concentration from that seen when the snap beans had been cooked without NaCl.

Table V. Effect of NaCl, added before or after cooking, on snap bean firmness

Treatments		Firmness	Liquor Ca	Liquor pectin
Cooking media	After retort	kg force	$\mu\text{g/ml}$	$\text{mg/ml}$
NaCl, 0.20 M	Held in can liquor	23	94	2.6
Distilled water	Held in can liquor	42	49	2.1
Distilled water	Held in can liquor plus <sup>a</sup> NaCl to give 0.20 M NaCl	30	86	2.1

<sup>a</sup>Solid salts were dissolved in liquor decanted from opened cans, then the liquor was returned to the beans. Beans were then held 3 days at 1 °C with occasional gentle mixing prior to texture measurement and liquor sampling. Source: Reproduced with permission from reference 18. Copyright 1983 Institute of Food Technologists.

It appears that the presence of monovalent salt during cooking enhanced both pectin solubilization and  $\text{Ca}^{++}$  displacement into the liquor, and that the use of the same concentration of salt after cooking in distilled water displaced  $\text{Ca}^{++}$  but did not increase the liquor pectin concentration. Salt-enhanced pectin solubilization required cooking, but enhanced  $\text{Ca}^{++}$  displacement took place apart from cooking. Both consequences of the presence of salts were associated with decreased canned bean firmness.

#### The effects of adding salts after cooking

The above results showed that two salt effects on firmness could be separated from each other. The influence on firmness of the  $\text{Ca}^{++}$  displacement action could be measured by determining the relative firmness, compared to unsoaked beans given a normalized value of 100, when cooked snap beans were equilibrated with a monovalent salt solution (19) by soaking at 1 °C for 3 days. As the concentration of NaCl in the soak solution was increased, Figure 1, the pod firmness decreased. But even at rather high concentrations of NaCl the

firmness of the beans was still appreciable. The loss in firmness was mirrored in increased soluble  $\text{Ca}^{++}$ , Figure 3. After soaking in 1 M NaCl about 25% of the original pod  $\text{Ca}^{++}$  remained bound to the pod tissue; this may represent strongly bound  $\text{Ca}^{++}$  that makes a particularly effective contribution to pod firmness.

The displacement effect of KCl in decreasing firmness of snap beans cooked earlier in distilled water can be seen in the bar chart of Figure 2. Bars not surmounted by the same letters were significantly different at the  $P < 0.05$  level. The soakings in the indicated solutions were for 3 days at  $1^{\circ}\text{C}$ . Here the effect of  $\text{CaCl}_2$  addition in increasing firmness is also shown. Pods that had been softened by KCl had much of their firmness restored by a subsequent  $\text{CaCl}_2$  solution soak. The effect of Na hexametaphosphate was to decrease the firmness to about 25% of that for the original canned beans. This material is an effective chelator of  $\text{Ca}^{++}$  and its use results in the solubilization of a large part of the pod pectin.

#### Addition of salts before cooking, corrected for $\text{Ca}^{++}$ displacement

The action of salts added before cooking, separate from the  $\text{Ca}^{++}$  displacement effect, can be obtained as the difference in values between pods cooked in salt solution and those cooked in distilled water, then soaked in salt solution. This number, after division by the value for pods cooked in distilled water and multiplication by 100, gives a normalized, dimensionless measure of salt effects elicited by cooking (20). For softening, there is obtained a "salt cook softening effect", and, for the solubilization of pectin, there is obtained a "salt cook pectin solubilizing effect". The results given in Table V can be calculated to show a salt cook softening effect of 17 and a salt cook pectin solubilizing effect of 24.

Table VI. Comparison of two blanch and cook time treatments that give cooked beans of similar firmness

Blanch temperature	$71^{\circ}\text{C}$	$90^{\circ}\text{C}$
Cook time, minutes at $115^{\circ}\text{C}$	40	5
Firmness, kg force, d. w. cook	53	48
Salt cook softening effect, 0.2 M NaCl	20	19
Liquor pectin, d. w. cook, mg/g	2.6	2.8
Salt cook pectin solubilizing effect, 0.2 M NaCl	77	55

From Ref (20).

The cook dependent salt effects were examined in beans receiving different blanch treatments which modified cooking behavior through differences in the degree of enzymatic demethoxylation (21, 22). The higher temperature blanch led to less pectin demethoxylation. Various combinations of cooking time and temperature were used. There was a slight depression of several tenths of a pH unit cause by the presence of NaCl during cooking, but this pH depression had no significant relation to the salt cook effects. A convenient manner of

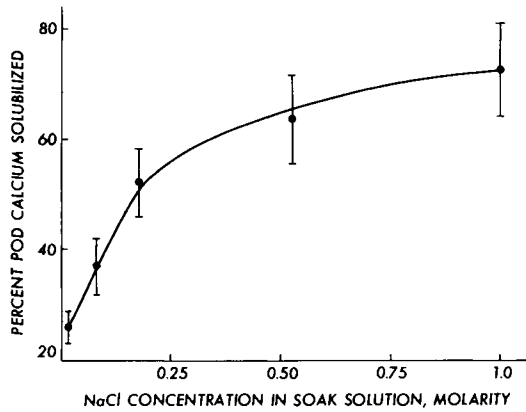


Figure 3. The effect of soaking canned snap beans in NaCl solutions on the % of the pod calcium solubilized by the soak. Reproduced with permission from Ref 19. Copyright 1984, 'Institute of Food Technologists'.



making comparisons between treatments that affect the softening rate is to compare canned products that have similar firmness values. This reduces the uncertainties in assessing the significance of firmness differences. The comparison given in Table VI (20) shows that 40 minutes cooking time was required for beans blanched at 71°C to reach a firmness similar to that obtained after 5 minutes cooking for beans blanched at 90°C. Therefore, the softening reaction(s) in beans that had received the lower temperature blanch were considerably slower than in the case of beans that had received the high temperature blanch. The salt cook softening effect was the same for both blanch treatments, indicating that the rate of the reaction(s) giving rise to the salt cook softening effect was affected by the blanch treatments in a similar manner as for the rate of softening in the distilled water cook.

Both treatments had similar concentrations of liquor pectin after the indicated cooking times. This is consistent with other findings that the extent of pectin solubilization is closely related to the degree of softening during cooking.

When longer cooking times were employed the salt cook effects became smaller (20); the values for firmness and liquor pectin obtained for beans cooked with salt came close to those for beans cooked in distilled water and subsequently soaked in salt solution. This suggested that the salt present during cooking was acting on the same type or fraction of pod pectin as was being solubilized during the distilled water cook. It is quite possible that the salt cook softening and pectin solubilizing effects are measures of the same reaction in cooking tissue.

This study also showed that  $\text{CaCl}_2$  had a softening effect masked by the commonly seen firming action of  $\text{Ca}^{++}$ , Table I and Ref (19). When bean pods, cooked earlier in either  $\text{CaCl}_2$  solution or distilled water, were soaked in the same type of salt solution in order to create similar cooked pod salt concentrations the beans cooked in distilled water had significantly firmer texture.

### Discussion

The ability of monovalent salts to increase softness apart from heating may be related to  $\text{Ca}^{++}$  displacement (23).  $\text{Ca}^{++}$  firms tissues in the absence of any heating, although its action is most easily seen in tissues that have lost their turgor (24). Addition of  $\text{Ca}^{++}$  increases the firmness of potato tissues while its removal softens them (9). Digestion of tissue pectin by pectolytic enzymes results in the solubilization of  $\text{Ca}^{++}$  (25). It appears that  $\text{Ca}^{++}$  has an important role in stabilizing and enhancing the association between pectin polymer chains that is necessary for binding tissue cells together and for tissue firmness.

Interactions between pectins and  $\text{Ca}^{++}$  can consist of both ionic interactions and coordination bonds. Ionic interactions involve negative groups on the pectin and are related to the degree of methoxylation (26). Pectin oxygen atoms interact with unfilled  $\text{Ca}^{++}$  orbitals to give coordination bonding and may result in configurations such as the 'egg box' structure proposed for  $\text{Ca}^{++}$ -pectin complexes by Rees and Welsh (27). The presence of carbonyl groups on the pectin enhances coordination (28). The action of the monovalent cations may be to compete with  $\text{Ca}^{++}$  for ionic and/or coordinate bonding to the pectins.

The enhancement of tissue softening and pectin solubilization during heating caused by the presence of salts may be associated with  $\beta$ -elimination pectin depolymerization (5, 29). However, there is very little direct evidence for this mechanism in intact tissues outside of some careful observations by Keijbets (30). Other analytical evidence is indirect and obtained from reactions carried out on purified vegetable tissue fractions (31, 32). Some workers have not seen evidence of significant  $\beta$ -elimination (33) and suggested that other mechanisms may have more important roles. The difficulties in detecting or dismissing  $\beta$ -elimination of pectin in heated whole vegetable tissues arise from the lack of specificity for the analytical methods combined with the presence of interfering materials in whole tissue extracts (30).

Despite these difficulties in measurement there is considerable indirect support for the  $\beta$ -elimination hypothesis. Conditions that increase the rate of  $\beta$ -elimination in model systems also increase the rate of softening during the heating of vegetables. Higher pH (32, 34) and higher methoxylation (21, 22, 35) lead to more rapid softening.

The participation of  $\beta$ -elimination in the salt cook effects is suggested by the indications that the same reaction(s) responsible for softening during cooking in distilled water is involved in producing the salt cook softening effect (20). Model system studies (31) have shown that salts enhance the rate of  $\beta$ -elimination at pH values close to 6.0, especially when present at molar concentrations the same or higher than the molar concentrations of uronide residues. Also suggestive is that demethoxylation by the use of a low temperature blanch slowed the rate of softening and pectin solubilization due to the salt cook effect (20).

However, the comparatively small salt cook softening effect brought about by  $\text{Ca}^{++}$  (20) was not consistent with work on model systems (31) which showed  $\text{Ca}^{++}$  to be very effective in enhancing  $\beta$ -elimination. Further research is needed to establish the relative importance of  $\beta$ -elimination as a mechanism for vegetable tissue softening during cooking.

In conclusion, there exists a significant interplay between salts and pectins in determining the firmness of cooked vegetables. At least two phenomena are involved in this, an interaction between monovalent salts and  $\text{Ca}^{++}$  affecting pectin matrix adhesive characteristics, and an enhancement by salts of softening and pectin solubilizing reactions associated with the heating process. These phenomena might be utilized in controlling the textural properties of cooked and canned vegetables.

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## Effects of Freezing and Frozen Storage on the Characteristics of Pectin Extracted from Cell Walls

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The effect of freezing and frozen storage on the texture of green beans peaches and strawberries has been assessed. The textural changes result from more than solely turgor pressure loss. The pectic component of the cell wall complex has been extracted and characterized as a function of the freezing method and frozen storage conditions. Changes in this pectic fraction could account for some of the textural alteration observed.

Texture is an important attribute of the eating quality of fruits and vegetables. An important aspect of texture is firmness. Texture or firmness result from a variety of contributions. Amongst these contributions are the turgor of an intact cell, and the strength of individual cell walls. It has often been remarked that a major textural consequence of freezing and frozen storage on fruits and vegetables is a loss of tissue firmness. Whilst it is known that freezing causes severe damage to the membranes of cells, and is therefore responsible for a loss of turgor, it is less clear whether there is a contribution to loss of firmness from the cell wall component. It should be remembered here that the softening of tissues during ripening has been shown to be largely a result of changes in the cell wall. In particular, it has been demonstrated that there is a correlation between the pectic component of cell walls, and the tissue firmness. In the study described here we wish to investigate whether some of the softening of plant tissues which accompanies freezing and frozen storage is related to changes in the cell wall, in particular whether there are related changes in the pectic fraction of the cell wall. This requires that we in some way assess texture, and that we also quantify and characterize the pectic materials of the tissue.

### Experimental Procedures

In order to assess texture, we have employed a back extrusion cell accessory on an Instron Universal Testing Machine. The cell,

illustrated in figure 1, is modified from a design described by Bourne and Moyer(1968). The Instron cross-head is moved at 100 mm/min. A sample of material, contained in the cup, is compressed as the plunger comes down. At length, the material extrudes back through the annulus between plunger and cup wall. A typical force-distance curve is shown. The rise reflects the compression, and the plateau of the back extrusion. We use the plateau force as a measure of firmness. Ten replicate measurements are performed. Repeatability is better than 4%.

In order to quantify and characterize the pectin component of the cell, we must use some procedure to prepare a stable cell wall fraction, and then follow some predetermined extraction scheme which allows for the separation of pectic materials of different characteristics. The overall amount of pectin in any situation is then determined by assaying for uronic acids. The extractions of the pectic fractions need not be exhaustive, but must be repeatable, since we wish to follow the changes which may take place in the fractions consequent upon freezing and frozen storage. The separation procedures we have employed are as follows. We have chosen alcoholic extraction of macerated thawed tissue as a method for the preparation of a cell wall material which will be stable, and suited to storage prior to further assay. Cell wall material was prepared according to a method modified from that of Ahmed and Labavitch (1977) by adding washing steps using chloroform/ methanol and acetone. A flow chart of the procedure is shown in figure 2. The wash steps were included as we found that material extracted using only alcohol tended to form a glassy, intractible material on drying. This was particularly true of strawberries. The material for extraction generally has been drained during the thawing procedure, and it is this drained, thawed material which is macerated. The drained liquid is collected separately, and identified as drip.

The extraction of pectic fractions from the cell wall material follows the traditional logic. First a water soluble fraction is prepared. This is obtained by taking 100mg of cell wall material and shaking it vigorously with 20ml of water. After standing for 5 minutes, the supernatant fluid is separated by centrifuge, and the solid material is treated with more water. The treatment is performed four times, and the supernatant fluids combined as the water - soluble pectic fraction (WSP). The residual solid is then used to prepare the chelator soluble fraction (CSP). A similar procedure is used to that already described, excepting that the solvent is 0.1M EDTA in 0.1M Tris, pH7. A final fraction, (HSP), is prepared by subjecting the residue of step 2 to dilute sodium hydroxide as solvent. Again, four stage extraction is performed. It should be noted that these procedures do not extract all the material of the cell wall preparation which contains uronic acid residues. The residue of step 3 is found still to contain uronic acid. This can only be brought into solution by using more extreme methods for solubilization. Replicate extractions are performed.

The materials obtained are analysed for pectin content by uronic acid assay. The method of Blumenkrantz and Asboe-Hansen (1973) is employed. Further characterization of the pectic material included assay for neutral sugar composition by the alditol acetate method of Albersheim et al (1967), which entails derivitization, and

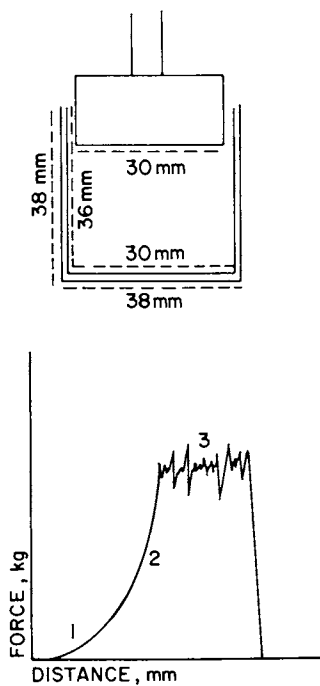


Figure 1. The back extrusion cell.

## AIS Preparation/Purification

Ethanol: Chloroform-Methanol: Acetone Procedure

1. Wash and trim raw material.  
↓
2. Weight 100 g raw material and place in Waring blender with 400 mls 70% ethanol.  
↓
3. Homogenize for 1 minute.  
↓
4. Transfer slurry to 50 ml round-bottom centrifuge tubes and cap.  
↓
5. Centrifuge at 19,000 x g (12,500 rpm, ss-34 rotor) for 10 minutes.  
↓
6. Discard supernatant and transfer pelleted material to a coarse sintered glass funnel.  
↓
7. Breakup pelleted material using a metal spatula.  
↓
8. Wash with 2 x 100 ml volumes 70% ethanol (applying vacuum after thorough mixing of the solids and solvent).  
↓
9. Wash with 3 x 100 ml volumes chloroform-methanol (1:1 v/v).  
↓
10. Wash with 3 x 100 ml volumes acetone.  
↓
11. Air dry residue.

Figure 2. Flow chart of extraction procedure.

determination of the acetylated neutral sugars, in our case by capillary GC. In some cases, the extracts were separated further using column chromatography, the column containing DEAE sephadex, and elution being by an ionic gradient. Some samples were then subjected to gel filtration on a 1.1 x 60cm Biogel P100 column, in a 40mM Acetate, 40 mM EDTA, 50 mM NaCl buffer, pH 6.5, in order to estimate their approximate molecular weight profiles.

### Materials

In order to study the effect of freezing on texture, and texturally related parameters, we have chosen three tissue systems. Green beans, strawberries and peaches. Green beans were of the Gallatin variety. Peaches were of the variety Halford. Two varieties of strawberry were studied, Aiko and Pajaro. All were obtained as fresh material, and processed in our pilot plant. Blanching, where required, utilized a steam blancher. Fast freezing used a Conrad freezer with an air blast temperature of -70 C. Products were frozen unwrapped in a single layer on open mesh trays. Immediately after freezing they were sealed into bags for storage. Slow freezing was in still air in a cold room. In this case the materials were sealed into bags prior to freezing.

### Results

Texture is an important attribute of the three tissues chosen for study. Since blanching is an appropriate pretreatment to freezing in some cases, we might first ask the question "What is the effect of blanching on texture?" As blanching is a partial cooking process, not surprisingly, tissue softening occurs. We have taken samples of green beans and blanched them for increasing times at 100 C. Not surprisingly, measurement of the back extrusion force shows increasing softening with blanch time. At the same time, the blanch liquor has been assayed for pectin content, determined as uronic acid residues. The pectin released from the tissue as a consequence of blanching has been computed. Figure 3 shows the relationship between texture and released pectin. Clearly, the loss in texture is accompanied by a release of pectin. Another factor in the softening, of course, is the loss in turgor which accompanies the thermal destruction of the integrity of the cell membranes. What happens if we freeze the green beans?

As can be seen from figures 4 and 5, the result of freezing is a reduction in back extrusion force, whether the unfrozen material be blanched or not. The greatest reduction in back extrusion force as a consequence of freezing is seen for unblanched tissue, and presumably reflects in large measure the loss in turgor. However, loss of turgor is not the sole cause of the reduction in back extrusion force. Consider...blanching, too, destroys turgor, and yet the reduction in back extrusion force as compared to fresh tissue is greater for unblanched, frozen tissue than for blanched, unfrozen tissue. Also, there is a reduction in back extrusion force in blanched tissue due to subsequent freezing and thawing. This reduction is more marked, relative to the initial back extrusion force of the blanched tissue, for the more extensive blanches. Since it is unlikely that the turgor contribution can account for



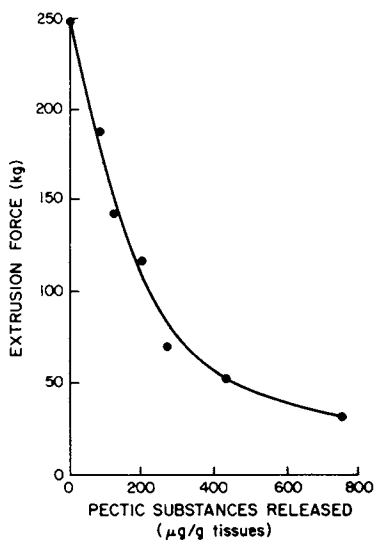


Figure 3. Relationship between texture and pectin release during blanching.

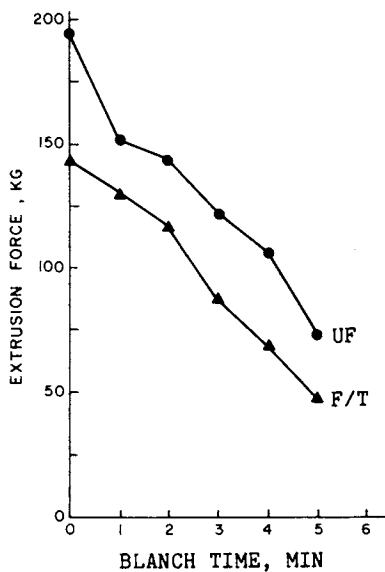


Figure 4. Effect of blanching and freezing on texture. UF refers to tissue which is not frozen after the blanch treatment, F/T to tissue which has undergone a freeze-thaw cycle after the blanch treatment.

this observation, it would appear that this loss in firmness is due to the cell wall contribution. Further, there is a continued decrease in tissue firmness as a consequence of extended frozen storage. It is therefore appropriate to follow the changes which take place in cell wall components as a function of freezing and frozen storage, and try to correlate these with observations relating to texture, tissue structure, etc. As already indicated, we have chosen to focus on the pectic fraction, since it is amenable to extraction and fractionation by reasonably simple methods. EDTA was chosen as chelator in part because we wished to perform our extractions under as mild conditions as possible, i.e. at room temperature, and close to neutrality, in order to minimize chemical change in the pectic fractions during their extraction. As can be seen from figure 6, the extraction efficiency of other commonly used chelators appears to be less than that of EDTA. This observation is discussed further by Reid and Carr (paper in preparation) and in Carr(1984).

Our results are best presented in stages. First we should consider the pectic composition of the unprocessed raw material. Then we should consider the changes which take place in the gross fractions as a consequence of processing and storage. To do this we need only to determine the amounts of pectin in each fraction. After this we should consider whether there have been any compositional changes in the pectin. This is best determined by using column techniques to fractionate the three categories of pectic material, and also by further analysing the pectins. If any changes are taking place as a consequence of processing, we would expect to see them reflected in at least some of these results.

Table 1 summarizes the data relating to the uronic acid content of individual pectic fractions from the three tissues both before and after processing. The data for the pectic fractions from unfrozen tissue include an assay for the uronic acid content of the residual material after the extraction of the three soluble fractions in order to confirm that we can account for all the uronic acid residues. The total uronic acid content of the original cell wall material in all cases defines 100%. As table 1 shows, the distributions of pectic fractions from the three tissues are very different. Also, the residual uronic acid content of the extracted cell wall material varies for these tissues. However, the residual assay indicates that we are accounting for most of the uronic acid containing material. The fractionation procedure extracts about 90% of the available uronic acid in Pajaro strawberry cell wall preparation, yet Aiko has an extraction of 70%. Only about 50% of the uronic acid in green bean cell wall preparation is extracted. The extraction efficiency for peaches increases during storage, suggesting that there is some change taking place.

Figure 7 illustrates the change in uronide content of the fractions obtained from the cell wall material as a function of the storage time at -20 C for the strawberry variety, Aiko. It is clear that the major change is in the uronic acid content of the water soluble fraction. This might be expected, if uronic acid containing wall components are constituents of the material lost as drip. In table 1a we compare the cell wall fractions obtained by homogenizing thawed tissue, from which the drip has been lost, and frozen tissue, which has lost no material. There is a loss of uronide in the

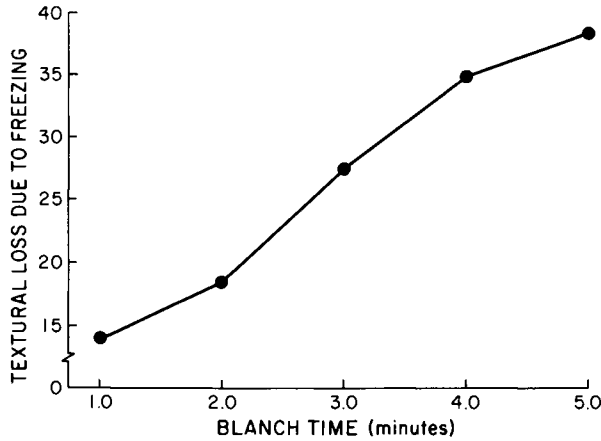


Figure 5. Texture loss due to freezing post blanch, calculated as the ratio of the loss in back extrusion force consequent upon freezing to the back extrusion force after blanching but prior to freezing.

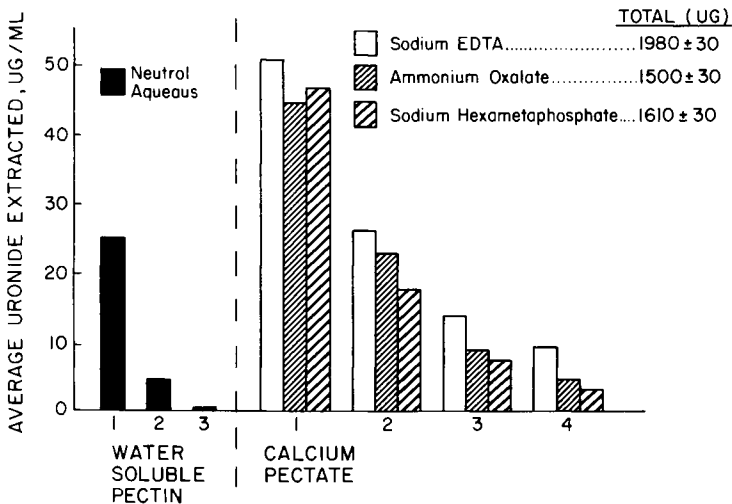


Figure 6. Extraction performance of different chelators at pH7 in AIS.

TABLE 1

(a) Strawberry blast frozen -20 C storage (variety Aiko)				
	unfrozen	1 week	10 mo	10 mo(*)
WSP	48.6	44.5%	31.2%	47.2%
CSP	14.1	15.4	17.4	14.8
HSP	9.5	9.8	12.6	10.1
Sum	72.2	69.7	61.2	72.1
(*) AIS prepared directly from frozen tissue, with no drip loss occurring, unlike regular preparation.				
(b) Strawberry slow frozen -12 C storage (variety Pajaro)				
	unfrozen	1 day	4 month	
WSP	44.63%	46.98%	47.17%	
CSP	24.80	19.48	18.43	
HSP	24.05	23.83	21.86	
Sum	93.48	89.79	87.46	
RESIDUAL	15			
(c) Peach unblanched -20 C storage				
	unfrozen	1 day	4 month	
WSP	23.56%	26.28%	23.74%	
CSP	8.86	9.27	11.82	
HSP	36.09	40.52	52.68	
Sum	68.51	76.07	88.24	
RESIDUAL	22			
(d) Green bean 2m blanch slow frozen -12 C storage				
	unfrozen	1 month		
WSP	8.81%	8.32%		
CSP	19.33	22.31		
HSP	21.33	18.85		
Sum	49.48	49.48		
RESIDUAL	36			

thawed tissue. Table 2 shows an analysis of cell wall uronide from thawed tissue, together with the uronide assayed in the drip collected during the thaw. Soluble uronide is indeed lost in the drip, leaving the cell wall material depleted of material which would otherwise be included in the water soluble fraction.

Figure 8 shows that there is a continuous loss of texture in strawberries during frozen storage. This is paralleled by the loss of soluble pectin. It still remains to be determined whether, and in what manner, the pectin of the three fractions is changing in character. Given that the most dramatic loss in pectic component of strawberries is in the WSP, figure 7, we have further characterized this fraction by subjecting it to fractionation using a DEAE sephadex column eluted by an increasing ionic gradient. An amount of solution containing about 7mg uronic acid is placed on the column, and eluted initially with 0.1M phosphate buffer, pH6.9. Once no more uronic acid material is found to elute, the gradient is started, the final eluant being 1.2M phosphate buffer, pH6.9. The remaining uronic acid on the column elutes in characteristic fashion. As can be seen from figures 9-11 there is a change in the elution characteristic of the WSP material as a function of storage time. We consider in particular the column bound material, that which does not elute until the ionic gradient is applied. These elution patterns are seen in figures 9b, 10b and 11b. The original elution patterns for the column bound material show two peaks, at about 0.3 to 0.4 M ionic strength and at about 0.6M ionic strength. As a result of storage, the second peak decreases in size, whilst the initial peak remains unchanged. The fraction lost is the more acidic material, and probably represents the primary rhamno-galacturonan backbone, as indicated by the significantly higher proportion of rhamnose found in DEAE column bound materials (table 3). Recent observations indicate that this rhamnose-rich uronide material is more slowly extracted from the AIS by water than is the material which elutes in the void volume of the DEAE column. To throw further light on the changes in this fraction, we have performed gel filtration analyses to obtain preliminary indications of molecular weights. WSP materials collected from unfrozen, 1day frozen and 4.5month frozen strawberry samples all voided a P100 column, indicating molecular weights in excess of 100,000, based on elution of globular proteins of known size. Interestingly, material precipitated from the drip loss fraction with ethanol also displayed a major high molecular weight peak, with in addition about 20% of the total uronide containing material eluting with the totally included volume of the column. This would correspond to a molecular weight around 10,000. The pectic material in the drip is a significant part of the pectin lost from the cell wall in long term storage (table 2). Since, during frozen storage, the drip loss increases, the nature of the uronide in the drip, and its change with time of storage, warrants further study. It would be useful to know how the proportions of the high and low molecular weight fractions vary with time of storage.

### Conclusions

Texture is affected by freezing in ways over and above the effect of freezing on turgor. This suggests that there is a clear

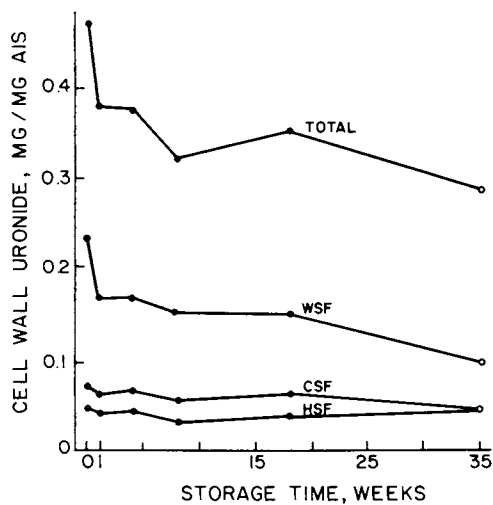


Figure 7. Uronide content of AIS fractions of Aiko strawberries after different frozen storage times. WSF represents water soluble fraction, CSF chelator soluble fraction and HSF alkali soluble fraction.

Table 2  
Strawberry drip analysis

Yield of uronide from 100g of fresh berry, frozen stored at  $-20^{\circ}\text{C}$  for 9mo, then thawed for 90 minutes at room temperature.

	Berry	Drip
Total fresh wt	78.4	21.7
ug uronide/ ug AIS	0.218	0.106
Uronide in fraction (g)	0.417	0.040

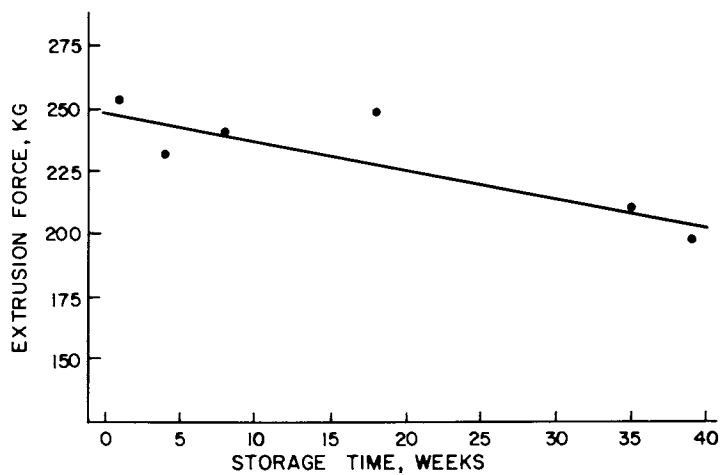


Figure 8. Texture loss in frozen storage of strawberries.

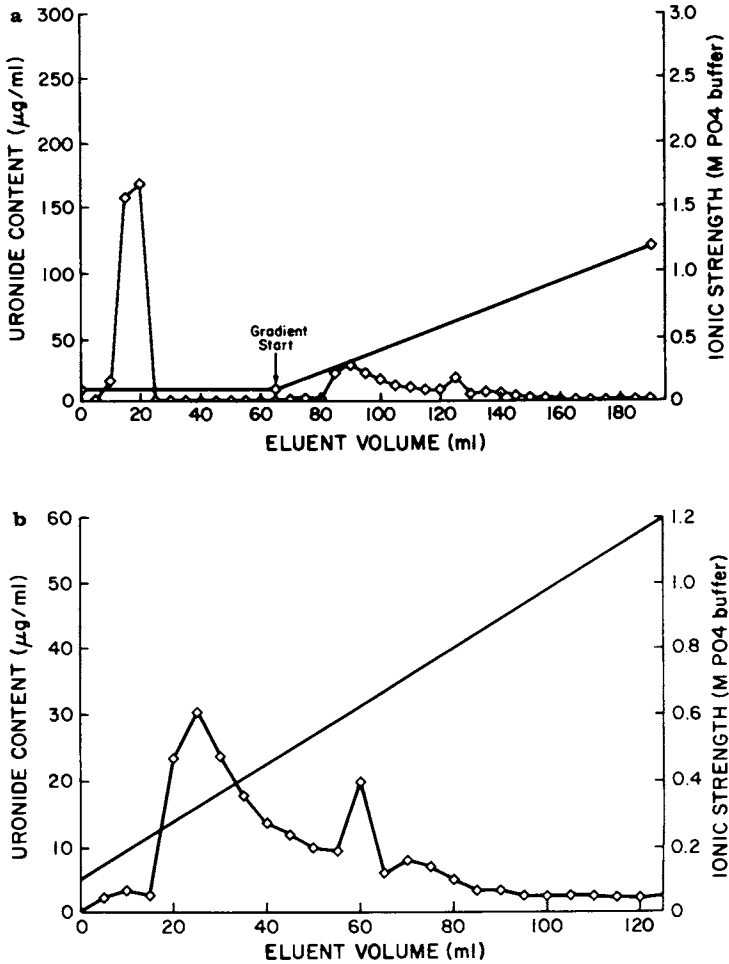


Figure 9. DEAE fractionation of WSP from unfrozen strawberries.  
 (a) complete fractionation profile  
 (b) elution of column bound material



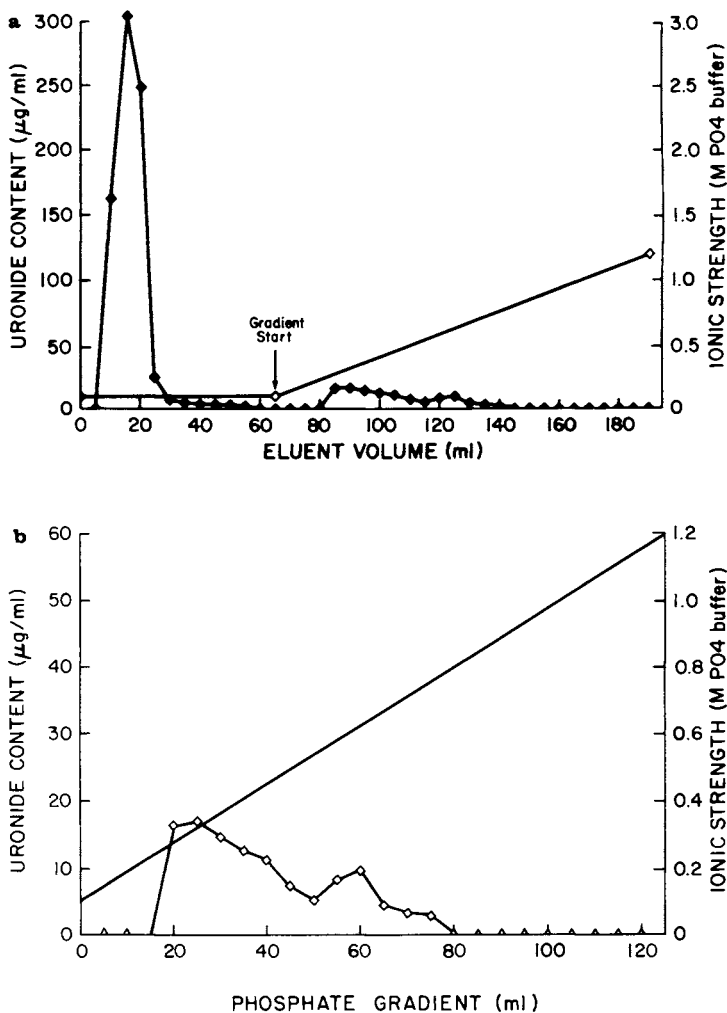
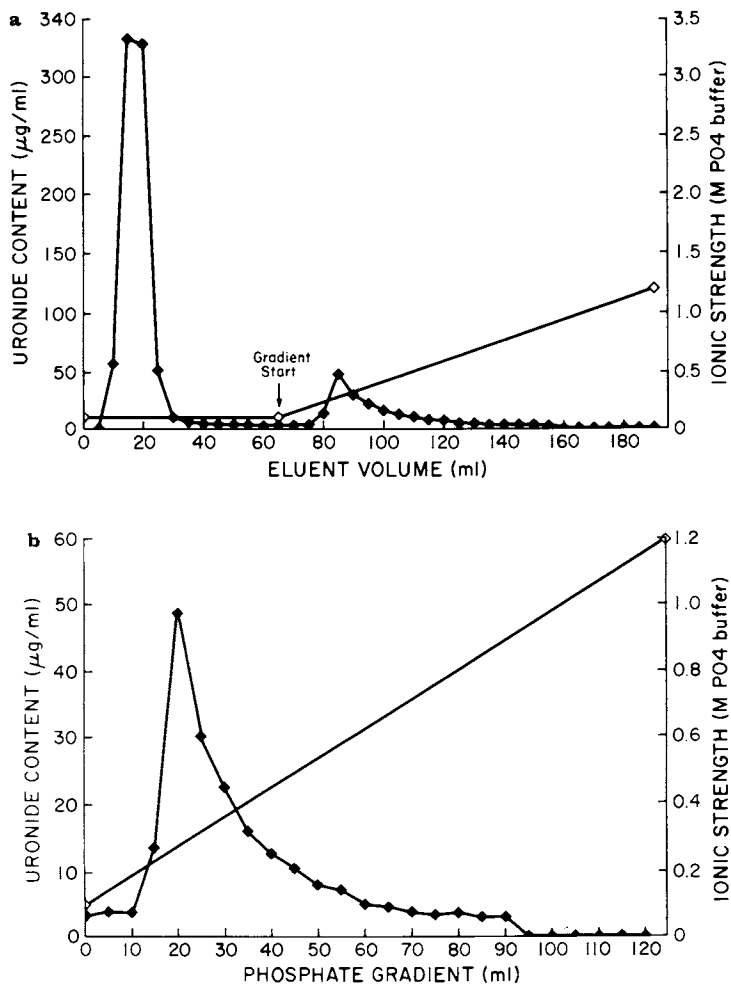


Figure 10. DEAE fractionation of WSP from 1 day frozen strawberries.

(a) complete fractionation profile

(b) elution of column bound material



**Figure 11.** DEAE fractionation of WSP from 4.5 month frozen strawberries.

(a) complete fractionation profile

(b) elution of column bound material

Table 3. Neutral sugars ratios from anion exchange chromatography fractions of strawberries WSP, still air frozen -12c storage.

sample tot neutral sugars : tot uronides	Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose
unfrozen unbound fraction	4.5	2.20	18.0	19.6	6.8	35.4	13.4
unfrozen bound fraction	29.1	3.1	24.3	5.6	5.7	19.6	12.6
1 day storage unbound fraction	5.3	3.0	15.3	15.7	4.9	40.1	15.7
1 day storage bound fraction	31.2	-	21.5	8.6	8.5	17.9	12.4
4.5 month unbound fraction	5.6	3.5	17.0	16.9	3.7	39.0	14.3
4.5 month bound fraction	30.1	1.2	20.7	3.9	2.1	19.5	21.6

contribution to texture from the cell wall material, a conclusion supported by abundant material following the changes in cell wall pectins during fruit ripening. Pectin is also an important contributor to the texture of tissues which have been frozen. Changes are seen in the pectic material as a consequence of freezing and frozen storage.

In strawberries, the WSP fraction, which we assume to be the fraction most loosely associated with the cell wall, shows the most dramatic change. There is a decrease in amount, paralleling a decrease in firmness. The compositional studies of the fraction suggest that the changes include some associated with the pectin rhamno-galacturonan backbone. The chemical change in the backbone is not yet clearly defined. Changes in the sugar distribution and in the uronic acid to neutral sugar ratios are not in evidence as we analyse the deae bound fractions from fruits at progressively longer storage. The molecular weight remains above the cut-off for a P100 column.

Further studies are in progress. These include investigation of changes in the degree of methyl esterification of the fractions. The composition of the other fractions is being determined. The uronide fraction of drip is being analysed. The effect of adding calcium, which should interact with pectin, to strawberries prior to freezing them is also being assessed. Initial data indicate that calcium alters the relative amount of pectin extractable into the different fractions, and also alters the back extrusion force, and the nature of the time dependence of texture on frozen storage.

#### Acknowledgments

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## Pectin Methylation Changes and Calcium Ion Effects on the Texture of Fresh, Fermented, and Acidified Cucumbers

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The commercial use of calcium ion in cucumber pickle products has stimulated efforts to better understand the mechanisms by which calcium affects cucumber texture. Recent results suggest that a high level of pectin methylation has little effect upon the ability of calcium to maintain the firmness of acidified cucumber tissue, while in fermented cucumbers maintenance of a minimum degree of methylation may be important to firmness retention. Efforts are being made to measure calcium binding characteristics in cucumber tissue and to obtain estimates of the effectiveness of calcium ion in inhibiting tissue degradation by pectolytic enzymes.

The ability of calcium ions to act as a firming agent in processed fruits and vegetables has been the subject of many studies over the years (1). Among the commodities in which calcium has been observed to cause firming are: snap beans (2), tomatoes (3), apples (4), carrots (5), apricots (6), and jalapeno peppers (7). Cucumbers are commercially preserved by fermentation (processed pickles), acidification and pasteurization (fresh-pack pickles) and refrigeration of mildly acidified fruit (refrigerated dills). Calcium ion has been found to be effective as a firming agent in all three types of products. It is now being used in most commercial cucumber pickle products. Investigations of the structure of cucumber cell walls and the interaction of calcium with the cell walls have followed the practical application of calcium.

There have been major advances in our understanding of the structure of plant cell walls over the past 15 years (8). However, it has not proven to be an easy task to explain specific textural changes which occur during ripening or processing of fruits and vegetables in terms of changes in the structures of cell wall polymers (9). This is perhaps not surprising since the detailed structures of cell wall polymers are proving to be very complex

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(8). There also appears to be great variability in the distribution of polysaccharides in different fruits and vegetables (10).

Recently, efforts have been directed toward trying to explain and ultimately control the textural changes that occur in cucumbers during processing and storage. Cucumbers have not been an exception in that it is difficult to see clear relationships between structural changes in the cell wall and texture effects in the tissue. Despite the difficulties which are encountered, the cucumber has several characteristics which make it a good model to investigate texture/cell wall structural relationships. The cucumber mesocarp is a large proportion of the total fruit tissue, and it can be isolated in quantity without great difficulty. The mesocarp tissue is relatively uniform in structure and contains little starch to interfere with the analysis of cell wall polysaccharides (11). A simple texture test has been devised which can be used with small pieces of cucumber tissue and which relates well with human perception of firmness (12). Finally, cucumbers can be obtained for experimental purposes throughout most of the year in a wide range of sizes.

This paper will give a brief background on textural investigations of cucumbers and cucumber products. Recent work on the structure of cucumber cell walls and initial efforts to determine texture/structure relationships will then be reviewed.

Cucumbers can be softened enzymatically by fungal polygalacturonases. They also contain natural pectolytic enzymes, though the conditions in which these enzymes contribute to fruit softening have not been determined. Finally, softening occurs slowly during storage of both pasteurized and fermented cucumbers, where polygalacturonases have been inactivated to nondetectable levels. The mechanism(s) of this post-processing softening has not been determined.

Bell and coworkers in the 1950's investigated the softening of cucumbers in commercial fermentations (13, 14, 15, 16). They found that softening of small size cucumbers was caused primarily by the presence of polygalacturonases in the fermentation brines, which degraded pectic substances in the fruit. These softening enzymes were primarily of fungal origin and were present on the cucumber fruits and flowers when they were put into fermentation tanks. Lampi et al. (17) attempted to measure changes in the pectic substances of cucumbers during fermentation, but were unable to show any consistent pattern of changes with the techniques available to them.

The use of calcium as a firming agent for cucumber products began in the 1960's with commercial trials conducted over several years. It was found that addition of 0.1%  $\text{CaCl}_2$  to pasteurized cucumber products resulted in a significant improvement in the retention of a firm texture during storage (18). This led to commercial use of  $\text{CaCl}_2$  in these pickle products. Fleming et al. (19) found that 0.1%  $\text{CaCl}_2$  also helped prevent firmness losses in fermented cucumber slices and in small whole cucumbers at low NaCl concentrations. Particularly important to the use of calcium in commercial cucumber fermentations was the finding by Buescher and coworkers (20, 21) that at  $\text{CaCl}_2$  concentrations up to 1%, whole cucumbers remained firm even when fungal polygalacturonases were intentionally added to the fermenting cucumbers (Table 1).

Table 1. Cucumber Firmness 30 Days After the Beginning of Fermentation as Influenced by NaCl, CaCl<sub>2</sub> and Polygalacturonase. From Buescher et al. (20)

Treatment	Pickle Firmness (kg) <sup>a</sup>
2% NaCl	
Control	7.9C
0.1 M CaCl <sub>2</sub>	9.6AB
Polygalacturonase	2.3E
0.1 M CaCl <sub>2</sub> + Polygalacturonase	8.9BC
5% NaCl	
Control	8.0C
0.1 M CaCl <sub>2</sub>	10.0A
Polygalacturonase	5.8D
0.1 M CaCl <sub>2</sub> + Polygalacturonase	10.0A

<sup>a</sup>Mean firmness of 18 pickles. Values with the same letters are not significantly different.

High calcium concentrations were also found to prevent breakdown of the locular tissue of large cucumbers during fermentation (22), presumably by preventing the degradation of pectin by cucumber polygalacturonase (23).

These observations have led to efforts to develop a better understanding of the structure of cucumber cell walls and the role that calcium ions play in improving texture. The cucumber cell wall contains about 30% cellulose (10), 15% pectin (24), and noncellulosic neutral sugars (10, 25, 26). Other than glucose, which is present primarily in cellulose, galactose and xylose are the most abundant neutral sugars in the cell wall. The degree of pectin methylation in cucumbers has been reported to be 65% in a Japanese fresh market cultivar (27) and 57% in 4 cm diameter pickling cucumbers (28). Preliminary evidence from this laboratory indicates that pectin methylation may increase during development of the cucumber fruit.

Demethylation of pectin has been the most obvious change observed in cell wall structure, both in the chilling injury of fresh cucumbers (27, 29) and during fermentation (25). Bell et al. (30) showed that cucumber plants contain pectinesterase in all parts of the plant, including the fruit. As part of a series of studies of chilling injury in cucumber fruits, Fukushima and Yamazaki (29) found that a decrease in hot, water-soluble, high methylation pectin and an increase in hot, water-insoluble, low methylation pectin occurred when fruit were stored at 0 or 5°C. They suggested that demethylation of the pectin by pectinmethyl-esterase resulted in a more rigid cell wall structure, and that this deesterification may be a common characteristic of chilling-sensitive plants (29).

Tang and McFeeters (25) investigated changes in the cell walls of cucumber mesocarp tissue when cucumbers were fermented in 6% NaCl, a procedure similar to commercial fermentations. Figure 1 shows that only small changes in the noncellulosic neutral sugars occurred during the experiment. The total pectic substances showed little change during fermentation and storage. The size of the pectin molecules in the major pectin fractions also showed almost no change. The average degree of polymerization of the major pectin fraction (acid-soluble pectin) isolated from fresh cucumbers was estimated to be 402 residues. After fermentation, the degree of polymerization of the EDTA-soluble fraction, which was the major pectin fraction after fermentation, was 403 after 3 months and declined slightly to 365 after 6 months. The major change observed was a large decrease in the acid-soluble pectin fraction during the fermentation period and an increase in the EDTA-soluble pectin during the same period (Figure 2). The acid-soluble pectin had a degree of esterification of 62%, while the EDTA-soluble material had a degree of esterification too low to measure. These results indicated that pectin deesterification was the major change to occur in the cell wall during fermentation. There were no substantial changes in the cell wall composition from 1 to 6 months after brining, even though there was nearly a 30% decline in tissue firmness when CaCl<sub>2</sub> was not added to the fruit. Thus, a situation was observed in which a substantial change in texture occurred in cucumber fruit without an obvious change in the structure of the cell wall.



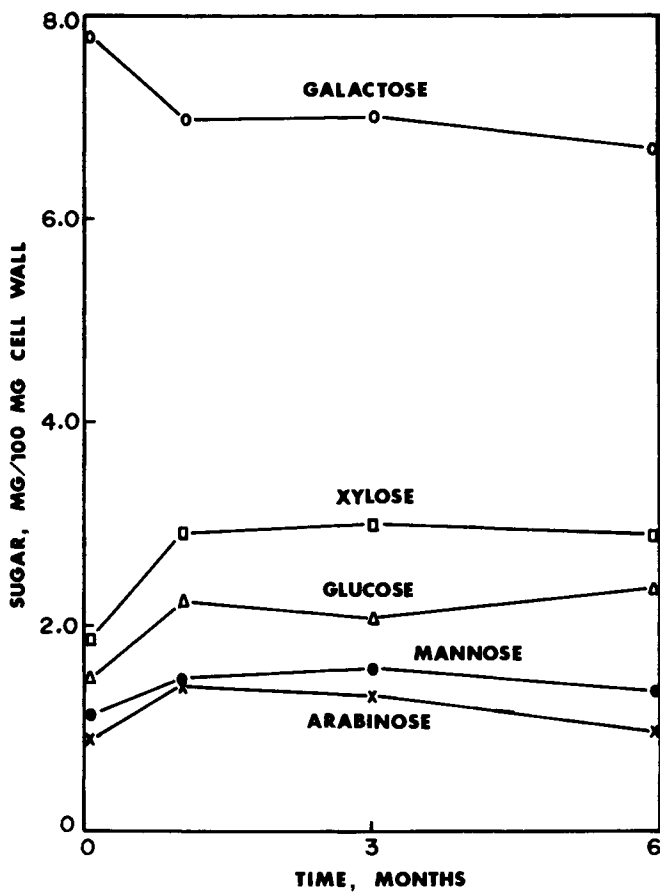


Figure 1. Changes in cell wall neutral sugar content during brining and storage of cucumbers.

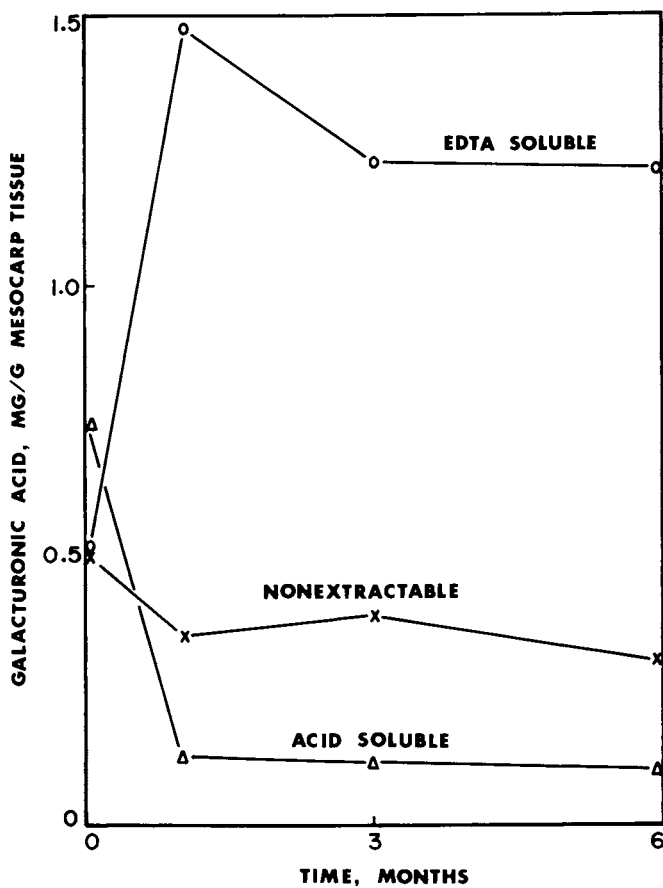


Figure 2. Changes in pectin fractions during fermentation and storage of cucumbers.

McFeeters et al. (28) recently investigated the effects of pectin methylation in nonfermented cucumber tissue when the degree of methylation was varied between 12 and 50%. Hudson and Buescher (31) have studied the effect of methylation on texture when the degree of esterification was in the range of 6 to 18%. McFeeters et al. (28) were able to vary the degree of methylation by blanching cucumber slices at different temperatures. Heating the slices at 66°C or less caused little or no inactivation of pectinesterase present in the tissue (30). During storage in pH 3.7, 2.0% NaCl brine, the pectin was extensively demethylated in these tissues (Figure 3). When slices were heated at 81°C, pectinesterase was inactivated. However, during storage, partial reactivation of the enzyme occurred. The result was an intermediate level of methylation. Finally, if cucumber tissue was heated in boiling water, only slight reactivation of pectinesterase activity occurred and the degree of methylation remained near 50%.

The effect of changing the degree of pectin methylation on the firmness of cucumber mesocarp tissue was determined over a 6-month storage period. When the slices were stored after blanching in a brine which contained 10 mM calcium ion, there were some texture differences, but the differences observed were not very large. The firmest texture was obtained when the tissue was blanched at intermediate temperatures, i.e. 66 and 81°C. Slices blanched at 99°C were less firm than the other treatments. However, much of the observed difference could be attributed to differences in the tissue firmness immediately after blanching, before the tissue was exposed to brine solutions. The rates of firmness loss during the 6-month storage period were similar, regardless of the degree of pectin methylation.

When the calcium concentration was varied in slices blanched at 54, 66, and 81°C, there was a very clear increase in firmness retention as the calcium concentration increased (Figure 4). However, the pattern of firmness changes was the same, regardless of blanch temperature. Thus, the results of these studies did not show any direct relationship between pectin methylation and firmness changes. Calcium ion was effective in preventing firmness loss during storage, regardless of the degree of pectin methylation. Studies of calcium ion binding by polypectate have shown that blocks of at least 14 consecutive demethylated carboxyl groups on adjacent polygalacturonan molecules are required for cooperative cross-linking to form an "eggbox" type structure (32, 33). The fact that calcium is effective in preventing softening, even at high degrees of pectin methylation, suggests that other types of polysaccharide/calcium interactions may be involved in the cucumber tissue. Calcium ion has been shown to form crystallizable coordination complexes with many mono- and disaccharides (34, 35). Cook and Bugg (35) have speculated upon the possible importance of calcium/galactose interactions in bone tissue. It may be useful to consider whether such interactions occur in plant cell walls.

Hudson and Buescher (31) have found a relationship between cucumber tissue firmness and the degree of methylation of the mesocarp tissue. When the pectin methylation was less than 13%, the firmness of the tissue declined as the degree of methylation

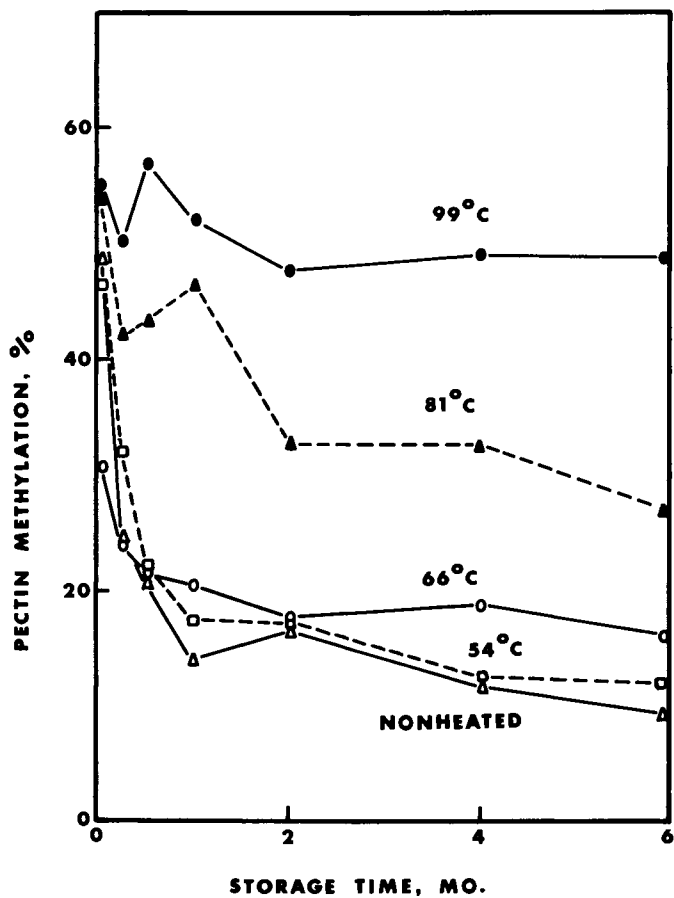


Figure 3. Effect of blanch temperature on changes in pectin methylation of the cucumber cell wall during storage.

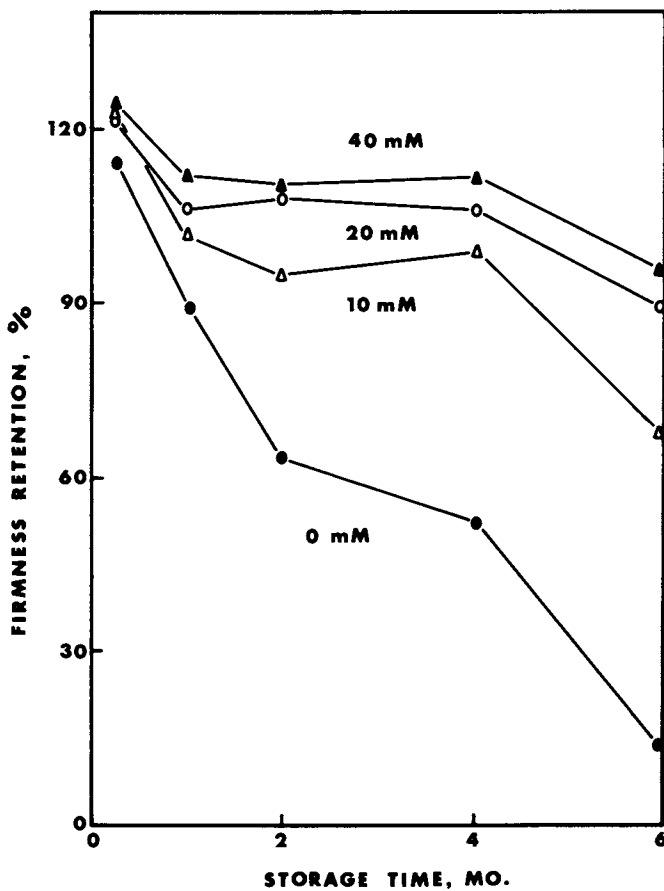


Figure 4. Effect of  $\text{CaCl}_2$  concentration on the firmness retention of cucumber slices blanched at  $81^\circ\text{C}$  for 3 min.

decreased. They suggest that this may be caused by a change in the conformation of the pectin molecules at very low esterification similar to that reported by Leeper and Dull (36) for pectin solutions. However, because it was necessary to compare treatments with and without calcium ions present, the texture differences observed may have been caused by factors other than differences in the degree of methylation.

Since we are interested in trying to better understand the interaction of metal ions with the cucumber cell wall, it was of interest to develop a technique to determine the extent and affinity of ion binding in cucumber tissue. Since it is generally thought that calcium is bound to plant tissues by interacting with the free carboxyl groups present in pectin, an effort has been made to relate calcium bound in cucumber mesocarp tissue to the concentration of the free carboxyl groups of pectin.

Pieces of mesocarp tissue were isolated from cucumbers and placed in a brine to give an equilibrated concentration of 0.6% acetic acid, 200 ppm  $\text{SO}_2$  (for preservation), and varying concentrations of calcium ion from 1 to 16 mM. After equilibration, the degree of pectin methylation in the mesocarp tissue was measured (24) and the concentration of free carboxyl groups in the tissue calculated. The concentration of calcium bound by the tissue was calculated as the difference in calcium concentration between the mesocarp tissue and brine solution as determined by a colorimetric procedure (37, 38). These data were used to construct a Scatchard plot to analyze both the moles of calcium bound per carboxyl group in the tissue and the affinity of calcium binding.

Figure 5 shows an example of the analysis of such a binding experiment. The intercept on the X-axis gave a ratio of 0.43 calcium ions bound per free carboxyl group in the tissue. If each divalent calcium ion were binding to two pectin carboxyl groups, a ratio of 0.5 would be expected. Analysis of the slope of the Scatchard curve, gave an affinity constant of 888 for calcium binding. Kohn (32) determined calcium binding constants as a function of the degree of pectin esterification for pectin solutions at neutral pH. Stability constants varied from <100 for highly methylated pectin to nearly 10,000 with very low degrees of methylation. The degree of esterification expected for pectin with a binding constant of 888 is 38% based upon his data. Table 2 shows that the esterification of the mesocarp samples varied with calcium concentration, but the predicted degree of esterification was within the observed range. These results suggest that this may be a useful approach to the analysis of ion binding in intact tissues under conditions similar to those found in fermented and acidified vegetable products. Additional data need to be obtained to determine whether a detailed analysis of binding of calcium and other ions in cucumber mesocarp tissue can help provide an understanding of the textural changes that occur in processed cucumbers.

The development of commercial applications of calcium addition to improve the textural qualities of fermented and acidified cucumber products has stimulated efforts to understand the mechanisms by which this ion affects the texture of cucumber

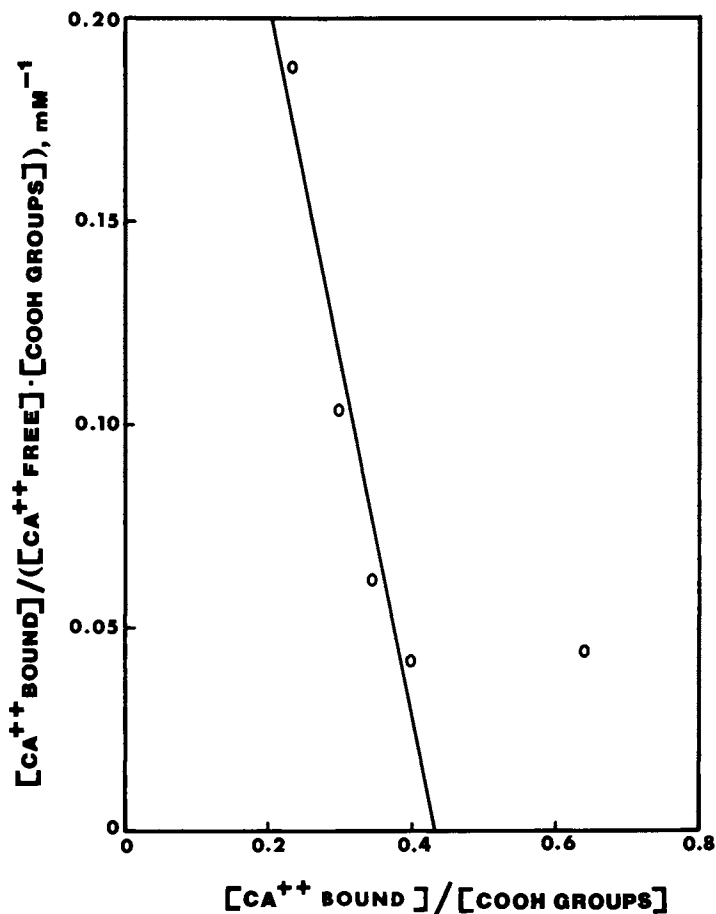


Figure 5. Scatchard plot of calcium binding to the free carboxyl groups of pectin in acidified cucumber mesocarp tissue.

Table 2. Degree of Pectin Methylation in Cucumber Mesocarp Tissue After Equilibration With Calcium Ions

Added $[Ca^{++}]$ (mM)	Degree of Pectin Methylation (%)
0	42.3
1.8	33.1
4.6	27.4
8.8	27.5
16.1	25.4

tissue. Procedures have been developed to measure the firmness of mesocarp tissue (12) and to measure pectin methylation in small samples of cell walls (24). Methods to quantitatively analyze ion binding by cucumber tissue are being developed. Techniques to analyze the neutral polysaccharides to plant cell walls are being used to determine changes in the neutral sugars of the wall during processing procedures (39). A number of interesting observations have been made concerning the effects of calcium ion and pectin methylation on the texture of cucumber tissue, but the structural basis for the textural effects remain to be explained.

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## Enzymic Lysis of Pectic Substances in Cell Walls: Some Implications for Fruit Juice Technology

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Detailed knowledge of the role of various polysaccharide degrading enzymes in cell wall degradation and of the chemical nature of the solubilized fragments enable improvement of enzyme applications in fruit and vegetable processing and development of new applications with balanced enzyme formulations. This is demonstrated for apple juice production and cloud stabilization in apricot nectar. The enzyme systems effective in the solubilization of apple and apricot cell wall pectin are identified and their role in technological applications are discussed. Possible implications of solubilized fragments on product quality (color, haze formation) are indicated.

Fruit juices are obtained from fruit pulps by mechanical separation (pressing, sieving, centrifuging) of cell liquid from cell wall fragments. For clear juices they are further clarified, for cloudy or pulpy juices only coarse and unedible particles are removed (1,2). The tissues of the edible parts of fruits and vegetables consist of parenchymatic cells which have a middle lamella consisting mainly of pectins, a primary wall which is a firm gel of pectin, cellulose, hemi-cellulose and some protein and sometimes a secondary wall in which cellulose and hemi-cellulose prevail (3,4). Technologically the pectic substances play the greatest rôle. They account for 0.5 to 4% of the weight of fresh material. When tissue is crushed this high molecular substance partly becomes water soluble and gives a high viscosity to the liquid phase and can form a protective colloid for cell fragments. Partly they remain in the pulp particles bound to cellulose fibrils by side chains of hemi-

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cellulose where they help to bind water. Pectins are complex heteropolysaccharides with a backbone of  $\alpha$ -1,4 bound galacturonic acids. Some of the uronic acid groups are esterified with methanol. In another contribution to this symposium structural features of pectins are discussed in more detail (5).

Pectin esterases (E.C. 3.1.1.11), endo-polygalacturonases (E.C. 3.2.1.15) and endo-pectin lyases (E.C. 4.2.2.10) are by far the most relevant pectic enzymes in fruit processing. Pectin esterases de-esterify pectins producing methanol and pectic acid. Endo-polygalacturonase and endo-pectin lyase are both depolymerases which split the glycosidic linkages in their preferred substrates either by hydrolysis (polygalacturonase) or by trans-elimination (pectin lyase). Endo-polygalacturonases hydrolyse low esterified pectins in a more or less random fashion. Endo-pectin lyases are the only depolymerases specific for highly esterified pectins which they degrade more or less at random. The combined action of pectin-esterase and polygalacturonase can also depolymerize high methoxyl pectins. Pectic enzymes are reviewed in more detail by Rombouts and Pilnik (6).

Pilnik et al (7) have noted that the combined action of pectinases and C-1 (1,4- $\beta$ -D-glucan cellobiohydrolase, E.C. 3.2.1.91) enriched cellulases are able to almost completely liquefy pulped fruits and vegetables. For the actual dissolution of the crystalline cellulose fibrils the C-1 enzyme is necessary which splits off cellobiose from the non-reducing end of the 1,4- $\beta$ -D-glucan and which needs some C-x (1,4- $\beta$ -D-glucan-glucanohydrolase, E.C. 3.2.1.4) to create such points of attack. Preparations rich in C-1 activity are usually obtained from *Trichoderma* spp. (8).

The extent and mode of bioconversions of pectic substances in situ, their solubilization and depolymerization effect consistency, cloud behaviour, pressing characteristics, juice release, soluble solids, clarification, haze formation and browning potential. We have been studying plant cell wall degradation with pure and well characterized enzymes and from the results of these studies we are now able to better define the various stages in cell wall degradation and to understand implications for fruit processing. This will be demonstrated in apple juice production and stabilization of apricot nectar.

### Apple juice

In processing of apples exogenous mould enzymes, mainly pectinases are frequently used. They are added to extracted juice, to facilitate filtration and prevent gelling in concentrated juice or they are added to the pulp to improve press yield, or added together with cellulase preparations to liquefy the pulp. (2). Information about the contribution of the various polysaccharide degrading enzymes in the degradation of apple cell walls has been obtained by detailed studies of degradation of apple cell wall preparations with pure enzymes (9,10,11) and by closely monitoring the chemical changes taking place in apple pulp and apple juice during processing (12). Our increased knowledge of the polysaccharide composition and structure of fruit cell walls (13-17) and their changes during ripening and storage (18-20) contributes also to the understanding of the processes. Table I shows the sugar composition of

the alcohol insoluble solids (AIS) prepared from apple cortical tissue and canned apricots and the composition of their pectin and hemi-cellulose fractions. These data illustrate the differences in cell wall composition of these fruits. Apricot cell walls were found to contain more anhydrogalacturonic acid (AGA) but less glucose than apple cell walls. The pectin fraction extracted from apricot cell walls was almost twice as large as the apple pectin fraction. For the cellulose content (data not shown) the reverse was the case. The hemi-cellulose fractions showed large differences in sugar composition, particularly in arabinose, mannose and galactose content.

Table I

Sugar composition of AIS prepared from apples and canned apricots and of the pectin and hemicellulose fraction prepared from AIS (sugar composition expressed as mole %).

Preparations	Composite sugars							
	% AIS	Rha/fuc	Arab	Xyl	Man	Gal	Glc	AGA
AIS:								
apple		2	13	6	2	7	42	28
apricot		2	16	5	3	6	32	35
Pectin:								
apple	27.2	1	23	2	-	4	1	68
apricot	52	2	20	1	-	4	1	73
Hemicellulose:								
apple	16.9	4	34	19	12	17	30	9
apricot	14.7	5	8	21	1	9	21	11

Ref: Voragen *et al*, (15) Siliha, (11).

Some results of the studies on the enzymatic degradation of apple cell wall preparations are summarized in Table II and III. They show the amounts of sugars released from apple AIS by pure pectic, cellulolytic and hemi-cellulolytic enzymes. From the pectic enzymes the combination of pectinesterase and endo-polygalacturonase (PE + PG) were found to release the largest amounts of neutral sugars and galacturonides followed by PL. PG alone released substantially less sugars. From these results it can be derived that degradation of highly esterified pectin (PE + PG, PL) coincides with an increased release of pectin associated sugars like arabinose, galactose and rhamnose.

C-1, an 1-4- $\beta$ -D-glucan cellobiolydrolase enriched cellulase preparation released in particular glucose and xylose (as cellobiose and xyloglucan fragments) and only minor amounts of galacturonides. A synergistic action was observed for the combination C-1 and PE + PG, overall 80% of all composite sugars of the cell walls were released resulting in the almost complete liquefaction of pulp particles. Endo-1,5- $\alpha$ -L-arabanase and endo-1,4- $\beta$ -D-galactanase were found to release mainly galactose and arabinose containing fragments. Galactanase however was also able to release 33% of the pectic material. The combination of arabanase and galactanase showed a cumulative

Table II

Amounts of neutral sugars and galacturonides released from apple AIS by pure enzymes. Amounts expressed in % of original amounts present, corrected for control.

Enzyme treatment	Neutral sugars released							AGA re-leased	Tot.sug. released
	Rha/fuc	Arab	Gal	Man	Xyl	Glc	Total		
PE (citrus)	--	--	--	--	--	--	--	--	0.1
PG	4	9	5	--	4	--	3	21	8
PL	19	39	27	--	<u>20</u>	4	17	57	28
PE+PG	25	52	34	--	<u>13</u>	--	18	75	33
C1	9	3	12	<u>26</u>	<u>20</u>	<u>22</u>	16	5	13
C1+PE+PG	<u>58</u>	<u>89</u>	<u>69</u>	<u>84</u>	<u>71</u>	<u>79</u>	<u>78</u>	<u>82</u>	<u>80</u>

Ref: (Voragen et al, (9)).

Table III

Amounts of neutral sugars and galacturonides released from apple AIS by pure enzymes. Amounts expressed in % of original amounts present, corrected for control.

Enzyme treatment	Neutral sugars released							AGA re-leased	Tot.sug. released
	Rha/fuc	Arab	Gal	Man	Xyl	Glc	Total		
PE+PG	25	52	34	--	13	--	18	75	33
Endo-Arab.	3	<u>34</u>	10	--	-	--	10	--	8
Endo-Gal	11	<u>18</u>	<u>33</u>	--	6	--	11	33	18
Arab+Gal	11	38	<u>31</u>	--	6	--	15	36	22
PE+PG+Arab+Gal	59	56	89	--	24	11	36	73	48

Ref: Voragen et al., (9,10)

effect. The combination of PE + PG with both arabanase and galactanase showed an increased release of all neutral sugars, but not to the extent as observed for the combination with C-1.

Chemical characteristics of pulp residues and juices obtained by pressing of enzyme treated and non-enzyme treated pulp and by centrifugation of liquefied pulp in laboratory scale experiments are summarized in Fig. 1. Pulp residues were analysed for AIS content, pectin content and cellulose content, from the juices the anhydrogalacturonic acid content was estimated before and after dialysis. The differences between these values represent oligomeric pectin fragments consisting of less than ca. 6 sugar units. The data show that pulp enzyming results in about 50% reduction of pectin in the press cake. A further reduction in pectin content and in addition a considerable reduction in AIS and cellulose content takes place during liquefaction. The polymeric, oligomeric and monomeric sugars in the

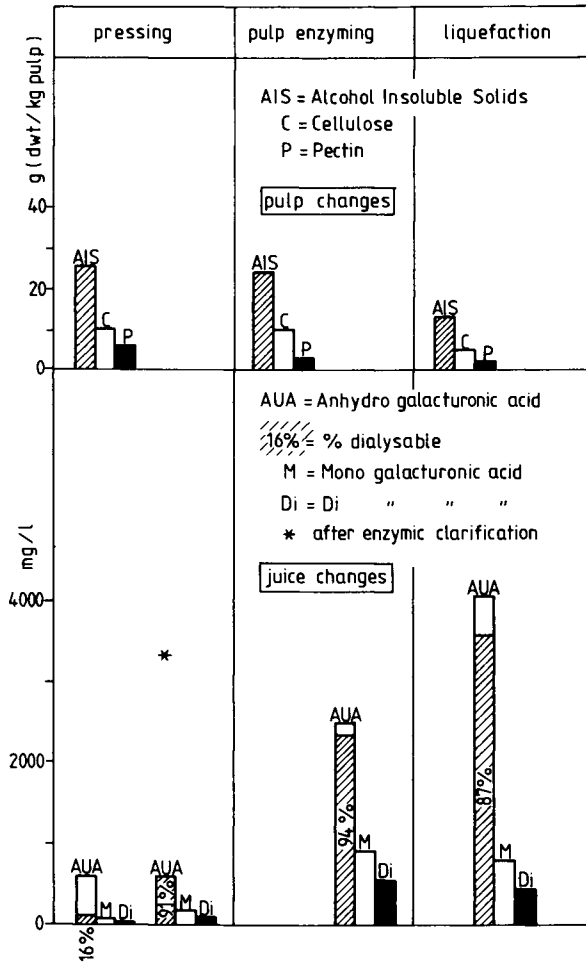


Figure 1. Changes in juice and pulp as a result of enzyme treatment of apple pulp.  
 Ref: Kolkman (12)

juices were further fractionated by gelfiltration chromatography on Biogel P-2. Fig. 2 shows the elution profile for press juice obtained from enzyme treated pulp. Mono - and oligalacturonic acid were isolated from the large excess of glucose, fructose and saccharose by ion-exchange chromatography as outlined in Fig. 2. It can be seen that the solubilized pectic material occurs mainly as monomeric and oligomeric galacturonides. From the absorbance at 232 nm measured for the oligogalacturonides it was derived that they are partially present in the unsaturated form. In all juices about 5% of the dimers and trimers were present in the unsaturated form, for tetramer this was 20-25%. They are typical reaction products of PL action. Preliminary studies have shown that oligogalacturonides and particular unsaturated oligogalacturonides are very reactive precursors for browning reactions (unpublished results). Table IV shows the overall galacturonide content estimated for differently processed juices, the release of soluble pectic polymers and oligomers was found to increase strongly by enzym action. A standard treatment of juice with bentonite and gelatine reduced the galacturonide content.

Table IV

Uronide content of apple juices (12°C Brix) estimated according to Kintner and van Buren (29)

Method of preparation	Uronide content mg/ml
Pressing	0.58
Water extraction	1.31
Pulp enzyming and pressing	2.51 (1.6)*
Enzymic liquefaction	4.32

\* After clarification with bentonite and gelatine  
Ref: Kolkman, (12).

The polysaccharides in the juice were isolated by ultrafiltration in a tubular system (PCI) equipped with a BX3 polysulfonmembrane (Mw cutoff 60,000 Dalton). The retentate and permeate fraction were then dialysed, centrifuged and freeze-dried. Fig. 3 shows a flow sheet of this process for liquefaction juice. From this juice ca. 0.3% of fresh apple weight was obtained as retentate fraction and ca. 0.1% as permeate fraction. Table V shows the sugar and glycosidic-linkage composition of the retentate fraction. The presence of an  $\alpha$ -1,5-arabinan with single unit or short arabinose containing side chains at C-2 and C-3 is evident (16,21). These arabinans are probably linked to C-4 of rhamnose which in turn is 1,2 linked in the rhamnagalacturonan backbone. This backbone further carries single unit and larger xylose containing side chains. Also the presence of 1,4 and 1,3/1,6 linked-galactan is indicated. The galacturonan was methylated for 42% and surprisingly a degree of acetylation of 60% was estimated (based on anhydrogalacturonic acid content). The retentate fraction was resistant to further degradation by pectic enzymes. With an arabinofuranosidase 80% of the arabinose could be removed, endo- $\beta$ -1,4-D galactanase was able to release some oligomeric galactose. The retentate fraction obviously represents hairy regions from pectin molecules (21).

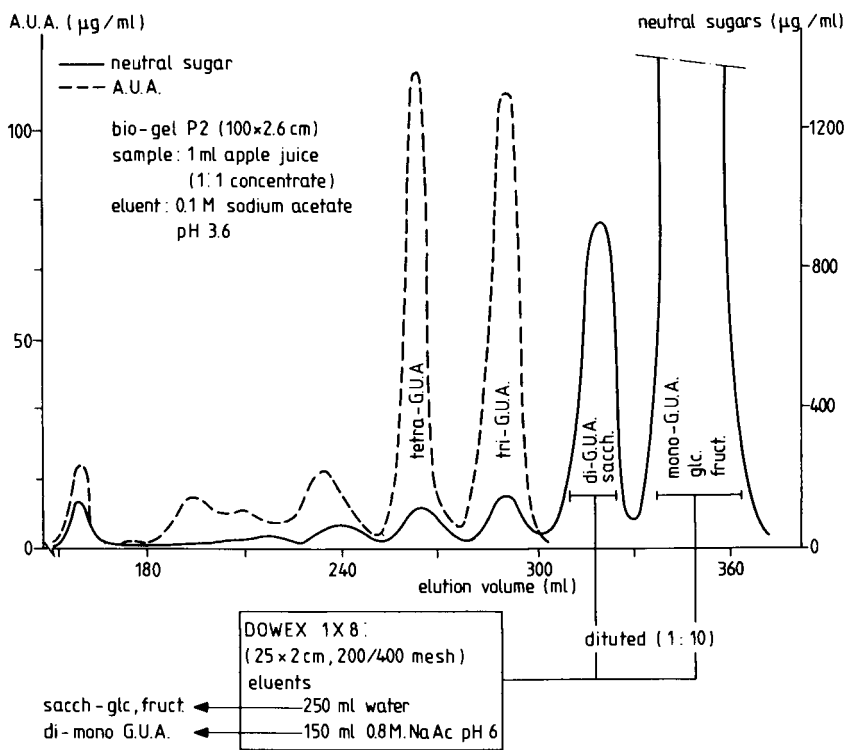


Figure 2. Analysis of oligomeric galacturonides by gelfiltration chromatography and ion-exchange chromatography. Sample: apple juice from enzyme treated pulp. Ref: Kolkman (12).



Table V  
Sugar and glycosidic linkage composition of ultrafiltration retentate.

Sugar composition (as mole %)		Glycosidic linkage composition (as mole %)	
Rhamnose	5.6	1,2,4-Rhap	100
Arabinose	55.1	T-Araf	25
		1,2-Araf	1
		1,3-Araf	8
		1,5-Araf	47
		1,3,5-Araf	11
		1,2,5-Araf	3
Xylose	8.4	1,2,3,5-Araf	5
		T-Xylp	65
		1,4-Xylp	32
		1,2-Xylp	3
Galactose	9.9	T-Galp	45
		1,3-Galp	19
		1,4-Galp	14
		1,6-Galp	4
		1,2,4-Galp	4
		1,3,6-Galp	14
Galacturonic acid	20.9	1,4-GalpA *	80
		1,3,4-GalpA*	20

\* Determined after carboxyl reduction.

Gelfiltration chromatography of the retentate fraction of liquefaction juice gave 2 overlapping peaks with similar uronide content and neutral sugar composition when buffer was used as eluent (Fig. 4b). However with water as eluent the negatively charged pectic polymers appeared in the void volume because they are excluded from the particle matrix due to their charge (22). A galacturonide free, almost pure arabinan representing 7% of the retentate eluted in the included volume (Fig. 4a). Table VI shows the glycosyl linkage composition of this arabinan fraction and of haze isolated from apple juice concentrate. In the retentate arabinan 15.7% of the arabinosyl units are branched, in haze arabinan this is only 4%. Haze arabinan has been identified as an almost linear  $\alpha$ -1,5-L-arabinan (10,23). Native arabinans are described as highly branched (13,14,16). Table VII summarizes the degree of branching estimated for arabinans in apple cell wall fractions and in fractions isolated from apple juices. From these data it is obvious that during enzyme treatment arabinofuranosidases commonly present in commercial pectinase preparations reduce the degree of branching and increase the chance for chain association resulting in haze formation. Several possibilities can be suggested for avoiding haze problems. Enzyme manufacturers can try to avoid the presence of arabinofuranosidase in their pectinase preparations or they can add an arabanase enzyme complex which degrades the branched arabinans completely. We are presently

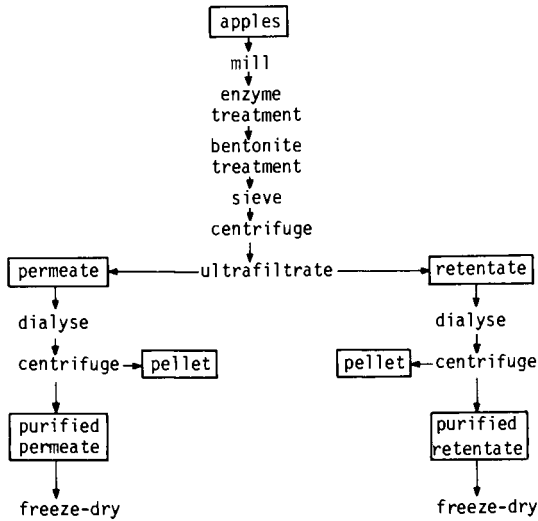


Figure 3. Scheme for the preparation of ultrafiltration permeate and retentate fractions from apple liquefaction juice.

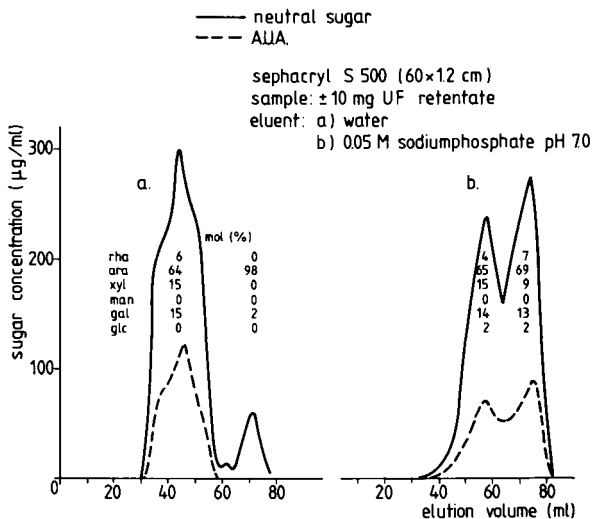


Figure 4. Gel filtration chromatography of the ultrafiltration retentate from apple liquefaction juice and sugar composition of fractions in mol %.

Table VI  
Glycosidic linkage composition for arabinosyl residues in arabinan fraction of ultrafiltration retentate and in haze.

Arabinosyl residue	Occurrence glycosyl linkage type in %	
	U.F. Retentate Arabinan	Haze
T-Araf	11.4	4.2
T-Arap	0.2	0.2
1,2-Araf	0.4	---
1,3-Araf	6.6	2.3
1.5-Araf	65.7	88.4
1,3,5-Araf	9.4	3
1,2,5-Araf	3.4	1
1,2,3,5-Araf	2.9	

Table VII  
Degree of branching (branched arabinosyl units in % of total arabinosyl units) of arabinan fractions isolated from apple cell walls and apple juice.

Apple cell wall	Degree of branching
Pectin fractions	36.5-49*
Hemi-cellulose fraction	30
<u>Apple juice</u>	
U.F. retentate (liquefaction)	19
Arabinan fraction retentate	15.7
Haze	4

\* (de Vries et al, 21).

studying a powerful arabinan degrading enzyme preparation produced by a fungus and kindly supplied by Gist Brocades (Delft, The Netherlands). Following largely isolation procedures as described by Kaji (30) we are able to isolate 3 enzymes active on branched (ex sugar beets) and linear (haze and ultra filtration retentate) arabinan (fig. 5). Two of them are arabinofuranosidases because of their activity on p-nitrophenylarabinofuranoside; type I is able to degrade oligomers and is only slightly active on branched arabinan. Type II readily debranches sugar beet arabinan and in addition it can also split arabinose from the  $\alpha$ -1,5-arabinan backbone starting from the non-reducing end. So type II can degrade branched arabinans completely on its own. The third enzyme is an endo- $\alpha$ -1,5-arabanase which is only active on linear arabinan, it can degrade haze and ultrafiltration retentate. Only in the presence of arabinofuranosidase activity it can completely degrade branched arabinan.

Empirical studies have shown that Pectinase preparations with a high activity on high esterified pectin were most effective in increasing juice yields (24,25). We understand now that such enzyme

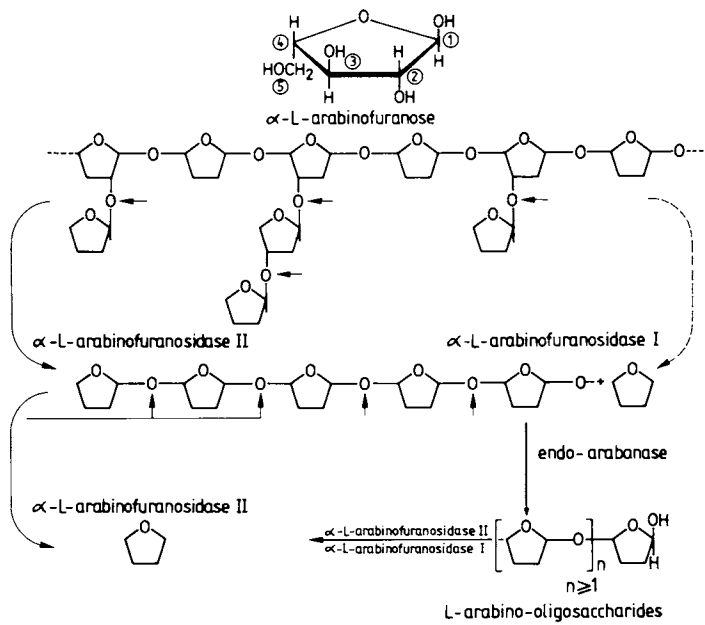


Figure 5. Points of attack of arabinan degrading enzymes.

formulations are able to solubilize the cell wall pectin very effectively and to drastically reduce the water holding capacity of the pulp. As a result juice release is facilitated. Galactanase might also play a rôle in this process. Over-enzyming will deteriorate the pressing characteristics of the pulp. The liquefaction process is a new way of making fruit juices based on the almost complete solubilization of all structural components of the cell wall by a combination of pectinases and C-1 rich cellulases. High juice yields with increased soluble solid contents have been obtained on an industrial scale. The increased AGA content in juices obtained by enzyme treatment and the released highly branched, arabinan rich pectin fragments and arabinan fragments in the liquefaction process make these juices more liable to color changes and haze formation. Addition of arabanases to the enzyme mix or ultrafiltration of the juice will solve the haze problem. Apricot cell walls form a different substrate for polysaccharide degrading enzymes. Studies on their enzymic degradation have helped to explain the empirical use of Pectinase preparations for the stabilization of fruit nectars.

#### Apricot nectar

Fruit nectars are beverages having a high content of fruit ingredients, sugars and sometimes acid. Depending on the fruit characteristics, cloudy (apricots, mango) or clear nectars (black currant, sour cherry, passion fruit) are made. An important quality factor of cloudy nectars is their cloud stability. Pectic enzymes have been used beneficially for stabilizing cloudy nectars (26,27). When apricot nectar is made by mixing finely screened pulp with water, sugar and acid but without stabilizing agents the particles tend to settle and even form quite strong gels with a clear supernatant. Siliha and Pilnik (11) have shown that this can be avoided by the action of specific pectolytic enzymes. Table VIII summarizes the effects of various pure and technical enzyme preparations on cloud stability of apricot nectars. These nectars were prepared as outlined in Fig. 6. Exo-arabanase (arabinofuranosidase II of Fig. 5) shows no effect, pure endo-polygalacturonase (PG) and commercial enzymes containing mainly PG have no effect on stability, they prevent gelling. PG with added fungal PE or PG + exo-arabanase, or pure pectin lyase (PL) or wide spectrum technical enzymes stabilize the cloud, prevent gelling and break the cell walls. Analysis of the sugars released from the cloud particles by pure enzymes and their combinations showed that the cloud stabilizing enzymes were particularly able to release galacturonides, arabinose and rhamnose from apricot cell walls in levels higher than found for apple cell walls (Table IX). Wide spectrum pectinase was the most effective, followed by PL, PG + fungal PE and PG + exo-arabanase. The addition of citrus PE to PG did not result in the release of more galacturonides. Exo-arabanase alone released almost exclusively arabinose, in combination with PG it enhanced release of neutral sugars and galacturonides. This indicates that the removal of pectic arabinan side chains permits PG to release more pectin fragments.

The pectic material in apricot puree has been isolated by sequential extraction with water, oxalate, dilute HCl and dilute alkali. It was established that the acid soluble fraction constituted 39% of the total galacturonides, 59% of total arabinose and 31% of total rhamnose. By ion-exchange chromatography it was shown that the

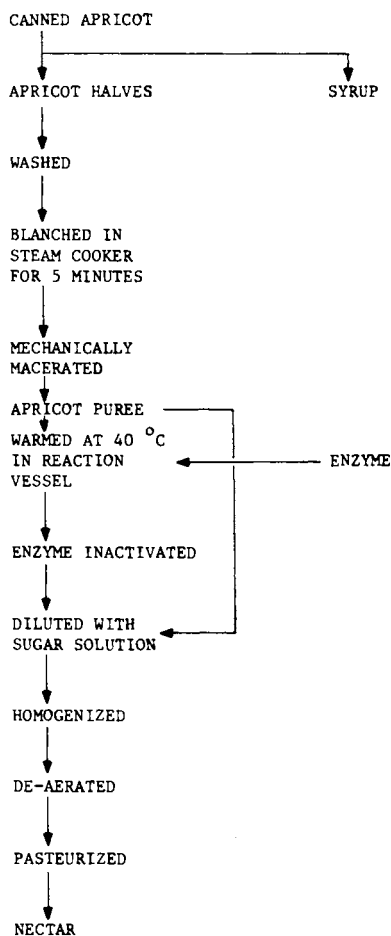


Figure 6. Scheme for the preparation of apricot nectar.  
Ref: Siliha (11).

neutral sugars were covalently linked to the pectic material (11). This makes this pectin fraction a good model substrate to study the action of the pure enzymes. Digests obtained by incubating this fraction with the various enzymes were chromatographed over Sephacryl S-300. The elution patterns in Fig. 7 show that PG can only partly breakdown this fraction whereas the other systems which do stabilize the nectar are able to achieve good to complete degradation. Obviously methoxyl groups and arabinan side chains protect the pectin molecules against PG action. If on or the other or both are removed or if pectin lyase is used which is not impeded by these groups the arabinogalacturonan is broken down and stability of the nectar is achieved. The arabinan rich pectin is an important cell wall component which must be degraded to achieve cloud stability. Microscopic examination has revealed that in the stable nectar systems the cell walls were desintegrated, density measurements showed that the specific density of the cloud particles was decreased and rheological measurements showed that the stabilizing enzymes increased the yield stress of the nectars.

Table VIII

Effect of pure and technical enzyme preparations on cloud stability of apricot nectars.

	Gel prevented	Stable cloud	Cell walls
Commercial PG	+	-	thinner
Pure PG	+	-	thinner
Pure PG+PE	+	+	broken
Pure PG+Arab	+	+	broken
Pure PL	+	+	broken
PX	+	+	broken

PX = Wide Spectrum pectinase

Table IX

Amounts of neutral sugars and galacturonides released from apricot puree by pure enzymes. Amounts expressed in % of original amounts present, corrected for control.

Enzyme treatment	Neutral sugars released							AGA re-Tot.sug. leased released	
	Rha/fuc	Arab	Gal	Man	Xyl	Glc	Total		
PG	46	57	22	--	--	--	15	81	33
PG+PE(C)	44	57	25				15	81	34
PG+PE(F)	60	74	31		6	6	23	91	42
PL	61	70	29		6		19	89	38
Exo-Arab	--	62	--	--	--	--	6	4	6
Exo-Arab									
+PG	58	81	34	5	7	4	23	86	40
PX	69	82	37	11	15	6	26	92	44

Ref: Siliha, (11). C = Isolated from Citrus fruit; F = Fungal origin; PX = Wide Spectrum pectinase

Based on the results presented here and results published by other scientists (2, 28) one can distinguish various stages in the enzymic degradation of parenchymal cell walls. In Table X these stages are described by the changes in the cell walls, the enzyme systems which bring about these changes and the technological effects obtained. Further examination of the mode of action of cell wall degrading enzymes, of the cell wall structure and its physico-chemical changes effected by enzymic action and of the chemical nature of the poly- and oligosaccharides solubilized can improve the application of enzymes in fruit and vegetable processing and enhance the development of new applications with specific enzyme formulations.

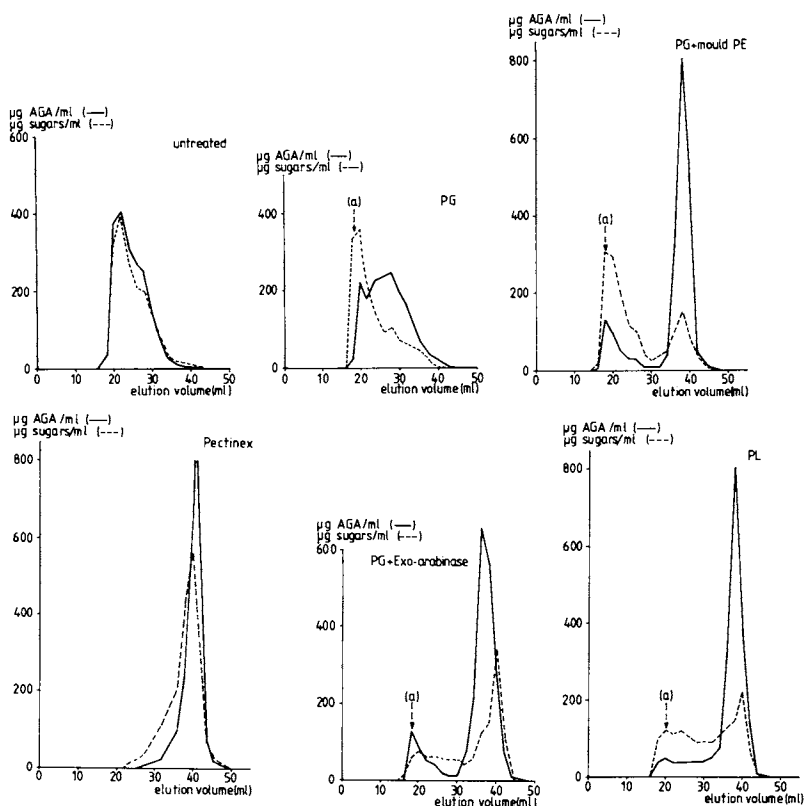


Figure 7. Gelfiltration chromatography of the HCl soluble pectin fraction, degraded by various enzyme systems on a Sephacryl S-300 column (68x1.05 cm), eluent 0.05 M phosphate buffer pH7. Ref: Siliha (11).



Table X  
Stages in enzymic cell wall degradation

Technol.effects	Changes in cell walls	Active enzyme(s)
Firming:	Saponification of cell wall pectin	PE (+Ca <sup>++</sup> )
Softening:	Limited degradation cell wall pectin	PG, PL, PAL
Maceration:	Limited degradation middle lamella pectin organized tissue→cell suspension	PG or PL
Desintegration: . juice release . cloud stabilization	Solubilization cell wall pectins and associated arabinans/galactans, cell wall fragmentation	PG + PE, and/or PL + hemicellulases (arabanases, galactanases)
Liquefaction:	Solubilization of all cell wall polysaccharides	C+PE+PG and/or PL
Saccharification:	Degradation of solubilized PS fragments to mono-saccharides	Hemicellulases Oligomerases Exo-carbohydrases Glycosidases
Cloudy juices: . cloud (de)stabilization . clarification	Saponification soluble/insoluble pectin Depolymerization soluble + insoluble pectin $\eta$ reduction	(inhibit. native) PE PE+PG, PL

- \* PE : Pectinesterase  
 PG : Polygalacturonase  
 PL : Pectin lyase  
 PAL : Pectic acid lyase  
 C : Cellulase

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## Effects of Pectin on Human Metabolism

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Recommendations have been made to include more fiber in the average U.S. diet. Pectin has been shown to lower blood cholesterol levels and the low density lipoprotein cholesterol fraction without changing levels of high density lipoprotein cholesterol or triglycerides. Pectin appears to be effective in reducing the postprandial rise in glucose in normal, obese and diabetic individuals when it is consumed with a meal or glucose tolerance test. The potentially detrimental effect of decreased mineral absorption in the presence of some high fiber diets has not been reported to occur with pectin consumption. It would appear that consumption of pectin in foods or as a supplement by hypercholesterolemic, diabetic or obese individuals should be of metabolic benefit without adverse mineral loss in the stool.

During the past few years there has been a great increase in interest in the type and amount of fiber in foods and the role of the various fibers that are consumed on human health. Part of the increased interest is due to the reported association of low fiber diets with diverticular disease of the colon, cancer of the colon and other diseases of the gastrointestinal tract (1,2), ischemic heart disease (3) and diabetes (4).

Two major approaches have been used to study the effects of fiber on humans: 1) to replace low-fiber foods with higher fiber foods and 2) to add refined fibers to self-selected or controlled diets. High pectin foods or refined pectin have been fed to man and animals alone or in combination with other fiber sources to investigate the effect of pectin on metabolic parameters.

Table I shows the pectin content of some foods, primarily fruits, and vegetables, as determined analytically (5). Pectin content of a single food source varies depending on several factors including analytical methodology, variety, maturity, harvest season, and cooking method. Citrus fruits, relatively high in pectin and low in calories, are commonly used as food sources of pectin.

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Table I. Pectin and Unavailable Carbohydrate Content of Fruits, Nuts, and Vegetables

	Dry matter	Pectin		Unavailable carbohydrate
		Ca Pectate	Carbazol	
Fresh weight of edible portion (%)				
<b>Fruit</b>				
Apple	15.9	0.78	0.45	1.7
Apricot	13.4	1.00	0.70	2.1
Banana	29.3	0.94		3.4
Blackberry	18.0	0.94	0.30	7.3
Blueberry	16.8		0.30	
Cherry	18.5	0.39	0.36	1.7
Fig	15.4	1.11		2.5
Grape	19.3	0.19	0.20	0.4
Grapefruit	9.3	3.90		0.6
Lemon	14.8	2.90		5.2
Loganberry	15.0	0.59		6.2
Orange	13.9	2.36		2.0
Peach	13.8	0.39	0.64	1.4
Pear	17.0	0.49	0.46	2.5
Pineapple	15.7	0.09		1.2
Plum	15.9	0.44	0.59	2.1
Raspberry	16.8	0.97	0.34	7.4
Rhubarb	5.8	0.44	0.34	2.6
Strawberry	11.1	0.75	0.50	2.2
Watermelon	7.4	0.18		
<b>Nuts</b>				
Peanut	95.5	5.98		8.1
Walnut	76.5	5.80		5.2
<b>Vegetable</b>				
Asparagus	7.6		0.22	1.5
Avocado	7.8	2.86		
Bean	8.4	0.70	0.55	3.0
Beet	12.9	0.91	0.42	3.1
Broccoli	9.2		0.49	4.2
Brussels Sprout	9.2		0.78	4.8
Cabbage, red	10.3		0.53	3.4
Carrot	10.2	2.00	0.96	2.9
Cauliflower	10.9		0.38	1.5
Cucumber	3.6	0.16	0.17	0.4
Eggplant	6.6	0.47		2.5
Garlic	38.7	1.11		
Kohlrabi	9.7		0.38	
Lettuce	4.8		0.34	1.4
Okra	11.1	1.53		
Onion	7.2	0.35	0.44	1.3
Pea	21.5		0.34	5.2
Pea, with pod		0.57		
Pepper, green	16.6	0.09		
Potato	24.2	0.83	0.34	2.1
Pumpkin	5.3	1.24	0.20	0.5
Radish	6.7		0.45	1.0
Rutabaga	13.0		0.80	
Soybean	90.0	3.45		
Spinach	14.9		0.33	6.3
Sweet potato	28.0	0.78		2.1
Tomato	6.6	0.20	0.30	1.5
Turnip	6.7	0.29		2.8
Yam	26.5	0.62		

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Apples, plums and peaches contain a highly methylated (95, 93 and 89% of total pectin, respectively) form of pectin (5).

During the more than two decades that pectin research has been carried out, two areas of investigation with potential benefit to human health have been in lipid metabolism (Table II) and carbohydrate metabolism (Table III). Pectin has been shown to decrease serum cholesterol in humans (6-11) as well as other animal species (10). High levels of serum cholesterol are generally considered to be a risk factor associated with ischemic heart disease (11-13). Consumption of pectin to reduce cholesterol levels in the blood might be expected to reduce the incidence of heart disease especially when serum cholesterol is elevated.

### Lipid Effects

The effect of pectin as part of a controlled diet has been reported. Keys et al (6) were able to show in serum cholesterol levels of normal men a 5% decrease by feeding 15 g of pectin per day for 3 weeks. The pectin was incorporated into a biscuit as part of the diet. Kay and Truswell (7) observed decreases in serum cholesterol, ranging from 5 to 26% (averaging 13%), in four male and five female subjects fed a controlled diet containing 15 g of pectin incorporated into a fruit gel for 3 weeks. Jenkins and coworkers (8) also observed a 13% average decrease in serum cholesterol in five men fed 30 g of pectin as part of a controlled diet for 3 weeks. Triglycerides and high density lipoprotein (HDL) cholesterol did not change significantly in this study. Inclusion of 21 g of apple or 20 g of carrots as the fiber source in a diet also decreased cholesterol although the change was not considered significant.

Stasse-Wolthuis et al (9) fed 62 subjects, 40 men and 22 women, a controlled low fiber diet for 2½ weeks followed by either a high fruit and vegetable diet, 37 g of bran, or 28 g of citrus pectin for 5 weeks. Pectin was incorporated into a dessert made from fruit juices and buttermilk. Serum cholesterol decreased significantly in the 15 subjects who consumed the pectin diet, decreased slightly in the fruit and vegetable group and increased in the high bran group. High density lipoprotein cholesterol did not change significantly in any of the groups. Challen et al (14) fed six males a controlled diet containing 36 g of pectin per day for 3 weeks. Cholesterol decreased an average of 10% in their subjects while other blood parameters studied including platelet aggregation and haemostasis did not change. Challen concluded that the effect of pectin on heart disease was probably not achieved through changes in platelet aggregation or haemostasis but through some other mechanism.

Raymond et al (15) fed a controlled formula diet containing 2 g pectin, 14 g cellulose, 39 g hemicellulose and 5 g lignin to six subjects for 4 weeks. No significant change in serum cholesterol was observed with the formula diet with or without cholesterol present in the diet.

The effect of adding pectin to self-selected diet has also been studied. As with the controlled diet studies, various amounts of pectin have been added to the self-selected diets.

Palmer and Dixon (16) fed a capsule containing 2 to 10 g of pectin per day for 4 weeks to 16 males with normal to high serum

cholesterol levels. When 6 g more of pectin per day was fed, a significant decrease in cholesterol averaging 6% was observed. Jenkins et al (17) fed 36 g of pectin per day in water to 12 men consuming self-selected diet for 4 weeks. Cholesterol decreased 12% after pectin in contrast to 16% with guar gum and no significant change with wheat bran.

Durrington and coworkers (18) fed 12 g pectin per day in juice to 12 men for 3 weeks. Serum cholesterol and apo- $\beta$  cholesterol significantly decreased, averaging 8% for the group, while triglycerides and very low density lipoproteins (VLDL) did not change significantly. Judd and Truswell (19) added 15 g of low methoxyl pectin or high methoxyl pectin in jelly to the self-selected diet of five healthy men and five healthy women. Subjects were given a copy of the first 3 weeks of recorded diet and asked to consume as close as possible the same diet for the second 3 weeks. Cholesterol decreased 16% in response to low methoxyl pectin and 18% after high methoxyl pectin. Serum cholesterol reductions produced by adding pectin to self-selected diets were also reported by Lopez et al (29) and Lagley and Thye (21), while Fahrenbach et al (22) observed no change in serum cholesterol.

Sable-Amplex et al (23) added 2 to 3 apples per day (containing 2 to 3 g pectin) for 4 weeks to self-selected diets and reported a significant decrease in cholesterol, significant decrease in low density lipoproteins (LDL) and an increase in HDL cholesterol and HDL ratio. Mahalko et al (24) added apple powder which contained approximately 2.5 to 6 g pectin per day to diets of non-insulin-dependent diabetics and reported either no change or some increase in serum cholesterol. Sable-Amplex suggested that the decreases they observed may be related to the presence of vitamin C or some other component in the apples acting synergistically and making the pectin more effective than powdered apple pectin alone.

The effect of pectin has also been studied in some hypercholesterolemic subjects. Ginter et al (25) fed 15 g pectin with 450 mg vitamin C for 6 weeks to 10 men and 11 women. Serum cholesterol decreased 9% in mildly hypercholesterolemic and 19% in severely hypercholesterolemic subjects. HDL cholesterol remained unchanged while changes in triglyceride levels varied. Nakamura et al (26) observed decreases in total cholesterol in 12 mildly hypercholesterolemic subjects fed 9 g pectin per day for 2 weeks similar to those reported by Ginter. In contrast Delbarre et al (27) observed no change in serum cholesterol levels in 10 hyperlipidemic subjects fed a hypolipemic diet with or without 6 g of pectin.

Schwardt et al (28) compared results after a self-selected diet alone, with cholestyramine added and with cholestyramine and 12 g apple pectin added for 12 weeks. Compared to the subjects self-selected diet alone, the addition of pectin plus cholestyramine decreased cholesterol 31% and LDL levels 35%, while the addition of only cholestyramine decreased cholesterol 19% and LDL levels 22%. Neither the HDL ratio nor triglycerides changed significantly from the prestudy levels.

Increased excretion of cholesterol is thought to be the mechanism for reducing serum cholesterol. Several investigators have reported increased fecal fat (7,19), sterols (7,9,19,26) or bile acids (7,9,29,30). Kay and Truswell (7) reported significant increases in fecal fat excretion, averaging 44%; fecal cholesterol,

Table II. Pectin and Serum Cholesterol in Man

Reference	Subjects	Time	Diet	Amount Pectin	Change in Cholesterol (% changes)
Keys et al, 1961	24 males	3 wk	controlled	15 g/d in biscuits	- 5%
Fahren Bach et al, 1965	23 subjects	7-9 wk		6-12 g/d	0
Palmer and Dixon, 1966	16 males	4 wk	self-selected	2-10 g/d in capsule	- 6% on 6 g or more/d -12%
Jenkins et al, 1975	12 males	4 wk	self-selected	36 g/d in water	-13%
Hopson, Lopez et al, 1975	3 subjects	5 wk	controlled	20-23 g/d	- 8%
Durrington et al, 1976	12 males	3 wk	self-selected	12 g/d in water or juice	-13%
Kay and Truswell, 1977	9 subjects	3 wk	controlled	15 g/d in fruit gell	0
Raymond et al, 1977	6 subjects	4 wk	controlled-	2 g/d with other fibers	0
Delbarre et al, 1977	10 hyperlipidemic subjects	6 wk	hypolipemic	6 g/d	0
Langley and Thye, 1977	11 males	4 wk	controlled	10 g/d	Significant Decrease
Jenkins et al, 1979	5 males	3 wk	controlled	30 g/d	-13%
	6 males	3 wk	controlled	21 g apple powder	- 7%
Ginter et al, 1979	21 mild hyper- cholesterolemic subjects	6 wk	self-selected	15 g/d with 450 mg Vit C	- 9%
	11 hypercholes- terolemic subjects	6 wk	self-selected	15 g/d with 450 mg Vit C	-19%
Stasse-Wolthuis et al, 1980	62 subjects	5 wk	controlled	15 g in dessert made with juices and buttermilk	-10%



Nakamura et al, 1982	12 mild hyper-cholesterolemic subjects	2 wk	9 g	-10%
Schwandt et al, 1982	6 severely hypercholesterolemic males	8 wk	12 g pectin/d 16 g cholestyramine	-31%
Judd and Truswell, 1982	10 subjects	3 wk	recorded self-15 g Low methoxyl/d in jelly selected 15 g High methoxyl/d in jelly	-16%
Sable-Amples et al, 1983 Challen et al, 1983	30 subjects 6 males	4 wk 3 wk	self-selected 2-3 apples/d controlled 36 g/d in bread diet	-14% -10%
Mahalho et al, 1984	10 subjects 8 subjects	4 wk 4 wk	self-selected 26 g apple powder self-selected 52 g apple powder	0 + 6%

Table III. Pectin and Serum Glucose and Insulin in Man

Reference	Subjects	Control Load Dose	Amount Pectin Added	Serum glucose	Response curve change from control Serum insulin
Jenkins et al, 1976	8 non insulin dependent diabetics and 3 insulin dependent	meal	10 g in jelly with 16 g guar gum in bread	Significant Decrease at 30 through 90 min. samples	Significant Decrease at 30 through 120 min. samples
Jenkins et al, 1977	13 normal subjects	meal	10 g in jelly without and with 16 g guar bread	Significant Decrease at 15 min. Non-Significant Decrease 30 through 90 min. samples. Guar gum further lowered response curve.	Significant Decrease at 15 through 45 min. samples
Leeds et al, 1977	5 gastric surgery patients with dumping syndrome	75 g glucose	10.5 g high methoxylol in water	Significant Decrease at 30 min. samples Improved retention of load in stomach	-----
Jenkins et al, 1978	6 non insulin dependent diabetics	50 g glucose 25 g xylose 15 g lactose 40 g lemon juice	14.5 g pectin in water	Non-Significant decrease from control load dose	Non-Significant decrease

Monnier et al, 1978	6 non insulin dependent diabetics	45 g glucose/ sq meter body surface	9 g pectin/ sq meter body surface	Significant Decrease at 30 through 60 min. samples	No Significant change from control
Holt et al, 1979	6 normal subjects	50 g glucose	14.5 g	Significant Decrease at 30 through 45 min. samples	-----
Labayle et al, 1980	23 gastric surgery patients	50 g glucose	10-20 g	Significant Decrease at 30 min. sample	-----
	3 hypoglycemic patients	-----	5 g with each meal	Hypoglycemia overtred	-----
Vaaler et al, 1980	8 insulin dependent diabetics	meal	15 g pectin in jam	Significant Decrease at 15 through 90 min. samples	-----
Poynard et al, 1980	7 insulin dependent diabetics	meal	7 g	Significant Decrease at 60 through 90 min. samples	Mean insulin delivery Significant Decrease 35% over 180 min.
Gold et al, 1980	6 normal subjects	100 g glucose meal	10 g in orange juice 10 g	No Significant change from control Significant Decrease at 60 and 90 min. samples	No Significant change from control No Significant change from control
Williams et al, 1980	13 non insulin dependent diabetics	meal	10 g as powder added to food	Significant Decrease at 60 min. sample Non Significant Decrease for remainder of curve	No Significant change from control

Continued on next page

Table III. (Continued)

Reference	Subjects	Control Load Dose	Amount Pectin Added	Response curve change from control Serum glucose	Serum insulin
Kanter et al, 1980	5 normal, 6 obese 5 latent diabetics	meal	10 g with 16 g guar gum test meal preceded by 3 days of added fiber/day	Significant Decrease in all subjects. Greatest change in obese and diabetic subjects	Significant Decrease in all subjects. Greatest change in obese and diabetic subjects
Bolton et al, 1981	10 normal males	50 g monosaccharides in 610 g orange juice (depectinated)	626 g orange (containing approximately 14 g pectin)	No Significant difference before 105 min. samples. Oranges were less hypoglycemic	Significant Decrease at 40 through 150 min. samples after oranges
Schwartz et al, 1983	7 normal	2 wk controlled diet	20 g/d	No Significant change from control. Gastric emptying time increased	-----
Mahalko et al, 1984	10 non insulin dependent diabetics 8 non insulin dependent diabetics	4 wk self- selected diet	26 g apple pectin/d 52 g apple pectin/d	1 subject had Significantly lower levels on both pectin supplements	-----

17%; and fecal bile acids, 33%, compared to control levels. Stasse-Wolthuis et al (9) reported significant increases in neutral steroids and bile acids in male but not in female subjects after pectin consumption. Cummings et al (29) reported significantly greater excretion of fatty acids (80%) and bile acids (35%) when five healthy men consumed for 6 weeks 36 g of pectin per day incorporated into a controlled diet compared to excretion during the control period. Ross and Leklem (30) fed eight men a controlled diet with or without 15 g citrus pectin for three 21 day periods in a double crossover design. Mean 7  $\alpha$ -dehydrozylase activity was increased 35%, mean neutral steroid concentration 8%, and total excretion of acid steroids 11%, when the pectin-containing diet was fed. Judd and Truswell (19) observed no difference in lipid response between the high and low methoxyl pectin and suggested that the ability of pectin to lower cholesterol lies in its gelling properties. Binding of bile acids or cholesterol to pectin has been reported in animal studies (10) and may reduce available cholesterol and lipids for absorption.

In vitro studies have suggested some chemical interactions including electrostatic and hydrogen bonding between intestinal lipids and pectin. Baig and Cerda (31) investigated human serum lipoprotein and grapefruit pectin interactions reporting a specific interaction between pectin and low density lipoproteins. Their results indicated that the interaction was of an electrostatic nature. Falk and Nagyvary (32) in an in vitro equilibrium dialysis study observed binding of pectin and lipid microemulsions and micelles of low charge density. Their results suggested a hydrogen bonding between the pectin carboxylic moieties and lipids which under optimum conditions would result in binding four times its weight in lipids. The mechanism involved in the action of pectin reducing serum cholesterol or cholesterol absorption is not clear at this time. Chemical binding as well as entrapment due to the viscous nature of pectin may both play a role in reducing lipid, cholesterol, or bile acid availability for absorption thereby decreasing serum cholesterol levels.

### Glycemic Effects

Unlike the experiments reporting the effect of pectin on serum lipids, most studies investigating the effect of pectin on glucose metabolism have been carried out in response to a meal or oral glucose tolerance test with and without pectin (33-44), rather than after adaptation to a pectin-free or pectin-containing diet. Studies reporting the effects of controlled dietary intake of pectin on glucose or insulin are limited (24,44,45). Most of the studies investigating the effect of pectin on glucose and insulin have been carried out with diabetic subjects, either insulin dependent (ID) or non-insulin dependent (NID) diabetics (38-43,45). Diabetics may benefit if either long-term consumption of pectin decreases blood glucose, or affects the amount of insulin required to control blood glucose levels by dietary means.

Jenkins et al (33) fed a meal including 10 g of pectin to 13 normal glycemic subjects. Compared to the control meal without the pectin present the glucose response curve was significantly lower at 15 minutes after the load and lower, although not

significantly, at the 30 through 90 minute portion of the response curve. When guar gum was added to the meal in addition to the pectin the response curve was significantly reduced at 15 and 30 minutes with the rest of the curve decreased but not significantly compared to the control. Insulin was similarly decreased in comparison to the control meal at 15 through 45 minutes with pectin and at 15 through 90 minutes with pectin and the guar. Levitt et al (34) fed a mixed meal with and without 5 g of pectin and 5 g of guar gum to 12 normal subjects and reported significant decreases in glucose at 15 and 30 minutes and glucagon response at 180 to 240 minutes in comparison to the control. Glucose and glucagon also tended to decrease in comparison to control levels over the remainder of the response curve while no changes in insulin or gastric inhibitory polypeptide response were observed.

Holt et al (35) and Gold et al (36) both gave oral glucose tolerance tests to normal glycemic subjects. Holt and coworkers reported significant decreases in serum glucose from control response levels at 30 and 45 minutes when 14.5 g of pectin was added, while Gold and coworkers observed no significant differences with 10 g of pectin added. Gold et al (36) did observe significant decreases in glucose at 60 and 90 minutes when the 10 g of pectin was consumed with a meal. Neither Holt nor Gold reported a significant change in insulin levels between the pectin-containing and control diet.

Bolton et al (37) compared glucose and insulin responses to oranges compared to depectinated orange juice, each containing equivalent amounts of monosaccharides. Glucose response after oranges was not significantly different from that after orange juice until 105 minutes after the load dose. Subjects had less hypoglycemia and insulin levels were significantly lower in response to oranges than to orange juice. Labayle et al (38) reported that when three hypoglycemic patients consumed 5 g of pectin with each meal, hypoglycemia was averted.

Schwartz et al (46) carried out one of the few feeding studies including pectin as the fiber source. The controlled diet was fed for 2 weeks with and without 20 g of pectin per day to seven normal glycemic subjects. No significant change in glucose response was observed compared to the diet without pectin but gastric emptying time was significantly increased with pectin. Gastric surgery patients also showed a significant increase in gastric emptying time when given 10 to 20 g of pectin with a glucose load dose (38,39).

Diabetics when tested with 7 to 10 g of pectin present in a meal or oral glucose tolerance test generally responded with lower serum glucose levels than when they were given the control meal or tolerance test without pectin. Jenkins et al (40) fed 10 g of pectin with 16 g of guar gum in a meal to eight NID diabetics and three ID diabetics. Both groups had significantly lower serum glucose response with pectin present, at 30 through 90 minutes in the NID and at 30 through 120 minutes in the ID diabetics. Insulin response in the NID diabetics was also significantly decreased from control response levels at 30 through 120 minutes. When Jenkins et al (41) fed 14.5 g pectin with simple sugars in an oral tolerance test to eight NID diabetics, no significant differences from control levels in either the glucose or insulin response curves were observed.

Monnier et al (42) and Williams et al (43) reported significant decreases in the serum glucose response curves of their NID diabetics from the control levels when pectin was present at 30 and 60 minutes and 60 minutes, respectively, with some non-significant decrease during the rest of the curve. Insulin was not significantly decreased in either experiment although Williams did report some delay in mean response. Levitt et al (34) reported the ingestion of the mixed meal with and without pectin and guar gum by NID diabetics significantly improved glucose tolerance without significant changes in insulin, glucagon or gastric inhibitory polypeptide when autonomic neuropathy was not present. However, when autonomic neuropathy was present in the diabetics glucose tolerance was not improved; insulin and gastric inhibitory polypeptide responses remained unchanged but glucagon levels were decreased.

Vaaler et al (44) and Poynard et al (45) fed 15 and 7 g of pectin, respectively, to ID diabetics in a meal. Both researchers reported significant decreases in serum glucose with pectin present compared to the meal without pectin. Vaaler observed the significant decrease 15 through 90 minutes after the meal while Poynard reported decreases at 60 through 90 minutes. Poynard et al (45) also measured insulin, and reported mean insulin delivery was significantly decreased, averaging 35% decrease over 180 minutes, when pectin was included compared to the control.

Kanter et al (47) fed a meal with and without pectin to several groups of subjects after they had been fed a controlled diet with and without 10 g of pectin and 16 g of guar gum for 3 days preceding the test meal. The five normal, six obese (130% ideal mean weight), five latent diabetics and six overt diabetics all showed some decrease in peak glucose response after the inclusion of the gums in the diet and meal. Normal subjects responded least to the presence of the gums, all decreases being non-significant, while the overt diabetics had the greatest response to the meal. Obese and diabetic subjects had significant decreases in serum glucose, the obese at 30 and 60 minutes after the meal and the diabetics at 0 through 90 minutes after the meal with the gums. Insulin response to the inclusion of pectin with guar gum in the diet and meal followed the same pattern as the serum glucose with the greatest decrease in the diabetic subjects and the least change in the normal subjects. Normal subjects had significant lowering of insulin response at 45 to 90 minutes after the meal containing pectin, obese subjects at 15 to 120 minutes; latent diabetics, 15 to 60 minutes; and overt diabetics 45 to 120 minutes compared to the response when pectin was not included in the meal. Mahalko et al (24) reported no significant change in serum glucose response to a low fiber formula following diets containing a low fiber white bread (control), 26 g or 52 g of the apple powder of dehydrated powdered apples as the pectin source. One subject, who consumed both levels of powdered apple, did respond with significant decreases in serum glucose. Mahalko suggested that this subject might be part of a subpopulation of persons with NID diabetes that responds to this fiber source. Gardner et al (48) were unable to find significant changes in hemoglobin  $A_{1c}$  or glycosylated serum protein in 17 ID diabetics fed 5 g of pectin with each meal and

at bedtime with their self-selected diet for 3 months. The authors felt these two parameters should have decreased since most studies report a decreased glucose response to a tolerance test when pectin was present.

#### Mineral Bioavailability

The effect of pectin consumption on mineral balance has not been extensively studied. Cummings et al (29) fed five normal men a controlled diet for 3 weeks followed by the controlled diet supplemented with 36 g of pectin. Calcium excretion was not significantly different between the two diet periods and all subjects were in calcium balance during the control and pectin supplemented period. Stasse-Wolthuis et al (9) after 5 weeks of feeding 15 g of pectin to their subjects did not observe any significant change in calcium, magnesium, sodium, or potassium excretion compared to levels after the control diet. Sandberg et al (50) fed six ileostomic patients 15 g of pectin a day in a double crossover; 4 days of control, 3 days of pectin supplementation and 3 days of control diet. After the pectin was consumed there was increased excretion of sodium, potassium, and iron while calcium, magnesium and zinc were not significantly changed from control levels. Apparent iron balance was significantly decreased. Monnier et al (51) reported iron absorption significantly decreased in eight patients with idiopathic hemochromatosis after a load dose of iron with 9 g of pectin per meter squared of body surface compared to retention of the load without pectin present. In vitro (51) pectin was found to have a high binding activity for iron averaging 83 to 92% at varying pH's.

Lei et al (52) fed nine normal subjects, seven women and two men, a controlled diet for 3 weeks with and without 15 g of pectin per day. The last 6 days of each period were used for sample collection. No significant differences were observed in excretion or apparent iron, zinc, or copper balance between the control or pectin supplemented diets. Baig et al (52) fed rats for 40 days one of four diets, low or adequate iron diets with or without 2% pectin. A load dose of  $Fe^{59}$  with or without pectin was given to the rats by gavage. The rats fed the low iron diets absorbed more iron than those on the adequate diets regardless of the presence or absence of pectin in the diet or load dose. Drews et al (53) fed adolescent boys a controlled diet with and without isolated fibers for 4 days each. Consumption of 14.2 g of pectin had no effect on the excretion or apparent balance of zinc, copper or magnesium.

Additional research needs to be carried out on mineral retention when pectin is present in the diet. Iron showed both decreased absorption and no significant differences from the control. In general the longer studies showed less differences in iron retention from control levels than did the load dose or very short studies. Other minerals studied did not significantly change when pectin was present in the studies reported.

#### Vitamin Bioavailability

Scant data are available on interactions between pectin and vitamin utilization. Ginter et al (25) added 450 mg of vitamin C per day



to the 15 g of pectin fed to hypercholesterolemic subjects. They reported a significant reduction in serum cholesterol, a greater reduction than anticipated on the basis of the amount of pectin added. The greater reduction in cholesterol levels was attributed to synergistic action between the vitamin C and the pectin. They hypothesized that vitamin C acted to stimulate the production of 7- $\alpha$ -hydroxycholesterol which subsequently increased bile acid production. Pectin in turn increased cholesterol and bile acid excretion by binding or entrapment.

Keltz et al (54) investigated vitamin C urinary excretion in relation to feeding 14.2 g of pectin per day with and without zinc supplementation. Vitamin C excretion decreased with pectin present in the diet with or without the zinc supplement. Increased utilization of vitamin C or decreased absorption or a combination of these factors was suggested as responsible for changing the vitamin C available for excretion.

Rat studies by Cullen and Oace (56) reported increased excretion of methylmalonic acid resulting in B<sub>12</sub> depletion in rats fed pectin. The authors suggested that pectin interfered with B<sub>12</sub> recycling possibly by stimulating microbial activity increasing the B<sub>12</sub> requirement when pectin was being degraded and thereby decreasing the B<sub>12</sub> availability for reabsorption. However, the addition of pectin to the diets of rats or chicks has not been found to have deleterious effects on the bioavailability of pyridoxine (57), folic acid (58) or Vitamin A or  $\beta$ -carotene (59). Additional research with animals and ultimately with man on the effects of pectin consumption on vitamin utilization and availability is needed. Too little research has been carried out to provide a definitive answer of the effects of pectin consumption over a long period of time.

#### Effect and Fate of Pectin in the Gastrointestinal Tract

Pectin appears to be better tolerated when it is included in the diet as a food source or completely hydrated in a food product (7) than when given as a powder mixed with water, juice or the subjects' own food (18). Subjects complained of the taste, gummy feel, and the stickiness in mouth and on teeth when powdered pectin was given while food sources, even those formulated by the researchers, were well accepted. Some side effects noted by some subjects in human studies included rectal flatulence, flatulence, abdominal distention and abdominal cramps.

In contrast with some other fiber sources such as wheat bran no significant decrease in mean transit times has been reported by most researchers (7,9,18,29,30) investigating the effect of pectin on bowel function or mineral bioavailability. Only one study (15) reported a significant decrease in transit time from a starting time averaging 63 hours. No significant difference in transit time between high and low methoxyl pectins was observed by Judd and Truswell (19).

Both increased frequency of defecation (7) and change in stool number (9,29) have been reported when pectin was added to the diet of human subjects. Significant increases in fecal wet or dry weight have been reported by some investigators (7,15,18,29) and no significant changes in fecal weight by others (9,30,59,60).

Most of the pectin consumed appears to be digested by normal human subjects (29,61,62). Pectin recovered in the feces has ranged from 0 to 25% (61,62). Most of the digestion (up to 80%) occurs in the large intestine with up to 40% occurring in the small intestine (61,63). It has been suggested that pectin added to the diet is digested by increased bacterial action (29,61).

Increased methane hydrogen, carbon dioxide and flatus volume have been reported with pectin consumption compared to a fiber free diet (64). Increases in fecal content of fatty acids (80%), nitrogen (47%), bile acids (35%) and total solids (28%) compared to control levels (29) were thought to be due to increased bacterial and intestinal cell mass. The degree of pectin digestion and cellular excretion would affect stool bulk and dry fecal weight resulting in the wide range reported in stool weight changes.

### Conclusion

Diets containing pectin incorporated into a food source appeared to be better tolerated and greater amounts could be consumed than when powder or capsules were consumed. The consumption of pectin by normal and hypercholesterolemic individuals has been reported in most studies to lower blood cholesterol levels without changing triglyceride or HDL levels. The greatest decreases in blood cholesterol were reported in hypercholesterolemic subjects. Low-fat diets and diets supplemented with less than 6 g of pectin per day generally produced the least change in blood cholesterol levels. Excretion of fecal fat, steroids, and bile acids were increased. Lipids are thought to be bound to or entrapped in the pectin gel so that they cannot be absorbed in the small intestine.

The consumption of pectin by normal and diabetic individuals has been reported to lower the glucose response curve after a load dose or meal. Insulin response curves were similarly decreased in responses to pectin in approximately two-thirds of the studies in which insulin was measured. Hypoglycemia and gastric dumping syndrome appear to be lessened or averted when pectin was consumed with the meal. Very few long-term studies have been carried out investigating pectin feeding in relation to glucose, insulin or other glucogenic parameters. More research is needed before pectin can be safely recommended to diabetics as a dietary means to reduce their blood glucose.

The effect of pectin consumption on mineral balance has not been studied extensively although the excretion and apparent balance of most minerals appears to be unaffected by dietary pectin. Iron utilization or absorption may be affected by pectin consumption but the longer feeding studies did not report any significant changes in apparent balance from control levels. Further long-term studies are needed. Interaction of pectin with vitamins has been studied little in man or animals. Vitamin C consumption with pectin may be beneficial to hypercholesterolemic persons. Vitamin B<sub>12</sub> absorption appears to be reduced with pectin while other vitamins studied showed no reduction in bioavailability. More research is needed to determine if pectin is beneficial, detrimental or benign when consumed as part of the diet.

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## Role of Pectin in Binding of Bile Acids to Carrot Fiber

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Carrot fiber, prepared as an alcohol-acetone insoluble residue of cell wall material, binds deoxycholate and chenodeoxycholate under physiological conditions with the release of protons. Removal of calcium pectate from this material by extraction with ammonium oxalate reduces the capacity of carrot fiber to bind bile acids. Calcium carboxymethyl cellulose exhibits similar binding activity, whereas free carboxymethyl cellulose shows no binding. Calcium pectate prepared from citrus pectin and dissolved in water was found to bind bile acids under conditions used with carrot fiber. These results suggest that binding occurs through formation of salt linkage between calcium pectate in the cell wall residue and a bile acid.

Carrot fiber has been shown to bind bile acids under physiological conditions (1,2). Our earlier investigations have established that co-binding of bile acids releases protons and can be related to the content of calcium in the fiber preparation (2). We have proposed that the binding of bile acids to carrot fiber, or cell wall residue rich in pectin, may involve salt linkages between calcium pectate and the carboxylate group of the bile acid. This report presents studies that furnish additional evidence that calcium pectate does have a role in the binding of bile acids to carrot fiber.

### Materials and Methods

Carrot fiber was prepared as an alcohol-acetone insoluble residue (AAIR) (2). Citrus pectin was obtained from Sigma. Other chemicals were reagent grade. The binding assay employed reverse phase HPLC determination of the concentration of bile acids in a buffered solution before and after contact with fiber. To eliminate interactions between calcium in the cell wall and the phosphate buffer in the original procedure (2), 0.05 M imidazole was used to buffer the bile acid solutions. The HPLC RI detector signals were processed through an Adalab A/D converter installed in an Apple IIe computer controlled by Chromatochart, a software product of Interactive Microware, Inc. A program in Applesoft

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was developed to average the data collected and to calculate the extent of binding.

Purification of Pectin. 1 G of citrus pectin, 37% methylated, was dissolved in 60 mL of water and heated at 60-65°C. for 1 hour. The solution was stirred overnight under refrigeration. After dilution with water to 100 mL, the solution was centrifuged at 30,000 g for 40 min to remove suspended material. The supernatant was then dialyzed against water for 2 days (4 changes per day). The solution recovered after dialysis was passed through a cationic exchange resin (IR-120, H<sup>+</sup>) column, 2.8 cm x 40 cm at a rate of about 12 drops per minute. The eluant was freeze-dried to give 0.76 g of purified pectin.

Preparation of Calcium Pectate. 0.5 G of purified pectin was dissolved in 100 ml of water. The solution was then dialyzed against dilute calcium hydroxide (0.2 g Ca(OH)<sub>2</sub> in 2.5 L water) for 24 hours in a cold room under nitrogen. A trace of sodium azide was added to prevent bacterial growth. The 125 mL solution was used directly for the binding assay.

Binding of Chenodeoxycholate to Calcium Pectate. 10.1 mg of chenodeoxycholate (CDC) and 2 mg of Na<sub>2</sub>CO<sub>3</sub> were dissolved in enough solution of the calcium pectate described above to fill a 5 mL volumetric flask. The pH was adjusted to 7.00 by addition of a few grains of Na<sub>2</sub>CO<sub>3</sub>. Another solution of CDC was prepared in like manner with just water. Fine control of pH was possible by the addition of a few small grains of citric acid. These two solutions were subjected to the reverse phase HPLC assay for binding (2). Concentration of CDC was determined by peak area using the integration program of Interactive Microware, Inc.'s Chromatochart.

Calcium Analysis. Materials for calcium analysis were ashed in an electric muffle furnace at 600°C. for 2 hours. The ash after weighing was dissolved in 4N nitric acid and the calcium content of the solution was determined by atomic absorption spectroscopy using standard methods.

Calcium Carboxymethyl Cellulose. 2 G of carboxymethyl cellulose (Whatman CM-32) was mixed for one hour at room temperature in 200 mL of 0.1N HCl. The material was filtered and mixed with 200 mL of water three times. The CMC-H<sup>+</sup> was next stirred in 200 mL of water containing 0.5 g of Ca(OH)<sub>2</sub> overnight in the cold room. The CMC-Ca<sup>++</sup> was also washed with 200 mL of water three times. The material was then dialyzed against water and recovered by freeze-drying.

Ammonium Oxalate Extraction of Carrot AAIR. 2.0 G of carrot AAIR was added to 1 L of a 1% ammonium oxalate solution, pH 5.00. The mixture was stirred and slowly heated to 80°C. over a period of one hour. The AAIR was recovered by filtration and

thoroughly washed with water. 1.46 G of material was recovered by freeze-drying. A calcium content of  $0.260 \pm 0.013\%$  was found.

Deoxycholate Extraction of Carrot AAIR. 2.5 G. of sodium deoxycholate (DC) was dissolved in 250 ml of water and the pH was adjusted to 7.30 with acetic acid. To this solution was added 5.0 g of carrot AAIR and an additional 50 mL of water in order to have free solution for mixing. After mechanical shaking for one hour at room temperature the mixture was filtered. The filtrate was acidified with 3.0 mL. of acetic acid and added to 2 L. of absolute ethanol. After storage overnight in the cold room the alcohol suspension was filtered. The residue was dissolved in about 100 ml of water and the solution was dialyzed. The dialyzed solution was freeze-dried to give 0.3 g of material.

This material was found from  $^{13}\text{C}$  NMR spectroscopy to consist largely of pectin and DC. Further purification was carried out by reprecipitation of the pectin with acidified ethanol, followed by dialysis and freeze-drying. The DC-free pectin was then used for measurement of CDC binding.

NMR Spectroscopy. CPMAS  $^{13}\text{C}$  NMR spectra were obtained with a JEOL FX60QS NMR spectrometer operating at 15.04 MHz. The  $^1\text{H}$  decoupling rf irradiation field strength was 11 G; the contact time was 0.5 sec and the recycle time was 1.5 sec. A spectral width of 8000 Hz and a sampling rate of 2k data points, zero filled to 4k, were used. Chemical shifts were assigned relative to the methyl resonances, 17.36 ppm, of hexamethylbenzene. Samples were spun at approximately 2.1 kHz and no spinning sidebands were observed.

### Results and Discussion

The alcohol-acetone insoluble residue (AAIR) prepared from carrot was revealed by scanning electron microscopy to be largely remnants of cell wall material as seen in Fig. 1. The composition of carrot alcohol insoluble residue has been described by Aspinall, *et al.* (4), who found a high content of pectin along with galactans, galactoarabans, and a new polyxylyronide. We found calcium in carrot AAIR to range up to near 1%. Plant cell walls are now believed to have, as integral structural components, calcium pectate (5). Robertson, *et al.* (1) found that alcohol insoluble residue (AIR) of carrot exhibits a large water holding capacity of from 13 to 32 g/g and has cationic exchange capacities of from 1.1 to 2.4 meq/g. This property largely derives from the group of pectins present. Aspinall, *et al.*, (4) have isolated from AIR different pectin species. A highly methylated pectin can be extracted from AIR by hot water. Ammonium oxalate can extract a large fraction of pectin, presumably calcium pectate. Removal of remaining pectin requires treatment with base (4); this latter pectin is believed to be covalently linked to other cell wall components by ester linkages.

We have established that carrot AAIR co-binds bile acids under physiological conditions (2). Furthermore, such binding



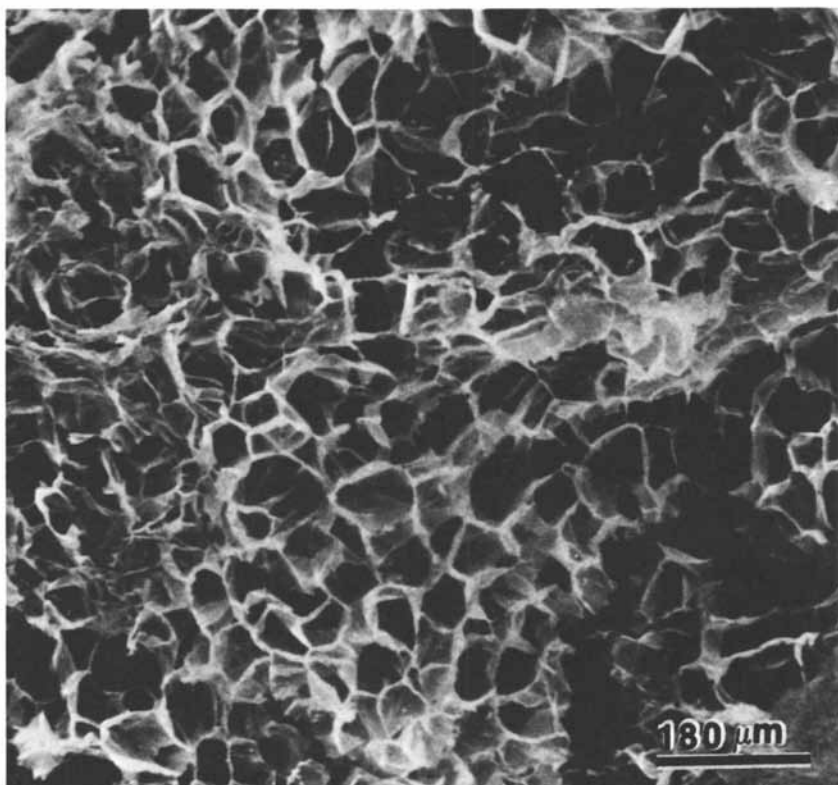


Figure 1. Scanning electron micrograph of carrot AAR, 240x. Material is residual cell walls.

releases protons and appears to be related to calcium content of the AAIR (2). Our earlier work has led us to suggest that binding of bile acids to carrot AAIR involves formation of calcium salt linkages between a bile acid and calcium pectate.

Treatment of carrot AAIR with ammonium oxalate resulted in removal of about 25% of the mass of the material. The calcium content of remaining AAIR was reduced from 0.92% to 0.26% (Table I). The AAIR after extraction with ammonium oxalate exhibits a reduced capacity to co-bind CDC and DC, as shown in Table I.

Table I. Effect of Ammonium Oxalate Extraction of Carrot AAIR on the Co-Binding of Chenodeoxycholate and Deoxycholate.

Fiber	%Ca	pH	%CDC <sup>a</sup>	%DC
AAIR <sup>b</sup>	.67	6.69	1.36 +/- .03	1.04 +/- .08
AAIR (AmOx) <sup>c</sup>	.26	6.68	.85 +/- .05	.37 +/- .05
AAIR	.67	6.39	1.79 +/- .10	1.61 +/- .05
AAIR (AmOx)	.26	6.48	1.07 +/- .19	.78 +/- .19
AAIR	.67	7.04	1.06 +/- .02	.45 +/- .03
AAIR (AmOx)	.26	7.18	.48 +/- .06	.00

<sup>a</sup>chenodeoxycholate, wt/wt dry fiber.

<sup>b</sup>alcohol, acetone insoluble residue, USDA grown carrots.

<sup>c</sup>residue after extraction with 1% ammonium oxalate, pH 5, 80°C., 1 hr (25% weight loss).

This effect is most pronounced for DC at the higher pH values. At the lowest pH, 6.68 (Table I, binding capacity is still appreciable even though over half the calcium had been extracted. These results indicate that calcium pectate has a role in the binding of bile salts to carrot AAIR and provide supporting evidence for the idea that the binding involves calcium salt linkages. Since calcium pectate appears to be a general plant cell wall structural polysaccharide, the observed binding of bile acids to a variety of plant fibers may involve salt linkages with calcium pectate (5,6).

To test the possibility that bile acids can bind to polysaccharides through calcium salt linkages, calcium carboxymethyl cellulose was prepared and assayed for bile acid binding activity. The results in Table II clearly indicate that binding can occur when carboxymethyl cellulose is in the calcium form. CM-32 has one carboxyl group per 4-5 glucopyranosyl units, based on a cation exchange capacity of 1 meq/g. These carboxyl groups are reported to be located on the surface of internal pockets of the carboxymethyl cellulose (7). These pockets are large enough to accommodate proteins and would therefore offer no restrictions to binding of bile acids. At 1.58 % Ca, the carboxymethyl cellulose used in our study might be ca.40% in the  $\text{CM-COO}^-(\text{Ca}^{++})^-(\text{HCO}_3)$  or 80% in the  $\text{CM-COO}^-(\text{Ca}^{++})^-00\text{C-CM}$  form.

Because of the statistical distribution of carboxylate groups in the carboxymethyl cellulose a mixture of these two forms probably obtains. The observed binding of CDC to calcium carboxymethyl cellulose very likely involves calcium salt linkages. This binding capacity also suggests that calcium carboxymethyl cellulose could be considered as a replacement for cholestyramine in the treatment of some gastro-intestinal disorders since carboxymethyl cellulose can be expected to have far less abrasive action on the intestinal wall.

Table II. Binding of Chenodeoxycholate to Calcium Carboxymethyl Cellulose<sup>a</sup>.

Fiber	pH	% Chenodeoxycholate, wt/wt fiber
CMC-Ca <sup>++</sup>	7.26	1.38 +/- .02
CMC-Ca <sup>++</sup>	7.45	1.26 +/- .02
CmC-Ca <sup>++</sup>	7.49	1.19 +/- .02
CMC	7.66	.00

<sup>a</sup>1.58 +/- .02 % Ca.

To further test the hypothesis that calcium pectate has a role in the binding of bile acids to carrot fiber, calcium pectate was prepared and assayed for binding activity. The direct preparation of calcium pectate, for example by titration, can lead to formation of a gel at the entry point of titrant where the local concentration of calcium ions is greatest. High concentrations of calcium pectate were avoided by dialyzing calcium ions into the pectin solution held within a low molecular weight cut-off, cellophane sac. The assay was made difficult by the necessity of maintaining a low concentration of pectin (0.4% w/v) to avoid gelation. Binding was measured from small differences in the chenodeoxycholate peak from the reverse phase chromatograms. The precision of measurement was thereby diminished. However, the data obtained in Table III do show that calcium pectate can bind CDC to a greater extent than sodium pectate.

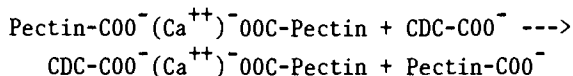
Table III. Binding of Chenodeoxycholate to Na Pectate and to Ca Pectate<sup>a</sup>

Substance	mg/ml	pH	mg CDC/ml	% Binding
Na Pectate	4.00	7.24	2.00	1.8 +/- .2
Ca Pectate	4.00	7.00	2.00	5.4 +/- .6
Ca Pectate	2.00	6.53	1.00	4.8 +/- .5

<sup>a</sup>citrus pectin, 70% methylated, Sigma, 3.3% Ca.

37% Methylated pectin contains nearly 3 meq/g of carboxylate groups. A 3.3% Ca content of the calcium pectate in

Table III corresponds to approximately 0.8 mM Ca/g. of polymer. This figure allows  $\text{-COO}^{\text{-}}(\text{Ca}^{\text{++}})^{\text{-}}\text{OOC-}$  intermolecular salt linkages in the calcium pectate, because the degree of methylation, according to Rees (8), is low enough to permit an "egg-box" structure that leads to dimerization and then to gel formation. The calcium pectate in our binding study was in a pre-gel state and may have formed calcium salt linkages with the bile acid through an exchange reaction as follows:



The release of the stronger carboxylate groups of pectin that this equation produces would account for the drop in pH that is observed upon binding of bile acids to carrot AAIR.

Pectin complexed with trivalent cations ( $\text{Al}^{\text{+++}}$  or  $\text{Fe}^{\text{+++}}$ ) has the demonstrated ability to bind biological anions through ion exchange (9). These complexes are hypocholesterolemic in rats and can interact with bile acids as well as anionic lipid micelles (9,10). Our studies favor a role for calcium pectate in the observed binding of bile acids to plant cell wall residue. It remains to be seen if calcium pectate will have a greater hypocholesterolemic effect than has been reported for commercial pectins in feeding studies (11-15).

During the course of bile acid binding assays of carrot AAIR it was noted that a peak emerged at the void volume during HPLC of the solution of bile acid that had been in contact with the fiber. We therefore suspected that the bile acid solution was solubilizing some small fraction of the AAIR. Accordingly, carrot AAIR was then extracted with sodium deoxycholate and a pectin fraction was isolated by alcohol precipitation of the acidified extract. Solid state CPMAS  $^{13}\text{C}$  NMR spectroscopy (Fig. 2) revealed this pectin to be 75% methylated and therefore similar to the highly methylated pectin that Aspinall, *et al.*, (4) isolated from carrot cell wall residue by hot water extraction. The pectin was found to bind  $6.39 \pm 0.26$  % CDC at pH 7.2. This pectin fraction requires further study because the data suggest that hydrophobic interactions may have a role in binding of bile acids to some highly methylated pectins. A combination of hydrophobic interactions and calcium salt linkages could possibly result in some very strong binding between bile acids and highly methylated calcium pectate.

Carrot AAIR can now be viewed as a dietary fiber with a growing list of beneficial properties. The large water holding capacity of this material (1) makes carrot fiber an effective, gentle bulking agent (6). The binding activity for bile acids that has been experimentally demonstrated (1,2) for carrot fiber (AAIR) offers a dietary means of controlling blood cholesterol levels (6,16-17). The calcium content of carrot fiber may be a significant dietary source of calcium for the senior population. In addition, some of the calcium of carrot AAIR may be released in the colon with the beneficial effect of removing free fatty acids as calcium salts (18). Carrot fiber, prepared as an

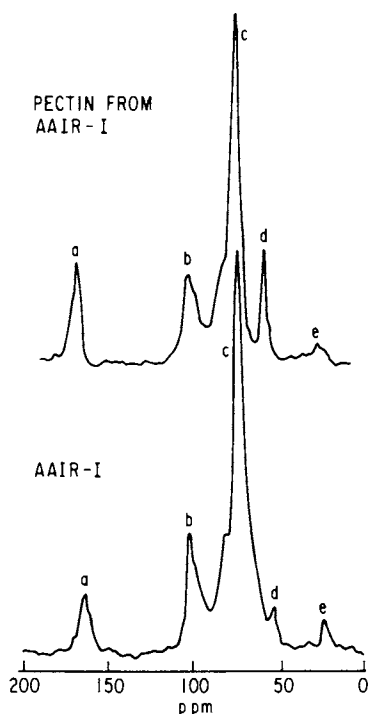


Figure 2. CPMAS<sup>13</sup>C NMR spectra for carrot AAIR and for pectin extracted from AAIR with deoxycholate. Chemical shifts (ppm): AAIR, 173.2 (a, carbonyl), 106.4 (b, anomeric), 73.4 (c, hydroxylated methylene), 53.7 (d, methoxy), 22.0 (e, methyl of acetyl); pectin, 172.1 (a), 102.5 (b), 71.4 (c), 54.0 (d) 22.0 (e); Sigma citrus pectin, 70% methylated (not shown), 171.9 (a), 101.0 (b), 71.2 (c) 54.0 (d), no (e).

alcohol insoluble cell wall residue, can be prepared easily and economically. Additional beneficial properties as a component of food formulations can be anticipated.

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## Author Index

- Barford, R. A., 22  
Beach, P., 103  
Behall, Kay, 248  
BeMiller, James N., 2  
Carr, J. M., 200  
Cesaro, A., 73  
Ciana, A., 73  
Crandall, Philip G., 88  
Damert, W. C., 22  
Davis, E., 103  
de Vries, J. A., 38  
Delben, F., 73  
Doner, Landis W., 13  
Fishman, Marshall L., 22  
Grasdalen, Hans, 117  
Hoagland, Peter D., 266  
Huber, Donald J., 141  
Ikkala, P., 103  
Irwin, Peter L., 175  
Labavitch, J. M., 200  
Lee, James H., 141  
Lundbye, M., 103  
McFeeters, Roger F., 217  
Northcote, D. H., 134  
Paoletti, S., 73  
Pepper, L., 22  
Pfeffer, Philip E., 266  
Phillips, J. G., 22  
Pilnik, W., 38,230  
Pressey, Russel, 157  
Reid, D. S., 200  
Reiser, Sheldon, 248  
Rinaudo, M., 61  
Rombouts, F. M., 38,49  
Sajjaanantakul, T., 200  
Schols, H. A., 230  
Sevilla, Michael D., 175  
Shieh, James J., 175  
Siliha, H. A. I., 230  
Smidsrød, Olav, 117  
Stoudt, Carla L., 175  
Thibault, J. F., 49,61  
Toft, Knut, 117  
Van Buren, Jerome P., 190  
Voragen, A. G. J., 38,230  
Wicker, Louise, 88

## Subject Index

- A
- Alginates  
  features of the monomer  
  sequence, 126  
  modulus measurements vs. structural  
  characteristics, 128,130f  
  structural  
  characteristics, 126,128,129t
- Apple juice processing  
  changes in juice and pulp after  
  enzyme treatment, 233,234f  
  degree of branching of arabinan  
  fractions, 237,239t  
  elution profile for press  
  juice, 235,236f  
  gel filtration chromatography of the  
  retentate fraction, 237,238f  
  mold enzyme degradation, 231-232  
  points of attack of arabinan-  
  degrading enzymes, 239-240f  
  preparation of ultrafiltration  
  permeate and retentate  
  fractions, 235,237,238f  
  role of pectinase in increasing  
  juice yields, 241
- Apple juice processing--Continued  
  sugar composition of  
  alcohol-insoluble  
  solids, 231-232t  
  sugar and glycosidic linkage  
  composition of the retentate  
  fraction, 235,237t  
  uronide content of apple  
  juices, 235t
- Apple pectins  
  degree of polymerization  
  distribution, 44,46f,47f  
  distribution methoxylated and free  
  carboxyl group, 44-47  
  extraction schematic, 39,40f  
  high-pressure LC, 44,46f  
  neutral sugar composition, 41,43f  
  neutral sugar distribution, 39-44
- Apricot nectar processing  
  effects of enzymes on cloud  
  stability, 241,243t  
  elution patterns of pectic  
  material, 243,244f  
  isolation of pectic material, 243  
  neutral sugars and galacturonides  
  released by pure  
  enzymes, 241,243t

## Author Index

- Barford, R. A., 22  
Beach, P., 103  
Behall, Kay, 248  
BeMiller, James N., 2  
Carr, J. M., 200  
Cesaro, A., 73  
Ciana, A., 73  
Crandall, Philip G., 88  
Damert, W. C., 22  
Davis, E., 103  
de Vries, J. A., 38  
Delben, F., 73  
Doner, Landis W., 13  
Fishman, Marshall L., 22  
Grasdalen, Hans, 117  
Hoagland, Peter D., 266  
Huber, Donald J., 141  
Ikkala, P., 103  
Irwin, Peter L., 175  
Labavitch, J. M., 200  
Lee, James H., 141  
Lundbye, M., 103  
McFeeters, Roger F., 217  
Northcote, D. H., 134  
Paoletti, S., 73  
Pepper, L., 22  
Pfeffer, Philip E., 266  
Phillips, J. G., 22  
Pilnik, W., 38,230  
Pressey, Russel, 157  
Reid, D. S., 200  
Reiser, Sheldon, 248  
Rinaudo, M., 61  
Rombouts, F. M., 38,49  
Sajjaanantakul, T., 200  
Schols, H. A., 230  
Sevilla, Michael D., 175  
Shieh, James J., 175  
Siliha, H. A. I., 230  
Smidsrød, Olav, 117  
Stoudt, Carla L., 175  
Thibault, J. F., 49,61  
Toft, Knut, 117  
Van Buren, Jerome P., 190  
Voragen, A. G. J., 38,230  
Wicker, Louise, 88

## Subject Index

- A
- Alginates  
  features of the monomer  
  sequence, 126  
  modulus measurements vs. structural  
  characteristics, 128,130f  
  structural  
  characteristics, 126,128,129t
- Apple juice processing  
  changes in juice and pulp after  
  enzyme treatment, 233,234f  
  degree of branching of arabinan  
  fractions, 237,239t  
  elution profile for press  
  juice, 235,236f  
  gel filtration chromatography of the  
  retentate fraction, 237,238f  
  mold enzyme degradation, 231-232  
  points of attack of arabinan-  
  degrading enzymes, 239-240f  
  preparation of ultrafiltration  
  permeate and retentate  
  fractions, 235,237,238f  
  role of pectinase in increasing  
  juice yields, 241
- Apple juice processing--Continued  
  sugar composition of  
  alcohol-insoluble  
  solids, 231-232t  
  sugar and glycosidic linkage  
  composition of the retentate  
  fraction, 235,237t  
  uronide content of apple  
  juices, 235t
- Apple pectins  
  degree of polymerization  
  distribution, 44,46f,47f  
  distribution methoxylated and free  
  carboxyl group, 44-47  
  extraction schematic, 39,40f  
  high-pressure LC, 44,46f  
  neutral sugar composition, 41,43f  
  neutral sugar distribution, 39-44
- Apricot nectar processing  
  effects of enzymes on cloud  
  stability, 241,243t  
  elution patterns of pectic  
  material, 243,244f  
  isolation of pectic material, 243  
  neutral sugars and galacturonides  
  released by pure  
  enzymes, 241,243t



Apricot nectar processing--Continued  
preparation of nectars, 241,242f

## B

Bile acid binding to carrot fiber  
ammonium oxalate extraction of  
  carrot alcohol-acetone insoluble  
  residue, 267-268,270t  
binding of chenodeoxycholate to  
  calcium (carboxymethyl)-  
  cellulose, 270,271t  
binding of chenodeoxycholate to  
  calcium pectate, 267,271t,272  
calcium (carboxymethyl)-  
  cellulose, 267  
calcium analysis, 267  
calcium effects, 268,270  
carbon-13 NMR spectroscopy of  
  pectin, 272,273f  
carrot fiber preparation, 266,267  
deoxycholate extraction of carrot  
  alcohol-acetone insoluble  
  residue, 268  
NMR spectroscopy, 268  
preparation of calcium  
  pectate, 267,271  
purification of pectin, 267  
scanning electron micrograph of  
  carrot alcohol-acetone insoluble  
  residue, 268,269f

## C

Calcium binding to pectins  
characteristics of the  
  pectins, 63,64f  
circular dichroism  
  measurements, 66,67f,68  
counterion activity  
  coefficients, 64-65,67f  
effective charge parameter, 68,69f  
egg-box model, 61-62  
influence of pattern of  
  esterification, 65-66,67f  
influence of polymer concentration  
  on calcium activity  
  coefficient, 65-66  
neutralization effects, 65,68,69f  
Calcium ion, effect on texture of  
  fruits and vegetables, 217-228  
Carbazole method, galacturonic acid  
  determination, 14  
Carbon-13 NMR spectroscopy,  
  galacturonic acid  
  determination, 16

Carrot fiber  
  beneficial properties, 272-273  
  binding of bile acids, 266-274  
Chemical properties of pectins  
  acid-catalyzed hydrolysis, 9  
   $\beta$ -elimination reaction, 9  
  deesterification, 9  
  depolymerization, 9  
  hydroxyl-group reactions, 9-10  
  lyases, 10  
  pectinesterases, 10  
  polygalacturonases, 10  
  transeliminases, 10  
Colloidal titration method,  
  galacturonic acid  
  determination, 15  
Colorimetric method, acetyl and  
  feruloyl ester determination, 18  
Concentric cylinder instruments, 97  
Conformation of pectins  
  formation of junction zones, 5  
  gels, 4-5  
Cucumbers  
  cell wall structure, 220  
  textural changes during processing  
  and storage, 218-228

## D

Decarboxylation with hydroiodic acid,  
  galacturonic acid  
  determination, 15  
Degree of amidation, definition, 3  
Degree of esterification,  
  definition, 3  
Degree of polymerization, number  
  average, 32,33t,34  
Destructive tests for gel strength  
  measurements  
  advantages, 96  
  finger test, 98  
  Herbstreith pectinometer  
  measurements, 99  
  Instron measurements, 99  
  Luers-Lochmuller pectinometer  
  measurements, 98-99  
  Tarr-Baker method, 98  
Dynamic testing, 97-98

## E

End-group titration, determination of  
  number-average molecular  
  weight, 26  
Endopectin lyases, role in fruit  
  processing, 231

Endopolygalacturonaese, role in fruit processing, 231  
 Enzymic cell wall degradation, stages, 244,245t

## F

Fiber effects on humans  
 approaches, 248  
 carbohydrate metabolism, 251,253-255t  
 effect and fate in the gastrointestinal tract, 261-262  
 glycemic effects, 257-260  
 lipid effects, 250,256  
 lipid metabolism, 250,251-252t  
 mineral bioavailability effects, 260  
 vitamin bioavailability effects, 260-261

## Firmness

influencing factors, 200  
 assessment--See Texture assessment

Foods, pectin content, 248,249t

## Fractionation of apple pectin

elution profile, 39,40f  
 enzymic degradation, 41,42f  
 ion-exchange chromatography, 41  
 neutral sugar distribution curves, 39,41,42f

## Fruit, ripening, cell wall

metabolism, 141-154

## Fruit juices

processing, 231-245

sources, 230

tissue composition, 230-231

## Fruit nectars

definition, 241

use of pectic enzymes for cloud stability, 241

## Fruit processing, pectic

processing, 231

## Fruit softening, effect of

endo-D-galacturonanase, 149,152

## G

## Galacturonic acid analysis in pectin

chemical methods 14-15

chromatographic methods, 16-17

physical methods, 15-16

## Gel strength, instruments for

measurement, 94,95t

## Gels, definition, 91

## GLC procedure

galacturonic acid determination, 16

neutral sugar determination, 19

methyl ester determination, 18

## H

Hairy region fractions, gel filtration profiles, 41,44,45f

Hemicelluloses, definition, 13

## Herbstreith pectinometer

internal strength vs. pectin

dosage, 110,111f

measurement of pectin breaking

strength, 99,105,108

photograph, 105,107f

## Herbstreith pectinometer

measurements, 99

## High-methoxyl pectins

categories, 89t

definition, 3

gelatin factors, 8

## High-performance size exclusion

chromatography, experimental

procedures, 24-26

## Human metabolism, effects of

pectin, 248-262

## I

## IFT sag method

effect of pH on measured sag of

jellies, 104-105,106f

instrumental

methods, 105,106-107f,108,109f

## Instron measurements, 99

## Internal gel strength influencing factors

jelly pH, 94

jelly test conditions, 94

molecular weight, 92

pectin type, 92,93f

## Internal gel strength instrumentation

creep compliance, 94,96

destructive test, 96,98-99

elastic limit, 94

nondestructive tests, 96-98

textural profiles, 94

## IR spectroscopy, galacturonic acid

determination, 15-16

## J

Jelly, definition, 89

Jelly grade, definition, 104

Jelly grade determination

IFT age method, 104-115

preparation of jellies, 104

Jelly manufacturer's needs, pectin

evaluation, 89-90

Jelly tester, 97

- Junction zones  
 alkaline depolymerization, 5,6f  
 formation models, 5
- L
- LC methods  
 galacturonic acid determination, 17  
 neutral sugar determination, 19  
 Length, number average, 34,35t  
 Low-methoxyl pectins  
 definition, 3  
 gelatin factors, 8  
 Luers-Lochmuller pectinometer  
 photograph, 105,106f  
 measurements, 98-99
- M
- Membrane osmometry, determination of  
 number-average molecular  
 weight, 26  
 Methyl, acetyl, and feruloyl  
 substitution in pectin, 17-18
- N
- Neutral sugar composition of pectin  
 determination, 19  
 list of sugars, 18  
 values, 41,43f  
 Neutral sugar residues, size, 54  
 Nondestructive tests for gel strength  
 measurements  
 advantages, 96  
 concentric cylinder instruments, 97  
 description, 96-97  
 dynamic testing, 97-98  
 IFT sag method, 96-97  
 jelly tester, 97  
 parallel plate measurements, 97  
 Wageningen sag methods, 97  
 Nucleotide sugar donors  
 formation, 135,136f  
 transport across the  
 membrane, 135,137
- P
- Parallel plate measurements, 97  
 Partition coefficients,  
 determination, 25-26
- Pectate  
 aggregation, 79,82  
 concentration determination, 84  
 definition, 2  
 dependence of osmotic pressure on  
 concentration, 74-75,76f  
 dependence of precipitate amount on  
 divalent ion addition, 79,81f,82  
 energetics of interaction with  
 divalent ions, 75,77  
 enthalpy data, 77,78f  
 interactions in aqueous  
 solutions, 73-86  
 molar ratio vs. ellipticity, 79,80f  
 molecular weight vs. bound divalent  
 ions, 82,83f  
 osmometric measurements, 84,86  
 pH-induced conformational  
 transition, 74-75  
 purification and neutralization, 84  
 rheology of mixed  $H^+-Ca^{2+}$   
 gels, 82,84,85f  
 structural properties, 79  
 volume change on addition of  
 divalent cations, 75,76f
- Pectic acid, definition, 2  
 Pectic polysaccharide, definition, 13  
 Pectin  
 alkaline deesterification, 62  
 analysis by uronic acid  
 assay, 201,204  
 apple, 38-47  
 characteristic parameters, 32,33t  
 characterization of samples, 62-63  
 chemical properties, 9-10  
 chromatograms for methyl-esterified  
 pectins, 28,29f  
 circular dichroism, 63  
 composition, 157-158  
 conformation, 4,5,6f  
 content in foods, 248,249t  
 definition, 3,14,90  
 enzyme degradation of  
 $\alpha$ -1,4-galacturonan, 158  
 enzymic deesterification, 62  
 extraction from cell wall  
 material, 201  
 formation during plant cell wall  
 growth, 134-139  
 glyceemic effects, 257-260  
 influence on internal gel  
 strength, 92,93f  
 interactions with counterions, 61-71  
 lipid effects, 250,256  
 methyl, acetyl, and feruloyl  
 substitution  
 determination, 17-18  
 mineral bioavailability effects, 260  
 model of types A-E, 41,43f  
 neutral sugar composition, 18  
 number-average degree of  
 polymerization, 32,33t,34  
 number-average length, 34,35t

- Pectate--Continued**  
 occurrence in plant materials, 157  
 physical properties, 5,7-9  
 potentiometry, 63  
 quantitative analysis of  
   galacturonic acid, 14  
   radii of gyration, 28,29t  
 role in binding of bile acids to  
   carrot fiber, 266-274  
 sample preparation, 62-63  
 self-disaggregation, 23-36  
 structure, 3-4,38-39,90  
 subdivision, 3  
 sugar beet, 49-59  
 theory of gel formation, 90-91  
 use in manufacture of jelly, 88  
 vitamin bioavailability  
   effects, 260-261
- Pectin-alginate systems**  
 determination of mechanical  
   properties of gels, 119  
 effect of calcium on gels, 120-121  
 effect of different ratios of pectin  
   and alginate, 121,126,127f  
 effects of D-glucono- $\delta$ -lactone  
   on gels, 121,122-125f  
 preparation of gels, 119  
 source of pectin and  
   alginate, 118-119,120t
- Pectin esterases, role in fruit**  
 processing, 231
- Pectin function in tomato ripening**  
 endo-D-galacturonase  
   activity, 145t  
 enzyme assays, 143  
 gel filtration  
   profiles, 143,145,146-147f,152  
 hemicellulose extraction and  
   analysis, 143,149,151f,154  
 ion-exchange  
   chromatography, 143,145,148f  
 measurement of total and soluble  
   pectins, 143  
 neutral sugar  
   analysis, 143,149,150f,153-154  
 percent esterification, 143  
 plant material, 142  
 preparation of ethanol-insoluble  
   solids, 142  
 total and soluble pectins in ethanol  
   powders, 144t,145
- Pectin gel network, 91**
- Pectin grading**  
 breaking strength vs. molecular  
   weight, 110,113,114f,115  
 breaking strength vs.  
   viscosity, 113,114f,115  
 comparison of various  
   methods, 108,115
- Pectin polymers**  
 chemical modification in the cell  
   wall, 139  
 compartmentalization within the  
   endomembrane system, 138
- Pectin polymers--Continued**  
 fusion with the plasmamembrane, 139  
 movement of vesicles, 138-139  
 synthesis within the endomembrane  
   system, 137
- Pectinate, definition, 2**
- Pectinic acid, definition, 2**
- Physical solubility of pectins**  
 function of structure, 7  
 gelation factors, 7-9  
 pH effects, 7  
 viscosities, 5,7  
 water solubilities, 5
- Plant cell wall**  
 channels for pectin movement and  
   synthesis, 135-139  
 formation of pectin, 134-135
- Polygalacturonase**  
 $\alpha$ -1,4-galacturonan cleavage, 158  
 characterization, 158-159  
 mechanism of degradation, 158
- Polygalacturonase converter**  
 effect on isoenzymes, 162  
 properties, 161-162  
 purification, 161t
- Polygalacturonase in fruits**  
 activity, 167,169,170f  
 isoenzymes, 169
- Polygalacturonase isoenzymes**  
 activity, 165,167  
 conversion of I to II, 159,161  
 converter, 161t,162,165,167  
 separation, 159,160f  
 similarities, 159
- Polygalacturonase in peaches**  
 activity, 167,169t  
 isoenzymes, 167  
 solubility, 167,168f,169
- Polygalacturonase in plant tissues**  
 activity, 169,171t  
 characterization, 171,172f,173t  
 isoenzymes, 169,171
- Polygalacturonase in tomatoes**  
 activity, 165,167  
 effect of pH on solubility, 162,163f  
 effect of salt on  
   solubility, 162,164f,165,166f  
 isoenzymes, 159  
 levels, 159
- Polysaccharides, universal calibration**  
 curves, 26,27f,28
- Polyuronides**  
 properties, 175-176  
 sequential cooperative ion binding  
   mechanism, 176-186

R

## Radii of gyration--Continued

- influencing factors, 28,30
- number average, 30,31t,32
- values, 28,29t

## Root-mean-square radii of gyration, determination, 25

## S

## Self-disaggregation of pectin

- experimental procedures, 23-26
- van't Hoff plots, 23,27f

## Sequential cooperative ion binding mechanism

- effect of competitive exchange on broadening, 183,184-185f,186
- evidence for a divalent cation-polyuronide aggregate structure, 182-183
- experimental conditions, 177
- paramagnetic ion couplings, 177,179
- paramagnetic ion dependency of line width, 179,180-181f,182
- preparation of tissues, 176-177
- titration of  $Mn^{2+}$ , 177,178f

## Serum cholesterol, mechanism for reduction, 256-257

## Softening of vegetables during heat processing

- effect of adding salts after cooking, 193f,194-195,196f
- effect of adding salts before cooking, corrected for  $Ca^{2+}$  displacement, 194t,195
- effect of  $\beta$ -elimination hypothesis
- effect of cooking time and temperature on firmness, 192,195t,197
- effect of K fertilization on firmness, 192t
- effect of leaching and NaCl on firmness, 192t,194t
- effect of NaCl added before or after cooking on firmness, 192,194t
- effect of salts present before cooking on cohesiveness, 191t
- effect of salts present before cooking on firmness, 191t
- effect of soaking on firmness, 192,193f
- role of  $Ca^{2+}$  displacement, 197,198

## Structure of pectin

- acetyl groups, 3-4
- linear chain, 3
- neutral sugars, 4
- types, 90

## Sugar beet pectin

- analytical methods, 50
- chromatography, 51

## Sugar beet pectin--Continued

- composition and properties, 52t
  - cross-linking and gelation, 51,53
  - degradation limits, 53t,54
  - degradation studies, 51,53t,54
  - effect of enzymic cross-linking on viscosity and molecular mass, 58t,59f
  - extraction and purification, 50
  - gel permeation chromatograms, 52-53,55f,57f
  - gelation through oxidation coupling, 56,58,59f
  - location of acetyl ester groups, 54,56,57f
  - neutral sugar content, 52t
  - new applications, 58
  - presence and location of feruloyl ester groups, 56,57f
  - source, 50
  - yields and anhydrogalacturonic content, 51,52t
- Sugar beet pulp, source of pectin, 49-50
- Synergistic gelling systems, pectin-alginate system, 117-131

## T

## Tarr-Baker method, 98

## Textural changes of cucumbers

- calcium as a firming agent, 218,219t,220
  - changes in cell wall neutral sugar content, 220,221f
  - changes in pectin fractions, 220,222f
  - effect of calcium concentration, 223,225f
  - effect of pectin methylation, 223,224f
  - enzymatic softening, 218
  - esterification vs. calcium concentration, 226,227t
  - fermentation, 220
  - firmness vs. degree of methylation, 223,226
  - Scatchard plot of calcium binding, 226,227f
- Texture assessment
- effect of blanching, 204,205f
  - effect of freezing, 204,205f,206,207f
  - elution profile vs. storage time, 209,212-214f,215t
  - extraction performance of different chelators, 206,207f
  - instrumentation, 200-201,202f
  - loss in frozen storage, 209,211f

- Texture assessment--Continued V  
pectic fractions before and after  
processing, 206,208t  
preparation and purification of  
pectin from cell wall  
material, 201,203f,204  
tissue systems used, 204  
uronide content vs. storage  
time, 206,210f,t
- Texture measurements  
internal strength curves, 108,109f  
Voland Stevens texture  
analyzer, 108,109f,110,111f
- Titration method, methyl ester  
determination, 18
- Tomato fruit, pectin function in  
texture modification, 142-154
- Vegetables, softening during heat  
processing, 190-198
- Voland Stevens texture analyzer,  
internal strength vs. pectin  
dosage, 108,109f,110,111f
- W
- Wageningen sag measurements, 97